Running head: actin organization requires villin

Author for correspondence:

Tijs Ketelaar
Laboratory of Cell Biology
Wageningen University
Droevendaalsesteeg 1
6708 PB Wageningen
The Netherlands

Email: tijs.ketelaar@wur.nl
Phone: +31 317 484329
Fax: +31 317 418094

Journal research area: Cell biology
Arabidopsis VILLIN2 and VILLIN3 are required for the generation of thick actin filament bundles and for directional organ growth

Hannie S. van der Honing¹, Henk Kieft¹, Anne Mie C. Emons¹,² and Tijs Ketelaar¹

¹Laboratory of Cell Biology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

²Department of Biomolecular Systems, FOM Institute for Atomic and Molecular Physics, Science Park 113, 1098 SG Amsterdam, The Netherlands
Abstract
In plant cells, actin filament bundles serve as tracks for myosin-dependent organelle movement and play a role in the organization of the cytoplasm. Although virtually all plant cells contain actin filament bundles, the role of the different actin bundling proteins remains largely unknown. In this study, we investigated the role of the actin bundling protein villin in Arabidopsis thaliana. We used Arabidopsis T-DNA insertion lines to generate a double mutant in which VLN2 and VLN3 transcripts are truncated. Leaves, stems, siliques and roots of vln2 vln3 double mutant plants are twisted, which is caused by local differences in cell length. Microscopy analysis of the actin cytoskeleton showed that in these double mutant plants, thin actin filament bundles are more abundant, while thick actin filament bundles are virtually absent. In contrast to full-length VLN3, truncated VLN3 lacking the headpiece region does not rescue the phenotype of the vln2 vln3 double mutant. Our results show that villin is involved in the generation of thick actin filament bundles in several cell types, and suggest that these bundles are involved in the regulation of coordinated cell expansion.
**Introduction**

The plant actin cytoskeleton plays an essential role in cell division, cytoplasmic organization, cytoplasmic streaming, cell growth, and consequently plant morphogenesis. Actin binding proteins modulate the formation and dynamics of filamentous actin, and its configuration. Among these proteins are the actin bundling proteins, which are able to cross-link adjacent actin filaments, resulting in bundles consisting of several parallel actin filaments (Thomas et al., 2009). In plant cells, bundling of actin filaments occurs (Thomas et al., 2009), which is likely mediated by actin bundling proteins. There are four known families of actin bundling proteins in plants: villins (Vidali et al., 1998; Klahre et al., 2000; Tominaga et al., 2000; Yokota et al., 2003; Huang et al., 2005; Yokota et al., 2005; Khurana et al., 2010; Zhang et al., 2010), fimbrins (Kovar et al., 2000; Kovar et al., 2001), formins (Cheung and Wu, 2004; Favery et al., 2004; Michelot et al., 2005; Ye et al., 2009), and LIM proteins (Thomas et al., 2006; Thomas et al., 2008; Wang et al., 2008; Papuga et al., 2010). In addition, elongation factor 1 alpha (Collings et al., 1994; Gungabissoon et al., 2001) has been shown to have actin filament bundling properties as well. The presence of these different actin bundling proteins suggests that their combined actions can result in several types of actin filament bundles, which differ in form and function (Thomas et al., 2009).

Although the role of the different actin bundling proteins in the generation of actin filament bundles is not yet known, it is clear that actin filament bundles fulfill several functions in plant cells. Actin filament bundles serve as the preferred tracks for myosin-dependent movement of organelles (Miller et al., 1999; Ketelaar et al., 2003; Holweg, 2007; Ye et al., 2009). Next to their function in cytoplasmic streaming, actin filament bundles have been shown to play a role in keeping the nucleus at a fixed position from the root hair tip (Ketelaar et al., 2002). Furthermore, actin filament bundles structure the cytoplasm. Their depolymerization causes collapse of cytoplasmic strands (Staiger et al., 1994; Shimmen et al., 1995; Valster et al., 1997; Hussey et al., 1998; Van Gestel et al., 2002; Higaki et al., 2006; Sheahan et al., 2007; van der Honing et al., 2010), and unbundling results in more, but thinner cytoplasmic strands (Tominaga et al., 2000; Ketelaar et al., 2002). Thus, actin filament bundles are required to maintain cytoplasmic strand size and number, i.e. the overall organization of the cytoplasm of plant cells.
The genome of *Arabidopsis thaliana* contains five villin genes, and the villins encoded by these genes are highly expressed in several tissues of *Arabidopsis* (Klahre et al., 2000). Plant villins are similar to vertebrate villins (Staiger and Hussey, 2004). Villins consist of a core (made up of six tandem subdomains) and a distinct C-terminal domain, which is referred to as the headpiece. Villin’s core shares structural homology to the actin binding protein gelsolin, which has Ca\(^{2+}\)-regulated actin filament nucleation, severing, and barbed end capping activity (Bryan and Kurth, 1984; Way et al., 1989). Both the core and the headpiece contain an actin filament binding domain, and the headpiece region of vertebrate villins has been shown to be essential for actin filament bundling (Glenney and Weber, 1981). This led to the hypothesis that villin bundles actin filaments by acting as a monomer, with the two actin filament binding domains – one present in the core, the other one in the headpiece – cross-linking two adjacent actin filaments (Glenney et al., 1981). However, one study suggests the presence of a third actin binding domain, which is present in the core (Hampton et al., 2008), while another study suggests that villin acts as a dimer (George et al., 2007). In addition to their ability to bundle actin filaments, vertebrate villins also show Ca\(^{2+}\)-dependent actin filament severing, nucleating and capping activity (Bretscher and Weber, 1980; Glenney et al., 1981; Glenney and Weber, 1981). Plant villins also possess actin filament barbed-end capping (Yokota et al., 2005; Zhang et al., 2010; Zhang et al., 2011), nucleating (Yokota et al., 2005) and severing (Khurana et al., 2010; Zhang et al., 2010; Zhang et al., 2011) activities *in vitro*. Zhang et al. (2011) show that the barbed-end capping and severing activities of Arabidopsis VLN4 are Ca\(^{2+}\) dependent. In plants, villin has been shown to play a role in organizing the cytoplasm in root hairs (Tominaga et al., 2000; Ketelaar et al., 2002) and pollen tubes (Zhang et al., 2011), as well as in nuclear positioning in root hairs (Ketelaar et al., 2002).

In this study, we analyzed the role of two *Arabidopsis* villins using lines with a T-DNA insertion in *VLN2*, *VLN3*, or both *VLN2* and *VLN3*. The *vlh2 vln3* double mutants show a clear anomaly in the growth direction of organs, suggesting problems with coordinated cell elongation. The actin cytoskeleton in the double mutants has a finer appearance, and thick bundles of actin filaments are virtually absent. GFP:VLN3 rescued the morphological phenotype and localizes to actin filament bundles in all cell
types studied. We further show that the headpiece region of VLN3 is important for the localization of VLN3 to actin filament bundles and for the regulation of directional organ growth. The data show that villin is involved in the generation of thick actin filament bundles and suggest that these bundles are important for coordination of cell expansion in different organs.
Results

T-DNA insertions in VLN2 and VLN3 result in a truncated transcript for both genes

A cladogram based on cDNA sequences shows that VLN2 (At2g41740) and VLN3 (At3g57410) belong to the same clade (Figure 1A), suggesting that they have arisen from a relatively recent genome duplication. They share 84% similarity in their cDNA sequences, and 80% similarity in their amino acid sequences. Both VLN2 and VLN3 are expressed in all organs (www.bar.utoronto.ca), with similar expression levels for both villins in most organs. VLN2 has a slightly higher expression level in mature pollen grains.

To test the biological role of VLN2 and VLN3, lines homozygous for T-DNA insertions in VLN2 and VLN3 were identified. The T-DNA insertions for both vln2 and vln3 (i.e. SAIL_813_H02 and SALK_117097, respectively) are located in exons (Figure 1B), at locations corresponding to the G6 domain in vln2, and to the linker domain between the G6 and headpiece domain in vln3 (Figure 1C) according to Klahre et al. (Klahre et al., 2000). The presence of VLN2 and VLN3 transcripts was tested using RT-PCR in both azygous (homozygous for the undisrupted gene) and mutant plants from the same population. Primer combinations before and after the inserts were used to test if transcripts were present, truncated, or absent. For both vln2 and vln3, transcripts corresponding to coding regions before the insert were present, but the region after the insert was not transcribed (Figure 1D). Thus, the presence of the inserts results in truncated transcripts for both VLN2 and VLN3. We generated a double mutant of these lines with truncated mRNA for both genes (Figure 1D).

Although the presence of the T-DNA inserts results in truncated transcripts for both VLN2 and VLN3, the corresponding proteins could be absent. We tested this by probing a protein gel blot of wild type Columbia-0 (Col-0), vln2, vln3, and vln2 vln3 root extracts with a polyclonal anti-lily villin antibody (Tominaga et al., 2000; Ketelaar et al., 2002; Khurana et al., 2010). This resulted in a band at a height corresponding to the predicted mass of VLN3 (107 kD) in Col-0 and vln2 extracts, but in vln3 and vln2 vln3 extracts, no band was visible (Figure 1E). The absence of a band at a height corresponding to a smaller protein shows that vln3 and vln2 vln3 do not contain a detectable amount of the truncated version of VLN3. Thus, although these
lines contain a truncated VLN3 transcript (Figure 1D), the VLN3 protein is absent or a truncated protein is produced at levels below the Western blotting detection limit. The absence of a villin band in the root extract of the vln3 plant shows that the antibody does not recognize the VLN2 protein. We conclude that while vln2 and vln2 vln3 might contain a truncated version of VLN2, vln3 and vln2 vln3 do not contain a detectable amount of the full length or truncated VLN3 protein.

**Arabidopsis plants homozygous for a T-DNA insertion in two villin genes display defects in directional organ growth**

Seedlings of single mutants of vln2 and vln3 do not show any developmental defects or delays, but those of vln2 vln3 double mutants have curly roots (Figure 2A). Plant growth and organ development in the double mutant occur at similar rates as those of azygous plants and single mutants, and production of viable seeds is unaffected by the presence of T-DNA insertions in both genes. However, rosette leaves of the double mutant are twisted (Figure 2B). In addition, stems of the vln2 vln3 double mutant are curly, and inflorescences show complete turns (Figure 2C, D). Both single mutants do not show this phenotype. The tops of the growing inflorescences of the double mutant are often (41 %; n = 17) oriented downward, while this never occurs in Col-0 plants (n = 22) and single mutants (n = 19 for vln2, and 17 for vln3; Figure 3A). Time lapse recording of Col-0 and vln2 vln3 plants shows that the rotational movements (circumnutation) of vln2 vln3 inflorescences differ from those of Col-0 inflorescences: in the double mutant, periods of normal circumnutation alternate with periods in which the circumnutation movements show larger amplitudes than those of Col-0 (Figure 2E; Supplemental Movie 1 online). Although hypocotyls from light grown seedlings do not show any defects, etiolated hypocotyls appear curly, similar to other organs (supplemental figure 1A, B online). These data suggest that the coordination of cell expansion in the organs is affected in vln2 vln3, resulting in the curly phenotype of roots, leaves, etiolated hypocotyls and inflorescences. Siliques and fruit stalks of vln2 vln3 are also curly (Figure 2C, D), and 59 % of the siliques were oriented at angles smaller than 90° with respect to the plant axis (n = 80), while this rarely occurred (with a maximum of 6 %) in Col-0 plants (n = 49) or single mutants (n = 50; Figure 3B). Despite the curly phenotype of leaves and fruit stalks, only the width of fruit stalk epidermal cells is slightly, but significantly, higher in the double mutant (0.99 ± 0.25 μm in vln2 vln3 compared to 0.88 ± 0.23 μm in Col-0; Student’s t test, P
The surface area, perimeter and circularity (Brembu et al., 2004) of abaxial leaf pavement cells \((n = 26\) for Col-0 plants, and \(61\) for vln2 vln3; Figure 3C) and the surface area, perimeter, length and circularity of fruit stalk epidermal cells \((n = 68\) for Col-0 plants, and \(67\) for vln2 vln3; Figure 3D) are not affected by the mutations in VLN2 and VLN3. The gravitropic response in the vln2 vln3 double mutant is not affected (supplemental figure 1C, D online). In addition, trichome size, shape and branch numbers are normal in the vln2 vln3 double mutant.

To determine the cause of the organ curling phenotype, we stained roots with 1 µg.ml\(^{-1}\) propidium iodide and measured cell lengths of fully grown root epidermal cells of different cell files of the inner and outer side of a curve in the root. In wild type roots, we did not observe these curves and did not find differences in cell lengths between cells of the different cell files \((193 \pm 30 \text{ µm per cell})\). The length of vln2 vln3 root epidermal cells is significantly different in cell files of the inner and outer side of a root curve (cell lengths in outer cell files: \(195 \pm 35 \text{ µm}\) and inner cell files \(182 \pm 40 \text{ µm}\)). Lengths of 20 root epidermal cells on an inner or outer side of a root curve were measured in 13 (outer side) and 10 (inner side) roots. The average cell lengths of the different roots were used to determine the significance of the difference. An unpaired T-test shows that the difference is highly significant \((P < 0.001)\). Thus, the organ curling occurs through local differences in cell expansion.

Although VLN2 and VLN3 are both expressed in root hairs (www.bar.utoronto.ca), we did not observe differences from Col-0 plants in root hair morphology (Supplemental Figure 2A online), nucleus to tip distance (Supplemental Figure 2B online), and growth rate (Supplemental Figure 2C online) of elongating root hairs of the double mutant. Thus, the mutations in VLN2 and VLN3 do not affect the growth and morphology of individual cells, such as root hairs and trichomes, but do result in defects in directional growth of roots, shoots, leaves and siliques. This suggests that the mutations affect coordinated cell elongation.

To confirm that the observed morphological phenotype is caused by the presence of the T-DNA inserts in VLN2 and VLN3, we complemented the mutant phenotype with genomic VLN2 and VLN3, under their endogenous promoters, and with PVLN3:VLN3 cDNA. In most transformants carrying these constructs the mutant phenotype was
fully rescued (genomic VLN2: 27 out of 34 independent transformants; genomic VLN3: 26 out of 29 independent transformants; PVLN3::VLN3 cDNA: 38 out of 45 independent transformants; Supplemental Figure 3 online). These results confirm that the defects in directional organ growth are caused by the combined mutations in VLN2 and VLN3. Thus, VLN2 and VLN3 play a redundant role in the regulation of directional organ growth.

**Thick actin filament bundles are virtually absent in vln2 vln3, while thin bundles are more abundant**

To investigate if the actin organization is affected by the presence of the T-DNA insertions in VLN2 and VLN3, we used GFP::FABD2 (Ketelaar et al., 2004) expression to visualize actin filaments in cells of the single mutants and the double mutant, and compared the actin organization with that of Col-0 cells. We experienced silencing problems when we tried to cross GFP::FABD2 expression into the vln2 vln3 double mutant (the F2 from vln2 and vln3 single mutants segregated as expected: approximately 25% of the plants were homozygous for vln2 or vln3 and 1:4 of the homozygous plants expressed GFP::FABD2. In the F2 of the cross of GFP::FABD2 and the vln2 vln3 double mutant, we found that approximately 1 in 16 plants showed the mutant phenotype as expected (115 out of 1889 F2 plants), but none of these plants showed GFP::FABD2 expression, where GFP::FABD2 expression is expected in 75% of the F2 lines homozygous for vln2 vln3. Over 90% of the F1 plants of this cross showed GFP::FABD2 expression). Hence, we resorted to transforming the 35S::GFP::FABD2 construct directly into the vln2 vln3 double mutant. This approach resulted in 202 independent transformants. Most (198) transformants did not show any GFP::FABD2 expression. Only 4 of these lines occasionally showed GFP::FABD2 expression in several cells. We used these lines to study actin organization in the vln2 vln3 double mutant. The actin organization in fully elongated hypocotyl epidermal cells of the single mutants is similar to that of wild type cells: thick, predominantly longitudinal actin filament bundles are interspersed with a more complex network of thinner bundles (Figure 4A-I). In the double mutant, however, the thick, longitudinal actin filament bundles are absent, and thinner bundles are more abundant. Fully elongated root and abaxial leaf epidermal cells of the double mutant also appear to contain more thin bundles of actin filaments than those in azyous plants and the single mutants, while thick actin filament bundles are absent (Figure 4J-L).
To quantify the observed differences in actin organization, we created intensity profiles of GFP fluorescence intensities of P35S:GFP:FABD expressing hypocotyl cells (n = 8 for Col-0 and vln2 vln3), in the middle of Z-projections of the cortical cytoplasm, perpendicular to the longitudinal cell axis (excluding the bright cell edges; Figure 4M-O). In these intensity profiles, high peaks represent brightly labeled actin filament bundles, while low peaks represent weakly labeled actin filament bundles (or perhaps single actin filaments, although it is unlikely that single actin filaments are detectable with the used set-up). We counted the number of peaks per µm, and distributed these peaks in three classes: high, medium and low grey levels (Figure 4P).

The frequency distribution of the number of peaks across the three classes was clearly different between Col-0 and vln2 vln3 hypocotyl cells. 17 % of the peaks in Col-0 cells belonged to the class with the highest intensity levels, representing thick actin filament bundles, while in vln2 vln3, only 2 % of the peaks represented this class (Figure 4O). Peaks with a low fluorescence intensity were more abundant in vln2 vln3 (70 %) than in Col-0 (47 %) cells. A Pearson’s chi-square test showed that the frequency distribution across the three classes was significantly different between Col-0 and vln2 vln3 cells (p < 0.001). The average number of peaks per µm was higher (t-test; p = 0.04) in the double mutant (0.82 ± 0.29) then in Col-0 cells (0.51 ± 0.26; Figure 4Q). Besides this analysis, we used the analysis method designed by Higaki et al. (2010) to assess differences in the degree of actin filament bundling. We determined skewness, which is a parameter that represents the amount of bundling (higher values indicate the presence of thicker bundles), and occupancy, which gives insight in the density of the actin cytoskeleton. Analysis was performed as described in the Methods section and resulted a skewness of 1.86 ± 0.29 in hypocotyl epidermal cells of Col-0 plants (n= 100 cells from 7 different plants) and 0.73 ± 0.31 in these cells of the vln2 vln3 mutant (n= 50 cells from 30 different plants). This difference is highly significant (unpaired T-test; P< 0.0001). The occupancy in hypocotyl epidermal cells of Col-0 plants was 0.23 ± 0.024 compared to 0.41 ± 0.079 in those of the vln2 vln3 mutant. Also this difference is highly significant (unpaired T-test; P< 0.0001). The skewness and occupancy of the actin cytoskeleton in vln2 and the vln3 single mutants did not differ significantly from that in Col-0 plants (vln2: skewness
1.95 ± 0.35 (unpaired T-test; P= 0.12) and occupancy 0.21 ± 0.130 (unpaired T-test; P= 0.14; n= 50 cells from 7 plants); vln3: skewness 1.80 ± 0.45 (unpaired T-test; P= 0.33) and occupancy 0.23 ± 0.031 (unpaired T-test; P= 1.0; n= 50 cells from 5 plants))

We conclude that cells of the double mutant, but not the single mutants, contain more thin bundles of actin filaments than those of Col-0 cells, while the average thickness of these bundles is thinner.

To test whether the defects in actin organization occur during cell expansion and correlate with the defects in organ growth, we studied the actin organization in elongating root epidermal cells in Col-0 and vln2 vln3 plants (Figure 5). Similar to the actin organization in fully grown cells, the skewness and occupancy of the actin cytoskeleton in these cells were significantly different (Col-0 skewness 1.34 ± 0.38 and occupancy 0.15 ± 0.009; vln2 vln3 skewness 0.43 ± 0.19 and occupancy 0.57 ± 0.11 (unpaired T-test; P< 0.0001 for both skewness and occupancy; Col-0: n = 50 cells from 7 different plants; vln2 vln3: n = 35 cells from 20 different plants).

**GFP:VLN3 labels some (bundles of) actin filaments**

To determine the subcellular localization of villin, we complemented the double mutant with **GFP:VLN3**, expressed under control of the endogenous promoter (**PVLN3:GFP:VLN3** genomic). Expression of this construct in the mutant rescued the phenotype in 88 out of 99 independent transformants, showing that the fusion protein is functional. GFP:VLN3 is present in all investigated cells: leaf, hypocotyl, and root epidermal cells, including root hairs (Figure 6). In all these cell types, GFP:VLN3 partly shows a cytoplasmic localization. Besides this cytoplasmic localization, GFP:VLN3 localizes to filamentous structures resembling (bundles of) actin filaments both in the cortical cytoplasm and in cytoplasmic strands of the cells studied (Figure 6A-D). Coexpression of **PVLN3:GFP:VLN3** and **P35S:mCherry:FABD2** in tobacco (*Nicotiana benthamiana*) leaves demonstrates that GFP:VLN3 and mCherry:FABD2 colocalize (Figure 6E-G), confirming that GFP:VLN3 localizes to actin filaments. Villin appears not to label all actin filament bundles equally strong (Figure 6E-G) In growing root hairs, GFP:VLN3 localizes to the long actin filament bundles oriented longitudinally to the cell’s long axis in the root hair tube (Figure 6D). These actin filament bundles do not penetrate the (sub)apical region. Image sequences of root epidermal cells collected at 5 s intervals (Figure 6H-J; Supplemental Movie 2 online),
or of root hairs at 30 s intervals (Figure 6K-M; Supplemental Movie 3 online) show that the actin filament bundles to which VLN3 localizes, relocate over time.

**The headpiece region of VLN3 is required for bundling of actin filaments**

Next to its actin filament bundling capacity, which is independent of Ca\(^{2+}\) levels (Khurana et al., 2010), VLN3 has actin filament severing properties, and this activity is Ca\(^{2+}\)-dependent (Khurana et al., 2010). Since the mutations in VLN2 and VLN3 result in an actin cytoskeleton organization that is virtually devoid of thick actin filament bundles, we propose that the absence of villin’s bundling activity plays a major role in causing the morphological phenotype. The fact that the mutations affect the actin cytoskeleton organization also at locations where Ca\(^{2+}\) is at the basal level, while VLN3 shows only severing activity at high Ca\(^{2+}\) concentrations (Khurana et al., 2010) is in agreement with the hypothesis that villin’s bundling rather than its severing activity causes the developmental problem in the double mutant. It is likely that plant villins require the headpiece region for actin filament bundling. Both the core and the headpiece region of vertebrate villins can bind to filamentous actin, and the headpiece region of vertebrate villin is crucial for its bundling capacity (Glenney and Weber, 1981). We therefore hypothesized that in plant cells, villin’s headpiece region plays an important role in the generation of actin filament bundles. To obtain more insight into the function of the headpiece region of *Arabidopsis* VLN3, we performed a complementation analysis with 3 different constructs lacking the DNA that encodes the villin headpiece region, all driven by the endogenous VLN3 promoter: *PVLN3:VLN3ΔHP* genomic, *PVLN3:GFP:VLN3ΔHP* genomic, and *PVLN3:GFP:VLN3ΔHP* cDNA. All these constructs were unable to rescue the phenotype (*PVLN3:VLN3ΔHP* genomic: 43 independent transformants; *PVLN3:GFP:VLN3ΔHP* genomic: 56 independent transformants; *PVLN3:GFP:VLN3ΔHP* cDNA: 34 independent transformants). In addition, in contrast to GFP:VLN3, which localizes to (bundles of) actin filaments, GFP:VLN3ΔHP fluorescence is equally distributed throughout the cytoplasm (Figure 7). To test whether the cytoplasmic fluorescence represents full length GFP:VLN3ΔHP, we performed Western blotting with an antibody against GFP. Figure 7A shows that plants expressing VLN3ΔHP produce a fusion protein of approximately 120 kD, which is the expected mass of GFP:VLN3ΔHP. This shows that the cytoplasmic distribution is due to the absence of the headpiece and not caused
by truncations in the GFP:VLN3ΔHP gene product. We conclude that VLN3 requires the headpiece region for a correct localization to actin filament bundles, for actin filament bundling, and for its function in directional organ growth.
Discussion
The actin cytoskeleton plays a key role in plant cell growth and morphogenesis. Although in virtually all plant cells actin filament bundling occurs (Thomas et al., 2009), it is unknown how actin filament bundles are generated by actin bundling proteins. In this study, we investigated the role of two villins in *Arabidopsis*, and show that the absence of these villins results in a low abundance of thick actin filament bundles. *vln2 vln3* plants have twisted leaves, stems, siliques and roots, implying an important role for villin in the regulation of directional organ growth. Truncated VLN3 lacking the headpiece region is, in contrast to full-length VLN3, not able to rescue the phenotype, and *in vitro* experiments show that the headpiece region is essential for actin filament bundling. These data show that villin is involved in the generation of actin filament bundles, and suggest that villin-mediated actin filament bundling is required for the regulation of coordinated cell expansion.

**VLN2 and VLN3 play a role in actin filament organization in *Arabidopsis***
The presence of the T-DNA insertions in *VLN2* and *VLN3* affects the actin organization in several cell types. In cells of the double mutant, thick actin filament bundles are virtually absent, whereas thin bundles are more abundant. The fact that the double mutant still contains thin actin filament bundles points to the combined action of VLN2 and VLN3 with that of another actin bundling protein in the generation of actin filament bundles in plant cells. Since *VLN5* is preferentially expressed in pollen (Zhang et al., 2010), VLN1 and VLN4 are good candidates to work cooperatively with VLN2 and VLN3. Alternatively, another class of actin bundling proteins could be involved in the generation of actin filament bundles in plant cells. In vertebrate cells, also different actin bundling proteins are generally present in the same actin filament bundles (Tilney et al., 1998; Bartles, 2000) and these proteins do not act redundantly. *In vitro* experiments showed that small rigid actin-bundling proteins can generate small bundles with a finite thickness of approximately 20 filaments (Claessens et al., 2008). Other actin bundling proteins were shown to be able to link these small bundles into larger bundles of several hundreds of actin filaments (Claessens et al., 2008). In plants, actin filament bundles could be generated in a comparable way. Villins might work coordinately with fimbrins (Kovar et al., 2000), formins (Cheung and Wu, 2004; Favery et al., 2004; Michelot et al., 2005; Ye et al., 2009), LIM proteins (Thomas et al., 2006; Thomas et
al., 2007), and/or elongation factor 1 alpha (Collings et al., 1994) in the formation of thick actin filament bundles. Consistent with this idea, fimbrin has been proposed to cross-link actin filament bundles generated by other actin bundling proteins, such as villin (Matova et al., 1999; Wu et al., 2010). The silencing of GFP:FABD2 expression in the vln2 vln3 double mutant that we observed could be a consequence of simultaneous inhibition of VLN2 and VLN3 expression and disruption of fimbrin binding to actin filaments by GFP:FABD2, which consists of the second actin binding domain of Arabidopsis fimbrin 1 (Ketelaar et al., 2004). Together, these disruptions may cause insurmountable actin bundling problems.

Interestingly, the vln2 vln3 double mutant does not display a trichome phenotype. Trichome development is strongly affected by disruption of the actin cytoskeleton, resulting in distorted trichomes (Szymanski et al., 1999; Mathur et al., 1999). The Arp2/3 complex has been identified as a key factor in actin organization in trichomes and trichome development (Le et al., 2003; Li et al., 2003; Mathur et al., 2003). Since actin defects cause a well-defined trichome phenotype that is absent in the vln2 vln3 double mutant, together with the lack of detectable PVLN3:GFP:VLN3 expression in trichomes, is unlikely that VLN2 and VLN3 contribute to actin organization during trichome development.

Besides villin’s role in actin filament bundling, it is likely to play additional roles in actin organization. In addition to its bundling capacity, which is independent on Ca\(^{2+}\) levels, VLN3 has been shown to have actin filament severing properties, and this activity is Ca\(^{2+}\)-dependent (Khurana et al., 2010). VLN4, which is expressed in root hairs (Zhang et al., 2011), and VLN5, which is highly expressed in pollen tubes (Zhang et al., 2010), have similar properties: they both bundle actin filaments in a Ca\(^{2+}\)-independent manner, but have actin filament severing capacity only at high (micromolar and millimolar) Ca\(^{2+}\) concentrations (Zhang et al., 2010). In addition, these villins have actin filament capping activity. The lily villin P-135-ABP has been shown to have actin filament nucleating, depolymerizing, and capping activity, and these activities were Ca\(^{2+}\)/calmodulin-dependent. Although the authors state that the nucleating capacity is probably not relevant in vivo (since the nucleation was not accelerated when G-actin was saturated with profilin, which is the case in plant cells), the depolymerizing and capping activity might enhance actin dynamics in the apical
region of tip-growing cells, where Ca$^{2+}$ is abundant (Yokota et al., 2005). Zhang et al. (2011) predicted that VLN4, which is involved in the generation and/or maintenance of actin filament bundles in the shank of root hairs (Zhang et al., 2011), participates in the regulation of actin cytoskeleton organization in the subapical and apical region of root hairs by its bundling, capping and/or severing activity. Likewise, VLN5 has been proposed to bundle actin filaments in the shank of pollen tubes, while enhancing actin dynamics in the apical region, by severing and capping of actin filaments (Zhang et al., 2010). VLN3 (and perhaps also VLN2) could, besides being involved in the generation of actin filament bundles, locally also play a role in enhancing actin dynamics.

Although the localization of GFP:VLN3 to (bundles of) actin filaments in the shank of root hairs shows that VLN3 is expressed in root hairs, root hair growth and morphology, which are very sensitive to changes in actin filament organization, are not affected by the mutations in VLN2 and VLN3. This might mean that the proteins act redundantly with another villin in root hairs. VLN5 is preferentially expressed in pollen and pollen tubes (Zhang et al., 2010), and therefore not likely to act redundantly with VLN2 and VLN3 in root hairs. VLN1, which is Ca$^{2+}$- independent, has only actin filament bundling capacity (Huang et al., 2005), and VLN3 can sever actin filament bundles in the presence of VLN1 (Khurana et al., 2010), showing that the activities of VLN1 and VLN3 are not completely redundant. If VLN2 and VLN3 act redundantly with another villin in root hairs, VLN4, which is involved in actin filament bundling in root hairs (Zhang et al., 2011), would therefore be the best candidate. Alternatively, the fact that root hair growth and morphology are not affected by the mutations in VLN2 and VLN3 could mean that these villins are not essential for root hair growth and morphology. In intercalary growing cells, VLN2 and VLN3 are essential for the organization of actin filaments. Thick actin filament bundles are virtually absent in cells of the vln2 vln3 double mutant, and our data show that villin requires the headpiece region for localizing to (bundles of) actin filaments in vivo. This implies that although villin may play additional roles in actin organization, for instance by actin filament severing, villin’s bundling capacity plays a major role in its function in actin filament organization.

**Actin filament organization is required for plant growth polarity**
The actin cytoskeleton plays a key role in plant cell growth. It plays a fundamental role in the delivery of growth materials to exocytosis sites (Miller et al., 1997; Geitmann and Emons, 2000; Vidali and Hepler, 2001), not only because (bundles of) actin filaments serve as tracks for cytoplasmic streaming, but also because they optimize the cytoplasmic organization for cell growth. In addition, fine F-actin is thought to be important for the filtering and delivery of Golgi-derived vesicles (Miller et al 1999) that contain cell wall matrix materials in their lumen and the enzymes for callose and cellulose production in their membrane. Our data show that the activities of VLN2 and VLN3 are required for the organization of the actin cytoskeleton. In the absence of VLN2 and VLN3 proteins, thick actin filament bundles are virtually absent, while fine bundles are more abundant. Cell shapes and sizes, and plant growth rates are similar in Col-0 and double mutant plants. This shows that the thick actin filament bundles that are absent in the double mutant are not essential for cell and plant growth. However, the wavy, twisted, appearance of several organs in the double mutant, and the larger amplitudes of the rotational movements (circumnutation) of double mutant inflorescences, point to a role for VLN2 and VLN3 in coordinated cell elongation. Indeed, the organ twisting in vln2 vln3 results from subtle changes in cell size in opposite locations of the organs, which we show for root epidermal cells. It is not clear how villin mediated actin filament bundling regulates coordinated cell expansion. We show that it does so by altering the organization of the actin cytoskeleton. The altered actin cytoskeleton organization in the double mutant might have effects on the direction of transport routes, and/or the proper allocation of Golgi vesicles in the vicinity of the plasma membrane. In conclusion, our results show that villin is involved in the generation of thick actin filament bundles and suggest that these bundles are, directly or indirectly, important for coordinated cell expansion.
Material and methods

Growth conditions, plant strains, allele characterization and creation of double mutants

Seeds were sterilized for 1 minute with 70%-ethanol, followed by a 3-5-minute treatment with 15-20% household bleach (4% hypochlorite) and 0.05% triton X-100. After sterilization, the seeds were stratified at 4°C for 2-4 days, and germinated on 0.5 MS plates containing 0.7% agarose. After 1 week, seedlings were transplanted to potting compost. For live cell visualization of root epidermal cells, hypocotyl epidermal cells and leaf pavement cells, seeds were germinated on 0.5 MS plates containing 1.5% agarose, which were placed at an oblique angle (approximately 15-30° off vertical). For root hair imaging, seeds were sown on tilted coverslips containing a thin 0.7% agarose layer of Hoaglands’ medium, covered with biofoil (Vivascience, Göttingen, Germany). Root hairs grew along the coverslip, and were imaged 3-4 days after planting. Colocalization of GFP:VLN3 and mCherry:FABD2 was performed by *Agrobacterium tumefaciens* -injection in tobacco (*Nicotiana benthamiana*) leaves as described by Bouwmeester et al. (PlosOne, accepted). All plants were grown at 25°C (16 h light, 8 h darkness).

The T-DNA insertion lines (both in a Col-0 background) for VLN2 (SAIL_813_H02) and VLN3 (SALK_117097) were obtained from the Nottingham Arabidopsis Stock Centre (NASC; (Scholl et al., 2000)). 4-6-week old leaves were used to isolate genomic DNA, which was used to confirm the T-DNA insertions (Supplemental Figure 4 online) by PCR using T-DNA left-border-specific primer LB3 (for SAIL_813_H02) or LBA1 (for SALK_117097) and VLN specific primers (Supplemental Figure 5 and Table 1 online) flanking the insertions. Homozygous mutants were identified in F3 progeny.

To analyze the expression of VLN2 and VLN3, RNA was extracted from leaves of the homozygous T-DNA insertion mutants using a QUIAGEN RNeasy Mini Kit. Total RNA was reverse transcribed into cDNA with Superscript II Reverse Transcriptase (Invitrogen), and eluted in 20 μL DEPC-treated H2O. A volume of 1 μL of the total cDNA was used in RT-PCR reactions using primer combinations designed for coding regions before and after the T-DNA inserts (Supplemental Figure 5 and Table 1 online).
Complementation analyses

Primers that included GATEWAY sequences (Invitrogen) were used to amplify genomic VLN3 including the promoter region (2299 bp upstream of the ATG) and terminator region (1228 bp including the stop codon) as annotated by The Arabidopsis Information Resource (TAIR; www.arabidopsis.org), which was recombined into pDONR207 (Invitrogen), followed by recombination into pMDC99 (Curtis and Grossniklaus, 2003). Genomic VLN3 including the promoter but lacking the headpiece encoding region and terminator region (which lacks the last 606 bp of coding region of VLN3 including introns) was recombined into pDONR207, followed by a recombination into pMDC32 (Curtis and Grossniklaus, 2003), from which we deleted the 2 x 35S promoter. The same adapted version of pMDC32 was used for recombination of VLN2 including the promoter (3902 bp upstream of the ATG) but lacking the stop codon and terminator region.

To express GFP:VLN3 and GFP:VLN3ΔHP in the vln2 vln3 double mutant, genomic VLN3 lacking the promoter and terminator region, as well as genomic VLN3 lacking the promoter, headpiece encoding region, and terminator region were amplified by PCR and recombined into pDONR207, followed by a recombination into pMDC43 (Curtis and Grossniklaus, 2003) of which the 2 x 35S was replaced by the endogenous VLN3 promoter. The same adapted version of pMDC43 was used for recombination (using pDONR221 (Invitrogen) as entry clone) of coding sequences of VLN3 and VLN3ΔHP, which were amplified from cDNA. All constructs were transformed into the vln2 vln3 double mutant by Agrobacterium tumefaciens mediated transformation using the floral dip method (Clough and Bent, 1998). Primer sequences are shown in Supplemental Table 2.

Phenotype analysis and confocal microscopy

To visualize the actin cytoskeleton in the single mutants, we crossed the vln2 and vln3 single mutants with wild-type Col-0 plants expressing P35S:GFP:FABD (Ketelaar et al., 2004). In the F2-generation, homozygous lines were identified by genotyping and selected for GFP:FABD2 expression. A double mutant line with GFP:FABD2 expression was obtained by Agrobacterium tumefaciens mediated transformation of
*P35S:GFP:FABD2* into the *vln2 vln3* double mutant using the floral dip method (Clough and Bent, 1998).

For live cell imaging of GFP:FABD2 and GFP:VLN3 localization, 3-5 day old plants were used. Root hairs were imaged with a I-LAS Spinning Disk Confocal System (Roper Scientific SAS, France) on a Nikon Eclipse Ti microscope using a 60x (N.A. 1.4) oil immersion objective. Root epidermal cells, hypocotyl epidermal cells, leaf pavement cells, and GFP:VLN3 and mCherry:FABD2 colocalization were imaged with an Axiovert 200M microscope (Zeiss, Jena, Germany) connected to a Zeiss LSM510 META confocal scanning system equipped with a 63x (N.A. 1.4) oil immersion objective. Cell dimensions of leaf and fruit stalk epidermal cells were imaged with a Nikon Eclipse 80i microscope, using a 10x (N.A. 0.3) objective, and traced in image J. Circularity reflects the ratio of cell area to cell perimeter, and is defined as $4\pi \text{Area}/\text{Perimeter}^2$ (Brembu et al., 2004; Vidali et al., 2007).

Thickness of actin filament bundles was quantified by creating intensity profiles of GFP fluorescence intensities of *P35S:GFP:FABD* expressing hypocotyl cells, in the middle of Z-projections of the cortical cytoplasm, perpendicular to the longitudinal cell axis (excluding the bright cell edges). To correct for differences in GFP:FABD2 intensity, we selected an area in which no actin filaments were visible, and subtracted the mean fluorescence intensity of this region from the fluorescence intensities of the intensity profile. This resulted in a new plot profile, which was used to distribute the peaks in three classes: low (0-40 arbitrary units), medium (40-80 arbitrary units) and high (8-120 arbitrary units) grey levels (8-bit files were used). Only peaks that were at least 10 units higher in fluorescence intensity then the intensities of the left and right basis of the peaks were included.

To determine skewness and occupancy of the actin cytoskeleton in hypocotyl epidermal cells, maximum intensity Z-projections we manually segmented cells such that cell borders were excluded. The resulting images were processed by using rolling ball background substraction (ball radius: 20 px) and Gaussian blurring (radius: 1 px). Further image analysis was performed as described by Higaki et al. (2010). For hypocotyl cells, sample sizes were 100 cells from 7 different plants for Col-0 and 50 cells from 35 different plants for *vln2 vln3*; and for growing root epidermal cells,
sample sizes were 50 cells from 7 different plants for Col-0 and 35 cells from 20
different plants for *vln2 vln3*: and for growing root epidermal cells). Two-tailed,
unpaired T-tests for calculation of significance.

**Accession numbers**

Sequence data from this article can be found in GenBank/EMBL data libraries or The
Arabidopsis Information Resource (TAIR) under accession numbers At2g41740 or
NP_565958.1 (*Arabidopsis* VLN2) and At3g57410 or NP_567048.1 (*Arabidopsis*
VLN3).
Supplemental material

Supplemental Figure 1: Etiolated hypocotyls of the vln2 vln3 double mutant curl (A and B) and root gravitropism is not affected in the vln2 vln3 double mutant (C and D).

Supplemental Figure 2: Phenotype analysis of mutant complementation with different constructs.

Supplemental Figure 3: the mutations in VLN2 and VLN3 do not affect root hair growth.

Supplemental Figure 4: Molecular characterization of villin T-DNA insertion alleles. For primer sequences, see Supplemental table 1.

Supplemental Figure 5: Locations of primers used for molecular characterization of villin T-DNA insertion alleles. For primer sequences see Supplemental table 2.

Supplemental Table 1: Sequences of primers used for molecular characterization of villin T-DNA insertion alleles.

Supplemental Table 2: Sequences of primers used for complementation experiments and bacterial protein expression.

Supplemental Movie 1: Time lapse recording of Col-0 and vln2 vln3 double mutant plants.

Supplemental Movie 2: Time lapse recording of GFP:VLN3 in root epidermal cells.

Supplemental Movie 3: Time lapse recording of GFP:VLN3 in root hairs.

Acknowledgements

We thank Chris Staiger (Department of Biological Sciences, Purdue University) for the kind gift of the anti-lily villin antibody (Tominaga et al., 2000) and for sharing unpublished results and Dr. Takumi Higaki for help with determining the skewness
parameter. Sjoerd Derksen is gratefully acknowledged for the images shown in Figure 2D, Klaas Bouwmeester for help with the leaf infiltration experiments, and Ying Zhang for assistance with root hair growing experiments.
Literature cited


Bretsch A, Weber K (1980) Villin is a major protein of the microvillus cytoskeleton which binds both G and F actin in a calcium-dependent manner. Cell 20: 839-847


Figure legends

**Figure 1.** Characterization of the *Arabidopsis* villin gene family and T-DNA insertions in *vln2* and *vln3*. (A) Cladogram of the Arabidopsis villins, based on cDNA sequences. (B) Locations of T-DNA inserts in *vln2* and *vln3*. Grey boxes represent exons, and horizontal lines represent introns. T-DNA inserts (arrowheads) are not drawn to scale. (C) Domain structure of villin. Arrowheads show locations corresponding to the locations of T-DNA inserts of *vln2* and *vln3*. (D) T-DNA insertions result in truncated transcripts in *vln2*, *vln3*, and *vln2 vln3*. Products could be amplified using a cDNA template using VLN specific primers before the inserts, but when both primers (*vln2*) or the reverse primer (*vln3*) were designated for coding regions after the insert (see Supplemental Figure 3 and Table 1 online), products could not be amplified. (E) A protein gel blot of Col-0, *vln2*, *vln3*, and *vln2 vln3* root extracts probed with lily anti-villin antibody (Tominaga et al., 2000) shows that *vln3* and *vln2 vln3* do not contain (a truncated version of) the VLN3 protein.

**Figure 2: Phenotype of vln2 vln3.** (A) Root morphology of azygous, *vln2*, *vln3* and *vln2 vln3* plants. Roots of both single mutants have the same appearance as azygous roots, but roots of double mutants grow in a curly, wavy manner. (B) Phenotype of 2-week-old plants. Leaves of the *vln2 vln3* double mutant are twisted, but in single mutants this twisting is absent. (C) Phenotype of 5-week-old plants. Branches of single mutants grow straight, similar to those of azygous plants, but in the double mutants, branches are curly, and even show complete twists (e.g. arrow). This twisting also occurs in the fruit stalks. (D) Twisting of double mutant branches and fruit stalks shown at a higher magnification. (E) The rotational movements (circumnutation) of *vln2 vln3* inflorescences have larger amplitudes than those of Col-0 inflorescences and are less regular (see also Supplemental Movie 1 online).

**Figure 3: Quantification of vln2 vln3 phenotype.** (A) 41 % (*n* = 17) of the tops of inflorescence meristems of *vln2 vln3* grow downward, while this never occurs in Col-0 (*n* = 22) and single mutant (*n* = 19 for *vln2*, and 17 for *vln3*) plants. (B) The angle of siliques with respect to the plant axis of the *vln2 vln3* double mutant is less regular than that of Col-0 and single mutant plants: siliques of *vln2 vln3* grow in all directions at similar frequencies, while those of Col-0 and single mutant plants preferentially
grow upward at an oblique angle. (C, D) Leaf pavement (C; \( n = 26 \) for Col-0 plants, and \( n = 61 \) for \( vln2 \ vln3 \)) and fruit stalk epidermal (D; \( n = 68 \) for azygous, and \( n = 67 \) for \( vln2 \ vln3 \)) cell dimensions of \( vln2 \ vln3 \) are similar (Student’s \( t \) tests, \( P > 0.05 \)) to those of Col-0 plants, except for fruit stalk epidermal cell width, which is significantly higher (Student’s \( t \) tests, \( P = 0.01 \)) to in \( vln2 \ vln3 \). Circularity reflects the ratio of cell area to cell perimeter, and is defined as \( 4\pi \text{Area}/\text{Perimeter}^2 \) (Vidali et al., 2007). Error bars in C and D represent standard deviations.

**Figure 4:** thick actin bundles are absent in \( vln2 \ vln3 \), but thin bundles of actin filaments are more prominent. (A-L) The actin organization (visualized with GFP:FABD2) in cells of both single mutants (D-I) is similar to that in Col-0 cells (A-C): thick bundles of actin filaments are alternated by a more complex network of thin (bundles of) actin filaments. In the double mutant (J-L), thick actin filament bundles appear to be absent, while thin actin filament bundles seem more prominent. (M-O) Representative intensity profiles of fluorescence intensity in a Col-0 (M) and a \( vln2 \ vln3 \) (N) fully elongated hypocotyl cell. High peaks represent thick actin filament bundles, while lower peaks represent thinner bundles. The yellow lines in M and N show the location of the intensity profile in figure O. (P) Frequency distribution of peaks belonging to 3 fluorescence intensity classes (determined for 6 cells for each genotype) in Col-0 and \( vln2 \ vln3 \). In Col-0 cells, peaks with a fluorescence intensity of 80-120 (representing thick actin filament bundles) are more abundant than in \( vln2 \ vln3 \), while peaks with a fluorescence intensity of 0-40 (representing thin(ner) actin filament bundles) are more abundant in \( vln2 \ vln3 \). (Q) The number of peaks per micrometer shown for Col-0 and \( vln2 \ vln3 \). \( vln2 \ vln3 \) cells contain significantly more (Student’s \( t \) test; \( p = 0.04 \)) actin filament bundles than Col-0 cells. Bars in A-N = 10 \( \mu \text{m} \). Error bars in Q represent standard errors.

**Figure 5:** The irregular organ growth phenotype correlates with defects in actin organization in expanding cells. The actin organization in elongating root epidermal cells of the \( vln2 \ vln3 \) mutant (B) is disrupted when compared to the actin organization of Col-0 (A) cells. The defects in actin organization resemble those in fully grown cells. The actin organization is visualized by GFP-FABD2 expression. Bars = 20 \( \mu \text{m} \).
Figure 6: GFP:VLN3, expressed under control of the VLN3 promoter, localizes to (bundles of) actin filaments. Representative images of complemented *vln2 vln3* plants show that besides a cytoplasmic localization, GFP:VLN3 decorates (bundles) of actin filaments in hypocotyl epidermal (A), leaf epidermal (B) and root epidermal (C, D) cells. These images show fully grown hypocotyl epidermal (A) and leaf epidermal cells (B) and elongating root epidermal cells (C, D). In growing root hairs (D), GFP:VLN3 localizes to the long actin filament bundles oriented longitudinally to the cell’s long axis in the root hair tube. (E-G) Coexpression of *PVLN3:GFP:VLN3* and *P35S:mCherry:FABD2* in tobacco (*Nicotiana benthamiana*) demonstrates that GFP:VLN3 (E) and mCherry:FABD2 (F) colocalize (e.g., arrows) in leaf epidermal cells, although GFP:VLN3 does not localize to all actin filaments (e.g., arrowheads). (G) Overlay of E and F (GFP:VLN3: green; mCerry:FABD2: magenta). Image sequences of elongating root epidermal cells (H-J; K-M: root hairs) of complemented *vln2 vln3* plants show that GFP:VLN3 localizes to (bundles of) actin filaments that reorganize over time. See also Supplemental Movies 2 and 3 online. Bar = 10 μm.

Figure 7: GFP:VLN3ΔHP, expressed under control of the VLN3 promoter, shows a cytoplasmic localization. Western blotting with an anti-GFP antibody reveals that GFP:VLN3 expressing plants express a fusion protein of approximately 137 kD, the expected mass of GFP:VLN3. GFP:VLN3ΔHP expressing plants express a fusion protein of approximately 120 kD, which is the expected mass of GFP:VLN3ΔHP (A). Representative images of leaf epidermal (B), hypocotyl epidermal (C), and root epidermal (D) cells (E: root hair) of *vln2 vln3* plants in which *GFP:VLN3ΔHP* is expressed. In these plants, which are not rescued, GFP:VLN3ΔHP fluorescence is equally distributed throughout the cytoplasm. Bar = 10 μm.