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The DC1-domain protein VACUOLELESS GAMETOPHYTES is essential for female and male gametophyte development in Arabidopsis

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Summary

In this work we identified VACUOLELESS GAMETOPHYTES (VLG) as a DC1 domain containing protein present in the endomembrane system essential for both female and male gametophyte development. *VLG* was originally annotated as a gene coding for an unknown function protein containing DC1 domains. DC1 domains are cysteine and histidine -rich zinc finger domains found exclusively in the plant Kingdom that have been named based on its similarity with the C1 domain present in protein kinase C (PKC). In Arabidopsis, both male and female gametophytes are characterized by the formation of a big vacuole early in development, which is absent in *vlg* mutant plants. As a consequence, development is arrested in embryo sacs and pollen grains at the first mitotic divisions. VLG is specifically located in multivesicular bodies or prevacuolar compartments and our results suggest that vesicular fusion is affected in the mutants, disrupting vacuole formation. Supporting this idea, AtPVA12 - a member of the SNARE vesicle associated protein family and previously related to a sterol binding protein was identified as a VLG interactor. A role for VLG is proposed mediating vesicular fusion in plants as part of the sterol trafficking machinery required for vacuole biogenesis in plants.

Introduction

Divergent C1 (DC1) domains are cysteine and histidine rich zinc finger domains found exclusively in the plant Kingdom. They have been termed based on its similarity with the C1 domain present in protein kinase C (PKC) and other mammalian proteins including protein kinase D, DAG kinases, RasGRPs, chimaerins and Munc13s (Brose et al., 2004). Originally discovered as diacylglycerol (DAG) and phorbol esters binding molecules, C1 domains have been also reported as membrane binding (Johnson et al., 2007) and protein-protein interaction modules (Colon-Gonzalez and Kazanietz, 2006). C1 domains have crucial roles in allosteric enzymatic activation of PKC (Ono et al., 1989) and in the targeting of PKC and other proteins to membranes (Lehel et al.,

1995; Mosior and Newton, 1995). C1 domains are absent in the plant kingdom but instead, a large number of genes coding for DC1 domain-containing proteins occur. Although DC1 domains could be possibly assuming C1 domain-related roles in plants, there are only few reports on DC1 domain-containing proteins but neither the function of the protein nor the role of the domains involved could be elucidated. In Arabidopsis, two different genes coding for DC1 domain-containing proteins, *At5g17960* and *ULI3 (At5g59920),* were reported as responsive to hormone and stress treatments (Bhaskar et al., 2015) and involved in UV-B-mediated signal transduction (Suesslin and Frohnmeyer, 2003), respectively. In *Capsicum annuum,* CaDC1, was identified as a positive regulator of SA-dependent plant defense responses (Hwang et al., 2013). CaDC1 is nuclear localized and binds both DNA and RNA (Hwang et al., 2013). In wheat, *TaCHP* was identified as a protein differentially expressed under salt stress. In addition, Arabidopsis plants expressing *TaCHP* exhibit high tolerance to salt stress (Li et al., 2010). In tobacco, *NtDC1a* and *NtDC1b*, were identified as early induced after treatment with a β-1,3-,1,6-oligoglucan -enriched fraction or laminarin (Shinya et al., 2007). More recently, GhCHR, a cotton DC1 protein was identified as a target of miRNVL5 and involved in the regulation of plant response to salt stress (Gao et al., 2016). Authors could not identify the Arabidopsis ortholog of miRNVL5, but recognized another DC1 protein, At2g44380, as a putative target of the cotton microRNA through a degradome sequencing technique (Gao et al., 2016).

In this work we identified VACUOLELESS GAMETOPHYTES (VLG) as a DC1 domain-containing protein present in the multivesicular bodies (MVB) essential for both female and male gametogenesis. Embryo sacs and pollen grains show arrest in *vlg* mutants during the first stages of gametophyte development. Remarkably, the big vacuole that characterizes the stage at which each gametophyte is arrested, is missing in both the embryo sac and the microspores. Through a yeast two-hybrid screening and bimolecular fluorescence complementation assays we identified AtPVA12 -a vesicle associated protein and member of the VAP33 subfamily of SNAREs as a VLG protein partner, suggesting that VLG might be part of a complex involved in vesicle fusion.

Results

VLG encodes a DC1 domain containing Protein

At2g17740 encodes a 248-amino acid long protein of unknown function, which was named VACUOLELESS GAMETOPHYTES (VLG) in this paper. Analysis of VLG in protein domain databases identified three DC1 or C1-like domains (PF03107 and IPR011424 in Pfam and Interpro domain databases respectively, Figure 1). DC1 domains resemble C1 domain (PF00130 in Pfam database and IPR002219- protein kinase C-like, phorbol ester/diacylglycerol-binding domain in Interpro database) but they have not been characterized and their function remains elusive. Scrutiny of the Protein Data Bank (PDB) using VLG as a query identified 1v5n, an unpublished NMR structure of the DC1 domain of a disulfide isomerase (PDI) like protein encoded by At1g60420 (Miyamoto et al., 2003). This structure contains 2 antiparallel β-sheet of a total of 3 β-strands and 2 α-helices and coordinates 2 Zn^{+2} ions through 3 cysteine and 5 histidine residues, some of them corresponding to conserved amino acids of the DC1 domain signature. Structure-based alignments of 1v5n with two available C1 domain structures, 1y8f from rat Munc13-1 (Shen et al., 2005) and 2yuu from human PKC delta type (Abe et al., 2007) showed a folding that is similar to DC1 and C1 domains. TM-align analysis yielded root-mean-square deviation values of 1.69 A and 1.85 A and TM-scores of 0.61066 and 0.65438, respectively (Figure S1a). Based on these results, a functional signature for DC1 domain could be proposed as $\mathbf{H}X_{1\text{-}2}\mathbf{H}X_{12\text{-}18}\mathbf{C}X_2\mathbf{C}X_{10\text{-}14}\mathbf{C}X_2\mathbf{C}X_4\mathbf{H}X_2\mathbf{C}$, where the first conserved His in DC1 domain substitutes the last Cys in C1 domain in the Zn^{+2} coordination (Figures S1 and S2).

Protein sequence alignment of VLG's DC1 domains, DC1 domain of the PDI-like protein encoded by *At1g60420* and C1 domains of rat Munc13 (Q62768) and human PKC delta (Q05655) also showed the conservation of the residues involved in Zn^{+2} coordination (Figure 1b). Modeling of VLG's DC1 domains using 1v5n as template shows also the structural similarity of the domains, with highly superimposed regions in the areas involved in Zn^{+2} coordination and the antiparallel β-sheet strands and more dissimilar regions in the area that constitute the loops connecting the Zn^{+2}-binding areas (Figure 1c).

Inspection of the Arabidopsis genome searching for DC1 domain-containing proteins yielded 140 proteins harboring between 1 and 7 DC1 domains. Phylogenetic analysis distributed them in 2 groups: one constituted by two PDI-like proteins, with a single C-terminal DC1 domain in addition to 2 or 3 thioredoxin like domains, and the second group with 138 proteins containing exclusively DC1 domains (Figure S3). These 138 proteins could be further divided into two main groups: one containing 122 proteins harboring between 4 and 6 DC1 domains and with a conserved WDG inter-domain motif, and the other group, with 16 mostly shorter and more divergent sequences. VLG belongs to this last group. An alignment of this last group of proteins and their domain structure is shown in Figure 1d.

vlg is a gametophytic mutation

To analyze *VLG* function, two Arabidopsis lines (Columbia [Col-0] ecotype) with T-DNA insertions located at the promoter region (SAIL_507_F09, named here *vlg-1*) and in the single exon (and GK-793C12, named here *vlg-2*) of the gene were studied (Figure 1e). After backcrossing to the Col-0 ecotype, the progeny of self-crossed plants was genotyped. No homozygous mutant plants were recovered in the offspring from either *vlg-1/VLG* or *vlg-2/VLG* self-pollinated plants. The ratio of hemizygous to wild type (WT) plants was 0.139 for *vlg-1/VLG* (n= 270) and 0.264 for *vlg-2/VLG* plants (n=129). The proportion of insertional mutants obtained in the progeny of self-fertilized *vlg/VLG* plants was lower than expected 3:1 Mendelian segregation ratio for diploid sporophytic or embryo lethal (2:1) mutants and suggested a gametophytic defect. The only visible difference observed between WT and hemizygous mutant plants was the size of the siliques. The siliques from the hemizygous mutant plants are shorter than WT (9.65 ± 0.53 mm for *VLG/vlg-1* and 10.45 ± 0.55 mm for *VLG/vlg-2*) compared to 14.15 ± 0.41 mm in WT plants. No other sporophytic phenotype was observed in the hemizygous plants, suggesting that *vlg* is a recessive mutation (Figure S4). When the siliques of *vlg/VLG* plants were analyzed, nearly half of the seeds looked aborted (45.5% for *vlg-1/VLG, n=1045;* and 41.3% for *vlg-2/VLG* plants, n=563, Figure 2), which suggested that *VLG* is a gene that is required for gametogenesis and/or seed development.

To evaluate the involvement of VLG in gametophyte development, reciprocal

crosses between *vlg-1* and *vlg-2* insertion lines and WT plants were performed and the transmission efficiency of the mutant allele was calculated (TE=mutant/WT offspring x 100, Table 1). The TE represents the percentage of gametes carrying the mutant allele that successfully transmit the mutation. Only 23.6 % of megagametophytes carrying *vlg-1* and 36.08 % of the megagametophytes carrying *vlg-2* were able to transmit the insertion to the next generation. The ratio obtained was significantly different from the 1:1 ratio expected for a mutation that does not affect transmission ($p<0.0001$, Chi square test), indicating that *vlg* affects female gametophyte development or function. In addition, male transmission was also affected. TE through the pollen was 12.4 % for *vlg-1* and 33.1 % for *vlg-2* (Table 1, significantly different from 1:1 ratio hypothesis, $p<0.0001$, Chi square test). Thus, both gametophytes are compromised by mutations in *VLG.*

To further confirm that mutations in *VLG* were indeed responsible for the defects observed, a genetic complementation assay of *vlg-1/VLG* and *vlg-2/VLG* plants using a using a construct containing VLG full length cDNA under its own promoter (*pVLG-VLG-GFP*) was performed.

Siliques of T3 plants that were hemizygous for the *vlg* allele and homozygous for the transgene were analyzed. Five lines were analyzed for each allele and in all cases the siliques showed seed sets similar to WT plants (Figure 2a, d-e).

Pollen development in *vlg* mutants is arrested before PMI

To analyze the basis of the deficiency in transmission through the male gametophyte, viability of pollen grains was analyzed using Alexander staining. About 44% (n=1135) of unviable pollen was detected in mature anthers in *vlg-1/VLG* plants while 29.9% of unviable pollen was obtained for *vlg-2/VLG* plants (n=1944), (Figure 3 and Figure S5). In *vlg-1/VLG* and *vlg-2/VLG* plants carrying the construct *pVLG-VLG-GFP* in homozygosis, viability reached values similar to WT resulting in 1.3% and 2% pollen aborts, respectively (Figure S5). In Arabidopsis, the development of the pollen grain (male gametophyte) begins with the expansion of the microspore, which is associated with the formation of a large vacuole (Twell, 2011). This vacuole displaces the microspore nucleus against the microspore wall. The nucleus undergoes a first mitosis (pollen mitosis I) which results in the formation of two dimorphic cells: a large,

vegetative cell and a small generative cell (bicellular pollen, Figure 3a). The generative cell is subsequently surrounded by the vegetative cell and divides again by mitosis (pollen mitosis II) to render two sperm cells that are completely enclosed within the vegetative cell cytoplasm (tricellular pollen, Figure 3a). To understand how the *vlg* mutation was affecting pollen viability, we analyzed pollen development in WT and hemizygous plants using DAPI staining in pollen from flowers at different developmental stages. To perform this study, flowers from the same inflorescence were analyzed. Stage 0 corresponds to the flower at anthesis and every correlative negative number corresponds to the flower located immediately above, as stated in (Durbarry et al., 2005). From stages -9 to -7, pollen present in the anthers of WT plants show only 1 nucleus, from stages -6 to -3 two nuclei and three nuclei from stages -2 to 0 (Figure S6). When *vlg-1/VLG* plants were analyzed, no differences were observed from stages -9 to -7, where most pollen was uninucleated. However, from stages -6 to 0 arrest in development was evident and a fraction of the pollen remained with one nucleus (Figure 3 and Figure S7). While 99% of the pollen present in WT flowers at stage -5 showed two-nuclei (n=561), in *vlg-1/VLG* plants 43% of developing pollen grains were found at the uninucleate stage (n=1243). A similar result was obtained for *vlg-2/VLG* plants, where 39.8% of the developing pollen grains were also found at the uninucleate stage (n=1941), while the remaining pollen grains showed two nuclei (Figure 3 and Figure S7). Additionally, those microspores were not polarized and the big vacuole characteristic of this stage of development was not present (Figure 3). To further confirm this observation, a staining with neutral red was performed, which revealed that while WT microspores show a large vacuole with a nucleus at a side (Figure 3i), mutant microspores only show small vacuoles that were distributed throughout the cytoplasm (Figure 3g). A large vacuole was not detected. These results suggest that *vlg* mutation prevents the formation of a big vacuole in the microspore which in turn might result in developmental arrest (Figure 3j-m).

Functional *VLG* is required for female gametogenesis

To investigate the basis of the transmission defect observed trough the female gametophyte, the phenotype of gametophytes in flowers from *vlg/VLG* plants was studied by DIC microscopy.

In Arabidopsis, the female gametophyte (also called embryo sac or megagametophyte), is a highly polarized structure that originates from a haploid megaspore called functional megaspore (FM, Figure 4). This functional megaspore, which is the remaining spore from the original tetrad of haploid cells produced after female meiosis, undergoes three successive mitotic nuclear divisions forming a syncytium of eight nuclei (stages FG2 to FG5; Figure 4). (Drews and Koltunow, 2011). Further differentiation gives rise to the seven cells that comprise the mature embryo sac (stage FG6, Figure 4a). Ovules from WT and *vlg/VLG* plants were collected from pistils of a size between 1.5 and 2 mm, where WT gametophytes should be at a FG5 stage (Cigliano et al., 2013), as later on mutant embryo sacs were found collapsed. At that stage, 89% of the embryo sacs in WT pistils were found at FG5 stage while 8.5% were found still at FG4 stage and 2.5% looked collapsed (n=334). However, around 45% of the embryo sacs present in *vlg/VLG* pistils were found arrested at the first stages of development, at FG1 or at FG2. In the case of the embryo sacs from *vlg-1/VLG* pistils, 36.52% were found at FG1 stage, 9.2% were found at FG2 stage, 1.2% looked collapsed and 53.08% of the embryo sacs were found at FG5 stage (n=229, Figure 4). For *vlg-2/VLG,* 32.7% were found at FG1*,* 8.8% were found at FG2 stage, 1.31% looked collapsed and 57.2% were at FG5 (n=305). No female gametophytes were found at FG3 stage, in which a large vacuole is formed between the two nuclei, suggesting that this key step might be affected in the mutants. As the fraction of aberrant embryo sacs detected in *vlg-2/VLG* was slightly higher than expected (43% versus 32% expected from the TE calculated above), it is probable that part of the female gametophytes found at FG1-FG2 stage, although severely delayed, are able to complete the developmental program.

Thus, *vlg* mutants showed severe defects in both megagametogenesis and in microgametogenesis; the formation of the large vacuole that is characteristic of both processes was not observed. The absence of a large vacuole at the uninucleate microspore stage might prevent polarization during microgametogenesis. On the other hand, as a central vacuole is not formed, progress from FG2 to FG3 stage is affected in the developing embryo sac.

Although the insertions in *VLG* severely compromised the development of both gametophytes, transmission of the mutation is not zero (table 1), indicating that we should be able to detect homozygous mutant plants in the self-progeny of hemizygous plants. As this was not the case, we investigated whether embryogenesis was affected by the mutation analyzing pistils from *vlg-2/VLG* plants (as this allele showed the highest transmission ratios), 72 hours after manual pollination with pollen from a *vlg-2/VLG* plant. Out of 174 ovules analyzed, 93 (54.06%) were found at an early globular stage, 68 (39.5%) were found arrested at early stages of gametogenesis and collapsing and 11 (6.4%) were found arrested at the zygotic stage (Figure S4). This result suggests that a fraction of the aborted seeds detected in the siliques might correspond to embryo lethality. In addition, post-embryonic seedling lethality is also possible and together with gametophytic defects and embryo lethality would explain the absence of homozygous mutant plants in the progeny of selfed hemizygous plants.

VLG is expressed in young sporophytic tissues

To study the temporal and spatial expression pattern of the VLG gene during sporophytical development, its expression was analyzed in transgenic plants carrying a VLG promoter-GUS reporter gene fusion (*ProVLG:GUS*). The expression of GUS driven by the 1104 bp region located upstream *VLG* translation start codon was analyzed at different stages of development in WT transgenic plants. GUS expression was detected in young seedlings at the hypocotyl, cotyledons and leaf primordia, where is primary located in regions of active cell division (Figure 5). In mature leaves as well as in sepals, expression was weaker and mainly confined to vascular tissues and high expression was detected in the filaments of mature flowers (Figure 5d). Localization of GUS in

the roots was excluded from the proliferation zone but high expression was detected in the elongation and differentiation zones, mainly associated to vascular tissues (Figure 5e-f). This expression pattern suggests that besides its role during gametogenesis, VLG might act also in other developmental processes in the sporophyte.

VLG is found both in female and male gametophytes and localizes to multivesicular bodies

To analyze VLG localization during mega and microgametogenesis, fusion protein VLG-GFP was followed in transgenic WT plants carrying the construct *VLG:GFP*. This construct was proven to be fully functional as was able to complement the phenotype observed in *vlg/VLG* plants (Figure 2 and Figure S5).

In ovules, VLG-GFP was detected both in the nucellus and inside the developing embryo sac, showing a punctate pattern (Figure 5). This pattern was observed from FG1 to FG6 (Figure 5g-j) when it can be detected in all cells composing the embryo sac. After fertilization, signal is detected in the zygote and in the developing endosperm (Figure 5k). 48 hours after pollination, the signal is faint and is not detectable 72 hour after pollination, suggesting that VLG might be required early during embryogenesis. In pollen grains, GFP was also detected in the polarized microspore and at the early bicellular stage, where it is confined to the periphery of the developing pollen grain (Figure 5n-q). At the late bicellular stage the signal is lower in the periphery and the protein is mainly detected towards the center of the pollen grain, following a punctate pattern as observed in embryo sacs (Figure 5p). At the tricellular stage the protein was again detected scattered in the pollen grain following a punctate pattern (Figure 5q). In addition, the same punctate pattern was also found in the pollen tube of germinating pollen grains (Figure 5r).

To define the nature of the punctate arrangement observed, we followed the endocytic trafficking of the lipophilic dye FM4-64 in roots of plants expressing the fusion protein VLG-GFP. After internalization, this styryl dye passes through endosomal compartments on their way to the tonoplast (Dettmer et al., 2006; Chow et al., 2008). The trans-Golgi network (TGN) and the prevacuolar

compartments (PVC) or multivesicular bodies (MVB) (PVC/MVB) are sequentially labeled, and for that reason they are defined as early or late endosomes respectively (Dettmer et al., 2006; Chow et al., 2008). After 30 min, FM4-64 could be detected in the membrane and internalized in early endosomal compartments (Figure S8). However, no co-localization with VLG could be detected. After two hours FM4-64 is starting to define the tonoplast. As it can be observed in Figure S8, VLG was found in discrete bodies near the vacuole membrane, but still no co-localization with FM4-64 was found. After four hours of incubation with FM4-64, the dye defines small vesicles surrounding the tonoplast in which VLG was localized (Figure 6). The localization and structure of these compartments indicate that VLG might localize to late endosomal compartments called prevacuolar compartments (PVC) or multivesicular bodies (MVB) that are composed by multiple luminal vesicles. To further confirm this localization we studied VLG co-localization with Rha1 (a plant Rab5 homologue) that is used as a PVCs/MVBs marker (Foresti et al., 2010), using a transient expression system in *Nicotiana benthamiana*. As it can be observed in Figure 6e-g, VLG co-localizes with the RFP-Rha1 marker, confirming its localization in the PVCs/MVBs compartment. In addition, no co-localization was found with ST-RFP (rat sialyltransferase fused to red fluorescent protein) which is used as a marker for the Golgi apparatus (Figure S8) (Jin et al., 2001; Speth et al., 2009).

VLG Interacts with PVA12, a protein member of the VAP33 subfamily of SNAREs and with LTL1, a GDSL-motif lipase

To gain a better understanding of VLG function, a high-throughput yeast two-hybrid (Y2H) screening to identify proteins capable of interacting with VLG was conducted (Hibrygenics, Paris, France). Among the putative interacting proteins identified, there were clones that correspond to proteins with a predicted localization that was associated either with the nucleus or with organelles such as the chloroplast (Table 2). That was the case for a SAP domain-containing protein (chloroplast), for a Rho termination factor (found in cytosol) and for a NAC domain containing protein 73 (NAC073, found in nucleus). As their localization did not overlap VLG´s subcellular localization, we did not pursue further experiments with those clones. On the other hand, we focused our

attention in two clones whose predicted localization might overlap VLG´s one *in vivo*. That was the case of PVA12, a VAMP-associated member of the VAP33 family of plant proteins that was previously reported as an interactor of the sterol-binding protein ORP3 (Saravanan et al., 2009), and of LTL1, a GDSL-motif lipase that was previously associated with salt resistance (Naranjo et al., 2006). Interestingly, proteins containing a GDSL-motif have been previously associated to the tonoplast (Carter et al., 2004) and mutants of *MODIFIED VACUOLE PHENOTYPE1* (*mvp1*), which encode a GDSL-lipase/esterase family protein, show an aberrant morphology of vacuolar membrane and Golgi bodies (Agee et al., 2010; Nakano et al., 2012).

To further confirm VLG-PVA12 and VLG-LTL1 interactions, we performed a bimolecular fluorescence complementation (BiFC) assay using transient expression in *N. benthamiana* leaf cells. We fused *VLG* and each of the candidate genes to the N- and C-terminal yellow fluorescent protein (YFP) fragments to generate VLG-N-YFP and PVA12-C-YFP or LTL1-C-YFP fusion constructs, respectively. The fusion constructs under control of the *CaMV 35S* promoter were transformed into *N. benthamiana* leaves and BiFC signals were detected. YFP was detected following a punctate pattern (Figure 7), suggesting that interaction is taking place in the endomembrane system. Control *N. benthamiana* leaves in which *VLG-N-YFP* was co-expressed with unfused C-YFP or unfused N-YFP was co-expressed with *PVA12-C-YFP* or with *LTL1-C-YFP* did not show any BiFC signal (Figure 7). An additional control experiment using a protein related with VLG (a DC1 domain-containing protein encoded by At2g44370 with 72.0% identity, Figure 7 and Figure S9) did not show positive results with PVA12 nor with LTL1, indicating that the interaction observed was specific.

As *vlg* mutants showed gametophytic defects, we analyzed if VLG´s interactors, PVA12 and LTL1, were also required for female or male gametogenesis. T-DNA insertional mutant lines (SALK_088746 for PVA12 and SALK_012570 for LTL1) were characterized for this purpose. We were able to find knockout homozygous mutant plants for the insertion in both *LTL1* and *PVA12* (Figure S10a), suggesting that these genes are not essential. Homozygous mutant plants did not show any obvious phenotype in the sporophyte and siliques show full seed set (Figure S10c). We also assessed

whether pollen tube germination or growth was affected by testing germination of pollen grains from hemizygous plants, as pollen tube growth has been shown to relay on normal vacuole morphology (Steinhorst et al., 2015). However, no defects were detected, suggesting that other proteins might function redundantly with LTL1 and PVA12 (Figure S10d). In agreement with this idea, there are two proteins among the GDSL esterase/lipase family, encoded by At5g18430 and At5g33370, with about 70% identity and over 80% of similarity of aminoacid sequence with LTL1. In addition, two other vesicle associated proteins PVA11, encoded by At3g60600 and PVA13, encoded by At4g00170 share 81% and 56% identity and 90% and 72% similarity respectively with PVA12 (Figure S11). Furthermore, *PVA11*, *LTL1* and their closest homologues are expressed in pollen and ovules (Figure S12).

Discussion

VLG is a modular protein containing three DC1 domains. DC1 domains occur exclusively in plants and resemble C1 domains, absent in plants but widely distributed in the animal kingdom. Structural similarities presented here between DC1 and C1 domains suggest that DC1 domains could have in plants one or more of the roles fulfilled by C1 domains in animals, as binding to signal molecules, membrane lipids or other proteins. Three dimensional analyses exposed a high similarity of structures between C1 and DC1 domains. A very similar folding was observed in the Zn^{+2} coordination and antiparallel β–sheet regions. On the other hand, connecting loops, that in C1 domains define the binding region of the domain (Zhang et al., 1995), resulted more dissimilar. These loop regions, not conserved neither in sequence nor in length in DC1 domains, may determine the specificity of binding also in DC1 domains, either to lipids or to proteins.

In this work we show that VLG is necessary for vacuole biogenesis during microgametogenesis and megagametogenesis, as evidenced in hemizygous lines carrying T-DNA insertions and by genetic complementation. Moreover, we also showed that the formation of a central vacuole is essential for gametophyte development progression. The formation of a big vacuole characterizes the steps at which each of the gametophytes was found arrested in *vlg* mutants. During microgametogenesis, free uninucleate microspores contain small

vacuoles around a centrally located nucleus. In more mature uninucleate microspores, the small vacuoles coalesce into one large vacuole that pushes the nucleus to one side of the microspore. However, when developing pollen was studied in *vlg/VLG* plants, nearly half of the microspores were found arrested at a point in which the large vacuole was not formed and only small vacuoles were visible, scattered in the cytoplasm surrounding a central nucleus. Coincidentally, the formation of a big vacuole that characterizes FG3 during female gametogenesis was never found in *vlg* mutant embryo sacs, suggesting that a similar process might be affected by the mutation in both gametophytes.

VLG localizes to plant prevacuolar compartments (PVCs) or multivesicular bodies (MVBs), which mediate protein trafficking to vacuoles in the secretory pathway. PVC/MVBs are also considered as late endosomes in the endocytic pathway in plants (Cui et al., 2016). In plants, the secretory and endocytic pathways merge in the trans-Golgi network (TGN) and their cargoes are passed on to the PVC/MVB which serves as intermediate compartments between the TGN and the vacuole, enabling proteins to recycle before their fusion with the vacuole (Cui et al., 2016). The specific membrane fusion between transport vesicles and target membranes is mediated by the SNARE (soluble *N*-ethyl-maleimide sensitive factor attachment protein receptors) (Chen and Scheller, 2001; Jürgens, 2004). In *A. thaliana*, at least 64 SNARE molecules have been identified (Uemura et al., 2004). SNARE-mediated vesicle trafficking has been related to VAMP-associated proteins (VAPs), which are highly conserved integral membrane proteins. Mammalian VAPs were shown to be involved in different cellular functions, including lipid transport, membrane trafficking, neurotransmitter release, stabilization of presynaptic microtubules, and unfolded protein response (Lev et al., 2008). The first VAP protein described, VAP33, was isolated as a 33 kDa VAMP-associated protein in *Aplysia californica* and it is involved in the VAMP-mediated neurotransmitter release at the plasma membrane (Skehel et al., 1995). VLG was found to interact with a plant VAMP-ASSOCIATED PROTEIN, PVA12. Arabidopsis has 10 VAPs grouped in the VAP33 subfamily of SNAREs that localize in the endomembrane system and have also been associated to the ER membrane, mediating ER-associated vesicular trafficking, docking and fusion (Saravanan et al., 2009). Particularly, PVA12 has been found to mediate the targeting of the oxysterol-binding protein

ORP3a to the endoplasmic reticulum (Saravanan et al., 2009). Interestingly, it was recently found that vacuole biogenesis and trafficking of tonoplast proteins and lipids can occur directly from the ER (Viotti et al., 2013). Thus, it was proposed that the ER might act as the main membrane source for vacuole biogenesis. In this scenario, VLG might be acting mediating sterol traficking from the ER to the tonoplast by interacting with PVA12. VLG might function both as a docking protein mantaining PVA12-ORP3a inside prevacuolar vesicles and facilitating their traficking by lowering energetic barriers required for vesicle fusion, as was showed for Munc13-1 C1 domain in mammals (Zhang et al., 1995), probably as part of a SNARE complex. Interestingly, ORP3 has been identified as an embryo sac mutant in a screening for female gametophyte mutants (Pagnussat et al., 2005). This suggest that even though we were not able to find a female gametophyte phenotype related to PVA12, it is probably that a similar process might be taking place in the embryo sac, involving a PVA with an overlapping function. In addition, VLG was also found to interact with LTL1, a GDSL-motif lipase, that was shown to increase salt tolerance when overexpress in yeast and plants (Naranjo et al., 2006). The Arabidopsis GDSL lipase protein family consist of 118 members, although some of them might not have lipase activity. This is the case of MODIFIED VACUOLE PHENOTYPE1 (MVP1), a putative myrosinase-associated protein previously annotated as a GDSL-motif lipase that localizes to the ER, ER bodies, and tonoplast (Agee et al., 2010). *mvp1* mutants show several vacuole-related phenotypes, as well as aberrant trafficking. In the particular case of LTL1, its subcellular localization was only infered by its sequence analysis. As LTL1 contains a highly hydrophobic transmembrane region included in a putative signal peptide it was proposed that LTL1 might be either a plasma membrane associated protein or a secreted protein (Naranjo et al., 2006). Thus, LTL1 could be either transiently associated to VLG in its secretory pathway or might have a more transcendental role in vacuole biogenesis as reported for MVP1. In any case, further studies are required to elucidate its specific role.

Altogether, our results demonstrate that central vacuole formation is essential for both female and male gametogenesis and place VLG as a key player in vacuole biogenesis. VLG´s interaction with PVA12 might directly or indirectly facilitate sterol traficking from the ER to the tonoplast, mediating their location to

PVC/MVB and enabling vesicule fusion, as was reported for C1 domain-containing proteins in animals (Zhang et al., 1995). The modular nature of DC1 proteins offers multiple binding sites that might both recruit proteins and interact with membrane lipids, which makes these proteins excellent candidates to regulate complex assembly during vacuole biogenesis or other processes related to vesicular trafficking and fusion. VLG interaction with PVA12 has been shown to be highly specific, as a close-related DC1 domain-containing protein was not able to interact (Figure 7). This suggests that DC1 domain-containing proteins might interact very specifically with their protein partners and that ligand recognition exists between different DC1 domains. As the Arabidopsis genome encodes 140 proteins harboring multiple DC1 domains and given the fact that some of them have been reported associated to precise developmental and environmental cues, these proteins might work as platforms for protein assemblies in precise cellular compartments and in response to different stimuli.

Experimental procedures

Plant materials and growth Conditions

VLG mutant lines SAIL_507_F09 (*vlg-1*) and GK-793C12 (*vlg-2*), both Columbia [Col-0] ecotype, were obtained from ABRC (Ohio State University, Ohio, USA) (Alonso et al., 2003). Both lines were backcrossed twice to the wild type Columbia prior to their use. Single insertion in At2g17740 after backcrosses and all genotypes were confirmed by PCR-based genotyping. When indicated, seeds were sterilized in 20% (v/v) sodium hypochlorite, washed with sterile water and plated on MS plates with 50 µg/mL kanamycin and/or 15 µg/mL sulfadiazine. Resistant (green) seedlings were then transferred onto soil and grown under the conditions described above.

For pollen tube germination, pollen grains were incubated overnight in a dark moisture chamber at 22ºC in 0.01% boric acid, 5 mM CaCl2, 5 mM KCl, 1 mM MgSO4, 10% sucrose pH 7.5 and 1.5% agarose, as described by Boavida and McCormick (Boavida and McCormick, 2007).

Molecular characterization of insertional lines

For *vlg-1*, the left border–genomic sequence junction was determined by PCR in plants showing glufosinate resistance using the T-DNA–specific primer LBb1 (5`-GCG TGG ACC GCT TGC TGC AAC T-3`) combined with the genomic sequence-specific primers vlg-1 RP (5`-TCA TCT TCA ACT TGG GCT TTG -3`) and LP (5`-TGC CCA TTA AAA CGG TTA CAC -3`). For vlg-2, the left border–genomic sequence junction was determined by PCR in plants showing sulfadiazine resistance using the T-DNA–specific primer GKGT8474 (5´-ATA ATA ACG CTG CGG ACA TCT ACA TTT T-3´) combined with the genomic sequence specific primer vlg-2 RP (5`-CCT CCT CTA TTC CCC ACA GAA -3`) and LP (5`-TGT TCA CTC CAT TTC CAC CA -3`).

Segregation analysis

For self-cross analysis, heterozygous plants were allowed to self-pollinate and progeny seed was collected. The F1 seed was germinated on selective growth medium containing 6 µg/mL glufosinate for *vlg-1* or 5.2 µ/ml of sulfadiazine for *vlg-2*, and the amount of resistant and sensitive plants were scored. Reciprocal crosses were performed as described previously (Pagnussat et al., 2005).

Constructs

Genomic DNA was extracted from rosette leaves as described (Capron et al., 2008). The *VLG:GFP* translational fusion was generated as follows: the *VLG* gene including the putative promoter region (1104 upstream the ATG codon) was amplified by PCR using the following combination of primers: Fw: (5'-CAC CCT GAT TTC AAG TAT ATG TTT ACG C-3´) and Rv: (5´-GAT CAT TTT AAG CAT ATG CTT TCT-3´). The amplicon was cloned into pENTR/TOPO (Invitrogen) using gateway technology and the sequence was verified. The resultant plasmid *pENTR-ProVLG-GFP* was subjected to the LR reaction using the destination vector pMDC107 (Curtis and Grossniklaus, 2003). The *ProVLG:GUS* construct was generated amplifying the *VLG* putative promoter region (1104 upstream the ATG codon) by PCR using the following combination of primers: Fw: (5'-CAC CCT GAT TTC AAG TAT ATG TTT ACG C-3´) and Rv: (5´-TAG AGG TAA TAT TCT TGA TTT GCT-3´). The amplicon was cloned into

pENTR/TOPO (Invitrogen) and its sequence was verified. The resultant plasmid *pENTR-ProVLG:GUS* was subjected to the LR reaction using the destination vector pMDC162 (Curtis and Grossniklaus, 2003). For *Pro35S-VLG-GFP* construct, *VLG* ORF was amplified via PCR using as template clone DKLAT2G17740 obtained from ABRC (Ohio State University, Ohio, USA) using the following combination of primers Fw: (5´-CAC CAT GGC CGC AAG AAA ACC GTC-3´) and Rv: (5´-GAT CAT TTT AAG CAT ATG CTT TCT-3´). The amplicon was cloned into pENTR/TOPO (Invitrogen) and its sequence was verified. The resultant plasmid *pENTR-Pro35S-VLG-GFP* was subjected to the LR reaction using the destination vector pMDC83 (Curtis and Grossniklaus, 2003).

Transformation of *Agrobacterium tumefaciens* and *Arabidopsis thaliana*

Vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Transformation into Col Arabidopsis wild-type plants was performed by the floral dip method (Clough and Bent, 1998). Transformants were selected based on their ability to survive in MS medium with 15 mg. L^{-1} hygromycin. Resistant (green seedlings with true leaves) were then transferred to soil and grown under the conditions described above.

Morphological and histological analyses

Flowers were collected before anthesis and pistils were dissected and cleared overnight in Hoyer's solution. Ovules were observed on a Zeiss Axioplan imaging 2 microscope under DIC optics. Images were captured on an Axiocam HRC CCD camera (Zeiss) using the Axiovision program (version 4.2).

For pollen viability determination, anthers were collected at anthesis and colored with Alexander's staining. Pollen developmental stages in wild type and mutant plants were analyzed for each successive bud in the same inflorescence, starting from the first open flower (termed +1) to ten following unopened buds (-1 to -10) (Lalanne and Twell, 2002). Buds were fixed in ethanol:acetic acid 3:1 until discoloration and stored at room temperature. Anthers were washed prior dissection and mounted into slides with DAPI staining solution (0.02 M citric acid, 0.16 M Na_2HPO_4, 0.2 µg mL^{-1}; Sigma-Aldrich) with 10% sucrose to avoid bursting. Pollen grains were observed on a

Zeiss Axioplan imaging 2 microscope under DIC optics.

For GUS staining, developing seedlings and inflorescences of p*VLG-GUS* were collected and incubated in GUS staining solution as already described (Pagnussat et al., 2007).

Vacuoles were stained with 0.01% w/v neutral red for 30 minutes as first described by Mahlberg (1972). Images were captured on an Axiocam HRC CCD camera (Zeiss) using the Axiovision program (version 4.2).

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Co- localization experiments in *N. benthamiana*

For subcellular localization studies, Agrobacterium strain GV3101 carrying *Pro35S-VLG-GFP* were infiltrated together with p19 (a gene silencing suppressor) and Agrobacterium cells carrying either the late endosome marker RFP-Rha1 or the Golgi apparatus marker St-RFP, in leaf epidermal cells of 5-week-old *N. benthamiana* plants. Sections of infiltrated leaves were analyzed 48 h after infiltration on a confocal microscopy (Nikon Eclipse C1 Plus Confocal microscope, (Nikon, Canada, Mississauga, Ontario, Canada) using EZ-C1 3.80 imaging software and Ti-Control).

Fluorescence microscopy and image analysis

Pistils were dissected in 10mM phosphate buffer on a microscopic slide and immediately observed under fluorescence microscope. Fluorescence detection was done on confocal microscopy (Nikon Eclipse C1 Plus Confocal microscope, (Nikon, Canada, Mississauga, Ontario, Canada) using EZ-C1 3.80 imaging software and Ti-Control).

Yeast two-hybrid screen

Yeast two-hybrid screen was performed by Hybrigenics Services, S.A.S., Paris, France. A DNA fragment encoding Arabidopsis At2g17740 (aa 1-248) was PCR-amplified and cloned into pB27 as C-terminal fusion to LexA DNA-binding domain (N-LexA-At2g17740-C) and used as a bait to screen Universal Arabidopsis Normalized library containing 3.2 millions of independent clones in pGADT7-RecAB vector. pB27 derive from the original pBTM116 (Vojtek and Hollenberg, 1995). For the LexA bait construct, 62 million clones were screened using a mating approach with YHGX13 (Y187 ade2-101::loxP-kanMX-loxP,

matα) and L40ΔGal4 (mata) yeast strains as previously described (Fromont-Racine et al., 1997). 102 His+ colonies were selected on a medium lacking tryptophan, leucine and histidine. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure. A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction as previously described (Formstecher et al., 2005).

BiFC analysis

VLG, *LTL1, PVA12 and AT2G44370* cDNA sequences were PCR amplified using the following primers: VLGFW (5'-CAC CGC TTC CCG CCC TTC AGT GAG AC-3'); VLGREV (5'-GTT AAT ATC TTA GAT CAT TTT AAG C-3´); PVA12FW (5'-CAC CAG TAA CGA GCT TCT CAC CAT-3´); PVA12REV (5'-TGT CCT CTT CAT AAT GTA TCC C-3´); LTL1FW (5'-CAC CAA TAT CAA TTG TTC TCC ATT AGG-3´); LTL1REV (5'-CTA GAT TTT AGA AGA ATC CAA TAG-3'); *AT2G44370*FW (5´-CAC CGG CTT CCC GCC CTT CAG TG-3´); *AT2G44370*REV (5'-TTA GAT CAA CTT GAG ACA AGC CTT-3´). PCR products were cloned into pENTR TOPO plasmids using GATEWAY technology (Invitrogen), following the manufacturer's protocol, sequenced and recombined through BP reaction into BiFC destination plasmids pUBN-YN and pUBN-YC (Grefen et al., 2010). The binary plasmids were then transformed into Agrobacterium strain GV3101 by electroporation. Split nYFP- and cYFP-tagged protein pairs and p19 (a gene silencing suppressor were co-expressed in *N. benthamiana* leaves by Agrobacterium-mediated inoculation. Plant leaves were examined 48 hs post infiltration on a confocal microscope (Nikon Eclipse C1 Plus Confocal microscope, (Nikon, Canada, Mississauga, Ontario, Canada) using EZ-C1 3.80 imaging software and Ti-Control).

Bioinformatics and phylogenetic analysis

Amino acid sequences of related proteins used here were obtained from GenBank (National Center for Biotechnology Information). The amino acid sequences were aligned using MEGA7 (version 7.0.14) (Kumar et al., 2016). Graphic display of identities was visualized using Geneious (version 9.1.4)

(http://www.geneious.com) based on identity matrix (Kearse et al., 2012). A phylogenetic tree was constructed using the neighbor-joining method and the default settings of MEGA7 (version 7.0.14) (Saitou and Nei, 1987; Kumar et al., 2016). The optimal tree (sum of branch length = 5,855) is shown in Figure 1. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown on the branches (Felsenstein, 1985). The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site (Zuckerkandl and Pauling, 1965). Modeling of VLG DC1 domains was performed on SWISS-MODEL Workspace (Biasini et al., 2014). Structure comparisons were performed using TM-align (version 20160521) (Zhang and Skolnick, 2005). Molecular graphics and modelling were performed with the UCSF Chimera package (Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco) (Pettersen et al., 2004).

Accession Numbers

Sequence data from this article can be found under the following accession numbers in the Arabidopsis Genome Initiative: At2g17740 (VLG), At2g45140 (PVA12), At1g60420 (PDI-like) or in the UniProt Knowledgebase: Q62768 (rat Munc13) and Q05655 (human PKC delta).

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Supporting Information Legends

Figure S1. Comparison of structures between C1 and DC1 domains.

Figure S2. Comparison of sequence conservation between C1 and DC1 domains.

Figure S3. Identification of DC1 domain-containing proteins in plants.

Figure S4. Phenotype of vlg/VLG plants.

Figure S5. Pollen viability reaches WT values in complemented *vlg/VLG* plants.

Figure S6. Nuclear constitution of pollen grains through flower developmental stages in *A. thaliana***.**

Figure S7. Pollen development is impaired in *vlg-2* mutants.

Figure S8. VLG-GFP does not co-localize with the Golgi apparatus marker St-RFP or with early endosomal compartments.

Figure S9. Alignment of DC1 domain containing proteins encoded by AT2G17740 (VLG) and AT2G44370.

Figure S10. Characterization of *pva12* and *ltl1* homozygous plants.

Figure S11. Alignments of GDSL-lipases and vesicle associated proteins showing high similarity with LTL1 (A) and PVA12 (B).

Figure S12. Identified VLG interacting proteins and their closest homologues are expressed in pollen and ovule.

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Tables

Table 1. Transmission efficiency of the *vlg-1* and *vlg-2* alleles in reciprocal crosses between mutant and WT plants.

Female	Male	Genotype of progeny† Number of individuals		Transmission efficiency of:	p*
		VLG/VLG	*vlg-1/VLG*	*vlg-1* gamete (%)	
vlg-1/VLG	*VLG/VLG*	207	49	♀23.6%	<0.0001
VLG/VLG	*vlg-1/VLG*	134	19	♂12.4%	<0.0001
		VLG/ VLG	*vlg-2/ VLG*	*vlg-2* gamete (%)	
vlg-2/VLG	*VLG/VLG*	147	83	♀36.08%	<0.0001
VLG/VLG	*vlg-2/VLG*	101	50	♂ 33.1%	<0.0001

* Chi-square test for a 1:1 segregation hypothesis.

Table 2. Identity of clones retrieved by a yeast two-hybrid screen using At2g17740 (aa 1-248) as a bait.

Locus	Annotation	Frame	PBS	Length in aa (interaction region)	Localization
AT2G45140	Plant VAMP-associated protein	IF	D	239 (1-239)	Endomembrane system, Cytosol, PM, ER*[1]
AT5G63460	SAP domain-containing protein	IF	A	162 (1-162)	Nucleus, chloroplast
AT4G18740	Rho termination factor	IF	A	245 (1-245)	Cytosol*[2]
AT4G28500	NAC domain containing protein 73 (NAC073)	IF	D	305 (20-305)	Nucleus*[3]
AT3G04290	GDSL-like lipase/acylhydrolase superfamily protein Li-tolerant lipase 1 (LTL1)	IF	D	366 (1-366)	secreted

*Denotes experimentally proved. Otherwise, means predicted.

[1] (Saravanan et al., 2009), [2] (Ito et al., 2011), [3] (Zhong et al., 2008).

PBS column shows confidence of interaction; A: Very high confidence in the interaction, C: good confidence, D: moderate confidence. In frame (IF) denotes fragments in the same frame as Gal4AD.

Figure Legends

Figure 1. *VLG* encodes a DC1 domain-containing protein.

(a) Translated ORF of VLG encodes a 248 amino acid protein with 3 DC1 domains.

(b) Alignment of VLG´s DC1 domains with DC1 domain of PDI-like protein and C1 domains of hPKC and rMunc.

(c) Structures of DC1 domain of PDI-like protein and modeled DC1 domains of VLG.

(d) Alignment of 16 Arabidopsis DC1 domain-containing proteins. Sequence logo showing amino acid conservation is shown. DC1 domains are framed.

(e) Graphical representation of the VLG locus (AT2G17740) in Chromosome 2 of Arabidopsis. Insertion points of vlg-1 and vlg-2 lines are shown. Darker boxes in the alignments correspond to more conserved regions.

Figure 2. *vlg/VLG* mutant plants have Reduced Seed Sets.

(a) Percentage of aborted seeds in siliques from Wild type*, vlg-1/VLG, vlg-2/* and complemented vlg-1/VLG and vlg-2/VLG plants. Data are the mean ± SE. Siliques were analyzed in five independent experiments (mean: 28 siliques; range: 23–32). Different letters denote statistical difference (one-way ANOVA $p<0.05$).

(b) Dissected silique of a WT plant showing viable seeds.

(c*) vlg-1/VLG* silique at a comparable stage. Aborted ovules are indicated by arrowheads.

(d) *vlg-2/VLG* silique at a comparable stage. Aborted ovules are indicated by arrowheads.

(e) Silique from a complemented *vlg-1/VLG plant* (*vlg-1/VLG ProVLG-VLG-GFP*) at a comparable stage. Aborted ovules are indicated by arrowheads.

(f) Silique from a complemented *vlg-2/VLG plant* (*vlg-2/VLG ProVLG-VLG-GFP*) at a comparable stage. Aborted ovules are indicated by arrowheads.

Figure 3. Pollen development is impaired in *vlg-1* mutants.

(a) Scheme showing the nuclear constitution of WT pollen grains through microgametogenesis developmental stages. PMI: pollen mitosis I; PMII: pollen

mitosis II.

(b) and (c) Pollen viability by means of Alexander's staining in anthers at anthesis in WT and *vlg-1* plants respectively. Inset: detail of isolated pollen grains.

(d) and (e) Alexander's staining of pollen grains from buds at stage -5 from WT and *vlg-1* plants respectively.

(f) and (h) DAPI staining of polarized nucleus (arrow) and central nucleus (arrowhead) respectively in microspores from buds at stage -9 in *vlg-1/VLG* plants.

(g) and (i) Bright field images of (f) and (h) respectively, showing a big vacuole by neutral red staining (arrow) in a polarized microspore, or small vacuoles in a microspore arrested right after release.

(j) and (l) Nuclear constitution by means of DAPI staining in pollen grains in buds at stage -5 from WT and *vlg-1* plants respectively. Nuclei are indicated by arrowheads. v, vegetative nucleus; g, generative nucleus.

(k) and (m) Bright field images of (j) and (l) respectively.

Bars = 100 μm in (b) and (c), 25 μm in (d) and (e), and 10 μm in (f) to (m).

Figure 4. Female gametogenesis is impaired in ovules from *vlg/VLG* plants.

(a) Scheme showing female gametophyte development.

(b) Embryo sac at stage FG5 from a *vlg-1/VLG* plant. Nuclei are indicated by arrowheads. V, vacuole.

(c) Embryo sac found at stage FG1 from a *vlg-1/VLG* plant displaying only one visible nucleus (arrowhead).

(d) Embryo sac at stage FG2 from a *vlg-1/VLG* plant, two nuclei are visible (arrowheads), no central vacuole was observed.

(e) Embryo sac at stage FG5 from a *vlg-2/VLG* plant. Nuclei are indicated by arrowheads. V, vacuole.

(f) Embryo sac from a *vlg-2/VLG* plant displaying only one visible nucleus (arrowhead).

(g) Embryo sac from a *vlg-2/VLG* plant showing only two nuclei (arrowheads). A central vacuole was not detected.

(h) Embryo sac at stage FG4 from a WT plant. Nuclei are indicated by arrowheads. V, vacuole.

(i) Embryo sac at stage FG5 from a WT plant. Nuclei visible in the focal plane shown are indicated by arrowheads. V, vacuole.

(j) Embryo sac at stage FG4 from a WT plant. Nuclei visible in the focal plane shown are indicated by arrowheads. V, vacuole.

Bars: 25 µm.

Figure 5. Promoter activity in different sporophytic organs and VLG-GFP localization in female and male gametophytes.

(a) to (c) GUS activity in *A. thaliana* transgenic seedlings carrying the *pVLG-GUS* construct.

(d) Promoter activity in an inflorescence. Inset: detail of an open flower showing *GUS* expression in the style and filaments.

(e) and (f) Promoter activity in a seedling primary root tip (e) and in a cross section (f) that corresponds approximately to the indicated section in (e).

(g) to (j) Confocal images of VLG-GFP localization in the female gametophyte at stages FG1 (g), FG2 (h), FG4 (i) and FG6 (j). Insets show overlay between DIC and GFP fluorescence. Four independent transgenic lines were used for this study (1.1, 1.2, 4.1, 4.2). For each developmental stage analyzed, three flowers were dissected. Pictures are representative of the results obtained in all lines analyzed.

(k) to (m) Confocal images of VLG-GFP localization in the female gametophyte at 24 hours after pollination (k), 48 hours after pollination (l) and 72 hours after pollination. Insets show overlay between DIC and GFP fluorescence. Four independent transgenic lines were used for this study (1.1, 1.2, 4.1, 4.2). For each developmental stage analyzed, three flowers were dissected. Pictures are representative of the results obtained in all lines analyzed. Z, zygote, E, embryo.

(n) to (q) Confocal images of VLG-GFP localization in the male gametophyte at released microspore (n), polarized microspore (o), bicellular (p) and tricellular (q) stages. Insets show overlay between DIC and GFP fluorescence. Four independent transgenic lines were used for this study (1.1, 1.2, 4.1, 4.2). For each developmental stage analyzed, three flowers were dissected. Pictures are representative of the results obtained in all lines analyzed.

(r) VLG-GFP localization in pollen tube. Inset shows overlay between DIC and GFP fluorescence. Four independent transgenic lines were used for this study (1.1, 1.2, 4.1, 4.2). The pictures shown is representative of the results obtained in all lines analyzed.

Bars = 25 μm in (g) to (j), 50 in (k) to (m), 5 μm in (n) to (q) and 25 μm in (r).

Figure 6. Subcellular localization of VLG-GFP to PVC/MVBs

(a) Localization of VLG-GFP in *A. thaliana* root cells stained with FM4-64.

(b) The same root cells shown in (A) displaying FM4-64 staining (magenta). Root cells were stained with FM4-64 for 10 min, washed, and observed 4 hours later.

(c) Merged image showing partial co-localization of FM4-64 and VLG-GFP in small vesicles surrounding the tonoplast. Inset: bright field image.

(d) Pearson and Spearman Correlation test (PSC) plugin for ImageJ revealing a pixel population with both green and magenta signals, confirming co-localization of FM4-64 and VLG-GFP.

(e) *N. benthamiana* epidermal cells transiently expressing VLG-GFP under the 35S CaMV promoter.

(f) The same cells shown in (e) displaying the localization of the prevacuolar compartment fluorescent marker RFP-Rha1 (magenta).

(g) Merged image showing co-localization of VLG-GFP and RFP-Rha1. Inset: bright field image.

(h) Pearson and Spearman Correlation test (PSC) plugin for ImageJ revealing co-localization of VLG-GFP and RFP-Rha1.

Bars: 10 μm.

Figure 7. VLG Interacts With LTL1 and PVA12. BiFC analysis of VLG interaction with PVA12 and LTL1 *in N. benthamiana leaves.*

(a-f) Representative images from confocal microscopy showing VLG-LTL1 interaction. Reconstituted YFP fluorescence (YFP) or DIC images of *N. benthamiana* leaf are shown in epidermal cells co-infiltrated with Agrobacterium harboring the indicated constructs.

(g-l) Representative images from confocal microscopy showing VLG-PVA12

interaction. Reconstituted YFP fluorescence (YFP) or DIC images of *N. benthamiana* leaf are shown in epidermal cells co-infiltrated with Agrobacterium harboring the indicated constructs.

(m-r) Representative images from confocal microscopy showing that PVA12 does not interact with the VLG related protein DC1. Reconstituted YFP fluorescence (YFP) or DIC images of *N. benthamiana* leaf are shown in epidermal cells co-infiltrated with Agrobacterium harboring the indicated constructs.

(s-x) Representative images from confocal microscopy showing that LTL1 does not interact with the VLG related protein DC1. Reconstituted YFP fluorescence (YFP) or DIC images of *N. benthamiana* leaf are shown in epidermal cells co-infiltrated with Agrobacterium harboring the indicated constructs.