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“Caracterización funcional de las proteínas MTV9
y MTV11 y su implicación en el tráfico vacuolar”

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Summary

This doctoral thesis addresses the characterization of MTV genes that are involved in vacuolar trafficking in Arabidopsis. The vacuole is an essential organelle for adaptative strategies of plants and has a great agronomic and biotechnological importance for its storage capacity of essential proteins for human nutrition or of recombinant proteins for biotechnological purposes.

In this thesis, two mutants modified in trafficking to vacuole (*mtv* mutants) have been studied and point mutations in the MTV9 / At1g24560 and MTV11 / At4g29380 genes have been identified as the responsables for the defects in vacuolar trafficking.

The MTV9 gene is plant-specific and encodes a protein that is located at the prevacuolar compartment, possibly directed to that compartment by the palmitoylation of a cysteine present at its C-terminal end. The overexpression of MTV9 provokes the aggregation of the pre-vacuolar compartments and the delocalization of SNARE proteins, interfering with the trafficking of proteins to vacuoles but not with the secretion of plasma membrane proteins. These results indicate that MTV9 is essential for the trafficking to vacuoles and could be involved in anchoring processes of vesicles or organelles with the prevacuolar compartment to mediate their fusion.

The MTV11 gene encodes for the ortholog of the VPS15 protein, which is part of a phosphatidylinositol-3-kinase (PI3K) complex, whose activity is required for all vacuolar related trafficking routes in yeasts and animals. The MTV11 gene is essential in plants and its disruption causes lethality in pollen. The mutant *mtv11-1* isolated in this thesis is a hypomorphic allele that has allowed to study the role of this gene in Arabidopsis growth and development. By using the biomarker 2xFYVE we have obtained evidence that the synthesis of phosphatidylinositol-3-phosphate is significantly decreased in the mutant, indicating that the mutant allele reduces the activity of the PI3K complex. The mutant *mtv11-1* has affected the trafficking of storage proteins in seeds reservoirs and the recycling of plasma membrane proteins. These defects cause a decrease in growth and alterations in phyllotaxis under normal growth conditions. Moreover, different assays to study resistance to abiotic stresses performed in the *mtv11-1* mutant, have shown that *mtv11-1* plants are more tolerant to high concentrations of arsenate, a compound that is

sequestered in vacuoles once it is reduced to arsenite in the cytosol. This discovery may have important implications for the development of improved plants in phytoremediation of soils contaminated with arsenic.

Resumen

Esta tesis doctoral se centra en la caracterización de genes *MTV* que están implicados en tráfico vacuolar en *Arabidopsis*. La vacuola es un orgánulo esencial en las estrategias adaptativas de las plantas y tiene una gran importancia agronómica y biotecnológica por su capacidad de almacenamiento de proteínas esenciales para la nutrición humana o de proteínas recombinantes de uso biotecnológico.

En esta tesis se han estudiado dos mutantes modificados en tráfico a vacuolas (mutantes *mtv*) y se han identificado mutaciones puntuales en los genes *MTV9/At1g24560* y *MTV11/At4g29380* como las causantes de los defectos en tráfico vacuolar.

El gen *MTV9* es específico de plantas y codifica una proteína que se localiza en el compartimento prevacuolar, posiblemente dirigida a ese compartimento por la palmitoilación de una cisteína presente en su extremo C-terminal. La sobreexpresión de *MTV9* provoca la agregación de los compartimentos prevacuolares y la deslocalización de proteínas SNARE, interfiriendo con el tráfico de proteínas a vacuolas pero no con la secreción de proteínas de membrana plasmática. Estos resultados indican que *MTV9* es esencial para el tráfico a vacuolas y podría estar implicado en procesos de anclaje de vesículas u orgánulos con el compartimento prevacuolar para mediar su fusión.

El gen *MTV11* codifica para el ortólogo de la proteína VPS15, que forma parte de un complejo fosfatidilinositol-3-kinasa (PI3K), cuya actividad es necesaria para todas las rutas de tráfico a vacuola en levaduras y animales. El gen *MTV11* es esencial en plantas y su disrupción causa letalidad en polen. El mutante *mtv11-1* aislado en esta tesis es un alelo hipomórfico que ha permitido estudiar el papel de este gen en el desarrollo de *Arabidopsis*. Mediante el uso del biomarcador 2xFYVE hemos obtenido evidencias de que la síntesis de fosfatidilinositol-3-fosfato está disminuida considerablemente en el mutante, indicando que el alelo mutante reduce la actividad del complejo PI3K. El mutante *mtv11-1* tiene afectado el tráfico de proteínas vacuolares de reserva en semillas y el reciclaje de proteínas de membrana plasmática en la vacuola. Estos defectos provocan una disminución en el crecimiento y alteraciones en la filotaxia en condiciones

normales de crecimiento. Ensayos de resistencia a estreses abióticos han demostrado que las plantas *mtv11-1* son más tolerantes a altas concentraciones de arsenato, un compuesto que se secuestra en vacuolas una vez es reducido a arsenito en el citosol. Este descubrimiento puede tener importantes implicaciones en el desarrollo de plantas mejoradas para la fitoremediación de suelos contaminados con arsénico.

Introduction

1. The endomembrane system in eukaryotes.

The pioneer studies by George E. Palade on insulin secretion within pancreatic cells visualized the intricate network of endomembrane compartments comprising the secretory pathway (Palade, 1975). This network of intracellular compartments is referred to as the endomembrane system and in plants consists of the endoplasmic reticulum (ER), the Golgi apparatus (GA), the trans-Golgi network/early endosome (TGN/EE), prevacuolar compartments (PVCs), also called multivesicular bodies (MVBs), and the vacuole (Neumann et al., 2003). The endomembrane system of eukaryotic cells allows spatial and temporal compartmentalization for the synthesis, sorting, delivery, and degradation of cellular components. Possessing different compartments provides unique environments for post-translational modifications and biochemical reactions that require specific conditions, such as a distinct pH. For example, the acidic pH of the vacuole/lysosome enables degradation of proteins.

Most proteins that enter the endomembrane system do so in the ER, and then move through the different compartments until reaching their final destination. Soluble proteins contain a hydrophobic signal peptide at the N-terminus that targets them to the lumen of the ER (Blobel and Dobberstein, 1975), and may leave the ER once they are quality-checked for proper folding. Transport between successive compartments can occur in three different ways: a) through a vesicle trafficking step; b) through maturation of one compartment into another; c) through heterotypic fusion between two consecutive compartments of the endomembrane system. Proteins leave the ER from specific locations called ER exit sites (ERES) packed inside COPII vesicles. COPII vesicles fuse at the *cis* side of the GA releasing their contents (Budnik and Stephens, 2009). Transport along the GA occurs through cisternal maturation in a *cis*-to-*trans* direction, coupled to bi-directional vesicle trafficking between the stacks (Orci et al., 1997). In the GA/TGN a major sorting event takes place that separates proteins targeted to the vacuolar pathway from

proteins targeted for secretion, packaging them in distinct vesicles. Secreted proteins follow a default route and are transported from the GA/TGN in secretory vesicles that fuse with the plasma membrane. Soluble proteins targeted to the vacuolar pathway have specific sorting signals that are recognized by vacuolar sorting receptors, which are transmembrane proteins that couple cargo recognition in the lumen of the compartment to recruitment of the machinery required for vesicle formation at the cytosolic side (Happel et al., 2004; Zouhar et al., 2010). Trafficking of vacuolar cargo between the TGN and the PVC may occur through a vesicle trafficking step or through maturation of the TGN into the PVC, without the proteins leaving the compartment (Huotari and Helenius, 2011). The PVC eventually fuses with the vacuole, and vacuolar proteins reach their final destination. In addition to the biosynthetic pathway to the vacuole, plasma membrane proteins and secreted cargo are endocytosed to be recycled back to the plasma membrane or to be degraded in the vacuole. Moreover, the machinery involved in biosynthetic or endocytic trafficking may be recycled to prior compartments for further rounds of forward transport.

2. Vesicle trafficking and the machinery involved

Vesicle trafficking between compartments can be subdivided in three consecutive reactions: a) cargo recruitment and vesicle formation at the donor organelle, mediated by cargo receptors and small GTPases that recruit coat proteins and effector proteins for vesicle budding; b) vesicle movement along the cytoskeleton mediated by motor proteins; c) fusion of the vesicle with the target organelle and cargo delivery mediated by tethering factors and SNARE proteins.

2.1. Vesicle formation machinery

For a vesicle to be formed, the major steps involved are cargo recognition by the sorting receptors, coat recruitment, membrane deformation and budding of the vesicle from the donor membrane.

- **Sorting receptors**

Before a nascent vesicle is formed, cargo selection occurs in the lumen of the organelle, where sorting receptors bind their cargo by recognizing sorting determinants. Sorting determinants in plants are encoded in the amino acid sequence of the cargo proteins (Gershlick et al., 2014). Receptors functioning in transport between ER and Golgi and the corresponding sorting motifs are conserved among eukaryotes. The COPII proteins SEC23-24 recognize signals for export from the ER (Yorimitsu et al., 2014) while the p24 cargo receptors and the ARF1 GTPase are involved in retrograde transport from the GA to the ER (Sun et al., 2007). In the TGN, vacuolar sorting receptors select cargo to be transported to the vacuole. In plants, two families of vacuolar sorting receptors have been described, the vacuolar sorting receptors (VSRs) and the receptor homology-transmembrane-RING H2 domain proteins (RMRs). Vacuolar sorting receptors (VSRs) are type 1 integral membrane proteins consisting of a large luminal N-terminal domain responsible for cargo binding, a transmembrane domain and a highly conserved cytosolic C-terminus that binds the sorting machinery. Research performed with different *vsr* mutants in *A. thaliana* points to a specialization within this family for transport of storage proteins and soluble proteins to the lytic vacuole (LV) (Lee et al., 2013; Shimada et al., 2003; Wang et al., 2011; Zouhar et al., 2010). The recognition and binding of VSRs to cargo proteins relies on amino acid motifs in the cargo proteins, whereas in mammals, cargo proteins contain mannose-6-phosphate modifications that are recognized by the sorting receptors (Ghosh et al., 2003). RMRs are also type 1 integral membrane proteins with a shorter luminal domain and a long C-terminal tail that contains a RING domain (Shimada et al., 2003; Wang et al., 2011; Zouhar et al., 2010). RMRs have been localized to

endosomal compartments (Park et al., 2005a) but their physiological role is still controversial (Bocock et al., 2009). Although RMRs bind vacuolar cargo *in vivo*, no defects in trafficking have been described in mutants of RMR genes (Kim et al., 2005; Lee et al., 2013; Zouhar et al., 2010).

- **Small GTPases**

Cargo proteins bound to their sorting receptors accumulate in discrete sites within a particular organelle, interacting with small GTPases that recruit cytosolic coat proteins to form a vesicle. Plant genomes encode small GTPases from the RAB, RHO, ARF, and RAN GTPase subfamilies, but no RAS GTPases have been identified (Vernoud et al., 2003). The RAB and ARF GTPases regulate the formation of vesicles on donor membranes and direct fusion with the target membrane (Figure 1). These proteins associate with membranes in the GTP-bound form, which prevents recognition by the Rab chaperone GDP-displacement inhibitor (GDI). Small GTPases cycle between active (GTP-bound) or inactive (GDP-bound) conformations, a cycle regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factor proteins (GEFs). GAPs inactivate small GTPases promoting the hydrolysis of GTP and GEFs exchange GDP by GTP in order to render them active again. Although this basic cycle is well-studied, it is unclear how these proteins associate with a particular membrane to perform their specific function (Nielsen et al., 2008).

Figure 1. Schematic representation of a role of small GTPases in vesicular fusion. The green objects represent two different conformations of a small GTPase. When it is bound to GTP (green squares) the protein promotes vesicle formation from the donor membrane and may also direct vesicle fusion with the acceptor membrane. GAP and GEF proteins associate with small GTPases producing GTP binding and GTP hydrolysis respectively; and GDI, acts as a chaperone that prevents the association of small GTPases binding the GDP form of small GTPases. Adapted from David Lambright website).

- **Coat proteins**

Four types of coated vesicles have been described in plants: clathrin-coated vesicles (CCVs), coat protein I (COPI)-coated vesicles, COPII-coated vesicles, and retromer coated vesicles (Figure 2). These coats are made up of cytosolic proteins that are recruited to the donor membrane of the

nascent vesicle. In addition to their role in shaping the vesicles, coats contain sequence motifs that recognize sorting receptors that ensure that both receptors and their cargoes will be included in the nascent vesicle before its scission. Once vesicles are released and transported by cytoskeletal elements, vesicles shed their coat to fuse with the subsequent compartment and eventually they fuse with their target compartment to release their content. Interestingly, vesicles without apparent coats forming from the Golgi or the ER and transporting vacuolar cargo have been described in plants (Fuji et al., 2016; Wang et al., 2010), and may represent alternative routes to reach the vacuole.

Figure 2. Coat recruitment structure of different trafficking vesicles. The model shows adaptor proteins that are responsible of cargo internalization; the GTPase enzymatic core, responsible for the identity and formation of the vesicles; and the outer layer of the vesicle that is decorated by coat proteins. Adapted from (Gurkan et al., 2006)

COPII vesicles are involved in anterograde ER to Golgi trafficking. The COP II coat comprises four proteins which are arranged as an internal receptor/cargo-binding dimer of SEC23 and SEC24 and an outer cage dimer of SEC31 and SEC13 (Stagg et al., 2007). These heterodimer complexes bind activated SAR1 GTPase, and together with SEC23-24 recruit the cargo protein in the ER exit sites where ER membrane is deformed to produce the nascent vesicle. SAR1 GTP hydrolysis and release of COPII coats precedes fusion with the Golgi (Yorimitsu et al., 2014).

CCVs are formed at the TGN and at the plasma membrane. Clathrin uses adaptor protein complexes (AP complexes) to connect with sorting receptors and their bound cargo. There are five AP complexes in animal and plant cells (Robinson and Pimpl, 2014). AP-1 and AP-2 recognize the conserved tyrosine motif present in sorting receptors and aid in the formation of CCVs at the plasma membrane (AP-2) and the TGN (AP-1). AP-3 also interacts with clathrin to transport membrane proteins to the PVC or directly to the vacuole (Chen et al., 2011; Dores et al., 2012; Sauer et al., 2013; Zwiewka et al., 2011).

COP I vesicles in plants are involved in retrograde transport from the Golgi to the ER and in intercisternal trafficking between the Golgi stacks (Hwang and Robinson, 2009). The COP I coat consists of two subunits: F-COPs and B-COPs. COP I vesicles interact with p24 proteins to transport proteins such as the ERD2 receptor back to the ER (Montesinos et al., 2014).

Retromer coated vesicles in plants are involved in retrograde transport of trafficking machinery (i.e: vacuolar sorting receptors) from the PVC to the TGN to perform further rounds of anterograde transport. The retromer complex recognizes sorting receptors through the VPS26-VPS35-VPS29 heterotrimer and together with sorting nexins bind phosphatidyl-inositol-3-

phosphate (PI3P), deforms the target membrane and performs the scission to form the corresponding vesicles for receptor recycling (McGough and Cullen, 2011).

Uncoated vesicles without apparent protein cover have been reported to mediate trafficking of storage proteins from the TGN to the vacuole in plants and in other organisms (Gershlick et al., 2014).

- **Proteins inducing membrane curvature**

Once the coat proteins have been recruited with the aid of small GTPases to the membrane surface, the membrane needs to be physically deformed for formation and scission of vesicles. Each cell compartment possesses a specific membrane composition, mainly differentiated by their phosphoinositide (PtdIns) composition, which allows specific binding of proteins to deform the membrane. PtdIns are modified through phosphorylation at different positions of the inositol ring and specific forms label the different membrane compartments of the cell (Di Paolo and De Camilli, 2006; Simon et al., 2014). Phosphatidylinositol-3-phosphate (PI3P), phosphatidylinositol-4,5-phosphate (PI4,5P), and phosphatidylinositol-4-phosphate (PI4P) are the most abundant PtdIns in plants (Simon et al., 2014) and their levels are tightly regulated by various kinases and phosphatases (Balla et al., 2009; De Matteis and Godi, 2004). Analysis of overexpression and knockout mutants in PIP kinase/phosphatase genes have shown that some trafficking steps are significantly affected when the balance of these PtdIns is not properly maintained (Novakova et al., 2014; Tejos et al., 2014). Several protein domains that bind PtdIns were identified within coat protein complexes, adaptor proteins or other proteins (e.g, EPSINs). The interaction between them and the membrane lipids aid in the efficient membrane bending and subsequent vesicle scission (Brett and Traub, 2006). Domains responsible for PtdIns binding and for inducing membrane curvature are the ENTH domain, ANTH domain, and BAR-domains (Zouhar and Sauer, 2014).

Proteins with these domains act in combination with coat proteins and dynamins to deform the membrane for budding and scission of the vesicles (Brett and Traub, 2006). PI4,5P is enriched at the neck of CCV where ENTH and ANTH domains bind, and PI3P binding proteins such as FREE1 or FYVE1 are responsible of cargo recruitment and its delivery into the MVBs (Gao et al., 2014).

2.2. Vesicle movement

The cytoskeleton serves as a highly dynamic platform to regulate the movement and the position of the different cell compartments. In plants, there are two types of cytoskeletal filaments, actin filaments (F-actin) formed by polymerization of actin and microtubules formed by polymerization of tubulin subunits. These filaments are dynamic structures capable of growth or disassembly (Nogales, 2010). Motor proteins mediate the movement of vesicles along these cytoskeleton tracks. The motor proteins contain a head domain responsible for ATPase activity that energizes the movement, a neck that aids the movement and regulate the ATPase activity, and a tail domain that binds to vesicles or organelles (Lee and Liu, 2004). The motor proteins attached to the actin polymers are called myosins and the ones attached to microtubules are divided into kinesins and dyneins. Kinesins move toward the plus end of microtubules and dyneins move to the minus end. Microtubule and actin filaments display different functions within the cell. In the case of plants, where cells are larger and highly vacuolated, the use of myosin motors for vesicle trafficking is preferred since they are more suitable to reach longer distances. In fact, only kinesin14 family has been associated with long distance transport in a retrograde manner (like mammalian dyneins) and the function of this protein depends on its interaction with actin (Jonsson et al., 2015; Lawrence et al., 2001; Reddy, 2001; Wickstead and Gull, 2007; Wilhelm J. Walter, 2015). Although experimental evidence of myosin association with vesicles is lacking, the localization data of certain plant myosins and the effect of actin disassembly suggests that myosins are responsible for

the movement of post-Golgi organelles (Avisar et al., 2012; Kim et al., 2005). In contrast, the delivery and organization of cellulose synthase complexes in the plasma membrane is governed by cortical microtubule filaments (Gutierrez et al., 2009).

2.3. Vesicle fusion

Once vesicles reach the target compartment, their membranes fuse to complete the transport process. The fusion may be transient (kiss and run) or permanent (Giraudo et al., 2005), in which case the vesicle membrane is incorporated into the target membrane. The major factors to accomplish membrane fusion are SNARE proteins (soluble NSF Attachment protein receptor), but many other cellular proteins are required to ensure proper fusion reactions *in vivo*.

Multisubunit tethering complexes and homodimeric coiled coil tethers

Tethering factors play an essential function in membrane fusion reactions by establishing the specific contacts between the donor and target membrane compartments (Dubuke and Munson, 2016; Yu and Hughson, 2010). These contacts rely on the functional cycle of Rab GTPases and the shedding of the protein coat that allows the formation of a pre-fusogenic complex. The multisubunit tethering complexes (MTCs) involved in the different trafficking steps of the biosynthetic pathway are: DSL1, for ER to Golgi trafficking; TRAPPI for Golgi to ER trafficking; COG, for intra Golgi trafficking; TRAPP II and GARP for anterograde and retrograde TGN to Golgi trafficking; CORVET for TGN to PVC trafficking and HOPS for PVC to vacuole trafficking. In addition to MTCs, several coiled coil proteins function as homodimeric tethering factors. In contrast to MTCs, these homodimeric tethering factors show little sequence conservation across different kingdoms (Kim and Bassham, 2011; Takahashi et al., 2010; Vukasinovic and Zarsky, 2016). Unraveling the mechanisms governing the interaction between these tethering factors, the

arriving vesicles and the SNAREs involved in membrane fusion is key to understand how competent fusogenic complexes are formed in the cell (Chia and Gleeson, 2014).

SNARE and SM proteins

SNARE proteins mediate the fusion between the target membrane and the vesicle, providing both specificity and energy to drive this process. SNAREs contain a coiled-coil region referred to as the SNARE motif domain, which is essential for binding to other SNAREs and is widely conserved among species (Weimbs et al., 1997). When a vesicle is tethered to a target compartment for fusion, SNARE proteins present in the two membranes form a tetrameric bundle of coiled helices that brings the membranes close together, eliminating the water interface, initiating the mixing of the lipids and eventually provoking the fusion between the membranes (Figure 3). It has been shown that the energy released in the formation of the tetrameric complex is sufficient to drive liposomal fusion (Wickner and Schekman, 2008). In vitro experiments have demonstrated that there are multiple combinations of SNAREs that are able to produce tetrameric bundles, but in vivo only a few combinations lead to a successful membrane fusion (Varlamov et al., 2004). In addition, it has been proposed that there are some members of the SNARE family that can actively inhibit membrane fusion by interacting with other SNARE proteins (Bielopolski et al., 2014) or that a non-functional SNARE complex could assemble in vivo and inhibit the fusion process (Di Sansebastiano, 2013; Varlamov et al., 2004)

The localization of SNARE proteins is usually predicted by sequence conservation to characterized SNAREs from other organisms, as no amino acid sequence has been identified to link a particular SNARE protein to its corresponding membrane (Sanderfoot et al., 2000; Scales et al., 2000). For instance, Qa (syntaxins) SNAREs are well conserved between yeast and plants and the different types localize in analogous compartments in both species. Only for the SYP1 SNARE class, it could

be assumed that its long transmembrane region targets this protein family to the plasma membrane (Brandizzi et al., 2002; Sanderfoot et al., 2000).

SM (Sec1-Munc18) proteins are soluble peripheral membrane proteins that interact with the syntaxin class of SNAREs to regulate the membrane fusion reaction (Hong and Lev, 2014). Syntaxins shift between open and closed conformations that are respectively, competent and not competent to form tetrameric complexes with other SNAREs and drive membrane fusion. Current evidence suggests that SM proteins may play a dual regulatory role in membrane fusion: by binding SNARE complex and completing fusion or by interacting with syntaxins and stabilizing the closed conformation to prevent SNARE complex formation and membrane fusion. Examples of both types of interactions have been described but a structural data about conformational changes that allow the fusion is still missing (Archbold et al., 2014). Four major classes of SM proteins are present in eukaryotes: SLY1, VPS45, VPS33 and SEC1. These four classes intervene in different trafficking steps and together with SNAREs provide the specificity to membrane fusion reactions in the cell.

Figure 3. Schematic diagram of a SNARE competent fusion complex. The R-SNARE protein necessary to form a tetrameric bundle with the Q-SNAREs residing at the target membrane is shown in blue. The interaction of SM proteins with syntaxins and the other SNAREs is also shown. The syntaxin-SM interaction is essential for the different steps of membrane fusion and this association is regulated by calcium ions.

3. The vacuole in plants. Content and physiological functions.

There are particularities of the plant endomembrane system that distinguish it from the mammalian and yeast systems, such as the lack of an intermediate compartment between the ER and the Golgi, the high motility of the Golgi stacks, the process of cell plate formation and the presence of large central vacuoles (Contento and Bassham, 2012; Dettmer et al., 2006; Kim et al., 2005; Robinson, 2014; Zouhar and Rojo, 2009). The presence of these large vacuoles in plants is an essential adaptation to the unique life style of these organisms (Rojo et al., 2001). These large vacuoles provide a high buffering capacity that maintains cytoplasmic homeostasis in the different environments where plants may happen to germinate. Moreover, large vacuoles allow for energetically cheap growth which is essential for these autotrophic organisms to explore the surroundings for nutrients, water and light (Zouhar and Rojo, 2009). Loosening of the cell wall by enzymes such as expansins coupled to the high turgor pressure provided by vacuoles drives cell expansion (Cosgrove, 2000; Wang and Ruan, 2010) in a process that is regulated by auxins (Lofke et al., 2015). The vacuole is a reservoir of ions and metabolites and it is crucial for detoxification and general cell homeostasis. It also stores proteins and soluble carbohydrates as reserves in seed and vegetative tissues (Marty, 1999) and hydrolytic enzymes that function in recycling and degradation of cellular contents. Many plant species contain specialized protein storage vacuoles in seed tissues, which accumulate high amounts of reserve proteins to be used during germination and seedling establishment (Muntz, 2007). The proteins stored in seeds are an essential agronomical commodity obtained from crops and constitutes the main protein source for human and animal nutrition (Herman and Larkins, 1999).

3.1. Lytic and storage vacuoles

It has been shown that plants may contain separate types of vacuoles within a single cell. For instance, seed cells have been shown to contain both lytic vacuoles (LVs) and protein storage vacuoles (PSVs) (Bolte et al., 2011; Frigerio et al., 2008). The PSV is unique to plants and is actually a compound organelle made up of three independent compartments: the crystalloid and matrix are separate structures that are responsible of storage protein accumulation, while the globoid contains phytic and oxalate crystals associated with metals that provide a stable environment for the accumulation of certain enzymes (Baud et al., 2008). It has been suggested that the globoid is a lytic compartment inside the PSV (Jiang and Sun, 2002). Aquaporin markers (tonoplast proteins) have been used successfully to show the existence of separate compartments (LV and PSV) at some stages of development or as fused compartments (central vacuole) in the majority of vegetative tissues (Frigerio et al., 2008). It has been proposed that the protein storage vacuole originates from ER-derived compartments during seed maturation (Viotti, 2014). Throughout embryo development the LV shrinks and appears as a membrane enclosed structure inside the PSV (Bolte et al., 2011; Frigerio et al., 2008). During seed germination the PSV is transformed into a LV through acidification and progressive degradation of the stored proteins (Zheng and Staehelin, 2011). It has also been suggested that the ER is the membrane source for LV formation (Viotti et al., 2013). In vegetative tissues, where only a central vacuole with lytic features is present, the pathways that transport storage proteins to the vacuole are still functional (Sanmartin et al., 2007). However, the physiological role of the PSV pathway in vegetative tissues remains obscure. Interestingly, some pathogenesis-related proteins, e.g. the pathogen related protein PR5 and various lectins expressed in vegetative cells share the same sorting determinants as seed vacuolar storage proteins and are likely sorted by the same pathways in vegetative tissues (Carter et al., 2004).

3.2. Biotechnological potential of the vacuole: phytoremediation and storage

The plant vacuole is the largest compartment in plant cells, which makes it a suitable target for biotechnological strategies aimed at improving the storage and the detoxification capacity of plants. For instance, in vegetative tissues, the overexpression of tonoplast transporters to increase the concentration gradient between the vacuole and the cytoplasm is one of the common strategies to engineer plants with properties such as heavy metal tolerance (Jan Stomph et al., 2009), salt tolerance, drought resistance (Park et al., 2005b), and accumulation of secondary metabolites (Butelli et al., 2008). The storage capacity of PSVs, which accumulate enormous amounts of proteins during seed maturation, makes them valuable biotechnological compartments to store recombinant proteins. One of the major drawbacks for recombinant protein expression in plant vacuoles is the saturation of the vacuolar trafficking capacity when cargo proteins are overexpressed, which leads to secretion of the cargo to the apoplast by bulk-flow mechanisms and limits the amount of protein produced (Denecke et al., 1990). A possible solution to this problem would be to increase expression of the trafficking machinery that is limiting for transport to the vacuole. However, the transcriptional regulation of the trafficking genes in plants is not yet well characterized and the transcription factors involved in their regulation remain mostly unknown. Finding those transcription factors would be crucial for activating coordinately the expression of trafficking genes and increasing the transport capacity (Pizarro and Norambuena, 2014). In mammals, such transcription factors involved in coordinated activation of the trafficking machinery have been already identified (Sardiello et al., 2009), supporting the idea that the capacity of trafficking pathways can be modulated through transcriptional regulation.

4. Vacuolar trafficking in plants. Routes and genes involved

Genetic and pharmacological analysis has revealed the existence of parallel pathways for trafficking to the vacuole in plants.

4.1. Chemical inhibitors of vacuolar trafficking

Pharmacological inhibition has provided evidence for alternative trafficking pathways to the vacuole being functional in plants. It was first shown that treatment with wortmannin, a phosphatidylinositol 3-kinase and 4-kinase inhibitor, selectively blocked vacuolar trafficking of barley lectin but not of sweet potato sporamin (Matsuoka et al., 1995). Wortmannin induces homotypic fusion of the PVC, resulting in its enlargement and malfunction, which leads to mistargeting of vacuolar cargo proteins to the apoplast (Jia et al., 2013). Brefeldin A (BFA) is a fungal toxin that inhibits BFA-sensitive ARF-GEFs and prevents vesicle formation at different steps of the intracellular trafficking pathways (Geldner et al., 2003). BFA treatment blocks vacuolar trafficking pathways but it does not affect the AP-3 dependent pathway, suggesting that the AP-3 derived vesicles formed at the Golgi bypass the PVC on its way to the vacuole (Feraru et al., 2010; Wolfenstetter et al., 2012).

4.2. Adaptor complexes define different pathways to the vacuole

The adaptor protein complexes (AP-complexes) mediate sorting of receptors and associated soluble cargo into specific vesicles for targeting to their particular destination. Plants contain five adaptor complexes (AP-1 to AP-5). The AP-2 complex is involved in endocytosis from the PM, (Gadeyne et al., 2014) while AP-1, AP-3 and AP-4 are involved in trafficking to the vacuole. AP-1 localizes at the TGN where it binds VSR1. Moreover, soluble vacuolar cargo sorted by VSRs is

abnormally secreted in *ap-1* mutants, demonstrating that AP-1 is involved in their proper transport to the vacuole (Happel et al., 2004; Park et al., 2013). Similarly, tonoplast proteins are mislocalized and the vacuole biogenesis is compromised in *ap-3* mutants, suggesting that the AP-3 complex also functions in vacuolar transport (Feraru et al., 2010; Zwiewka et al., 2011). Recently, it was shown that the AP-4 complex resides on the TGN separately from the AP-1 complex, where it also binds VSR receptors and is required for vacuolar transport of seed globulins (Fuji et al., 2015), suggesting that, as is the case in animals and yeast, the AP-4 pathway is an alternative route to the canonical AP-1 dependent pathway for transport of vacuolar sorting receptors and their cargoes to the vacuole (Gershlick et al., 2014).

4.3. Gene expansion for alternative pathways

The special characteristics of the plant vacuole may have required an expansion in the repertoire of genes involved in their biogenesis and function, including in the machinery for vacuolar trafficking. For instance, Arabidopsis encodes four Qb VTI SNAREs (VTI11, VTI12, VTI13 and VTI14) in contrast to the single Vti1p protein encoded by the yeast genome. Moreover, there is evidence that this expansion has resulted in functional specialization. VTI11 resides at the PVC and forms a SNARE complex with SYP2 and SYP5 syntaxins, whereas VTI12 is located at the TGN in complex with SYP4 and SYP6 syntaxins (Sanderfoot et al., 2001). Mutations in VTI11 and VTI12 affect trafficking of distinct vacuolar cargoes, supporting that they act in parallel routes to the vacuole (Sanmartin et al., 2007).

Another example of gene expansion and specialization in the vacuolar trafficking machinery is the Rab5 GTPase subfamily. Arabidopsis encodes two conventional Rab5-type GTPases ARA7 and RHA1 and a plant specific isoform, ARA6 (Beck et al., 2012; Ebine et al., 2011). In pollen tubes ARA7 and ARA6 containing vesicles are affected differently by the actin

depolymerizing drug latrunculin B (LatB) (Zhang et al., 2010). ARA7 and RHA1 localize to the PVC and are involved in vacuolar trafficking. ARA6 localizes to the PVC and also the plasma membrane in a complex with SYP121 and VAMP727, consistent with a dual function in vacuolar trafficking and in secretion. The common activator of these RAB GTPases is the guanine nucleotide exchange factor VPS9. Interestingly, overexpression of constitutively active ARA7 but not ARA6 complements the root growth defects of a *vps9* mutant (Goh et al., 2007). Moreover, the *ara7* and the *rha1* knockout mutants exacerbate the developmental defects of a *syp22* mutant whereas the *ara6* mutant rescues them (Ebine et al., 2011), supporting that ARA6 has different functions from the conventional Rab5 GTPases in plants. Moreover, by studying the MON1-CCZ complex that functions as a GEF for Rab7 GTPases, evidence for three independent routes to the vacuole has been provided: 1) a route that involves the maturation of RAB5 endosomes into RAB7 late endosomes; 2) an AP-3 dependent route that does not involve RAB5 nor RAB7; 3) a RAB5-dependent and AP-3 independent route (Cui et al., 2014; Ebine et al., 2011; Ebine et al., 2014; Singh et al., 2014).

5. Genetic screens to study trafficking to the vacuole

5. 1. Genetic screens for trafficking genes in yeast

The first genes encoding trafficking machinery were isolated in genetic screens in *Saccharomyces cerevisiae* in the late 1970s. The *sec* mutants that were isolated in a temperature sensitive screen, showed at restrictive temperature (37°C) aberrant intracellular membranous organelles filled with proteins that normally destined to the vacuole or plasma membrane. Moreover, cell expansion and division were affected because of the defects in secretion of cell wall remodeling enzymes. By measuring the activity of apoplastic enzymes such as invertase and acid phosphatase, the *sec1* and

sec2 mutants with compromised secretion of these enzymes were isolated. The accumulation of secretory proteins in endomembrane structures in these *sec* mutants led to changes in cell density in the mutants, which was subsequently used to isolate novel mutants (Novick et al., 1980; Novick and Schekman, 1979). In this way, *sec* mutants belonging to 21 complementation groups were found (Novick et al., 1981; Novick et al., 1980; Sata et al., 1998; Schekman and Novick, 2004).

A screen for *vacuolar protein sorting* (*vps*) mutants was set up to identify genes specifically involved in trafficking to the vacuole. The screen searched for *vps* mutants that abnormally secreted the vacuolar carboxypeptidase Y, using a *pep4leu2* double mutant background (Stevens et al., 1982). The *pep4* mutation prevents processing and activation of proCPY inside the vacuole, while the *leu2* mutation renders the strain auxotrophic for leucine. In the screening, a medium containing the N-CBZ-L-phenylalanyl-L-leucine dipeptide was used. In the *vps* mutants, secreted proCPY is processed by periplasmic peptidases and then cleaves the dipeptide, allowing growth in Leu- media (Rothman and Stevens, 1986). 41 *vps* mutants were isolated that classified into six different phenotypic classes (A to F), depending on the alterations in morphology of the different endomembrane compartments (Banta et al., 1988; Raymond et al., 1992). An independent screen for *vacuolar morphology* (*vam*) mutants resistant to cloroquine, which accumulates in the vacuole and provokes toxicity, yielded nine *vam* mutants (Wada et al., 1992). In 2002, a genome-wide reverse genetic screen using a collection of 4653 homozygous diploid gene deletion yeast strains and scoring for CPY secretion by western blot yielded 93 new VPS genes (Bonangelino et al., 2002).

5.2. Genetic screens for trafficking genes in plants

Plasma membrane transporters such as PIN1 (auxin efflux membrane protein) and tonoplast transporters such as TIP2;1 (vacuolar aquaporin) fused to fluorescent proteins have been used as markers to identify mutants with altered subcellular distribution (Avila et al., 2003; Feraru et al.,

2010). PIN1 is endocytosed and then either recycled back to the plasma membrane or targeted to the vacuole for degradation. In a screen of a mutagenized population of M2 plants expressing PIN1-GFP, two *pat* mutants (protein altered trafficking) showing PIN1 accumulation in intracellular endosomes were isolated. Their characterization revealed that the defective trafficking was due to mutations in AP-3 components, demonstrating that AP-3 was required for vacuolar targeting of PIN1 and for lytic vacuole biogenesis (Feraru et al., 2010; Zwiewka et al., 2011). In a screen of a mutagenized population of M2 plants expressing GFP-TIP2;1 (Avila et al., 2003), two of them were characterized: the *mvp1* mutant that shows altered ER morphology and retention of vacuolar and secretory proteins in the ER (Agee et al., 2010) and the *csp-1* mutant that affects vacuole morphology and cell shape (Chary et al., 2008). A screen of mutagenized plants containing the Golgi associated protein sialyl transferase fused to GFP (ST-GFP) led to the identification of the *gom8* mutant showing an aberrant ER morphology due to a missense mutation in RHD3, a GTPase involved in morphogenesis of the ER (Stefano et al., 2012).

5.3. Genetic screens for trafficking to the plant vacuole

Several genetic screens have been developed in plants specifically aimed at identifying vacuolar trafficking genes. Based on the observation that a mutant in the vacuolar sorting receptor *VSR1* secreted storage proteins (12S globulins and 2S albumins) that accumulated as unprocessed precursors (Shimada et al., 2003), a collection of 28 000 T-DNA mutants was screened to identify mutants defective in processing of storage proteins. This rendered eight mutants, referred as *maigo* mutants (Li et al., 2013; Shimada et al., 2006; Takagi et al., 2013; Takahashi et al., 2010), four of which have been already characterized. The first *MAIGO* gene identified was *VPS29*, coding for a subunit of the retromer complex. This mutant affects VSR recycling from the PVC and as a consequence storage proteins are secreted (Shimada et al., 2006). In addition to this, the group of

Dr. Hara-Nishimura developed another screen based on the increased fluorescence observed in mutants that secrete the artificial vacuolar cargo GFP-CT24, which consists of GFP fused to the C-terminal sorting signal of the soybean storage protein conglycinin (Nishizawa et al., 2003). From this screening, more than 100 mutants showing a *green fluorescent seed* phenotype (*gfs* mutants), indicative of missorting of the transgene GFP-CT24 to the apoplast, were isolated. *GFS1* was shown to encode VSR1, *GFS2* encoded KAM2 (*gfs2*), a membrane protein involved in endocytic processes, and *GFS9* encoded a protein required for anthocyanin accumulation in the vacuoles. The *gfs9* mutant shows that alterations in trafficking of vacuolar proteins can affect the deposition of other vacuolar content (Fuji et al., 2007; Ichino et al., 2014).

Barley lectin is a vacuolar protein that contains a C-terminal vacuolar sorting signal (Ct-VSS) that is necessary and sufficient to target soluble endomembrane cargo into the vacuole (Bednarek and Raikhel, 1991; Rojo et al., 2001). CLAVATA3 (CLV3) is an extracellular ligand that activates the CLAVATA signaling pathway to restrict the size of the stem cell pool in the shoot apical and flower meristems of *Arabidopsis* (Fletcher et al., 1999). A genetic screen for vacuolar trafficking mutants was set up using a chimeric fusion protein between CLV3 and the barley lectin Ct-VSS expressed under a constitutive 35S promoter (*VAC2* construct). In wild type plants the *VAC2* protein (CLV3-CtVSS) accumulates in the vacuole where it is inactive (Rojo et al., 2001). In mutants where trafficking to the vacuole is impaired, *VAC2* is secreted to the apoplast, where it reduces the pool of stem cells in the shoot apical meristem and may eventually terminate it (Sanmartin et al., 2007). With this assay, *modified trafficking to the vacuole* (*mtv*) mutants with terminated meristems were isolated. In a preliminary screen four mutants (*mtv1* to *mtv4*) were found. *MTV1* encodes an epsin related protein; *MTV2* encodes the vacuolar sorting receptor VSR4; *MTV3* encodes the phosphoinositide phosphatase PTEN2b and *MTV4* encodes the ARF-GAP AGD5 (Sauer et al., 2013). Mutants in some of the paralogues of these genes also had *mtv* phenotypes,

namely the *vsr1*, *vsr3*, *agd12* and *pten2ba* mutants. In addition, reverse genetic analysis showed that mutants in the SNARE VTI12 and in its SM-regulator VPS45 also secreted the VAC2 protein resulting in terminated meristems (Sanmartin et al., 2007; Zouhar et al., 2009).

5.4. Screens using chemical genomics

The techniques that combine the use of chemical libraries of compounds with the effect of these compounds *in vivo* are referred as chemical genomics, and represent a powerful tool to dissect essential and/or redundant cell mechanisms. A small molecule can target all members of the same protein family and reveal an effect that would not be detected if only one member was disrupted. Furthermore, small molecules could target essential genes that are lethal when knocked out (Hicks and Raikhel, 2012). Sortins are inhibitors of vacuolar trafficking discovered in a chemical genomics screen by measuring CPY secretion in yeast. Some of the isolated sortins were subsequently shown to alter vacuolar trafficking and/or vacuolar morphology in plants (Zouhar et al., 2004). Genetic screens have yielded mutants hypersensitive to certain sortins in yeast and plants (Chanda et al., 2009; Norambuena et al., 2008; Rosado et al., 2011). In a screen for compounds causing defects in gravitropism in *Arabidopsis*, 34 molecules causing altered gravitropic response and aberrant morphology of the endomembrane system were isolated (Surpin et al., 2005; Surpin et al., 2003; Yano et al., 2003). Another example of a small molecule derived from chemical genomics that affects trafficking is endosidin1. Using pollen expressing GFP-RIP1 fluorescent marker and analyzing its germination *in vitro* led to the discovery of a family of drugs called endosidins. Some of them caused mislocalization of GFP-RIP1 and resulted in increased cytosolic localization of GFP-RIP1, inappropriate localization at the tip, and loss of polar growth. RIP1 is a plasma membrane protein that interacts with ROP and localizes at the tip of pollen tubes. The activity of these two proteins is essential for polar growth that is necessary for pollen germination. Indeed, pollen

grains treated with endosidin1 are not able to grow in a polar manner, and RIP1-GFP is mislocalized to the cytoplasm (Robert et al., 2008).

Materials and Methods

1. Biological material

1.1. Bacterial strains

- For propagation of various plasmids the DH5 α strain of *Escherichia coli* (*E.coli*) was used.
- For recombinant protein expression in bacterial cultures the BL21 (DE3) RosettaTM *E.coli* strain was used.
- For propagation and purification of GatewayTM plasmids carrying the *ccdB* gene the DB3.1 *E.coli* strain was used.
- For transient expression in *Nicotiana benthamiana* and stable transformation of *Arabidopsis thaliana* the C58C1 strain of *Agrobacterium tumefaciens* harbouring the pGV2260 plasmid was used.

1.2. Yeast strains

- *Saccharomyces cerevisiae* AH109: *MATa*, *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4 Δ* , *gal80 Δ* (ClontechTM). This strain is used for auxotrophy dependent yeast to hybrid (Y2H) experiments and mating purposes with Y187 strain.
- *Saccharomyces cerevisiae* Y187: *MAT α* , *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3, 112*, *gal4 Δ* , *met-*, *gal80 Δ* (ClontechTM). This strain is used for auxotrophy dependent Y2H experiments and mating purposes with AH109 strain.
- For yeast two-hybrid screening, we used a Y2H library made from cDNAs derived from RNA isolated from plants starved for phosphate (Puga et al., 2014) cloned in the pGADT7-Rec vector (ClontechTM) following the Matchmaker protocol PT3529-1 (ClontechTM) and transformed into the Y187 strain.

1.3. Plant material

- *Arabidopsis thaliana* (L. Heynh), accessions Columbia-0 (Col-0) and Landsberg *erecta* (Ler).
- *Nicotiana benthamiana* for transient expression assays (Table 1)
- *Arabidopsis thaliana* transgenic lines in Col-0 background (Table 1).
- *Arabidopsis thaliana* T-DNA mutant alleles in Col-0 background (Table 2).
- The *mtv9-1* and *mtv11-1* mutants were isolated from an ethyl methanesulfonate (EMS) mutagenized population derived from the L1 transgenic line expressing the VAC2 transgene, which consists of the CLV3 protein (At2g27250) fused to the barley lectin vacuolar sorting signal expressed under 35S constitutive promoter (Rojo et al., 2002), stably transformed in a *clv3-2* mutant in *Ler* background (Sanmartin et al., 2007). Therefore, the original *mtv* mutants obtained by forward genetics are in *Ler clv3-2* background.
- *Arabidopsis thaliana* subcellular marker lines: wave2R (mCherry-RabF2b/ARA7), wave2Y (YFP-ARA7), wave13R (mCherry-VTI12), wave22R (mCherry-SYP32), wave7R (mCherry-RHA1) (Geldner et al., 2009); SYP61-CFP (Drakakaki et al., 2012); VHA1-RFP (Dettmer et al., 2006), YFP-2xFYVE (Vermeer et al., 2006).
- Subcellular markers used for protoplast transfection and microscopy analyses: SYP41-CFP, mRFP-VSR2, ARA7Q69L-RFP, ARA7-RFP, mRFP-SYP61, ManI-RFP (Golgi Marker), mRFP-SYP61, Aleurain-RFP, RFP-SCAMP1 and RFP-VIT1 were used when cargo sorting was analyzed. These subcellular markers were generated at Liwen Jiang's lab using pBI221 gatewayTM compatible destination vector.

1.4 Plasmids

- pDONR207 and pDONR221 GatewayTM plasmids were used to generate entry clones (Invitrogen).

An integration reaction is carried out by homologous recombination between the PCR product carrying attB flanks (recombination site for bacteriophage λ), attB1 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTNN-(sequence of interest)-3') and attB2 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTN-(sequence of interest)-3') and the pDONR207 or pDONR221 containing the attP sites. This results in the formation of an entry clone that can be recombined into destination vectors carrying attR sites. The positive clones are selected based on its resistance to the respective antibiotic marker present in the plasmid used (gentamycin in case of pDONR207 and kanamycin in case of pDONR221).

- pGADT7: GatewayTM-compatible destination vector used in the yeast two-hybrid (Y2H) experiments (Chini et al., 2007). The protein of interest is fused upstream to an HA epitope and downstream of the GAL4 activation domain (AD) under control of the T7 promoter.

- pGBKT7: GatewayTM-compatible destination vector used in the yeast two-hybrid (Y2H) experiments (Chini et al., 2007). The protein of interest is fused downstream of the GAL4 binding domain (BD) and upstream to a c-myc epitope under the control of the T7 promoter.

- Binary plasmids (destination vectors): pGWB3, pGWB5, pGWB6, pGWB14 (Nakagawa et al., 2007), and p-UBQ10-N-RFP (Grefen et al., 2010). pGWBs are GatewayTM-compatible vectors and present hygromycin and kanamycin resistant genes. pGWB5 and pGWB6 allow translational fusion of a protein of interest with GFP at the C-terminus and the N-terminus, respectively. The recombinant protein expression is driven by the 35S constitutive promoter. In case of pGWB3 the promoter of the gene of interest is fused to the β -glucuronidase gene (*GUS* gene). The destination vector pGWB14 carries the 35S constitutive promoter in frame with the gene of interest tagged with three constitutive HA polypeptides.

- pBI221 modified plasmid was used for protoplast transformation (Gao et al., 2014). This plasmid carries an Ampicillin resistant gene and allows LR GatewayTM recombination reaction using Gateway compatible entry vectors (collaboration with Jinbo Shen at Liwen Jiang's lab, CUHK).
- Plasmid for recombinant protein expression: pDEST17 (GatewayTM plasmid, Invitrogen). This plasmid carries a T7 based promoter, recognized by the T7 polymerase. The T7 polymerase is encoded by the BL21 (DE3) bacterial genome and its expression is inducible by IPTG. The BL21 (DE3) rosetta strain used for expression is optimized for the codon usage of eukaryotic genes. The resulting recombinant protein is tagged with six histidines at its C-terminal part to allow protein detection and purification.
- Plasmid for inducible expression in plants: pMDC7, a GatewayTM-compatible destination vector for estradiol-induced expression of the protein of interest *in planta* (Curtis and Grossniklaus, 2003).

2. Culture methods

- Bacterial culture methods

The different *Escherichia coli* strains were incubated overnight at 37 °C in Luria Bertani (LB) media with appropriate antibiotics. LB media is made of bacto-tryptone (10 g/l), yeast extract (5 g/l), sodium chloride (10 g/l) and for culture in solid media, agar was added (3%, 18 g/l). The working concentrations for the different antibiotics were: ampicillin (100 µg/mL), gentamicin (50 µg/mL), hygromycin (40 µg/mL), kanamycin (50 µg/mL), rifampicin (50 µg/mL), spectinomycin (50 µg/mL), streptomycin (10 µg/mL) and tetracycline (5 µg/mL).

For protein expression in *E. coli*, colonies were grown in liquid LB until reaching an optical density of 0.6, induced with 0.4 mM IPTG, and aliquots were taken for protein extraction at different times.

The strains of *Agrobacterium tumefaciens* C58C1 were incubated during 2 days at 28°C in YEP media (10 g bacto-peptone, 5 g of NaCl, and 10 g of yeast extract per liter) with appropriate antibiotics.

In order to preserve *E.coli* and *A. tumefaciens* transformant lines during short periods of time, strains were kept at 4°C in solid LB media with adequate antibiotics. For longer periods of time the cells were resuspended in a 20% glycerol solution and kept at –80°C.

Table 1. Constructs used for Nicotiana Benthamiana transient expression, Arabidopsis thaliana stable transformation, and/or yeast transformation

construct	sequence origin	plasmid used	antibiotic selection	observations
35S::GFP-MTV9	genomic DNA	pGWB6 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	absence of phenotype, crossed with subcellular marker lines (F1 progeny was analyzed)
35S::MTV9-GFP	genomic DNA	pGWB5 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	silencing of GFP in all tested positive transformants
35S::GFP-NterMTV9	cDNA	pGWB6 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	used for transient expression in <i>N.Benthamiana</i>
35S::GFP-CORECterMTV9	cDNA	pGWB6 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	used for transient expression in <i>N.Benthamiana</i>
35S::GFP-COREMTV9	cDNA	pGWB6 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	used for transient expression in <i>N.Benthamiana</i>
35S::GFP-CterMTV9	cDNA	pGWB6 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	used for transient expression in <i>N.Benthamiana</i>
35S::NterCOREMTV9	cDNA	pGWB6 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	used for transient expression in <i>N.Benthamiana</i>
T7::6XHis-NterCOREMTV9	cDNA	pDEST17 (Invitrogen)	Amp ^r	used for protein expression in <i>E.coli</i> upon IPTG induction
1470up::GUS (MTV9 promoter)	genomic DNA (intergenic)	pGWB3 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	1200 bp before MTV9 initiation codon (includes 5'UTR)
500up::GUS (MTV9 promoter)	genomic DNA (intergenic)	pGWB3 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	500 bp before MTV9 initiation codon (includes 5'UTR)
35S::MTV9-3xHA	genomic DNA	pGWB14 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	absence of phenotype in 27 homozygous lines for the insertion
XVE::Hyg ^r ::lexA::MTV9	genomic DNA	pMDC7 (Curtis and Grossniklaus, 2003)	Spect ^r , Hyg ^r	absence of phenotype in 7 independent lines after estradiol induction
35S::MTV9-MTV11	genomic DNA	pGWB6 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	absence of phenotype in <i>A.thaliana</i> positive transformants
35S::MTV11-GFP	genomic DNA	pGWB5 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	silencing of GFP in all tested positive transformants
2000up::GFP	genomic DNA (intergenic)	pGWB4 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	GFP signal at the nucleus in transiently transformed <i>N.Benthamiana</i> leaves
500up::GFP	genomic DNA (intergenic)	pGWB4 (Nagakawa et al., 2007)	Kan ^r , Hyg ^r	no expression of GFP in transiently transformed <i>N.Benthamiana</i> leaves
UBQ10::RFP-STV9	cDNA	pUBC-CFP-Dest (Grefen et al., 2010)	Spect ^r	used for transient expression in <i>N.Benthamiana</i>
T7::BD-MTV9	cDNA	pGBKT7		used for yeast-two-hybrid experiments
T7::AD-MTV9	cDNA	pGADT7		used for yeast-two-hybrid experiments

- Yeast culture methods

The AH109 and Y187 strains of *S. cerevisiae* were grown in YPAD and YPD media, respectively (provided by ClontechTM). The liquid YPAD medium is a standard rich YPD medium (20 g/L peptone/tryptone, 10 g/L yeast extract, 20 g/L glucose with pH adjusted to 5.8) supplemented with 40 mg/L adenine to allow growth of strains carrying the *ade2* mutation. The corresponding solid media was supplemented with 20 g/L of bactoagar (DuchefaTM). Transformation of both AH109 and Y187 strains with pGADT7 and pGBKT7 plasmids led to normal growth of yeast cells on SD medium. The SD medium (synthetically defined medium) is minimal media that is routinely used for culturing *S. cerevisiae*. SD base supplies everything that a yeast cell needs to survive

(including 20g/L of glucose and 6,7 g/L of yeast extract as carbon sources and nitrogen sources, respectively) and it is supplemented with the amino acid drop-out (DO) mix and its pH adjusted to 5.8 with KOH. In addition, 20 g/L bactoagar were added for the SD solid media.

SD-WL: The SD media without tryptophan and leucine, prepared with the -Trp/-Leu DO Supplement (Clontech, No. 630417).

SD-WLH : The SD media without tryptophan, leucine and histidine, prepared with the -Leu/-Trp/-His/ DO Supplement (Clontech, No. 630419).

SD-WLA: The SD media without tryptophan, leucine and adenine, prepared with the -Ade/-His/-Leu/-Trp DO Supplement (Clontech, No. 630428) and supplemented with 0.002% histidine.

SD-WLHA: The SD media without tryptophan, leucine, histidine and adenine, prepared with the -Ade/-His/-Leu/-Trp DO Supplement (Clontech, No. 630428).

3-AT (3-amino-1,2,4-triazol): this compound is a competitive inhibitor for histidine biosynthesis and it is added in case of bait autoactivation or to assess the strength of protein-protein interactions in the yeast two hybrid assays, which use histidine auxotrophy as a marker for protein-protein interactions.

- *Arabidopsis in vitro* culture

Arabidopsis seeds were surface sterilized with a mixture of 70% ethanol and 0,05% Tween-20 during 10 minutes. After this time, seeds were washed 3 times with sterilized water and were cold treated for uniform germination during 3 days at 4°C in darkness. Seeds were germinated on ½ MS media (Murashige and Skoog media, DuchefaTM) supplemented with 1% sucrose, and 0,7% agar or 1,2% for horizontal or vertical growth, respectively. The growth chambers were set to long day photoperiod conditions (16 hours of light and 8 hours of darkness) with a 60 % relative humidity.

- Assays in arsenic limiting conditions

Seeds were surface sterilized and cold treated during 2 days at 4°C in darkness. Seeds were sown onto 1% agar vertical plates containing low phosphate Johnson media (12,5 µM phosphate) (Johnson CM, 1957) and grown for five days. Subsequently, seedlings were transferred to low phosphate Johnson media supplemented with or without 10 or 20 µM arsenate and root growth in the new plates was measured 3 days after transferring the plants.

- Cultivation of *Arabidopsis thaliana* in soil

Ten days old seedlings were transplanted into a mix of soil and vermiculite (ratio 3:1). Plants were grown in the greenhouse at 22°C with a photoperiod of 16 hours of light and 8 hours of darkness for long-day conditions, and 8 hours of light and 16 hours of darkness for short-day conditions.

3. Methods for bacterial, yeast and plant transformation

- Bacterial transformation

Transformation of competent DB3.1 and DH5α *E. coli* strains was carried out by heat-shock protocols as previously described (Sambrook, 1989). Competent *E. coli* cells were prepared using a calcium chloride treatment (Hanahan, 1985). Transformation of competent *A. tumefaciens* C58C1 strain was carried out as described in (Weigel, 2002). Competent *Agrobacterium* cells were generated using a freezing method and calcium chloride (Holsters et al., 1978). Transformed *E. coli* and *A. tumefaciens* strains were plated on selective media (LB with corresponding antibiotics) and incubated overnight at 37 °C or 48 hours at 28 °C, respectively.

- Transformation of *Arabidopsis thaliana*

A. thaliana plants were grown in soil during 20-25 days in long-day conditions before their transformation. Young inflorescences were immersed in a suspension of *A. tumefaciens* transformed with the construct of interest resuspended in Murashige and Skoog (MS) medium supplemented with 5 % of sucrose, 0.02 % of Silwet L77 (a surfactant agent) and 50 mM of benzylaminopurine (BAP, a cytokinin) for 10 minutes (Bechtold, 1993). After immersion plants were set in a tray covered with plastic for 2 days and then the plastic was removed and plants were kept in the greenhouse until seeds were harvested. The collected seeds were sown in MS medium with the corresponding antibiotics for selection of the resistant transformants.

- Agroinfiltration of *Nicotiana benthamiana* leaves

Three to four week old *N. benthamiana* plants were used for transient transformation experiments. Bacterial suspensions of *A. tumefaciens* transformed with the corresponding constructs were used for infiltration of the abaxial side of *N. benthamiana* leaves as previously described (Sparkes et al., 2006). To enhance the expression of a protein of interest, the leaves were co-transformed with Agrobacterium carrying the pBin61-P19 plasmid that encodes the p19 protein of tomato bushy stunt virus, a suppressor of post-transcriptional gene silencing (Voinnet, 2003). After 2 or 3 days of infiltration, leaves were analyzed by confocal microscopy.

- *Saccharomyces cerevisiae* transformation

S. cerevisiae cells were transformed as described in Matchmaker Gal4 Two-Hybrid User Manual (ClontechTM). Briefly, cultured yeast cells are incubated overnight and then refreshed by dilution in fresh media and subsequent growth until reaching a 0.6-0.7 OD value, followed by a polyethylene

glycol (PEG)/LiAc-based preparation of competent yeast cells. For plasmid transformation we used a heat shock based protocol where yeast cells are incubated at 30 °C for 30 min, mixed with DMSO (5 % volume) and incubated for 15 min at 42 °C before plating onto selective media.

4.Genetic interactions and phenotypic analyses

- Arabidopsis thaliana crosses

Recipient flowers at stage 13 (when the petals were barely visible) were emasculated with fine forceps and pollinized by gently brushing the mature anthers of donor plant onto the stigma.

- Flowering time analyses

To quantify the differences in flowering time between *mtv9-2* and Col-0, three independent experiments were carried out. Rosette and cauline leaves were counted at the time of flowering, considered as the time when the first flowered opened and petals were visible. A t-student test was used for statistical comparison of the mean flowering time (counted as number of leaves) in *mtv9-2* and Col-0.

4. Nucleic acid analysis and extraction

- Extraction of plasmid DNA from bacteria

To isolate plasmids, we used the Wizard *Plus* SV Minipreps DNA Purification System (Promega™).

- Plant DNA isolation

For standard PCR analyses, *A. thaliana* genomic DNA was extracted following an isopropanol-based method described previously (Doyle, 1990). DNA samples intended for whole genome sequencing were prepared using the plant DNeasy kit (QIAGEN™). This method ensures longer

DNA fragments and allows successful library preparation for the next generation sequencing (NGS). In case of *mtv9-1*, one gram of young leaves collected from twenty five plants showing the *mtv* phenotype (meristem terminated) were used for DNA isolation and the NGS.

- Gel/PCR DNA fragments extraction kit

DNA fragments separated by agarose gel electrophoresis or DNA amplified in a polymerase chain reactions (PCR), were purified using QIAquick Gel Extraction Kit (QIAGEN).

- Extraction of plasmid DNA from yeast

To isolate plasmids from yeast, we utilized previously described methodology (Hoffman and Winston, 1987). For sequencing the plasmids, were retransformed them into the standard DH5 α *E. coli* strain and subsequently isolated by a method described in the section 4.1.

- Amplification of DNA fragments

Taq polymerase (Roche) was used for standard PCR amplifications and the high-fidelity Phusion DNA polymerase (Roche) for cloning purposes. Primers used for PCR amplifications throughout this work are listed in the Tables 2 and 3.

Table 2. Primers used for mutant genotyping

primers used for PCR genotyping		gene, mutant line	observations
name	sequence		
Lbgarlic	TAGCATCTGAATTCATAACCAATCTCGATACAC		primer for T-DNA sail lines
mtv9-2RP	TTCCTCGCATATGTTGGTTTC	At1g24560, SAIL_24_C10	
mtv9-2LP	TTAGAGAGCTTGCCTTGATGC	At1g24560, SAIL_24_C10	
mtv9-3LP	TGGGTGTGGGAAACATCTTAC	At1g24560, SAIL_670_H06	
mtv9-3RP	CATTTCTTGCTTTGTTGCC	At1g24560, SAIL_670_H06	
stv9-2RP	GCTCGGATACTCCTATCTCCG	At3g49055, SAIL_193_D05	
stv9-2LP	TGCATTACCAAAATCGAAGC	At3g49055, SAIL_193_D05	
stv9-1LP	AGGGCTTCAAAATCCATGTCAC	At3g49055, SALK_029261	
stv9-1RP	AAGAAGTGGGGTTGAGGAAAG	At3g49055, SALK_029261	
LB643	GCAATCAGCTGTTGCCGTCCTCACTGGTTG		primer for T-DNA salk lines
LB 1.3	ATTTTGCCGATTTCCGGAAC		primer for T-DNA salk lines
mtv1LP	CTGCTGCCATTCTCATTCTTC	At3g16270, SALK_061811	
mtv1RP	CAATTTCTCAAAGGGCA	At3g16270, SALK_061811	
mtv3 LP	TTCCCAATCTAAAAATGTATCTC	At3g52850, SALK_114721	
mtv3 RP	CTTGTTGAGTTTCAAGA	At3g52850, SALK_114721	
mtv4 LP	ACAACAACCACTCCAAGTGG	At5g54310, SALK_079928	
mtv4 RP	AATGAAAATCCCTATGGGACG	At5g54310, SALK_079928	
vti11LP	CAAGACTTGACGCGTCAGAGACAG	At5g39510 (fast neutron mutagenized seed)	
vti11RP	GTTTCATCTCCTCGTCATG	At5g39510 (fast neutron mutagenized seed)	
MPO	GAGCAGGTGCAAGAAGGTCC-	primer for vti11/zig-1 T-DNA line	
vti12 LB	TCTGACGTGACAGTGGGTCTCCTGCCTGCG	At1g26670	
vti12 RB	TGGGAAAACCTGGCGTTACCAACTTAAT	At1g26670	
vsr1 LP	AGAACAGCCAATTGAAGCAAGCATTGTG		Ws background
vsr1 RP	CACATGAGGCTTTTCCAAGAGTCGCAGAA		Ws background
JL202	CATTTTATAATAACGCTGCGGACATCTAC		primer for T-DNA of vsr1 (Ws background)
vsr4 LP	CGTCGTCGATATCTGTTTGACTTTGAATA	at2g14720, SALK_094467	
vsr4 RP	CAGAAGTCACATGGACACAATTGACAAGA	at2g14720, SALK_094467	
ara4 RP	GATGGAATTAATTCATGCGC	At2g43130, SAIL_119_B07	
ara4 LP	ATCTCCCGAATAACCATCTCG	At2g43130, SAIL_119_B07	
vps34-2 RP	AAACCATTCCCTCCACCTATG	At1g60490, SALK_065645	
vps34-2 LP	CAACCAAAATATGAGCATCCC	At1g60490, SALK_065645	

Table 3. Primers used for gene cloning in gateway modified destination vectors

name	sequence
ATG_MTV9	GGG ACA AGT TTG TAC AAA AAA GCA GGC TGC ATG GCT AAC GGA GCC GAT GA
STOP_MTV9	GGG ACC ACT TTG TAC AAG AAA GCT GGG TC TCA TAG TAG AGG TTC AGC GAG T
noSTOP_MTV9	GGG ACC ACT TTG TAC AAG AAA GCT GGG TC TAG TAG AGG TTC AGC GAG TTC C
500UP_MTV9	GGG ACA AGT TTG TAC AAA AAA GCA GGC TGC CCT GTT GAA TGA TCT CGC TCC TA
5UTR_MTV9	GGG ACC ACT TTG TAC AAG AAA GCT GGG TC CTC ACC GTG CGA TCA GTG AAG
1470UP_MTV9	GGG ACA AGT TTG TAC AAA AAA GCA GGC TGC CGG TTT GGA TTT TAT GTG CAC CA
NTERMTV9stop	GGG ACC ACT TTG TAC AAG AAA GCT GGG TC TCA CCT AGC TTC ATT CCT CGA
COREMTV9forward	GGG ACA AGT TTG TAC AAA AAA GCA GGC TGC ATGGAGCAGATGGACC AACGG
CTERMTVforward	GGG ACA AGT TTG TAC AAA AAA GCA GGC TGC ATG CAGCTCGAGATCGTCGAACT
MTV9(CORE-STOP)	GGG ACA AGT TTG TAC AAA AAA GCA GGC TGC CAGCTCGAGATCGTCGAACT
primers for STV9 cloning, in bold STV9 annealing sequences are indicated	
name	sequence
ATG_STV9	GGG ACA AGT TTG TAC AAA AAA GCA GGC TGC ATGGATGTTAAAGATACCGAA
STOP_STV9	GG ACC ACT TTG TAC AAG AAA GCT GGG TC TCACGCATCATATCGTAACAATG
noSTOP_STV9	GGG ACC ACT TTG TAC AAG AAA GCT GGG TC CGCATCATATCGTAACAATGC
primers for sequencing Y2H candidates	
name	sequence
ad22cF	CTATTCGATGATGAAGATACCCC
ad21t7	GCTAATACGACTCACTATAGGGTTTTTCAGTATCTACGATTC
primers for MTV11 cloning, MTV11 promotor cloning, in bold MTV11 annealing sequences are indicated	
name	sequence
ATG_MTV11	GGG ACA AGT TTG TAC AAA AAA GCA GGC TGC ATG GGA AAC AAA ATC GCT CGT A
STOP_MTV11	GGG ACC ACT TTG TAC AAG AAA GCT GGG TC TTA CTT CCA GAC CTT TAT GGC TCC
noSTOP_MTV11	GGG ACC ACT TTG TAC AAG AAA GCT GGG TC CTT CCA GAC CTT TAT GGC TCC AT
UTR_MTV11	GGG ACC ACT TTG TAC AAG AAA GCT GGG TC CGA AGA CTT TTT AAA AAT TAA AAT TCT TTG TCC TTC GA
2000up_MTV11	GGG ACA AGT TTG TAC AAA AAA GCA GGC TGC TGA TCT CTT AGT CAC CTT GTA G
500up_MTV11	GGG ACA AGT TTG TAC AAA AAA GCA GGC TGC TCC AGT GAC AAC CAT TGTA
primers for sequencing MTV11 and the different T-DNA lines ordered	
name	sequence
16MTV11_23_F	GCCGGAGACGATTCTCTTTA
16MTV11_1139_R	GGTGCAACCAAAACAAAGG
16MTV11_548_F	TTGGCAAGAGACTGATAAAGCA
16MTV11_2135_R	GCCGATCTCATCACCTGACT
16MTV11_1530_F	TTTCTTGAGGGTCAGCCACT
16mtv11_3160_R	TCCACACTTCTCTGGCCAAC
16mtv11_2562_F	GTTGCCTTGCTTTCTGATCC
16mtv11_4140_R	TTTCTCTGCTTTCTGGCTCA
16mtv11_3539_F	AGAATTATGGCGAAACAGC
16mtv11_5153_R	TTGTAACCTAACAGAAAAACCA
16mtv11_4533_F	GGAGCCTCTGATGGTGTGAT
16mtv11_6159_R	CAATGGAAAAATCTGTACAAGGAA

- Gel electrophoresis of nucleic acids

DNA fragments obtained by PCR or the fragment analysis derived from plasmid digestion were visualised by agarose gel electrophoresis using ethidium bromide in 1x TBE buffer (50 mM Tris, 1 mM EDTA, pH 8).

- Sequence analysis of DNA

Library preparation and the NGS sequencing of the DNA from the pool of *mtv9-1* plants was done by the Genome Center at the Max Planck Institute for Developmental Biology. Reads were mapped onto the TAIR10 genome and a region that had only reads from Landsberg accession was identified. Within this region, EMS type polymorphisms (G/A or C/T) producing changes in the open reading frame were selected. For sequence alignment of MTV9 and STV9 the GENOMATIX program alignment tool was used (<http://www.genomatix.de/cgi-bin/dialign/dialign.pl>).

5. Protein analyses

- SDS-PAGE protein analyses

Arabidopsis thaliana and *Nicotiana benthamiana* plant samples were frozen in liquid nitrogen and homogenized in Laemmli loading buffer (Laemmli, 1970). The samples were boiled during ten minutes and after a short centrifugation to pellet cell debris they were loaded on the gels. To visualize seed storage proteins, gels were stained with Coomassie Brilliant Blue. For western blot analysis, proteins were transferred onto ECL-nitrocellulose membranes (GE Healthcare Life Sciences). The membranes were first incubated in blocking solution (5% non-fat milk in TBS+0.1% Tween-20) to avoid unspecific protein binding, and then incubated with primary antibodies diluted in 1% non-fat milk in TBS+0.1% Tween-20, washed five times in TBS+0.1% Tween-20 buffer, incubated with secondary antibodies fused to horseradish peroxidase diluted in 1% non-fat milk in

TBS+0.1% Tween-20, washed five times in TBS+0.1% Tween-20 buffer, incubated with ECL reagents and exposed on films for detection of the proteins.

- Expression of a MTV9 fragment for antigen production

We expressed a N-terminal fragment of the MTV9 protein (N-terCORE) in *E.coli*. Analysis of the distribution in soluble and insoluble fractions showed that the protein was mostly found in inclusion bodies. 50 mL of a bacterial culture from a colony that overexpressed the MTV9 fragment were resuspended in 5 mL 50 mM TRIS buffer solution containing 2 mM EDTA at pH= 8.2 and lysed by ultrasound with a sonicating probe, centrifuged at 13,000 rpm for 15 minutes at 4°C and the resulting pellet was resuspended in Laemmli loading buffer and subjected to SDS-PAGE. The gel band containing the recombinant MTV9 fragment was cut and used to immunize rabbits.

- Antibody production and purification

Once we were able to induce a sufficient amount of the N-terCORE MTV9 fragment, we sent the gel band corresponding to this fragment for antibody production. Five different rabbits from Pineda Antikörper Service (<http://www.pineda-abservice.de/main.php>) were pretested for lack of cross-reaction with Arabidopsis proteins. After initial immunization and three boost shots we obtained a serum that recognized the endogenous MTV9 protein and was utilized for western blotting analyses. We further purified the serum by binding to MTV9 protein transferred into nitrocellulose membranes and used it for immunoelectron microscopy analyses. Briefly, the N-terCORE fragment was run and transferred to an ECL nitrocellulose membrane and the band of interest was cut out. A 0.1 M glycine pH=2,5 solution was used to remove poorly bound proteins during 5 min and the membrane was washed two times with 1xTBS 0,1% Tween buffer. The membrane was blocked with 3% BSA 1x TBS solution during 1 hour, and after washing 500 µL of

MTV9 antibody were allowed to bind the membrane during 3 hours at room temperature. The supernatant was saved and the membrane was incubated with 500 μ L of the glycine solution to elute the MTV9 purified antibody. After 10 min the glycine solution was neutralized with 23 μ L of TRIS 1M pH=8 and this was the sample used for MTV9 immunoelectron microscopy analyses.

- Analyses of Arabidopsis apoplastic fluid

To isolate the protein content of the Arabidopsis apoplast, one gram of 5 week old rosette leaves grown in short day conditions was used. These leaves were vacuum infiltrated with 50 mL of sodium phosphate buffer, pH 8.0, containing 150 mM NaCl for 15 min, breaking down the vacuum every 3 min. After infiltration, leaves were carefully dried with a tissue paper and the apoplastic fluid was collected by low-speed centrifugation (900 x g). The isolated apoplastic fluid was mixed with Laemmli loading buffer and processed as described in the section 5.1.

- Sucrose density gradients

To analyze localization of various protein markers to distinct endomembrane populations, protein samples from flower tissues or 10 days old seedlings were separated on sucrose density gradients. The samples ($\approx$ 1-1.5 g) were lysed in 8 ml of 50 mM Hepes-KOH, pH 6.5/5 mM EDTA/13.7% (wt/vol) sucrose/0.1 mM phenylmethylsulfonyl fluoride/1 mM DTT/ 1x Complete Protease Inhibitor Cocktail (Roche), centrifuged (1,000g and 4 °C for 10 min) and the supernatant was collected. 1.5 mL of the supernatant were layered on top of the 9-mL line step gradient, as previously described (Sanderfoot et al., 1998). Gradients were centrifuged for 2 h at 100,000 x g in a Beckman SW40Ti rotor at 4°C. Subsequently, 0.5 mL fractions were collected from the top of the tube and the proteins were precipitated in 10% trichloroacetic acid (TCA), washed in 90% acetone

and dried. The protein pellets were resuspended in the Laemmli loading buffer, processed as described in the SDS-PAGE analysis section, subjected to electrophoresis followed by immunoblotting.

- Differential centrifugation of Arabidopsis microsomal fractions

Arabidopsis seedlings were grown in the liquid MS for 10 days under long-day conditions. The plants (≈ 1 -1.5 g) were carefully dried with a tissue paper and homogenized in extraction buffer (100 mM Tris-HCl, pH 7.5, 400 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, 1X Complete Protease Inhibitor Cocktail) using a mortar and pestle, followed by centrifugation at 1,000 x g for 15 min resulting in a pellet (P1) and a corresponding supernatant (S1) (Bassham and Raikhel, 1998). The S1 supernatant was centrifuged at 3,000 x g for 15 min to produce a membrane pellet (P3). The S3 supernatant was subsequently centrifuged at 16,000 x g for 15 min to produce a membrane pellet (P16). 100,000 x g for 30 min to yield a membrane pellet (P100) and a soluble fraction (S100). Pellets and supernatants were resuspended in the Laemmli extraction buffer and analyzed by SDS-PAGE and immunoblotting.

- Extraction of MTV9 from membranes

1-1.5 grams of Arabidopsis seedlings were processed as described in the previous section to yield the S1 fraction, which was directly subjected to 100,000 x g ultracentrifugation. The isolated P100 membrane pellets were resuspended in 200 μ L of the HE extraction buffer containing 0.1 M Na_2CO_3 , 1 M NaCl, 2 M urea, or 1% (v/v) Triton X-100, respectively, and incubated for 2 h on ice. Samples were subjected to a second ultracentrifugation at 100,000 x g and the resulting pellets were resuspended in the Laemmli loading buffer. Supernatants were precipitated using 10% TCA and also resuspended in the Laemmli loading buffer and processed as in the 5.1. section. Samples

were subsequently analyzed by SDS-PAGE and immunoblotting.

- Co-immunoprecipitation analyses

Microsomes obtained from Arabidopsis seedlings were homogenized in 500 µL of extraction buffer (100 mM Tris-HCl, pH 7.5, 400 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, 1X Complete Protease Inhibitor Cocktail), 0.2 % NP-40, and 1% bovine serum albumin (BSA). Extracts were centrifuged twice during 1 minute at 13,000 rpm at 4°C. Supernatants were mixed with 15 µL of anti-MTV9 antibody and incubated for two hours at 4°C. In a new test tube in which 150 µL of protein A resin (Roche) were added and the protein extract was gently shaken for 2 hours at 4°C. Subsequently, the tubes were centrifuged at 13,000 rpm for 30 seconds. The resin was washed three times with the extraction buffer supplemented with 1% BSA, and also with HE buffer lacking BSA. Afterwards, the resin was resuspended in the Laemmli loading buffer and boiled for 10 minutes. Samples were analyzed by SDS-PAGE and immunoblotting.

- Antibodies

Different primary antibodies were used and these were diluted 1:1000 but express indication: anti-GFP, anti-MTV9, anti-Aleurain (Ahmed et al., 2000); anti-CPY (Rojo et al., 2003); anti-SYP21 (da Silva Conceicao et al., 1997), anti-VSR (Sohn et al., 2003), anti-VTI11, anti-VTI12 (Surpin et al., 2003), anti-SEC12 (Bar-Peled and Raikhel, 1997), anti-PR5 (Palacin et al., 2010), anti-VacPeroX (Sanmartin et al., 2007), anti-TGG1 (Ueda et al., 2006), anti-BiP (Santa Cruz Biotechnology sc-33757). Secondary antibodies fused to horseradish peroxidase were used at 1:10000 dilutions (anti-rabbit, anti-goat, anti-mouse).

6. Microscopic techniques

6.1. Confocal microscopy

Confocal images were acquired by Leica TCS SP2 and Leica TCS SP5 multispectral confocal microscopes (Leica Microsystems) with a 63x water-immersion objective, recording images from laser lines of 405, 488, and 561 nm wavelengths. LAS AF v.2.3.6 software was used for image compilation. Image processing including colocalization of single color protoplast images were done with ImageJ and Photoshop (<http://www.macbiophotonics.ca/imagej/>). *A. thaliana* seedlings visualized in confocal imaging were grown for 3-6 days at 22°C with a 60 % of humidity and 16-hours photoperiod with a fluorescent light of a $100 \mu\text{mol m}^{-2}\text{s}^{-1}$ intensity. We visualized root epidermal cells in *A. thaliana* and epidermal cells of the abaxial side of leaves in *N. benthamiana* plants.

6.2. Chemical treatments

A. thaliana seedlings and culture protoplasts were treated chemically with BFA and wortmannin to check possible defects in the endomembrane system and in cargo trafficking. BFA and wortmannin were diluted in DMSO at 50 μM and 33 μM , respectively. Seedlings were incubated for 2 hours in these concentrations before microscope examination.

Results

1. Identification of novel *mtv* mutants

Seeds from the L1 transgenic line containing a single VAC2 insertion in chromosome 3 (17.1 Mb) were mutagenized with EMS and sown in 48 trays that were collected separately in 48 pools of M2 seeds. M2 plants were screened for mutants showing prematurely terminated shoot apical and flower meristems. In a partial screen of some of the pools, eight mutants (*mtv1-mtv4* and *mtv8-mtv11*) with prematurely terminated vegetative, inflorescence and flower meristems (*mtv* phenotype) were isolated. Using this same strategy, three other mutants (*mtv5-mtv7*) have been identified and characterized in the laboratory of Dr. Natasha Raikhel (Rosado et al., 2011; Sohn et al., 2007). The mutations responsible for the *mtv* phenotype in four of these mutants were previously identified: *mtv1/At3g16270* (pool 40); *mtv2/vsr4/At2g14720* (pool 43); *mtv3/pten2a/At3g19420* (pool 37) and *mtv4/agd5/At5g54310* (pool 46) (Sauer et al., 2013; Zouhar et al., 2010); PhD Thesis of María Otilia Delgadillo). In this work, we characterized two other mutants from that initial screen, *mtv9* (pool 31) and *mtv11* (pool 32) and identified the corresponding genes through map-based cloning. Recently, in the lab we have carried out a saturating screen of approximately 2000 M2 plants from each of the pools and isolated 121 new putative mutants with a strong *mtv* phenotype. We have identified by next generation sequencing the causative mutation of 20 of these *mtv* mutants, and found an additional allele of *mtv9* (pool 48).

2. MAP-based cloning of *mtv9*

The *mtv9-1* mutant was isolated from pool 31. The F1 plants from a cross between the *mtv9-1* mutant and Col-0 had a wild type phenotype. Moreover, the proportion of plants with a strong *mtv* phenotype in the F2 mapping population (157 out of 2011 plants) was consistent with the expected ratio for a recessive mutation unlinked to the VAC2 transgene producing a strong *mtv*

phenotype when both the mutation and the *VAC2* transgene were in homozygosis. Hence, for rough mapping of the *mtv9-1* mutation, we performed bulk-segregant analysis with F2 plants displaying the *mtv* phenotype. We isolated DNA from a pool of 49 plants with *mtv* phenotype and scanned the five chromosomes for regions showing an enrichment in *Ler* SSLPs. In this way, we found that the AthZFPG SSLP marker (chromosome I) was highly enriched for the *Ler* polymorphism in the pool of mutant DNA. Genotyping of the 49 individual mutant plants revealed that 97 of the 98 chromosomes had the AthZFPG *Ler* SSLP, indicating close linkage to the *mtv9* mutation. Moreover, the analysis of these plants with markers around AthZFPG delimited a 1 Mb region containing the mutation. We sequenced the candidate genes in that region *VTI12*/At1g26670, *ISTL2* /At1g25420, and *TNO1*/At1g24460 that encode proteins with previously reported roles in vesicular trafficking (Kim and Bassham, 2011), but no mutations were identified in those genes. Fine mapping restricted the position of *MTV9* to 0.1 Mb interval containing 34-genes (At1g24320–At1g24640) between the BOF316 marker and the AthZPFG SSLP markers (Figure 1A). However, no obvious candidates were apparent in that interval, so we performed deep sequencing of a DNA sample from a pool of 30 plants with *mtv* phenotype from the F2 mapping population (Figure 1B). In the interval defined through fine-mapping, we found a single EMS (G/A) mutation that generated a premature stop codon in the first exon of At1g24560, a gene of unknown function (Figure 5A). This EMS mutant allele was named *mtv9-1*.

Figure 1. *mtv9* identification through map-based cloning. (A) *mtv9* mapped to chromosome 1 in an interval between BOF316 (8,63 Mb; 3 recombinant chromosomes) and AthZPG (8,73 Mb; 1 recombinant chromosome) SSLP markers. (B) The DNA from a pool of 30 plants with *mtv* phenotype was deep sequenced and the percentage of reads with Col-0 or Ler SNPs along chromosome 1 is shown as the black line. The red line marks equal percentage of Ler and Col-0 SNPs, above the red line marks enrichment in Col-0 SNPs and below the line enrichment in Ler SNPs. The region encircled has reads with 100% Ler SNPs.

In order to confirm that the mutation of At1g24560 is responsible for the observed *mtv* phenotype, two additional T-DNA mutant alleles were analyzed. The *mtv9-2* allele (SAIL_24_C10) has a T-DNA insertion in the first exon of the At1g24560 gene and the *mtv9-3* mutant (SAIL_670_H06) has a T-DNA insertion in the fourth intron (Figure 5A). Plants homozygous for these T-DNA insertions were isolated and crossed with VAC2 line. The analysis of the two F2 populations showed that plants homozygous for *mtv9-2* or *mtv9-3* and at least one copy of the VAC2 transgene had terminated meristems, reproducing the phenotype of *mtv9-1* plants (Figure 5B). Moreover, we identified an additional *mtv9* allele in an *mtv* mutant isolated from pool 48, which contained an EMS mutation that disrupted the splice acceptor site of the third intron of the *MTV9* gene (*mtv9-4* allele). Altogether, the analysis of these four independent alleles strongly supports that *MTV9* is required for vacuolar trafficking of VAC2 and that eliminating its function results in VAC2 secretion and consequent premature meristem termination.

Figure 5. Independent mutant alleles for At1g24560 show *mtv* phenotype in the presence of VAC2. (A) A schematic representation of the *MTV9* gene locus, showing the positions of the four isolated *mtv9* mutant alleles. The *mtv9-1* and *mtv9-4* are EMS-induced point mutants while the *mtv9-2* and *mtv9-3* are T-DNA insertional mutants. (B) Confirmation of meristem termination in *mtv9-2* and *mtv9-3* mutants expressing the VAC2 transgene.

3. *MTV9* is a plant specific gene with a putative coiled-coil domain.

A search for homologous proteins through sequence alignment search tools (Altschul et al., 1990; Gish and States, 1993) showed that there are no homologues of *MTV9* present outside the plant kingdom. In contrast, close homologues of *MTV9* were found in all land plants, including in *Selaginella moellendorffii*, *Physcomitrella patens* and *Marchantia polymorpha*. The Arabidopsis genome has also a paralogous gene (At3g49055) that we named *STV9*. Interestingly, seed plants contain both *MTV9* and *STV9* paralogues whereas non-seed plants like *S. moellendorffii* and *P. patens* only contain the *MTV9* paralogue (Figure 6).

Figure 6. Phylogenetic analysis of MTV9 homologues in Arabidopsis, rice, Physcomitrella and Selaginella.

The Arabidopsis STV9 protein has 27% overall identity (46% similarity) with MTV9, and homology is maximal in the C-terminal half (Figure 7). We analyzed whether STV9 was also involved in trafficking of VAC2 by crossing the transgene into T-DNA knockout mutants for *STV9* gene (SAIL_193_D05 and SALK_029261). However, the homozygous *stv9* mutant did not display meristem termination in the presence of the VAC2 transgene, indicating that STV9 has a different function than MTV9.

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MTV9      MANGADEDAVLSDVESDEPAPVVLKDSPREEASDRITELIAELDREKKAREAAESSKSE 60
STV9      -----MDVKDTGINRSDTPISDQD-----HDFRQWNHSS 29

MTV9      LQVSFNRLKALAVEEAIKRDESKRERDEALKEKENLTNELENVNKGKDEMSKKLDEALRS 120
STV9      LDSNFLSLRSQIFEASRRTDLIRVNHELFHER-----DALRR 67

MTV9      RDGLKAEIENSSHMLVSGIEKISGKVSSFKNFSNGGLPKSQKYTGLTSAVGVIKRTNEI 180
STV9      RN----- 69

MTV9      VEELVRQIDTTAKSRNEAREQMDQRNYEIAIEVSQLESAISNLRLEVAEKASIVDDLERG 240
STV9      -----SELEAGILEVMIREEMKRD 89

MTV9      VSEKERIAELEKGNLEKVSLLEGEVVELKQLVDEYDGKLKTMELKMVAQRPLMDQLNL 300
STV9      LEVSKETVSELEGEAKEKTLLS-----DIADY 117

MTV9      VSRIHDQLYEVVRIVDGNSSEQSDLSESFFMPQETEMEENIRASLAGMESIFELTKVVSG 360
STV9      VRSMEDRLSKLIRCLNEENVP-----EERGRKLETKEYNSKSILELVKEVVT 165

MTV9      KAQSLVEEKSHELKNLNETVGLLVKEKEHIGTLLSALSKRVIGEQPSQKRELFQAAENG 420
STV9      KLETFQESTKKKKMELSRSVEFLEENRDINVLRAALFEKQTAEKQLKEMNDQKGLALL 225

MTV9      LRDGGTDSKFAKLLKDGKVQDSRSDNHDHSKEDNEIYSLASTLENIVKASQLEIVELQH 480
STV9      QIAGRGLQRIGFGLGESVESESTGNIANEEE--NGVVIAEKTMKKLRQEVSQLKI 283

MTV9      LLEASREETSSLRKQLDTQTKELNQMRQIEELKEKERIANENVEGLMTDIAAEEEITR 540
STV9      SLEESRLEEVLRKVTEEQAKLAENTVYINKLQNQEKFLAQNVEELVKAIREAESEVSR 343

MTV9      WKVAAEQEAAAGGAVEQDFTSQLYVLKEELEAKQAIESEKKLKFKEETAAAMGARDA 600
STV9      WREACELEVEAGQREVEVRDQLIAVLKSEVEKLSALARSEGKLKKEELAKAAMVAEA 403

MTV9      AESRLRLADNRATKLRERIQELNRKVEELETHRDMNTSNRARYACWPWQLLGIDFVGSRR 660
STV9      AEKSRLARRIAQLLSRIEHLYRQLEEAESTERRRGKFRVWCWPMRFTAASTATA 463

MTV9      IESGQESANEMELAEPLL 678
STV9      TGSSSYTSNRALLRYDA- 480

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Figure 7. Alignment of Arabidopsis MTV9 and STV9 protein sequences. Red color indicates sequence identity and blue color indicates sequence similarity.

Structural sequence analysis showed that MTV9 has no previously described functional domain (Wang et al., 2007). However, the Pfam server predicted MTV9 to contain coiled-coil

domains along its sequence (Finn et al., 2016) (Figure 8). This is consistent with a prior prediction of MTV9 being a coiled-coil protein (Gardiner et al., 2011).

Figure 8. MTV9 is predicted to contain coiled-coil domains. Predicted coiled-coil domains in MTV9 are marked as green rectangles.

Coiled-coil domains are present in homodimeric membrane tethering factors (Chia and Gleeson, 2014), involved in intracellular trafficking throughout eukaryotes. To analyze whether MTV9 could also homodimerize, we tested for interaction by the yeast two-hybrid assay. This assay showed that MTV9 can interact with itself (Figure 9) and may thus homodimerize *in vivo*.

Figure 9. MTV9 interacts with itself in yeast two hybrid assays. pGKB7-MTV9 was cotransformed into AH109 *S.cerevisiae* strain with pGAD empty vector and pGAD-MTV9. The cotransformants were plated on synthetic selection media to analyze the protein interaction and its strength.

4. *MTV9* expression in RNA-seq datasets

The ATH1 microarray platform commonly used for transcriptomic analysis in *Arabidopsis* does not contain a probe for *MTV9*, but we could analyze its expression in public RNA-seq datasets using the GENEVESTIGATOR server. *MTV9* is a medium to highly expressed gene in most tissues. Interestingly, gene co-expression analysis showed that *MTV9* is highly co-expressed with genes involved or potentially related with intracellular trafficking: among the 25 genes most co-expressed with *MTV9* we found *VCL1/VPS16* (Rojo et al., 2001), *VPS39*, the golgin *GC5*, *ALIX* (Cardona-Lopez et al., 2015), *Dynamin2B*, and four proteins with phosphatidylinositol binding domains.

5. *MTV9* promoter activity

To study the *MTV9* expression profile with cellular resolution, we studied the activity of two promoter constructs (500 bp or 1200 bp fragments upstream from the start codon) driving expression of the GUS reporter gene (Figure 10). We analyzed several independent transgenic lines for each promoter construct and they showed similar GUS expression patterns. GUS staining was first detectable in floral tissues, suggesting that *MTV9* expression is maximal in those tissues, in particular in stamens and in the petal abscission zone. In young seedlings, we observed strong GUS expression in the hypocotyl. Transversal cross section of the stem showed that *MTV9* is expressed in the epidermis, the cortex and the vascular bundles (Figure 10D). In roots and mature leaves, *MTV9* expression is also detectable in the vasculature and in leaf trichomes (Fig. 7G), but not in root hairs. We also observed expression in the shoot apical meristem, which is consistent with the mutation affecting trafficking of *VAC2* there and resulting in meristem termination (Figure 10I). In contrast, the expression in root apical meristems was very low. Intriguingly, *MTV9* was

expressed specifically in the quiescent center (QC) (Figure 10L), suggesting that those cells may have special requirements for this gene. We also investigated if treatments with hormones (auxins, cytokinins, brassinosteroids, gibberellins and jasmonates) or with specific inhibitors of trafficking (wortmannin and BFA) would alter *MTV9* expression. However, we could not observe any significant changes compared to mock treated plants.

Figure 10. Tissue expression of the *ProMTV9:GUS* transgene. (A) flower meristem. (B) flower tissue. (C) Gynoecium with pollen grains on its pistil. (D) Transversal cut of the stem. (E) 10 days-old seedling showing *MTV9* expression mainly in the hypocotyl. (F) Cotyledon showing *MTV9* expression in the vascular tissue. (G) Trichomes in adult leaf express *MTV9*. (H) Root-hypocotyl junction showing expression at the hypocotyl and the vascular tissue of the root. (I) Shoot apical meristem in a 10 days-old seedling. (J) Root showing *MTV9* expression in the vascular tissue. (K) 15 days-old seedling showing expression in the older parts of the root. (L) *MTV9* expression at the quiescent center (QC, arrowhead).

6.MTV9 localizes primarily at the PVC

MTV9 may be recruited to the one of the compartments of the vacuolar pathway to exert its function in vesicular trafficking. To determine the subcellular localization of MTV9, we analyzed various versions of the protein fused to the GFP. We cloned the MTV9 coding sequence in the pGWB5 and pGWB6 vectors to produce in frame fusions with GFP sequence at the N-terminus and the C-terminus of MTV9 expressed under the control of the constitutive 35S promoter (Figure 11A). Transient expression in *Nicotiana benthamiana* leaves showed that both versions localized in a punctate pattern throughout the cytosol. Moreover, transfection of Arabidopsis protoplasts rendered a similar localization pattern (Figure 11B). These findings suggest that, firstly, MTV9 is associated with an endomembrane compartment, and secondly, that the localization is not affected by a translational fusion to the GFP, as both N-terminal and C-terminal versions showed the same apparent distribution.

Figure 11. MTV9 shows a punctate pattern in transiently transformed *Arabidopsis* protoplasts and *Nicotiana* cells. (A) *Nicotiana benthamiana* pavement cells agroinfiltrated with 35S-GFP-MTV9 (left) and 35S:MTV9-GFP (right). (B) *Arabidopsis* leaf cell protoplasts transfected with 35S:MTV9-YFP (left) and 35S:GFP-MTV9 (right). Scale bars: 10 μ m.

To determine what compartment(s) corresponded to the observed punctate pattern, we studied the co-localization of GFP-MTV9 with markers of different endomembrane compartments, in collaboration with the group of Dr. Liwen Jiang from the Chinese University of Hong Kong. We co-transformed well-established markers developed in Dr. Jiang's lab with GFP-MTV9 in *Arabidopsis* protoplasts. We observed complete co-localization of GFP-MTV9 with the PVC-marker VSR2-RFP (Figure 12A), indicating that MTV9 is localized at steady state primarily there. In protoplasts with moderate expression of GFP-MTV9, the GFP signal was completely separate from the punctate signal observed for the TGN markers SYP41-CFP, and the Golgi markers ManI-RFP (Figure 12A and 13), consistent with MTV9 being localized in the PVC. Interestingly, we observed that in protoplasts overexpressing GFP-MTV9, the RFP-VSR2 signal concentrated in larger dots (Figure 12B). In order to discard possible effects of the GFP fusion and to confirm that MTV9 overexpression is responsible for this apparent PVC enlargement, MTV9 fused to a small HA-tag was co-transformed with RFP-VSR2. This experiment also showed accumulation of RFP-VSR2 in enlarged dots, confirming that the effect of the MTV9 overexpression is independent of the GFP fusion at the N-terminus.

Figure 12. MTV9 localizes at the PVC. (A) Protoplasts were co-transformed with GFP-MTV9 and RFP-VSR2 (PVC marker), Man1-RFP (Golgi marker) or SYP41-CFP (TGN marker). (B) A protoplast showing high levels of GFP-MTV9 expression and PVC aggregation. Scale bars, 10 μ m.

To complement the data from the transient expression systems, we generated stably transformed Arabidopsis plants expressing *35S-GFP-MTV9* (pGWB6 plasmid). In these lines, GFP

fluorescence was distributed in a punctate pattern, similar to what we observed in protoplasts. Many of the lines showed transgene silencing and unfortunately, lines showing high expression were not identified, so the effect of overexpression could not be assessed. For subcellular localization studies we crossed in markers for different compartments (Geldner et al., 2009). The PVC markers RHA1-RFP (wave7 line) and ARA7-RFP (wave2 line) co-localized largely with GFP-MTV9 (Figure 13B,C). Moreover, no co-localization was obvious with the TGN marker Vha1-RFP (Figure 13A).

Figure 13. MTV9 overexpression aggregates the PVC in Arabidopsis stable transformed plants. (A) F1 plants expressing GFP-MTV9 and the TGN marker Vha1-RFP. (B) F1 plants expressing GFP-MTV9 and the PVC marker Rha1-RFP. (C) F1 plants expressing PVC markers ARA7-YFP and RHA1-RFP. (D), F1 plants expressing GFP-MTV9 and the PVC marker ARA7-RFP. Scale bars correspond to 2,5 μ m and 10 μ m in the case of D panel.

Together, the results from transient expression in *Arabidopsis* protoplasts or from stable expression in transgenic plants are coincident and support that MTV9 associates with the PVC.

7. The conserved C-terminal domain of MTV9 is responsible for targeting to the PVC

Analysis of the MTV9 primary sequence in the TMHMM and TMPRED servers for prediction of transmembrane domains and in the SignalP 4.1 Server for prediction of signal peptides (Petersen et al., 2011) suggest that MTV9 protein does not contain a signal peptide nor a transmembrane domain for association with membranes. To identify the sequences responsible for the targeting to the PVC we dissected the MTV9 coding sequence in three non-overlapping constructs, fused them to GFP (Figure 14A) and analyzed their localization in *N. benthamiana* leaves (Figure 14B) and in *Arabidopsis* protoplasts (Figure 15). The fragments analyzed were NT (amino acids 1-199), CORE (amino acids 200-472) and CT (amino acids 473-678). The localization of NT-GFP, CORE-GFP and NT-CORE-GFP was cytosolic, and no association with membrane compartments was apparent. In contrast, the CORE-CT-GFP and the CT-GFP constructs presented a punctate pattern similar to the full length MTV9 (Figure 14B). Similar results were obtained in *Arabidopsis* protoplasts transformed with these constructs (Figure 15). These results suggest that the CT fragment contains the necessary information for targeting MTV9 to the PVC. The CT is the most conserved region among MTV9 homologues and the STV9 paralogue, which also localizes to endosomes (Figure 14C), indicating that the CT is involved in membrane recruitment and that this is essential for the function of these proteins.

Figure 14. The CT domain is necessary for correct localization of MTV9. (A), different deletion constructs of MTV9 cloned into the pGWB6 vector. (B), GFP-localization of the different deletion forms of MTV9 in *N. benthamiana* leaves. (C), i) STV9 construct that carries RFP fused to STV9 cDNA under the control of the constitutive promoter UBQ10, ii) localization of RFP-STV9 in *N. benthamiana* leaf pavement cells. Scale bars correspond to 10 μm and 2,5 μm in the case of STV9 picture magnification.

Figure 15. The CT domain directs localization at the PVC. (A), N-terminal part of MTN9 co-transformed with RFP-VSR2. (B), N-terminal part and CORE domain of MTN9 co-transformed with RFP-VSR2. (C), CORE domain of MTN9 co-transformed with RFP-VSR2. (D), CORE domain and C-terminal part of MTN9 co-transformed with RFP-VSR2. (E), C-terminal part of MTN9 co-transformed with RFP-VSR2. Scale bars, 10 μm.

8. *MTV9* overexpression perturbs transport of vacuolar cargo but not of PM proteins

As noted above, *MTV9* overexpression in protoplasts caused the RFP-VSR2 signal to concentrate in larger dots, whereas moderate expression did not cause this apparent PVC enlargement (Figure 12). Co-transformation experiments with the TGN syntaxin markers RFP-SYP61 and SYP41-CFP showed that in protoplast expressing moderate amounts of GFP-*MTV9*, the GFP-*MTV9* signal at the PVC was separated from RFP-SYP61 and SYP41-CFP signal at the TGN (Figure 16A). Interestingly however, when GFP-*MTV9* was strongly overexpressed, SYP61 but not SYP41 was re-localized in the enlarged GFP-*MTV9* positive compartments (Figure 16B).

A

B

Figure 16. MTV9 overexpression leads to SYP61 mislocalization. (A). Protoplasts expressing moderate levels of GFP-MTV9 co-transformed with SYP41-CFP or RFP-SYP61. (B), Protoplasts expressing high levels of GFP-MTV9 co-transformed with SYP41-CFP or RFP-SYP61. Scale bars, 10 μ m.

We reasoned that PVC enlargement and SNARE mislocalization caused by MTV9 overexpression should interfere with the functionality of the compartment and with protein trafficking to the vacuole. To test this, we co-transformed Arabidopsis protoplasts with GFP-MTV9

and a soluble vacuolar cargo (Aleurain-RFP), a tonoplast membrane vacuolar cargo (RFP-VIT1) and a plasma membrane cargo (RFP-SCAMP1). Aleurain is a thiol protease with an N-terminal vacuolar sorting signal that has been widely used as a model soluble vacuolar cargo. When transformed into *Arabidopsis* protoplast, it labels the lumen of the vacuole. When it is co-transformed with GFP-MTV9, Aleurain-RFP also reaches the vacuole but there is also some retention in the enlarged PVCs together with MTV9-GFP (Figure 17), suggesting that trafficking to the vacuole is partly disrupted. Soluble and membrane cargoes may use different routes to reach the vacuole, so we analyzed how transport of the tonoplast membrane marker was affected by co-expression with MTV9-GFP. Interestingly, RFP-VIT1 was mostly found in the enlarged PVCs together with GFP-MTV9 (Figure 17) and almost none of the protein reached the tonoplast. Hence, the interference with targeting of this membrane cargo was higher than with aleurain, possibly because soluble cargo can enter vesicles from alternative vacuolar transport pathways. To test if the effects were specific to vacuolar proteins, we co-transformed the PM marker RFP-SCAMP1 with MTV9-GFP. Importantly, SCAMP1-RFP was properly targeted to the PM, indicating that only trafficking to the vacuole was disrupted. This is consistent with MTV9 overexpression affecting the functionality of the PVC, a late endosome specific for the vacuolar transport route.

Figure 17. Trafficking to the vacuole is disrupted when MTV9 is overexpressed. Arabidopsis protoplasts expressing GFP-MTV9 and the vacuolar soluble cargo Aleurain-RFP, the plasma membrane protein RFP-SCAMP or the tonoplast protein RFP-VIT1. Scale bars, 10 μ m.

9. PVC aggregation by *MTV9* overexpression is not affected by wortmannin

Wortmannin, a PI3 kinase inhibitor, causes homotypic fusion of PVCs leading to the formation of greatly enlarged PVCs that appear as spherical compartments with a limiting membrane that can be resolved from the lumen in a light microscope (Figure 18A). This is different from the enlarged PVCs formed in MTV9-GFP overexpressing protoplasts, which have a more amorphous shape and the lumen is not distinguished. This suggests that the mechanism causing PVC enlargement in the MTV9-GFP overexpressing protoplasts is different from that of wortmannin and may instead reflect an aggregation of non-fusing PVCs. This process of aggregation of intracellular

compartments in certain cytosolic domains is also observed in plants treated with BFA, which induces the formation of the so-called BFA bodies that are large aggregates of Golgi and TGN cisternae in the cytosol. Given that wortmannin and MTV9 overexpression were both affecting the PVC, we analyzed the effect of their combined action. Interestingly, wortmannin treatment had no effect on PVC morphology in protoplasts (Figure 18A) or in transgenic lines overexpressing MTV9-GFP (Figure 19), indicating that the MTV9-induced aggregation of the PVC prevented wortmannin-induced homotypic fusion. To confirm this, we co-transformed protoplast with a constitutively active form of ARA7(Q69L) that also causes homotypic fusion of PVCs and mimics the effect of wortmannin treatment (Jia et al., 2013). Protoplasts transformed with ARA7(Q69L)-RFP induced the formation of large ring-like PVCs, but this effect was lost when co-transformed with MTV9-GFP, which led to PVC aggregation (Figure 18B).

Figure 18. PVC aggregates caused by GFP-MTV9 overexpression are not affected by wortmannin treatments or by constitutively active ARA7. (A) Arabidopsis protoplasts co-transformed with GFP-MTV9 and RFP-VSR2 (left panels) and treated with wortmannin do not produce ring-like structures observed in RFP-VSR2 transformed protoplasts treated with wortmannin (right panels). (B) GFP-MTV9 overexpression changes the ring-like structures of ARA7 (Q69L)

transformed protoplasts (left panels), and aggregates the PVC in ARA7-RFP transformed protoplasts (right panels). Scale bars, 10 μm .

Together, these experiments suggest that MTV9 overexpression causes a rearrangement of the PVCs into aggregates, which impedes normal functionality of the PVC and blocks wortmannin- and ARA7(Q69L)-induced homotypic fusion of the compartment.

Figure 19. Wortmannin did not produce enlarged ring-like PVCs in MTV9oe plants. In *Arabidopsis* plants transformed with ARA7-YFP the PVCs show the typical ring shape (arrowhead) after treatment with 33 μM wortmannin treatment. In contrast, in plants expressing GFP-MTV9 the aggregated PVCs do not respond to wortmannin treatment. Scale bars correspond to 10 μm (control: mock treated plants) and 2,5 μm (Wm: wortmannin treated plants).

10. Characterization of antibodies against MTV9

To characterize the endogenous MTV9 protein, we raised polyclonal antibodies against the first 472 aminoacids (NT-CORE fragment) of MTV9. We cloned the fragment in the pDEST17 plasmid and expressed it in *Escherichia coli*. Figure 20 shows that within three hours of induction there was high expression of the recombinant protein that accumulated almost entirely in inclusion bodies.

Figure 20. Recombinant expression of the NT-CORE MTV9 fragment (amino acids 1-472) in *E. coli*. Protein extracts of the *E. coli* cultures induced with IPTG for 0, 1.5 and 3 h were analyzed by SDS-page and coomassie staining. i: insoluble fraction; s: soluble fraction.

We purified inclusion bodies containing high concentration of the recombinant protein and used them to immunize rabbits. The obtained sera showed a specific recognition of a protein band of the expected size (90 kDa) in samples from Wild-type (Wt) plants that was absent in extracts from *mtv9-2* mutant plants (Figure 21), indicating that it corresponds to the MTV9 protein.

Figure 21. The MTV9 antibody specifically recognizes a protein band absent in *mtv9-2* protein extracts. The asterisk indicates the protein band specifically recognized by the MTV9 antibody in stem and flower protein extracts.

11. MTV9 associates with membranes that correspond to the TGN and the PVC

To gain insight into the localization of the endogenous MTV9 protein, we carried out subcellular fractionation studies using the antibody for detection. First we performed differential centrifugation experiments with samples from Wt seedlings. As shown in Figure 22A, the MTV9 protein was found in the pellet fractions, consistent with association with endomembrane compartments. The fractionation pattern was very similar to that of SYP21, a Qa-SNARE residing at the PVC. To determine how MTV9 associates with membranes, we isolated microsomal membranes and tested for extraction with different agents, using SYP21, an integral membrane protein, and VPS45, a peripheral membrane protein, as controls. Interestingly, MTV9 behaved similarly to SYP21 and could only be efficiently extracted from membranes with Triton X-100. In contrast, VPS45 was also extracted in the presence of urea. These results indicate that MTV9 is likely inserted into the membrane (Figure 22B). However, MTV9 does not contain transmembrane domains, so the insertion should be through post-translational modification with lipid anchors.. Interestingly, it has been recently reported that MTV9 may be palmitoylated (Hemsley et al., 2013) which could serve to attach the protein to membranes. Palmitoylation normally occurs at cysteine residues and MTV9 contains a single cysteine that is present in the CT domain, and may thus promote PVC targeting of the protein.

Figure 22. MTV9 is tightly associated with membranes (A) Differential centrifugation of protein samples from 10 days-old Col-0 or *mtv9-2* seedlings. The numbers indicate centrifugal force applied to the protein sample (in thousands *g*), the “S” indicates soluble, and “P” indicates the pellet fraction. (B) 100,000*g* membrane pellet from an Arabidopsis 10 days-old seedlings extract was resuspended in HE buffer alone, or in 2 M urea, 1 M NaCl, 0.1 M Na₂CO₃, or 1% Triton X-100, respectively. Insoluble material was re-pelleted, and soluble and pellet fractions were analyzed by SDS-PAGE with anti-MTV9, anti-SYP21 (integral membrane protein localized to the PVC) and anti-VPS45 (peripheral membrane protein localized to the TGN).

In sucrose gradients, the majority of MTV9 was found in fractions containing the PVC resident proteins VTI11 and SYP21, while VSR proteins that cycle between the TGN and the PVC only partially overlapped with MTV9 (Figure 23). The subcellular fractionation pattern of MTV9 (Figures 22 and 23) is consistent with the localization of the GFP fusion constructs in the PVC and supports that the endogenous MTV9 localizes to that compartment.

Figure 23. MTV9 associates with membrane fractions that correspond to the prevacuolar compartment. Sucrose gradient of ten days olds seedlings from Col-0 were analyzed by western blots with antibodies against MTV9 and VTI11, VSR, and SYP21, the previously characterized organelle markers.

12. MTV9 and VTI11 function in separate vacuolar trafficking pathways

None of the four isolated *mtv9* mutant alleles presented obvious phenotypes in the absence of VAC2, except for a slight but significant delay in flowering time (Figure 24). To test if this lack of phenotype was due to the gene redundancy of the *STV9* gene, we isolated and characterized two T-DNA insertions in that gene. The *stv9* mutant alleles did not cause secretion of VAC2 when the transgene was crossed in, arguing against *STV9* having the same functions as MTV9. Moreover, an *mtv9-2 stv9-1* double mutant, which is predicted to carry null alleles in both genes, did not display any other developmental phenotype than the delayed flowering already observed in single *mtv9-2* mutants. Hence, it appears that MTV9 and STV9 are not essential for plant viability or for normal development.

Figure 24. *mtv9-2* flowering time is slightly delayed under long day conditions. (A) 4 week old plants from *mtv9-2* and Col-0 backgrounds showing the *mtv9-2* flowering delay (B) The average number of rosette leaves or rosette plus cauline leaves at the time of petal emergence in the first flower (54 plants per genotype). The error bars represent the standard deviation. An unpaired Student's t-test was used to compare the means. Two asterisks: p-value<0.001; three asterisks: p-value<0.0001.

The lack of any obvious phenotype in the *mtv9-2 stv9-1* double mutants is striking considering the conservation of the *MTV9* gene family in plants and the drastic effect of *MTV9* overexpression on PVC morphology and functionality. A possible explanation would be that there are *MTV9*-independent pathways to the vacuole that mask the effects of blocking the *MTV9*-dependent pathway. To gain genetic evidence for this, we crossed *mtv9* with previously

characterized vacuolar trafficking mutants (*mtv1*, *mtv2/vsr4*, *vsr1*, *mtv3*, *mtv4*, *mtv11*, *vti12* and *vti11*), with the rationale that alterations in parallel pathways should have a synergistic effect when combined with the *mtv9* mutations. The double mutants from all the crosses did not show any increased phenotypic changes in standard growth conditions relative to the respective parental single mutants, except for the *mtv9 vti11* double mutant that showed more severe dwarfism than the single *vti11* mutant (Figure 25 and 26).

Figure 25. Growth phenotypes of single and double mutant combinations. Only the *vti11mtv9* mutant shows synergistic effects on growth inhibition relative to the single mutant parents.

Figure 26. *mtv9* enhances the phenotype of *vti11* mutant. The pictures correspond to 6 week old plants grown under long day conditions.

Analysis of seed storage precursor accumulation in the double mutants generated did not reveal defects in processing in any of the mutants (Figure 27), indicating that at least in seeds, vacuolar trafficking was still proceeding efficiently. In contrast, in leaves we could detect abnormal secretion of a vacuolar peroxidase in the *mtv9-2 vti11* mutant, consistent with vacuolar trafficking being synergistically perturbed in vegetative tissues of the double mutant. Considering that *mtv9-*

2 and *vti11* are likely null alleles, these results suggest that MTV9 and VTI11 function in independent vacuolar trafficking pathways.

Figure 27. *mtv9* does not alter the trafficking of storage proteins in seeds. The lanes contain Col-0 seed protein extract as a control and seed extracts from *mtv9-2* mutant and indicated double mutants of *mtv9-2*.

13. *MTV11* encodes a homologue of yeast VPS15.

The *mtv11-1* mutant was isolated from pool 32. The F1 plants from a cross between *mtv11-1* and Col-0 had a wild type phenotype, and in the F2 population, the proportion of plants with *mtv* phenotype was consistent with a recessive mutation. We performed bulk-segregant analysis with DNA from a pool of 50 F2 *mtv* plants and found a region of chromosome IV showing enrichment in Ler SSLPs. Fine mapping restricted the interval containing the *mtv11-1* mutation to a 0.26 Mb region (between 14.40 and 14.66 Mb) (Figure 28).

Figure 28. *mtv11* mapped to a 0.26 Mb region on chromosome IV. The number of recombinant plants for the different molecular markers is indicated. 5 recombinant plants delimited a region between 14.4 and 14.66 Mb containing the *mtv11* mutation.

The region delimited through mapping contained the At4g29380 gene that encodes a homologue of *VPS15*, an essential gene for vacuolar trafficking and autophagy in yeast and animals (Anding and Baehrecke, 2015; Herman and Emr, 1990; Lindmo et al., 2008). Sequencing of At4g29380 in *mtv11-1* plants revealed an EMS (G/A) mutation that disrupted the splice acceptor site of the 7th intron (Figure 29C).

Figure 29. *mtv11* encodes VPS15 protein. A) Domains present in MTV11/AtVPS15 B) Terminated shoot meristem phenotype of the *mtv11-1* mutant, C) Schematic representation of the *MTV11/AtVPS15* gene locus, showing the *mtv11-1* EMS mutation at the splice acceptor site of the 7th intron of AtVPS15 and the *mtv11-2* T-DNA insertion.

Analysis by RT-PCR revealed that most of the At4g29380/AtVPS15 transcripts in the *mtv11-1* plants retained the 7th intron (Figure 30), which introduces an early stop codon that deletes most of the coding sequence of the protein. Moreover, we also detected a significant amount of mRNA molecules in the mutant with the 7th intron spliced out using an alternative splice acceptor site 4 nucleotides downstream of the original one, which causes a frameshift and also leads to an early stop codon. These two splice variants (7th intron retention and alternative splice acceptor site in exon 8) would produce a truncated MTV11/VPS15 protein containing only the kinase domain. Interestingly, we also observed in *mtv11-1* skipping of the eighth exon by using an intron acceptor site in the 8th intron. This exon skipping event maintains the reading frame and the resultant transcript would produce a truncated protein containing the kinase domain and three of the four WD.

Figure 30. The *mtv11-1* mutant accumulates differentially spliced transcripts. (A) RT-PCR analysis of the retention of the 7th intron of *MTV11* in Wt and *mtv11-1* mutant plants with flanking primers. (B) Schematic representation of the Wt *MTV11/AtVPS15* protein and the two truncated versions encoded by the differentially spliced transcripts detected in *mtv11-1* plants.

Intriguingly, null mutations in *AtVPS15* show pollen lethality and thus homozygous mutants cannot be recovered (Wang et al., 2012; Xu et al., 2011), indicating that the *mtv11-1* allele retains partial activity of the gene. To confirm that the mutation in *At4g29380/AtVPS15* was the cause of the *mtv* phenotype, we crossed a heterozygous T-DNA null mutant (*atvps15-2/Salk_004719*) with *mtv11-1* homozygous for *VAC2*. In the F1, we observed plants either with Wt or with *mtv* phenotype. Genotyping using a CAPS marker for *mtv11-1* and flanking primers for the T-DNA insertion showed that plants with *mtv* phenotype corresponded to *mtv11-1/atvps15-2* plants and plants with Wt phenotype to *mtv11-1/MTV11* plants. Hence, *mtv11-1* and *atvps15-2* are allelic, which confirms that the activity of *MTV11/AtVPS15* is necessary for transport of *VAC2* to the vacuole.

To analyze the phenotype of *mtv11-1* plants in the absence of *VAC2* or other secondary EMS mutations, we backcrossed the plant 5 times with the parental *Ler* genotype and segregated out the *VAC2* transgene. The *mtv11-1* showed a reduced growth rate in soil (Figure 31) and an altered phyllotaxis, possibly due to alterations in vacuolar turnover of PIN auxin-efflux transporters.

Figure 31. Growth phenotype of Wt (*Ler*) and *mtv11-1* plants.

We analyzed if transport of endogenous seed storage proteins was also affected in the mutant. As shown in Figure 32, the *mtv11-1* mutant accumulated precursors of 12S globulins, indicating that transport of these storage proteins to the vacuole was hindered (Fig 32A). Protein storage vacuoles (PSV) in seeds are autofluorescent and can thus be visualized by fluorescence microscopy. Interestingly, PSVs in *mtv11-1* embryos had lower fluorescence levels than in Wt embryos (Figure 32B), consistent with lower levels of protein accumulation. We conclude for these analyses that MTV11/AtVPS15 is required for vacuolar trafficking of both exogenous and endogenous vacuolar cargo.

Figure 32. MTV11 is involved in transport of 12S globulins. (A) Total seed protein extracts showing accumulation of precursor form of 12S globulin (p12S) in *mtv11* (left panel). (B) Autofluorescence of protein storage vacuoles in hypocotyl cells from mature Wt and *mtv11-1* embryos. Scale bar 10 μ m.

14. MTV11/AtVPS15 localizes to endosomal compartments

To study the localization of MTV11/AtVPS15, we fused GFP at the N and C-terminal end of the protein and expressed these constructs in *Nicotiana benthamiana* leaves. GFP-AtVPS15 showed a cytosolic localization whereas AtVPS15-GFP showed both cytosolic and punctate pattern (Figure 33), suggesting that there are soluble and membrane bound pools of the fusion protein. Interestingly, MTV11 has an N-terminal glycine residue that is conserved in VPS15 homologues from plants, animals and fungi. Moreover, it has been reported that the N-terminal glycine is myristoylated in yeast VPS15 (Herman and Emr, 1990). This myristoylation motif would be maintained in the AtVPS15-GFP but not in GFP-AtVPS15, explaining the difference in subcellular localization and suggesting that addition of myristic acid is necessary for reversible attachment of MTV11 to membranes. When expressed in Arabidopsis protoplasts, GFP-AtVPS15 was also cytosolic. In protoplasts, AtVPS15-GFP showed mainly a cytosolic pattern and only in a few cells

some punctate structures could be observed. Stable *35S-AtVPS15-GFP* transgenic lines were generated but they had very low levels of transgene expression and could not be used for co-localization experiments. Hence, we have yet to identify the compartments where AtVPS15-GFP localizes.

Figure 33. Subcellular localization of GFP-tagged MTV11/AtVPS15 in tobacco leaf cells. MTV11/AtVPS15 was tagged with GFP at the N-terminal (GFP-AtVPS15) or C-terminal (AtVPS15-GFP) of the protein and expressed in *Nicotiana benthamiana* leaf epidermis cells. Scale bar = 100 μ m.

15. The *mtv11-1* mutant has reduced PI3P levels

VPS15 is a subunit of phosphatidylinositol 3-kinase from class III (PIK3C3) that is essential for membrane recruitment of the complex and for activation of the catalytic VPS34 subunit (Stack and Emr, 1994; Stack et al., 1993). The activated PIK3C3 phosphorylates phosphatidylinositol (PI) at D-3 positions to form PI3P. The localized synthesis of PI3P functions as a signal for membrane recruitment of effector proteins involved in vesicle trafficking and autophagy. The specificity of

Arabidopsis thaliana PIK3C3 is still unknown. To determine if MTV11 is involved in regulating PI3P levels *in vivo*, we made use of the PI3P biosensor line YFP-2xFYVE, consisting on YFP fused to tandem dimer of the FYVE domain of mouse Hsr, which specifically binds PI3P, under the control of the constitutive 35S promoter (Vermeer et al., 2006). We crossed transgenic lines expressing 2xFYVE (in Col-0 background) with *mtv11-1* mutants backcrossed 4 times into Col-0 background and obtained in the F2 *mtv11-1* plants homozygous for 2xFYVE. Comparison of the fluorescence pattern between these plants and the parental YFP-2xFYVE line showed clearly reduced fluorescence in the *mtv11-1* mutants. Whereas in Wt plants, YFP-2xFYVE strongly labelled numerous endosomes, in many cases forming large aggregates, in *mtv11-1* plants overall fluorescence was lower and the 2xFYVE labeled endosomes remained mostly isolated (Figure 34). These results support that MTV11/AtVPS15 functions as an activator of the VPS34 PI3P kinase in plants. Consistent with this, AtVPS15 interacts with AtVPS34 in yeast two hybrid assays (Wang et al., 2012) and defects in pollen germination of *vps15* mutants are rescued with external application of PI3P (Xu et al., 2011). The reduction in PI3P levels in endosomes would interfere with recruitment of trafficking effectors, explaining why vacuolar transport is perturbed in the *mtv11-1* mutant.

Figure 34. Altered distribution and reduced levels of PI3P in endosomes of *mtv11-1* revealed by the YFP-2xFYVE biosensor. Scale bar 25 μ m.

16. The *mtv11-1* mutants show increased growth in arsenate containing media.

Many toxic compounds are sequestered into the vacuoles for detoxification. We thus wondered if defective vacuolar trafficking in *mtv9* and *mtv11* mutants would affect vacuolar-mediated detoxification capacity. To test this, we focused on arsenic, one of the most toxic compounds in soil. Arsenic is found in soils mainly as arsenate [As(V)] that is transported into cells by phosphate transporters present in the PM. In the cytosol arsenate is rapidly reduced into arsenite [As(III)], complexed to soluble thiols, and transported into the vacuole for detoxification (Catarche et al., 2007; Sanchez-Bermejo et al., 2014). To determine if arsenic tolerance was altered in *mtv9* and *mtv11* mutants, we measured in collaboration with the group of Dr. Antonio Leyva at the CNB the root growth dynamics of Wt and mutant plants under different concentrations of As(V). We compared *mtv9-2* with its Wt background (Col-0) and *mtv11-1* (backcrossed 5 times into Ler) with

its Wt background (*Ler*). We did not find any differences in As(V)-induced root growth inhibition between Wt and *mtv9-2* plants, but strong differences were observed between *mtv11-1* and its Wt control in media c containing high As(V) concentration (Figure 35). The *mtv11-1* plants showed significant lower levels of root growth inhibition, indicating that they have increased tolerance to As(V) even though trafficking to vacuoles is disturbed.

Figure 35. *mtv11-1* shows enhanced growth in arsenic containing media. Root growth of the genotypes indicated in the graphs was measured in media with low phosphate (-Pi: 12,5 μM phosphate) or in the same media with 10 or 20 μM As(V). The average growth (relative to growth of the Wt in -Pi media) is shown. Error bars: standard deviation. Asterisks: significant difference relative to the Wt in that media ($p < 0.001$; Student's t-test).

A possible explanation to this surprising result, considering that perturbations to vacuoles were expected to interfere with detoxification, is that MTV11 participates in other trafficking routes that require PI3P and would be necessary for As(V) uptake into the plant. For instance, enhanced tolerance would be expected if the *mtv11-1* mutation interfered with targeting of phosphate transporters to the PM. To test this, we crossed the *mtv11-1* mutant with a marker line expressing the high affinity phosphate transporter PHT1;1 fused to GFP under the control of the 35S promoter (Gonzalez et al., 2005). With low phosphate in the media (-Pi: 12,5 μ M phosphate), PHT1;1-GFP was found primarily in the PM, both in Wt plants and in *mtv11-1* mutants. The presence of 30 μ M As(V) in -Pi media induced turnover of PHT1;1-GFP from the PM in Wt plants (Figure 36) as previously reported (Castrillo et al., 2013). Interestingly, the *mtv11-1* plants showed higher levels of PHT1;1-GFP in the PM after exposure to As(V) and in addition they showed strong accumulation of PHT1;1-GFP in endocytic compartments. Similarly, in phosphate rich media (+Pi: 1 mM phosphate), *mtv11-1* plants showed higher levels of PHT1;1-GFP in the PM than Wt plants and also significant accumulation in endocytic compartments. The detection of PHT1;1-GFP in endocytic compartments is likely due to the perturbations in vacuolar trafficking, which would delay targeting of the protein to the vacuole for degradation (Cardona-Lopez et al., 2015). Importantly, these results indicate that the higher As(V) tolerance of *mtv11-1* plants cannot be ascribed to lower levels of PHT transporters in the PM, but may instead be due to alterations in the vacuole that somehow favor As(III) uptake into that compartment. A possibility is that the concentration of arsenite conjugate transporters in the tonoplast is relatively increased in the mutant or that lower levels of protein accumulation permit higher arsenite uptake.

Figure 36. PHT1;1 is retained in endosomal compartments in *mtv11-1* background. Subcellular distribution of PHT1;1-GFP in *Wt* and *mtv11-1* roots. The distribution of PHT1;1-GFP in low phosphate medium (-Pi), in high phosphate medium (+Pi) and in low phosphate medium 24h after adding 30 μ M As(V) (-Pi+ As(V) 24 h) is shown. Scale bar 2,5 μ m.

Discussion

1. Phenotypic consequences of disrupting *MTV9* activity

Map-based cloning of the *mtv9* mutant revealed that defective trafficking of the VAC2 protein was due to a point mutation introducing an early stop codon in At1g24560, a gene of unknown function. Homologues of MTV9/At1g24560 are not present outside the plant kingdom or in algae. In contrast, all land plants contain homologues of MTV9, including *Physcomitrella patens* and *Marchantia polymorpha*. This could indicate that MTV9 may have functions related with the specific needs of land plants, such as resistance to salinity or drought, as described for ARA6 (Ebine et al., 2011). Vacuolar trafficking is an essential cellular process, and disruption of vacuolar trafficking machinery often leads to plant lethality or severe growth defects (Niihama et al., 2005; Rojo et al., 2001; Sanmartin et al., 2007; Sauer et al., 2013). The *mtv9* mutants have weak phenotypes, but there is a paralogue of MTV9 in Arabidopsis, the *STV9/At3g49055* gene, which could be genetically redundant with MTV9. However, the *stv9* mutant does not have terminated meristems in the presence of the VAC2 transgene, indicating that its activity is not essential for trafficking of this cargo, possibly due to redundancy from MTV9. Moreover, *mtv9 stv9* double mutants do not display any additional phenotypes in normal growth conditions relative to the *mtv9* single mutants, supporting that the genes do not have an essential function under standard growth conditions. However, it is possible that phenotypes may become apparent in conditions requiring optimal vacuolar trafficking capacity or under stress conditions. Moreover, it is also possible that the alleles analyzed may still retain some gene activity, although this is unlikely because the T-DNAs are inserted in exons and should eliminate most of the coding sequences. Another possibility is that the cargo transported through the MTV9/STV9-dependent pathway may be rerouted to an MTV9/STV9-independent pathway and still reach the vacuole in the *mtv9 stv9* mutant, which would explain why phenotypes are not clearly observable. Furthermore, MTV9 and STV9 may cooperate with other proteins to achieve a particular trafficking reaction (see below),

and strong defects may only become evident if both factors are disrupted. An example of this would be the *MTV1* and *MTV4* genes, which belong to distinct gene families but may both be involved in clathrin coated vesicle formation at the TGN. Only when both genes are mutated, phenotypic consequences at the whole plant scale are evident (Sauer et al., 2013).

2. Functional domains in MTV9

A search for known domains in the Pfam website suggests that the MTV9 protein contains coiled-coil domains along its sequence. Coiled-coiled domains may be involved in homo or hetero oligomerization and may also function as molecular spacers (Truebestein and Leonard, 2016). Coiled-coil domains are found in homodimeric membrane tethering factors such as golgins (Chia and Gleeson, 2014). MTV9 homodimerizes and could also function as a membrane tethering factor at the PVC. Indeed, the PVC aggregation caused by MTV9 overexpression supports that it may tether incoming vesicles or endosomal compartments for homotypic or heterotypic fusion. Interestingly, MTV9 is highly co-expressed with VCL1 and VPS39, which are components of the CORVET and HOPS multisubunit tethering complexes that regulate homotypic fusion of endosomes to form the PVC and the heterotypic fusion of the PVC with the vacuole in the final step of the vacuolar trafficking pathway (Chia and Gleeson, 2014; Rojo et al., 2001; Rojo et al., 2003). Homodimerizing membrane tethering factors work at long ranges (Chia and Gleeson, 2014), while multisubunit tethering complexes function at shorter distances. It is thus possible that MTV9 cooperates with the CORVET and/or the HOPS complexes to mediate membrane fusion at the PVC. Then, an explanation to the lack of phenotype of the *mtv9* mutants could be that *MTV9* improves the efficiency or speeds up tethering, but is not essential for the process. In contrast, the CORVET and HOPS complexes appear to be essential for tethering, and knockout mutations abrogate vacuole formation and impair plant viability (Rojo et al., 2001). However, we have recently isolated

a novel *mtv* mutant with a hypomorphic mutation in a *VPS39*-like gene from the TRAP1 clade (Klinger et al., 2013). Yeast *VPS39* is a subunit of the HOPS complex that is distantly related to yeast *VPS3*, a subunit of the class C core vacuole/endosome tethering (CORVET) complex (Klinger et al., 2013). Interestingly, animal and plant genomes contain several close homologues of *VPS39*, but not of *VPS3*. It has been proposed that the human *VPS39*-like homologue TRAP1 could substitute for *VPS3* in the CORVET complex (Lachmann et al., 2014, Klinger et al., 2013). It is thus possible that the Arabidopsis *VPS39*-like gene also assumes the function of the *VPS3* subunit in the CORVET complex, which functions as a Rab5 effector and coordinates membrane fusion at early/late endosomes. Knockout mutations in this *VPS39*-like gene are embryonic lethal, but the viable hypomorphic allele that we have isolated will allow us to test whether indeed it is part of the CORVET complex, and whether this complex cooperates with MTV9 for tethering incoming vesicles at the PVC. If that were the case, we should find strong genetic interaction in a double *mtv9 vps39-L* mutant.

The fact that MTV9 and STV9 genes are specific for land plants suggests that these organisms may have exclusive needs that require the function of these genes. Because MTV9 homologues are present in basal plants like *Physcomitrella* and *Marchantia*, it implies that the plant specific needs predate the appearance of seed plants. In that regard it is telling that *MTV9* is not required for vacuolar trafficking of storage proteins in seeds, but it is required for vacuolar trafficking in vegetative cells. Clearly, vacuoles in seed cells and in vegetative cells are strikingly different (Zouhar and Rojo, 2009) and so is the organization of intracellular compartments, so the trafficking requirements may be different. Indeed, a key adaptation of land plants was the acquisition in vegetative cells of large vacuoles that occupy most of the cellular volume, in contrast to algal, yeast and mammalian cells that contain small vacuoles. The large vacuoles in land plants allow for energetically cheap growth that is essential for these autotrophic organisms to explore

the surrounding environment for light, water and nutrients. In addition, the large vacuoles also serve to maintain cellular homeostasis by acting as a buffering compartment (Zouhar and Rojo, 2009). It is evident that having such large vacuoles alters considerably the organization of the cytosol and the organelles within it, and therefore the trafficking between the different compartments. It is then foreseeable the need for novel tethering factors that help vesicles to navigate this new cellular landscape and promote efficient membrane fusion reactions. In this regard, MTV9 (and STV9) may have been selected to perform an activity essential for effective vacuolar trafficking in these novel cellular settings. However, in standard growth conditions, we have not detected any deleterious consequences of knocking out MTV9 and STV9, arguing that the activity of these genes is largely irrelevant for normal functioning of the plant. The challenge then is to find if there are conditions in which altering this their activity has phenotypic consequences for the plant. A possibility is that their activity is especially important for the response to stresses specifically encountered by land plants, such as salt and drought stress, or for processes that involve active vacuole dynamics, such as stomatal movements. Therefore, it will then be important to test all the double mutants generated, and especially the *mtv9stv9* double mutant, under a battery of stress conditions to find conditions in which the activity of this gene family has an essential role. As mentioned above, *Marchantia polymorpha* has a single *MTV9* gene and gene disruption is simple in that organism, so it would be a good alternative model to test how MTV9 knock out affects plant development and physiology in this basal plant.

3. MTV9 localization

Subcellular fractionation of endogenous MTV9 protein and confocal analysis of GFP fusion constructs suggest that MTV9 localizes primarily at the PVC. MTV9 does not contain a signal peptide or transmembrane domains, indicating that it associates with the PVC from the cytosolic

side, possibly through post-translational modifications or through interaction with PVC resident proteins. Recently, a proteomic study identified MTV9 as a putative palmitoylated protein (Hemsley et al., 2013), suggesting that palmyotylation could mediate its targeting to the PVC. Palmitoylation is a reversible post-translational modification that covalently attaches palmitic acid, a saturated 16-carbon fatty acid, in most cases to a cysteine residue. This modification confers hydrophobicity to the protein favoring the association with membranes (Levental et al., 2010). Our analysis revealed that MTV9 could only be extracted efficiently from membranes with detergent but not with buffers used to extract peripheral membrane associated proteins. This pattern of extraction resembles that of ERBIN, a human protein that is recruited to plasma membrane via palmitoylation of two cysteines (Izawa et al., 2008), which supports that membrane association of MTV9 may be mediated through palmitoylation. In this regard, we have determined that the PVC-targeting motif of MTV9 resides in the C-terminal CT domain (aminoacids 473-678), which contains the only cysteine residue present in MTV9. We thus propose that recruitment of MTV9 to the PVC may be mediated by reversible palmitoylation of that cysteine residue. Importantly, the cysteine residue and the next two amino acids define a motif (CWP motif) that is strictly conserved in all the MTV9 orthologues present in plants, suggesting that it is key for their function. The CWP motif is also present in the STV9 paralogues, which are also localized in endosomal compartments. Further characterization of the CT domain, including site directed mutagenesis of the CWP domain, will clarify what motifs are necessary for MTV9 and STV9 localization and function. A full characterization of the phenotypes caused by MTV9 and STV9 knockout will be necessary to then analyze what functions can be complemented by the different mutant versions of the proteins.

4. *MTV9* mRNA expression

Expression studies can provide important hints to uncover gene function. For example, *MTV4/AGD5* is expressed specifically in the petal abscission zone of flowers, and, accordingly, *mtv4* mutants have a *nevershed* phenotype of delayed petal abscission (Liljegren et al., 2009). Interestingly, co-expression analysis shows that *MTV9* is highly co-expressed with several trafficking genes, including *VCL1*, *VPS39* and *ALIX*, which are involved in PVC to vacuole transport. This suggests that coordinated expression of these genes in particular conditions and tissues is important for proper regulation of vacuolar trafficking. Promoter analysis supports that *MTV9* is not expressed ubiquitously throughout the plant, but has a rather specific spatial expression pattern. In the mature root it is expressed in the vasculature and in root tips it is specific for the quiescent center cells. In leaves, maximal expression is found in the vasculature and in trichomes. *MTV9* is also expressed in the hypocotyl, in the shoots and in the SAM, consistent with the mutation of *MTV9* provoking *VAC2* secretion in the SAM and leading to premature termination. Cross sections of shoots reveal highest expression also in the vasculature, indicating that *MTV9* may have a function related with conductive tissues. The specific expression of *MTV9* in these cell types and tissues will inform the search for phenotypes in the *mtv9* knockout plants. For instance, the vasculature plays a key role in the transition to flowering by transmitting information from the leaves (the florigen signal, nutritional status, etc.) to the SAM to promote the switch from vegetative to reproductive growth, so it is interesting that *mtv9* mutants show delayed flowering. In contrast, no expression of *MTV9* was observed in seeds, which is consistent with the lack of effect of the *mtv9* mutation in those tissues.

5. On the activity of the *mtv11-1* allele

Phosphatidylinositol 3-kinases (PI3Ks) are key enzymes involved in many aspects of cell biology and physiology in organisms from all eukaryotic kingdoms. Three classes of PI3Ks are present in animal cells, and each class has a characteristic substrate specificity and a set of cellular functions. Only the PI3Ks from class III (PIK3C3 class) are conserved across kingdoms, and in yeast and plant genomes they are the sole PI3Ks present. PIK3C3 have an invariant core formed by a catalytic subunit (VPS34) and a regulatory subunit (VPS15). This core interacts with VPS30, Atg14 and Atg38 to form the PIK3C3-C1 complex involved in autophagy, with VPS30 and VPS38 to form the PIK3C3-C2 complex involved in all the vacuolar trafficking pathways in yeast (biosynthetic, endocytic, autophagic and the cytoplasm-to vacuole pathways) and with GPA1 and Atg18 to form the PIK3C3-C3 complex involved in pheromone signaling (Reidick et al., 2017). The PIK3C3-C2 complex synthesizes phosphatidylinositol 3-phosphate (PI3P) at specific domains of the membrane to recruit effector proteins containing PI3P binding domains, most often PX or FYVE domains, involved in different steps of the vacuolar trafficking pathway. Using the synthetic biosensor YFP-2XFYVE it was reported that PI3P in Arabidopsis cells is gradually distributed in the TGN, the PVC and the tonoplast (Vermeer et al., 2006) consistent with VPS15 and the PI3K functioning specifically in the vacuolar trafficking pathway. Importantly, the *mtv11-1* mutation reduces but does not abolish the accumulation of PI3P in endosomes, indicating that the PI3K activity is affected but not blocked. Indeed, Arabidopsis null mutants in *AtVPS34* or *AtVPS15* are gametophytic lethal (Lee et al., 2008; Wang et al., 2012; Xu et al., 2011). In the *mtv11-1* mutant we detected abnormally spliced *AtVPS15* transcripts encoding two types of truncated proteins, one containing only the N-terminal kinase domain and another containing the N-terminal kinase domain fused to three of the C-terminal WD domains. Most likely, it is this latter fusion protein that retains sufficient activity to allow plant viability. Recently, a structure of the PIK3C3-C2

complex at 4.4 Å resolution was reported (Rostislavleva et al., 2015). VPS15 and VPS34 intertwine in an antiparallel fashion, with all three domains of VPS15 (Kinase, HEAT and WD domains) establishing contacts with VPS34. The catalytic domains of both proteins contact each other, whereas the WD domain of VPS15 bridges the VPS34C2 domain and the VPS30/VPS38 BARA domains. The central region of VPS15, which is missing in the truncated *mtv11-1* protein, is a linker domain that establishes further contacts with VPS34, VPS30 and VPS38. This linker domain adopts a folded V shape so that the N-terminal kinase domain and the C-terminal WD domains are actually nearby in the tertiary structure. Hence, the truncated protein could still maintain the interactions with the VPS34 kinase domain at one end and bridge the VPS34 C2 domain and the VPS30/VPS38 BARA domains at the other end. This could explain why this truncated fusion protein lacking the entire central region would still maintain activity of the PIK3C3-C2 complex. The study of the *mtv11-1* mutant allele may then reveal important information on the structural-functional relationship of the PIK3C3-C2 complex. The complex containing the truncated AtVPS15 protein encoded by the mutant allele is probably still correctly targeted to membranes, since the myristoylation motif of AtVPS15 is intact. However, the complex may be less stable, because of reduced interaction strength between the subunits. Alternatively, the activity of the catalytic VPS34 subunit may be compromised. Several catalytic elements of VPS34 contact the VPS15 kinase domain (Rostislavleva et al., 2015) so the deletion of the linker domain may perturb these contacts and affect the activity of VPS34. Analyzing how the truncated protein reduces but does not abolish activity of the PIK3C3-C2 complex will advance our understanding on how this complex synthesizes PI3P on target membranes to direct effector recruitment and membrane trafficking reactions.

6. MTV9 and MTV11, trafficking factors at the PVC

All the *MTV* genes isolated until now encode proteins localized in post-Golgi compartments of the biosynthetic pathway. VTI12, AtVPS45, MTV1 and MTV4 are localized at the TGN and in clathrin coated vesicles (Sanmartin et al., 2007; Sauer et al., 2013; Zouhar et al., 2009) and MTV2/VSR4, VSR1 and VSR3 cycle between the TGN and the PVC (Zouhar et al., 2010). MTV9 is also localized at the PVC, where it may function in tethering membranes for fusion. The compartment(s) where MTV11 is localized is yet unidentified, but considering that PIP3, the product of the PIK3C3-C2 complex, is enriched in membranes of the TGN, PVC and vacuole, and that MTV11-GFP displays a punctate pattern, we can assume that MTV11 is associated mainly with the TGN and the PVC. Why does the *mtv* screen only unveil post-Golgi steps of vacuolar trafficking? These steps are specific for transport to the vacuole and mutations affecting them should not interfere with trafficking to the PM. Moreover, the presence of independent routes to the vacuole in plants means that when one of the pathways is blocked, transport may still proceed through an alternate pathway. In contrast, mutations in earlier steps of the secretory pathway, at the ER or the Golgi, would affect transport both to the vacuole and the PM, and would be more detrimental to plant viability. Early plant lethality precludes isolation of the *mtv* mutants, which are detected relatively late in development. Indeed, some of the *mtv* mutants isolated correspond to hypomorphic alleles of essential genes that when knocked out cause pollen or embryo lethality. Moreover, the *mtv* screen relies on the abnormal secretion of VAC2 and if both transport to the vacuole and the PM would be affected, then VAC2 would be retained intracellularly and would not produce an *mtv* phenotype. These features favor the identification of mutants in the late secretory pathway, and explains why all the mutants isolated thus far are in genes acting in post-Golgi compartments.

7. Arsenate phytoremediation

Although in standard growth media or in soil the *mtv11-1* shows a growth delay, in media containing high As(V) concentration, *mtv11-1* has significantly increased growth relative to Wt plants, suggesting that it is more tolerant to this toxic metalloid. The *mtv11-1* mutant shows clear accumulation of the PM PHT1;1 high affinity phosphate (and As(V)) transporter in endocytic organelles. This retention in endosomes is consistent with vacuolar transport being perturbed in the mutant. This likely impairs proper turnover of the transporter in the vacuole and may increase their recycling into the PM. Indeed, we found increased levels of PHT1;1 in the PM in media containing As(V), which normally induces the internalization and vacuolar degradation of these phosphate transporters (Castrillo et al., 2013). The failure to recycle properly these transporters into the vacuole and their accumulation in the PM implies that the *mtv11-1* mutant may in fact uptake more As(V) than Wt plants, but still be more tolerant to this toxic metalloid. If indeed this were the case, understanding how the *mtv11-1* mutant achieves As(V) hyper-accumulation and higher tolerance would be of great interest for the bioremediation field. A possibility is that the alterations in the vacuolar pathway allow for higher levels of As(III) sequestration in the vacuole. Measuring As(V) and As(III) levels in whole tissues and in isolated vacuoles (Zouhar, 2017) will clarify if the *mtv11-1* mutant hyper-accumulates As(V) and has increased vacuolar sequestration capacity for As(III). It will also be interesting to analyze if the reduced activity of the PIK3C3-C2 complex directly results in higher As(V) tolerance. In that case this complex could be a target to breed plants with improved phytoremediation properties.

Conclusions

- 1) The analysis of four independent *mtv9* alleles indicates that *MTV9* is essential for vacuolar trafficking of VAC2 in vegetative tissues.
- 2) *MTV9* encodes a coiled-coil protein that homodimerizes.
- 3) *MTV9* is localized primarily at the prevacuolar compartment.
- 4) *MTV9* overexpression alters PVC morphology and blocks vacuolar trafficking but not transport to the plasma membrane.
- 5) The CT domain of *MTV9* contains the PVC targeting information.
- 6) The *mtv11* mutant phenotype is caused by a point mutation in a splice acceptor site of the 7th intron of the *AtVPS15* gene.
- 7) The hypomorphic *mtv11-1* allele accumulates alternatively spliced *AtVPS15* transcripts, including a transcript that skips exon 8 and retains the open reading frame.
- 8) The activity of the *AtVPS15* is required for trafficking of endogenous vacuolar storage proteins in seed tissues.
- 9) The *mtv11-1* mutation causes reduced PIP3 levels in endosomes.
- 10) The *mtv11-1* mutant retains more PHT1;1 in the plasma membrane but is more tolerant to As(V) in the media, suggesting that it combines tolerance with hyper accumulation.

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