Protein phosphatase 2A regulates the nuclear accumulation of the Arabidopsis bZIP protein VIP1 under hypo-osmotic stress

Daisuke Tsugama1,2,* Hyuk Sung Yoon1,2, Kaien Fujino2, Shenkui Liu3 and Tetsuo Takano1
1 Asian Natural Environmental Science Center (ANESC), The University of Tokyo, 1-1-1 Midori-cho, Nishitokyo-shi, Tokyo 188-0002, Japan
2 Laboratory of Crop Physiology, Research Faculty of Agriculture, Hokkaido University, Kita 9 Nishi 9 Kita-ku, Sapporo-shi, Hokkaido 060-8589, Japan
3 State Key Laboratory of Subtropical Silviculture, Zhejiang A & F University, Lin’an, Hangzhou 311300, PR China

* Correspondence: dtsugama@anesc.u-tokyo.ac.jp

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Abstract

VIP1 is a bZIP transcription factor in Arabidopsis thaliana. When cells are exposed to mechanical stress, VIP1 transiently accumulates in the nucleus, where it regulates the expression of its target genes and suppresses mechanical stress-induced root waving. The nuclear–cytoplasmic shuttling of VIP1 is regulated by phosphorylation and calcium-dependent signaling, but specific regulators of these processes remain to be identified. Here, inhibitors of protein phosphatase 2A (PP2A) are shown to inhibit both the mechanical stress-induced dephosphorylation and nuclear accumulation of VIP1. The PP2A B subunit, which recruits substrates of PP2A holoenzyme, is classified into B, B', B'', and B''' families. Using bimolecular fluorescence complementation, in vitro pull-down, and yeast two-hybrid assays, we show that VIP1 interacts with at least two of the six members of the Arabidopsis PP2A B''-family subunit, which have calcium-binding EF-hand motifs. VIP1AAA, a constitutively nuclear-localized VIP1 variant with substitutions in putative phosphorylation sites of VIP1, suppressed the root waving induced by VIP1–SRDX (a repression domain-fused variant of VIP1). These results support the idea that VIP1 is dephosphorylated by PP2A and that the dephosphorylation suppresses the root waving. The phosphorylation sites of VIP1 and its homologs were narrowed down by in vitro phosphorylation, yeast two-hybrid, and protein subcellular localization assays.

Keywords: Arabidopsis thaliana, calcium, mechanical stress, nuclear–cytoplasmic shuttling, protein phosphatase 2A, root tropisms, transcription factor

Introduction

VIP1 (VirE2-INTERACTING PROTEIN 1) is a basic leucine zipper (bZIP) transcription factor in Arabidopsis thaliana and has pleiotropic roles in Agrobacterium responses [Tzfira et al., 2001, 2002, 2004; Lacroix et al., 2005; Li et al., 2005; Djamei et al., 2007; Pitzschke et al., 2009, although this is questioned (Shi et al., 2014; Lapham et al., 2018)], biotic stress responses (Lapham et al., 2018), and sulfur deficiency responses (Wu et al., 2010). One of VIP1’s roles is to regulate a mechanical stress response in roots. One response to mechanical stress is touch-induced root waving, in which the root bends...
when it encounters an immovable object. VIP1–SRDX is a variant of VIP1 that has the transcriptional repression domain SRDX in the C-terminus. Overexpression of VIP1–SRDX disturbs the expression of VIP1 target genes and enhances touch-induced root waving (Tsugama et al., 2016a). Under steady-state conditions, VIP1 is phosphorylated and retained in the cytoplasm by 14–3–3 proteins (Tsugama et al., 2012a; Takeo and Ito, 2017). When cells are either touched or exposed to hypo-osmotic stress, which at least partially mimics mechanical stress (Shih et al., 2014), VIP1 transiently accumulates in the nucleus (Tsugama et al., 2012a, 2016b). Together, these findings support the idea that VIP1 suppresses touch-induced root bending after such bending is initiated. The hypo-osmotic stress induces VIP1 dephosphorylation and dissociation from 14–3–3 proteins, thereby causing the VIP1 nuclear accumulation (Takeo and Ito, 2017). The hypo-osmotic stress-induced nuclear accumulation of VIP1 also requires calcium signaling. This calcium signaling appears to be mediated by calmodulins, which bind calcium with their EF-hand motifs, but is not mediated by either the mecanosensitive calcium channel MCA1 (MID1-COMPLEMENTING ACTIVITY 1) or its close homolog MCA2 (Tsugama et al., 2018a). Specific regulators of the mechanical stress-induced dephosphorylation and nuclear localization of VIP1 remain to be identified. Identification and characterization of such regulators should help to better understand how plants respond to mechanical stress.

Arabidopsis has 12 members of Group I bZIP proteins, which consist of VIP1 and its close homologs (Jakoby et al., 2002; Tsugama et al., 2014). Of the genes encoding the group I bZIP proteins, five are hardly expressed in any tissues studied, whereas the other seven (VIP1, bZIP29, bZIP30, bZIP59, bZIP69, bZIP52, and bZIP18) are expressed in roots, leaves, flowers, shoot apical meristems, and root apical meristems (Tsugama et al., 2014; Cheng et al., 2017). Most of the proteins encoded by these seven genes share similar subcellular localization patterns and DNA binding specificity (Tsugama et al., 2014, 2016a). The VIP1 bZIP29 double knockout does not affect touch-induced root bending or any other plant phenotype (Tsugama et al., 2016a). Like VIP1–SRDX overexpression, the expression of bZIP29–SRDX fusion protein with the bZIP29 promoter enhances touch-induced root bending (Van Leene et al., 2016). Together, these findings support the idea that the group I bZIP proteins function redundantly in the mechanical stress response. Protein phosphatase 2A (PP2A) B*-family subunits, which can recruit substrates of the PP2A holoenzyme, were shown to interact with one of the group I bZIP proteins, bZIP29. This raises the possibility that not only VIP1 but also bZIP29 and other group I bZIP proteins are regulated by phosphorylation (Van Leene et al., 2016). However, the relevance of phosphorylation to these proteins remains to be elucidated.

Here, the nuclear accumulation of VIP1 and its close homologs under hypo-osmotic conditions is shown to be inhibited by okadaic acid and cantharidin, both of which strongly bind and inhibit the catalytic subunits of PP2A and protein phosphatase 4 (PP4), and less strongly inhibit protein phosphatase 1 (PP1) (for effects of okadaic acid and cantharidin, see Bialojan and Takai, 1988; Takai et al., 1992; Brewis et al., 1993; Honkanen, 1993). We also found that VIP1 interacts with PP2A B*-family subunits and that a VIP1 mutant with substitutions at the putative phosphorylation sites suppresses the VIP1–SRDX-dependent root bending. These results suggest that VIP1 dephosphorylation is a key event in the response of plants to mechanical stress.

### Materials and methods

**Plant materials, plant growth conditions, and evaluation of root VGIs**

*Arabidopsis thaliana* ecotype Col-0 was used as the wild-type control for all experiments. The seeds of the transgenic lines (#1 and #2) expressing the green fluorescent protein (GFP)–VIP1AAA fusion protein (Takeo and Ito, 2017) were provided by Dr Takeshi Ito (Hiroshima University, Japan). To confirm the expression of GFP–VIP1AAA, seedlings of ~10 d old of these lines were subjected to microscopy with a BX50 epifluorescence microscope (Olympus, Tokyo, Japan). Using this microscope, GFP–VIP1AAA could be detected without any treatment, even though GFP–VIP1AAA was under the control of an alcohol-inducible promoter (see Supplementary Fig. S1 at JXB online), suggesting that GFP–VIP1AAA is expressed at least at the seedling stage in these lines. The seeds of the *atb*δ mutant, which has a T-DNA insertion in one of the PP2A B*-subunit genes, *ATB*δ, and those of the *mlo4-11 mlo11-4 mlo14-7 (mlo4 11 14) triple mutant (Chen et al., 2009) were provided by the Arabidopsis Biological Resource Center (ABRC, https://abrc.osu.edu/). The ABRC stock number of *atb*δ is SALK_051304 and that of *mlo4 11 14* is CS66565. Transgenic Arabidopsis lines overexpressing GFP, VIP1–GFP, VIP1S115A–GFP, NLS–VIP1–GFP [a VIP1 variant fused to the nuclear localization signal (NLS) and GFP], bZIP50–GFP, bZIP29–GFP, bZIP52–GFP, bZIP69–GFP, bZIP30–GFP, and VIP1–SRDX were prepared as previously described (Tsugama et al., 2012a, 2014, 2016a). The transgenic line expressing either GFP or VIP1–GFP in the VIP1–SRDX-overexpressing (VIP1–SRDXox) background was prepared as previously described (Tsugama et al., 2016a). To maintain plants, seeds were surface-sterilized with 10% (v/v) sodium hypochlorite, and sown on medium containing 0.8% (w/v) agar (Fujifilm Wako Pure Chemical Co., Osaka, Japan), 0.5 × Murashige and Skoog (MS) salts (Fujifilm Wako) (Murashige and Skoog, 1962), 1% (w/v) sucrose (Fujifilm Wako), and 2 mM MES (Dojindo Laboratories Co., Mashiki-machi, Japan), pH 5.8, and incubated at 22 °C under a 16 h light/8 h dark photoperiod (light intensity: 120 µmol m−2 s−1) for 2–3 weeks. Plants were then transferred to rockwool cubes, and further grown at 22 °C under the same photoperiod with regular watering until they developed seeds.

To express either NLS–VIP1–GFP or GFP–VIP1AAA in the VIP1–SRDXox background, either the NLS–VIP1–GFP-overexpressing plants (line #4, which has a high NLS–VIP1–GFP expression level; Tsugama et al., 2014) or GFP–VIP1AAA-expressing plants (#1 and #2 described above) (as the pollen parents) were crossed with the VIP1–SRDXox plants (line #7, which has a severe root bending phenotype; Tsugama et al., 2016a) (as the pod parents). To express VIP1–GFP in either the *atb*δ background or *mlo4 11 14*, either *atb*δ or *mlo4 11 14* plants (as the pod parents) were crossed with VIP1–GFP-overexpressing (VIP1–GFPox) plants (as the pollen parents). GFP signals in the resulting F1 and F2 plants were briefly analyzed with the BX51 epifluorescence microscope as described below, and the plants with the GFP signals were further grown until flowering. Pieces (~1×1 mm²) of their cauline leaves were then sampled, and genomic DNA was extracted from them as previously described (Tsugama et al., 2018a). These DNA samples were used as templates for the genomic PCR with primers shown in Supplementary Table S1 to analyze the VIP1–GFP region and the T-DNA insertion in *ATB*δ, *MLO4*, *MLO11*, and *MLO14*. The plants that were confirmed to express VIP1–GFP in either the *atb*δ background or *mlo4 11 14* by these analyses were used for detection of VIP1–GFP signals under a hypo-osmotic condition.
To evaluate root vertical growth indices (VGIs), plants were grown for 10 d on MS agar medium [1.2% (w/v) agar, 0.5× MS salts, 1% (w/v) sucrose, 0.5 g 1 L MES, pH 5.8] tilted at a 60° angle. The plants were then photographed, and the lengths of the primary roots and of the vertical projection from those roots were measured with ImageJ software (Schneider et al., 2012). The vertical projection lengths were then divided by the primary root lengths to obtain the VGIs as previously described (Grabov et al., 2005).

Analyses of the subcellular localization of GFP-fused proteins

To examine the effects of okadaic acid and cantharidin, 10-day-old seedlings of the transgenic lines expressing GFP fusion proteins were incubated for 20 min at room temperature in a hypotonic solution (20 mM Tris–HCl, pH 7.5) with or without 5 µM okadaic acid (Fujifilm Wako) or 50 µM cantharidin (Fujifilm Wako). To examine the effects of inhibitors of protein kinases, those seedlings were incubated for 20 min at room temperature in the hypotonic solution, transferred to the hypotonic solution with or without 1 µM staurosporine (Fujifilm Wako) or 1 µM K-252a (Fujifilm Wako), and further incubated for 60 min at room temperature. GFP signals were detected with an epifluorescence microscope [BX51 (Olympus) equipped with the CCD camera DP73 (Olympus)]. The fluorescence mirror unit U-MWIB2 (Olympus) was used to image GFP. Images were processed with GIMP (http://www.gimp.org/) and Inkscape (http://www.inkscape.org).

To examine the effects of point mutations in VIP1 and bZIP52, the coding sequences (CDSs) for VIP1 (Arabidopsis Genome Initiative accession number: AT1G43700.1) variants with Ser→Ala substitutions at positions 35, 115, 149, and 151 of VIP1, and those for bZIP52 (AT1G66850.1) variants with Ser→Ala substitutions at positions 38, 40, 115, and 117 of bZIP52 were generated by PCR as described in Supplementary Table S3. The PCR products with the VIP1 variants were digested with Spel and SalI, and those with the bZIP52 variants were digested with XbaI and SalI. These DNA fragments were inserted into the XbaI–SalI site of the pHBl121-35SMCS-GFP vector (Tsugama et al., 2012b). The resulting constructs were introduced via agroinfiltration into lettuce (Lactuca sativa) leaf cells as previously described (Wroblewski et al., 2005, 2016a). The transformed lettuce cells were incubated in darkness at room temperature for 48 h, and GFP signals were detected as described above. Images were processed with GIMP and Inkscape.

Phos-tag SDS–PAGE and western blotting

Ten-day-old seedlings of the transgenic lines expressing GFP fusion proteins were treated with okadaic acid and cantharidin, staurosporine, or K-252a as described above. These plants (five plants for each line and each treatment) were then immediately submerged in 500 µl of cell lysis solution [0.1 M EDTA, 0.125 M Tris–HCl, pH 7.4, 10% (v/v) 2-mercaptoethanol, 4% (w/v) SDS, 5% (v/v) glycerol, and 0.005% (w/v) bromophenol blue] containing the anti-GFP pAb antibody (MBL Co., Ltd., Nagoya, Japan) at a concentration of 0.05% (v/v). The membrane was washed three times with TBST, and incubated for 30 min at room temperature in TBST containing the anti-IgG (H+L chain) (rabbit) pAb-HRP (horseradish peroxidase) antibody (MBL) at a concentration of 0.02% (v/v). The membrane was then washed three times with TBST, and signals were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA) and LumiVision Pro imager (Asin Seiki, Nagoya, Japan). Images were processed with GIMP and Inkscape.

Quantitative reverse transcription–PCR (qRT–PCR)

Ten-day-old seedlings were frozen in liquid nitrogen and ground to a fine powder with a pestle and mortar. Total RNA was extracted with a Nucleospin RNA Plant Kit (Macherey–Nagel Düren, Germany). cDNA was synthesized from 500 ng of total RNA with ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and oligo(dT)15 primer. The resulting cDNA solutions were diluted 5-fold with distilled water. The qRT–PCR was run with these cDNA solutions as templates, GoTaq qPCR Master Mix (Promega, Fitchburg, WI, USA), the CFX Connect real-time PCR detection system (Bio-Rad, Hercules, CA, USA), and primer pairs for either UBQ5 (AT3G26250.1) or VIP1–SRDX (above), and the primers for UBQ5 were 5′-GACGCCCTCATCTGGTCC-3′ and 5′-CCACAGTTGCGTTAG-3′, and that for VIP1–SRDX was 5′-AGGCGCCGGGATGAACTGAATG-3′ and 5′-CTATGTCGGAGCCCAAACCTTATGCTAAATCAGTCAGTGCGAC-3′. Relative expression levels were calculated using the comparative cycle threshold method with UBQ5 as the internal control.

Bimolecular fluorescence complementation (BiFC)

The CDSs of ATB6 (AT1G28900.1) and FAAS (AT5G18580.1) were amplified by PCR using primers listed in Supplementary Table S2 and the cDNA sample prepared as described above. The PCR products were digested with KpnI and SpeI, and inserted into the KpnI–SpeI site of the pBS-35SMCS-cYFP vector (Tsugama et al., 2012b). The resulting constructs were also introduced into onion epidermal cells with the Biolistic PDS-1000/He particle delivery system (Bio-Rad, Hercules, CA, USA). Cells were then incubated for 12 h at room temperature, and BiFC signals were observed using the BX50 epifluorescence microscope (Olympus) equipped with the fluorescence mirror units U-MGFPHQ (Olympus) (for detecting BiFC signals) and an ORCA-ER-1394 digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Images were processed with GIMP and Inkscape.

In vitro phosphorylation assays

The CDS of CPK21 (AT4G04720.1) was amplified by PCR using primers listed in Supplementary Table S2, and the cDNA sample prepared as described above. The PCR product was digested with BamHI and inserted into the BamHI site of pMAL-c5E (New England Biolabs, Ipswich, MA, USA), generating pBS-35S-ATB6-cYFP and pBS-35S-FAAS-cYFP. The CDS of VIP1 was inserted into the pBS-35SMCS-nYFP-2 vector as previously described (Tsugama et al., 2012b, 2014), generating pBS-35S-VIP1-nYFP. Either pBS-35S-nYFP-2 or pBS-35S-VIP1-nYFP (500 ng) was mixed with pBS-35S-MCS-cYFP, pBS-35S-ATB6-cYFP, or pBS-35S-FAAS-cYFP (500 ng), and bound to gold particles. These constructs were co-introduced into onion epidermal cells with the Biostic PDS-1000/He particle delivery system (Bio-Rad, Hercules, CA, USA). Cells were then incubated for 12 h at room temperature, and BiFC signals were observed using the BX50 epifluorescence microscope (Olympus) equipped with the fluorescence mirror units U-MGFPHQ (Olympus) (for detecting BiFC signals) and an ORCA-ER-1394 digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Images were processed with GIMP and Inkscape.
incubated at room temperature for 30 min, and centrifuged at 13 000 g for 5 min. MBP–CPK21 in the supernatant was bound to amylase resin (New England Biolabs), according to the manufacturer's instructions. The resin was washed three times with TBS, and resuspended in TBS containing 10 mM maltose to elute MBP–CPK21.

The CDSs of VIP1 variants with mutations in the putative phosphorylation sites were obtained by PCR, using the primers listed in Supplementary Table S3 and the pBl121-35SMCS-GFP constructs with VIP1 variants (see 'Analyses of subcellular localization of GFP-fused proteins'). The PCR products were digested with EcoRI and Sall, and inserted into the EcoRI–Sall site of the pGEX-5X-1 vector. The resulting constructs were transformed into BL21(DE3), and the glutathione S-transferase (GST)-fused VIP1 variants were all induced in *E. coli* cells and extracted from them as MBP–CPK21.

The GST-fused VIP1 variants were bound to glutathione Sepharose 4B resin (GE Healthcare) according to the manufacturer's instructions. The resin was then washed three times with TBS, and resuspended in a protein elution solution (20 mM reduced glutathione and 50 mM Tris–HCl, pH 8.0). The suspensions were centrifuged for 3 min at 10 000 g at room temperature, and supernatants were used as the solutions containing purified GST-fused proteins. For the phosphorylation reaction, CaCl₂ and ATP were added to the solution containing MBP–CPK21 to obtain final concentrations of 20 mM and 2 mM, respectively. The resulting solution was mixed with the solutions containing the purified GST fusion proteins in a 1:1 volume ratio, and incubated at room temperature for 30 min. Proteins in these solutions were separated by SDS–PAGE, transferred to Hybond-P membrane, and reacted with Phos-tag biotin BTL-104 (Fujifilm Wako), according to the manufacturer's instructions. The membrane was then washed three times with TBS, and incubated for 30 min at room temperature in TBS containing 10 mM maltose to purify the GST-fused proteins. For the phosphorylation reaction, CaCl₂ and ATP were added to the solution containing MBP–CPK21 to obtain final concentrations of 20 mM and 2 mM, respectively. The resulting solution was mixed with the solutions containing the purified GST fusion proteins in a 1:1 volume ratio, and incubated at room temperature for 30 min. Proteins in these solutions were separated by SDS–PAGE, transferred to Hybond-P membrane, and reacted with Phos-tag biotin BTL-104 (Fujifilm Wako), according to the manufacturer's instructions. The membrane was then washed three times with TBS, and incubated for 30 min at room temperature in TBS containing HRP–conjugated streptavidin (Thermo Fisher Scientific) at a concentration of 0.1% (v/v). The membrane was then washed three times with TBS, and signals were detected as described in 'Subcellular localization of GFP-fused proteins'. The membrane was then washed three times with TBS, and signals were detected as described in 'Phos-tag SDS–PAGE and western blotting'. After signal detection, the membrane was incubated at room temperature for 20 min in TBS containing 0.05% (v/v). The membrane was then washed three times with TBS, and signals were detected as described in 'Phos-tag SDS–PAGE and western blotting'. The GST-fused proteins in the phosphorylation reaction mixtures were detected by western blotting with peroxidase-conjugated anti-GST monoclonal antibody (Fujifilm Wako). Images were processed with GIMP and Inkscape. Relative signal intensities of the His-tagged proteins were obtained with ImageJ.

**Yeast two-hybrid (Y2H) assays**

The CDSs of *ATB''δ* and *FASS* were amplified by PCR, using the primers listed in Supplementary Table S2, and the cDNA sample was prepared as described above. The PCR product with *ATB''δ* was digested with *Ncol* and *XhoI*, and that with *FASS* was digested with *Ncol* and *SalI*. These DNA fragments were inserted into the *Ncol*–*XhoI* site of the pGADT7-Rec vector, and into the *Ncol*–*SalI* site of the pGBK7 T7 vector (Takara Bio USA, Inc., Mountain View, CA, USA), generating pGAD-ATB''δ, pGAD-FASS, pGBK-ATB''δ, and pGBK-FASS. The CDSs of GRF6 (AT5G10450.4), GRF8 (AT5G56430.3), BSL1 (AT4G0380.1), *ATB''α* (AT5G3470.1), and *ATB''β* (AT3G09880.1) were amplified by PCR using the primers listed in Supplementary Table S2 and the cDNA sample was prepared as described above. The PCR products for GRF6 and GRF8 were digested with *EcoRI* and *SalI*, and inserted into the *EcoRI*–*SalI* site of pGBK7 T7. The PCR products for BSL1, *ATB''α*, and *ATB''β* were digested with *XhoI* and *SalI*, and inserted into the *SalI* site of pGBK7 T7. The CDSs of VIP1 and bZIP52 variants with mutations in the putative phosphorylation sites were obtained by PCR using the primers listed in Supplementary Table S3 and the pBl121-35SMCS-GFP constructs with such VIP1 and bZIP52 variants (see 'Analyses of subcellular localization of GFP-fused proteins'). The resulting PCR products were digested with *Ncol* and *SalI*, and inserted into the *Ncol*–*SalI* site of pGADT7-Rec. The pGAD7 T7 construct with VIP1 and the pGBK7 T7 construct with FASS were prepared as previously described (Tsugama et al., 2012b). The pGBK7 T7 constructs with *ATB''α* and *ATB''β* were prepared as previously described (Tsugama et al., 2018). One of the pGAD7 T7 constructs and one of the pGBK7 T7 constructs were co-transformed into the yeast strain AH109 (Takara Bio USA). At least five colonies of transformed cells for each combination of constructs were streaked on synthetic dextrose medium lacking histidine and adenine to examine protein–protein interaction-dependent activation of reporter genes.

**Multiple alignment and motif scanning**

CLUSTAL W (Thompson et al., 1994) was used to generate the multiple alignment of amino acid sequences of ATB''δ, FASS, and ATB''β, and that of the amino acid sequences of VIP1, bZIP29, bZIP30, bZIP52, bZIP59, and bZIP69. The 14-3-3 interaction motifs <R[^DE]{0,2}> were amplified by PCR using the primers listed in Supplementary Table S1 and the pBI121-35SMCS-GFP constructs with each VIP1 and bZIP52 variant (see 'Analyses of subcellular localization of GFP-fused proteins'). The resulting PCR products were digested with *Ncol* and *SalI*, and inserted into the *Ncol*–*SalI* site of the pGAD7 T7 construct with VIP1 and the pGBK7 T7 construct with FASS were prepared as previously described (Tsugama et al., 2012b). The pGBK7 T7 constructs with *ATB''α* and *ATB''β* were prepared as previously described (Tsugama et al., 2018). One of the pGAD7 T7 constructs and one of the pGBK7 T7 constructs were co-transformed into the yeast strain AH109 (Takara Bio USA). At least five colonies of transformed cells for each combination of constructs were streaked on synthetic dextrose medium lacking histidine and adenine to examine protein–protein interaction-dependent activation of reporter genes.

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were identified with the Eukaryotic Linear Motif resource (http://elm.eu.org; Puntervoll et al., 2003; Dinkel et al., 2016) with the accession number ELME000417.

**Accession numbers**

Details regarding the sequences of the genes used in this study can be obtained with the following Arabidopsis Genome Initiative accession numbers: AT1G06070 (bZIP69), AT1G06850 (bZIP52), AT1G3700 (VIP1), AT2G21230 (bZIP39), AT2G31370 (bZIP59), AT2G40620 (bZIP18), AT4G03080 (BSL1), AT1G51690 (ATB8), AT1G17720 (ATB5), AT5G3470 (ATB8), AT3G09880 (ATB5), AT5G18580 (EASS), AT5G44090 (ATB8), AT1G03960 (ATB5), AT1G54450 (ATB5), AT5G28900 (ATB8), AT5G28850 (ATB5), AT1G11000 (MLO4), AT1G26700 (MLO14), AT5G53760 (MLO11), AT5G10450 (GRF6), AT5G65430 (GRF8), AT4G04720 (CPK21), and AT3G62250 (UBQ5).

**Results and Discussion**

**VIP1 is dephosphorylated by PP2A and interacts with PP2A B''-family subunits**

When transgenic plants overexpressing VIP1–GFP fusion protein (VIP1–GFPoX plants) were incubated for 20 min in a hypotonic solution, VIP1–GFP was detected in the nucleus (Fig. 1A, top panel). However, when either okadaic acid or cantharidin was present in the hypotonic solution, VIP1–GFP was mainly detected in the cytoplasm and rarely in the nucleus (Fig. 1A, middle and bottom panels). When VIP1–GFPoX plants were incubated for 20 min in a hypotonic solution to accumulate VIP1–GFP in the nucleus, and then incubated for 60 min in another hypotonic solution (‘second hypotonic solution’), VIP1–GFP was detected mainly in the cytoplasm (Fig. 1B, top panel). However, when either of two protein kinase inhibitors (staurosporine or K-252a) was present in the second hypotonic solution, VIP1–GFP was detected mainly in the nucleus (Fig. 1B, middle and bottom panels). Both the okadaic acid and cantharidin treatments decreased the mobility of VIP1–GFP in Phos–tag SDS–PAGE (Fig. 1C), whereas both the staurosporine and K-252a treatments increased the mobility of VIP1–GFP (Fig. 1D). These results confirm that dephosphorylation of VIP1 is necessary for its nuclear accumulation, and suggest that the VIP1 dephosphorylation is catalyzed by PP1, PP2A, or PP4.

The nuclear accumulation of VIP1–GFP requires calcium signaling and, among components of PP1, PP2A, and PP4, only the PP2A B''-family subunits of calcium-binding EF-hand motifs (Farkas et al., 2007). This led us to examine the interactions between VIP1 and two of the Arabidopsis PP2A B''-family subunits. Arabidopsis has six putative PP2A B''-family subunits [FASS (also known as TONNEAU 2 or EMBRYO DEFECTIVE 40) and ATB''α–ε]. ATB''α–ε are phylogenetically close to each other, and distinct from FASS (Supplementary Fig. S2). Among the six genes encoding these proteins, FASS is most strongly expressed, and even the FASS single knockout causes embryonic lethality (Torres-Ruiz and Jurgens, 1994; Cheng et al., 2017). All the other genes (ATB''α–ε) are also moderately expressed in at least the roots, leaves, and flowers (Cheng et al., 2017). FASS, which has strong effects on plant growth, and ATB''δ, which can represent ATB''α–ε, were chosen for the interaction assays with VIP1.

**Fig. 1.** Okadaic acid, cantharidin, and protein kinase inhibitors affect subcellular localization and phosphorylation states of VIP1. (A) Effects of okadaic acid and cantharidin on the hypotonic solution-induced nuclear accumulation of VIP1–GFP. Transgenic plants expressing VIP1–GFP were incubated for 20 min in a hypotonic solution containing either the protein phosphatase inhibitor (okadaic acid or cantharidin) or no inhibitor (‘Mock’), and GFP signals in their roots were detected by fluorescence microscopy. The experiment was performed three times with at least five individual plants for each treatment, and representative results are presented. Scale bars=100 µm. (B) Effects of protein kinase inhibitors on the cytoplasmic accumulation of VIP1–GFP. The VIP1–GFP-expressing plants were incubated for 20 min in a hypotonic solution to induce the nuclear accumulation of VIP1–GFP. The plants with nuclear-localized VIP1–GFP (‘Nuc. VIP1–GFP’) were then transferred to hypotonic solution containing either a protein kinase inhibitor (staurosporine or K-252a) or no inhibitor (‘Mock’), and incubated for 60 min. GFP signals in their roots were then detected by fluorescence microscopy. The experiment was performed three times with more than five individual plants for each treatment, and representative results are presented. Scale bars=100 µm. (C) Effects of okadaic acid and cantharidin on the phosphorylation states of VIP1–GFP. The VIP1–GFP-expressing plants were treated as described in (A). Proteins were then extracted, and separated using an acrylamide gel with or without Phos-tag acrylamide (+ Phos-tag or - Phos-tag, respectively). VIP1–GFP was detected by western blotting with an anti-GFP antibody. The arrowhead indicates the position of the phosphorylated form of VIP1–GFP. The experiment was performed three times, and a representative result is presented. (D) Effects of protein kinase inhibitors on the phosphorylation states of VIP1–GFP. The VIP1–GFP-expressing plants were treated as described in (B). VIP1–GFP in these plants was detected as described in (C). The arrowhead indicates the position of the phosphorylated form of VIP1–GFP. The experiment was performed three times, and a representative result is presented. (This figure is available in colour at JXB online.)
In BiFC assays, fluorescent signals were detected in the cytoplasm when the VIP1–nYFP (N-terminal half of yellow fluorescent protein) fusion protein was co-expressed with either the ATB''δ–cYFP (C-terminal half of YFP) fusion protein or FASS–cYFP in onion epidermal cells (Fig. 2A), which supports the idea that VIP1 interacts with PP2A B'' subunits in the cytoplasm in plant cells. In pull-down assays using GST–VIP1 fusion protein, the His–ATB''δ fusion protein and His–FASS were pulled down by GST–VIP1 but not by GST alone. The intensities of His–ATB''δ and His–FASS were both decreased when a divalent cation chelator (EDTA) was present in the reaction mixture (Fig. 2B), suggesting that VIP1 and PP2A B''-family subunits interact in vitro in a calcium-dependent manner. In a similar assay, the signal intensity of His–FASS was higher when His–FASS was reacted with the GST-fused VIP1C (amino acids 165–341 of VIP1) than when it was reacted with GST-fused VIP1N (amino acids 1–186 of VIP1) (Fig. 2C). This suggests that PP2A B''-family subunits interact more strongly with VIP1C than with VIP1N. This is consistent with a previous finding that calmodulins also interact more strongly with VIP1C than with VIP1N (Tsugama et al., 2018a). In Y2H assays, co-expression of the GAL4 activation domain (BD)-fused VIP1C with either the GAL4 activation domain (AD)–ATB''δ fusion protein or AD–FASS enabled yeast cells to survive on the selection medium (Fig. 2D), confirming that VIP1C can interact with PP2A B''-family subunits. BD–ATB''δ and BD–FASS fusion proteins both enabled yeast cells to survive on the selection medium (Supplementary Fig. S3), suggesting that these proteins can self-activate reporter genes and that they cannot be used to study protein–protein interactions with this Y2H system. BD–VIP1 and BD–VIP1N also self-activate reporter genes in the same Y2H system and cannot be used to study protein–protein interactions with Y2H (Tsugama et al., 2012a). It was therefore impossible to test the full-length VIP1 for Y2H interactions with ATB''δ and FASS. BD-fused forms of several proteins, including BSL1 (a protein consisting of the N-terminal Kelch-type β propeller and the C-terminal PP1-like domain), ATBα, ATBβ (PP2A B-family subunits), and ATB''α and ATB''β (PP2A B''-family subunits), were also subjected to similar Y2H assays with AD–VIP1, but none of them enabled yeast cells to survive on the selection medium (Supplementary Fig. S4). This supports the idea that VIP1 can interact only with PP2A B''-family subunits among PP1 and PP2A components. Taken together, it is likely that either PP2A or PP4 rather than PP1 promotes the dephosphorylation and the nuclear accumulation of VIP1.

![Fig. 2. VIP1 interacts with ATB''δ and FASS.](image-url)

(A) BiFC assays. nYFP-fused VIP1 was co-expressed with either cYFP-fused ATB''δ or cYFP-fused FASS in onion epidermal cells, and YFP signals were detected by fluorescence microscopy. Results with either nYFP alone or cYFP alone (‘Alone’) are shown as negative controls. For each combination of proteins, the experiment was performed three times, and a representative result is presented. Scale bars=100 μm. (B) In vitro pull-down assays. The presence and absence of GST–VIP1, CaCl₂, and EDTA in reaction mixtures are indicated as ‘+’ and ‘−’, respectively. For ‘GST–VIP1’, GST alone instead of GST–VIP1 was used for the reaction. Signals of His-ATB''δ and His-FASS were detected by western blotting with HisProbe-HRP, and signals of GST–VIP1 were detected by western blotting with an anti-GST antibody. GST–VIP1 was detected as ladder, probably as a result of protein degradation and/or unexpected termination of translation. The numbers on the right side of the images indicate molecular mass. Relative signal intensities (%) are presented on the bottoms of the images. The experiment was performed three times, and a representative result is presented. (C) An in vitro pull-down assay with truncated forms of VIP1. ‘N’ and ‘C’ indicate VIP1N (amino acids 1–186 of VIP1, ~18 kDa) and VIP1C (amino acids 165–341 of VIP1, ~20 kDa), respectively, which were used as GST-fused proteins for the assays. For ‘GST–VIP1−’, GST alone was used. His-FASS and the GST-fused proteins were detected as described in (B). Their approximate positions on the blot are indicated by ‘GST–VIP1N/C’ with an arrowhead. Relative signal intensities (%) are presented on the bottoms of the images. The experiment was performed three times, and a representative result is presented. (D) Y2H assays. Either AD–ATB''δ or AD–FASS was co-expressed with BD–VIP1C in the yeast strain AH109. The yeast cells were streaked onto medium either containing (Ade/His +) or lacking adenine and histidine (Ade/His −). The cells were then incubated for 5 d at 28 °C, and photographed. For each combination of proteins, at least five individual colonies were assessed, and representative results are presented. The results with AD alone and BD alone are presented as negative controls. (This figure is available in colour at JXB online.)
Sufficient changes in gene expression (Tsugama et al., 2016) and VIP1AAA, a VIP1 variant with Ser→Ala substitutions at all of these sites, is constitutively localized in the nucleus when expressed under the control of an alcohol-inducible promoter (Takeo and Ito, 2017; see also Supplementary Fig. S1). NLS–VIP1, another VIP1 variant with a Simian virus 40 NLS at the N-terminus, is also constitutively localized in the nucleus when expressed under the control of the Cauliflower mosaic virus 35S promoter (Tsugama et al., 2014). These proteins were expressed in a VIP1–SRDX-overexpressing (VIP1–SRDXox) background to examine whether they as well as VIP1–GFP can counteract the VIP1–SRDX–dependent enhancement of root waving. Signals of GFP–VIP1AAA in a resulting plant line were detectable even in the absence of exogenous addition of alcohol (Supplementary Fig. S1), suggesting that the alcohol-inducible promoter used can cause leaky expression of its downstream gene. The root VGI of seedlings of the transgenic plants with either the NLS–VIP1–GFP fusion protein or GFP–VIP1AAA was higher than that of plants expressing GFP alone in the VIP1–SRDXox background (‘control plants’) (Fig. 4A, B; Supplementary Fig. S5A). VIP1–SRDX expression in these plant lines was not significantly different (Fig. 4C). These results support the idea that the dephosphorylation of VIP1 and the subsequent nuclear accumulation of VIP1 suppress root waving. The root length of the seedlings of the VIP1–GFP-expressing plants and that of the NLS–VIP1–GFP-expressing plants were both similar to the root length of the control plants. However, the root length of the seedlings of the GFP–VIP1AAA-expressing plants was shorter than the root length of the control plants (Fig. 4D; Supplementary Fig. S5B). Similarly, plants expressing GFP–VIP1AAA in the wild-type background exhibited shorter roots than plants expressing GFP alone in the wild-type background (Supplementary Fig. S6). These results suggest that unlike either VIP1–GFP or NLS–VIP1, VIP1AAA can inhibit root growth at the seedling stage regardless of the genetic background. This raises the possibility that phosphorylation of VIP1 regulates not only VIP1 subcellular localization but also expression of VIP1 target genes. No difference in phenotype was observed between these lines at later developmental stages.

**Key phosphorylation sites of VIP1**

When transgenic plants expressing GFP-fused forms of several group I bZIP proteins (bZIP29, bZIP30, bZIP52, bZIP59, and bZIP69), as well as VIP1–GFP, were incubated for 10–20 min in a hypotonic solution, all of these proteins were detected in the nucleus (Tsugama et al., 2014, 2016a). These proteins were incubated in a hypotonic solution with cantharidin, GFP-fused forms of bZIP29, bZIP30, bZIP52, bZIP59, and bZIP69 were detected mainly in the cytoplasm and not in the nucleus (Fig. 5), suggesting that the PP2A-dependent dephosphorylation is also necessary for the nuclear accumulation of at least these group I bZIP proteins. VIP1 has three canonical 14–3–3 interaction motifs at the positions corresponding to S35, S115, and S151 (Takeo and Ito, 2017). Two of these interaction motifs, those containing S35 and S151, appear to be shared by bZIP29, bZIP30, bZIP52, bZIP59, and bZIP69, whereas the motif containing S115 does not (Supplementary Fig. S7). VIP1 variants

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**Fig. 3.** CPK21 phosphorylates VIP1 in vitro. (A) An in vitro phosphorylation assay. The presence and absence of GST–VIP1 and MBP–CPK21 in reaction mixtures are indicated as ‘+’ and ‘−’, respectively. GST–VIP1 in the top image was detected by western blotting with an anti-GST antibody, and MBP–CPK21 in the middle panel was detected with an anti-MBP antibody. ‘P–MBP–CPK21’ and ‘P–GST–VIP1’ indicate phosphorylated MBP–CPK21 and phosphorylated GST–VIP1, respectively, and were detected by western blotting with Phos-tag biotin. The numbers on the right side of the images indicate molecular mass. The experiment was performed three times, and a representative result is presented. (B) An in vitro pull-down assay with phosphorylated GST–VIP1. GST–VIP1 was reacted with either MBP alone (‘Phosphorylation reaction with MBP–CPK21’ ‘−’ or MBP–CPK21 (+’), and subjected to a pull-down assay with His-FASS as described in the legend of Fig. 2B. Relative signal intensities (%) are presented on the bottom of the images. The experiment was performed three times, and a representative result is presented.

CPK21 phosphorylates VIP1 in vitro. GST–VIP1, after it had been pre-reacted with MBP–CPK21, pulled down His-FASS, as did GST–VIP1 after it had been pre-reacted with MBP alone (Fig. 3B). This suggests that phosphorylation of VIP1 does not affect its interactions with PP2A B” subunits. This is consistent with the findings that the putative phosphorylation sites of VIP1 are present in its N-terminal region (Takeo and Ito, 2017) and that the C-terminal region is where the PP2A B” subunits bind (Fig. 3C).

Dephosphorylation of VIP1 causes suppression of root bending

In the absence of hypo-osmotic or mechanical stress, VIP1–GFP is hardly detectable in the nucleus even in wavy roots, but it suppresses the VIP1–SRDX–dependent enhancement of root waving. This is probably because even under such a condition, a small amount of VIP1–GFP is present in the nucleus and causes sufficient changes in gene expression (Tsugama et al., 2016a). Serine residues at positions 35, 115, and 151 (S35, S115, and S151, respectively) of VIP1 are putative phosphorylation sites,
and bZIP52 variants with the Ser→Ala substitution at those sites (Table 1) were generated to narrow down key phosphorylation sites of these proteins. In western blotting with Phos-tag biotin, neither VIP1S35 115A nor VIP1QA, which has the Ser→Alan substitutions at S35, S115, S151, and the serine at position 149 [S149, which was identified as a phosphorylation site in previous phosphoproteome analyses (Nakagami et al., 2010; Hoehenwarter et al., 2013)], was detectable even after they were reacted with MBP–CPK21 (Fig. 6A), suggesting that CPK21 primarily phosphorylates S35 and S115 of VIP1 in vitro. In Y2H assays, AD–VIP1 enabled yeast cells to survive on the selection medium when co-expressed with a BD-fused form of GRF6, one of the 14-3-3 proteins of Arabidopsis, but AD-fused forms of the VIP1 variants with the Ser→Ala

Fig. 4. VIP1 variants that are constitutively localized in the nucleus suppress VIP1–SRDX-dependent root waving. (A, B, and D) Root growth of the transgenic plants expressing GFP, VIP1–GFP, NLS–VIP1–GFP, or GFP–VIP1AAA in the VIP1–SRDX-overexpressing (+ VIP1–SRDX) background. GFP–VIP1AAA was introduced into the VIP1–SRDX-overexpressing background from two individual GFP–VIP1AAA-expressing lines (#1 and #2), and data for line #2 are presented (see Supplementary Fig. S5 for line #1). These plants were grown for 10 d on agar medium tilted at a 60° angle and photographed to obtain the root vertical growth indices (VGIs). (A) Phenotypes of these 10-day-old seedlings. Scale bar=5 mm. (B) VGIs of these plants. For each box, the top and bottom edges and the middle line indicate the quartiles, and the vertical bar corresponds to the data range (n=70 for GFP-expressing plants; 46 for VIP1–GFP-expressing plants; 46 for NLS–VIP1–GFP-expressing plants; 29 for GFP–VIP1AAA-expressing plants #2). Data with the same lower case letters are not significantly different (P>0.05) according to the Games–Howell test. (C) Expression levels of VIP1–SRDX. Plants were grown as described above, and used for RNA extraction and cDNA synthesis for RT–PCR. Data are means ±SD of three biological replicates. Data with the same lower case letters are not significantly different (P>0.05) according to the Tukey–Kramer test. (D) Length of the primary roots of these plants. Data are the same as those used to obtain the VGIs, and are presented as means ±SD. Data with the same lower case letters are not significantly different (P>0.05) according to the Games–Howell test.
substitution at S35 (QA and S35A) did not (Fig. 6B, left panels). The results of Y2H assays with bZIP52 variants and GRF6 (Fig. 6B, right) were similar to those of assays with the VIP1 variants. The results of Y2H assays with another Arabidopsis 14–3–3 protein, GRF8 (Supplementary Fig. S8), were also similar to those of assays with GRF6. These results raise the possibility that S35 of VIP1 and the corresponding residues of group I bZIP proteins are the only phosphorylation sites interacting with 14–3–3 proteins in the Y2H system. Consistent with a previous study (Takeo and Ito, 2017), the VIP1QA–GFP fusion protein was detected only in the nucleus when transiently expressed in lettuce epidermal cells. In the same system, the VIP1S35 115A–GFP fusion protein was also detected mainly in the nucleus, whereas the other GFP-fused VIP1 variants with the Ser→Ala substitutions were detected mainly in the cytoplasm (Fig. 6C, left panels). Similar results were obtained when VIP1 variants with Ser→Asp (phosphomimetic) substitutions at S35, S115, and S151 were used (Supplementary Fig. S9). These results suggest that S35 and S115 are more

### Table 1. VIP1 variants and bZIP52 variants with the Ser→Ala substitution at putative phosphorylation sites

<table>
<thead>
<tr>
<th>Protein</th>
<th>Variant name</th>
<th>Positions with Ser→Ala substitution</th>
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<td>S35 115A</td>
<td>35, 115</td>
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<tr>
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<td>QA</td>
<td>35, 115, 149, 151</td>
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<tr>
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<tr>
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<td>DA</td>
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</tr>
<tr>
<td>VIP1</td>
<td>S115 149 151A</td>
<td>115, 149, 151</td>
</tr>
<tr>
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<td>QA</td>
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<td>DA2 (S115 117A)a</td>
<td>115, 117</td>
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a The variant name in parentheses is equivalent to the preceding variant name (either DA1 or DA2).

Fig. 6. Narrowing down phosphorylation sites relevant to functions of VIP1 and bZIP52. VIP1 variants and bZIP52 variants with the Ser→Ala substitution were used for the analyses (see Table 1 for their definitions). (A) An in vitro phosphorylation assay with the VIP1 variants (S35 115A and QA). GST-fused forms of these proteins as well as GST alone (‘GST-VIP1 -’) and GST–VIP1 (‘GST-VIP1 WT’) were reacted with MBP–CPK21, and detected as described in the legend of Fig. 3A. ‘P-GST-VIP1’ indicates phosphorylated GST–VIP1. The numbers on the right side of the images indicate molecular mass. The experiment was performed three times, and a representative result is presented. (B) Y2H interactions between GRF6 and variants of VIP1 and bZIP52. GAL4 DNA-binding domain (BD)-fused GRF6 was co-expressed with an AD-fused variant of either VIP1 or bZIP52. The numbers on the right side of the images indicate molecular mass. The experiment was performed three times, and a representative result is presented. (C) Images of signals of GFP-fused VIP1 variants (left images) and bZIP52 variants (right images) transiently expressed in lettuce epidermal cells. For each protein variant, the transient expression experiment was performed three times, and a representative result is presented. Scale bars=100 μm. (This figure is available in colour at JXB online.)
frequently phosphorylated and/or more strongly interact with 14–3–3 proteins than S151. In addition, they suggest that the factor retaining VIP1 in the cytoplasm is phosphorylation itself and not the negative charge or hydrophilicity associated with phosphorylation. Both the bZIP52S38 40A–GFP fusion protein and bZIP52S115 117A–GFP were detected mainly in the cytoplasm, whereas bZIP52Q2A was detected only in the nucleus when they were expressed in lettuce epidermal cells (Fig. 6C, right panels). Together, these results raise the possibility that, although the number and positions of the 14–3–3 interaction motifs are not completely conserved among the group I bZIP proteins, many of these motifs promote the cytoplasmic retention of those proteins to some extent.

Mechanical stress sensor upstream of VIP1 remains to be identified

The mlo4-4 mlo11-4 mlo14-7 triple mutant (mlo4 11 14), which lacks the seven-transmembrane proteins MLO4, MLO11, and MLO14, exhibits enhanced touch-induced root bending under calcium-deficient conditions (Chen et al., 2009; Bidzinski et al., 2014). We therefore hypothesized that these proteins are possible mechanical stress sensors that initiate calcium signaling upstream of VIP1. VIP1–GFP was expressed in either mlo4 11 14 or the atb''δ mutant, which has a T-DNA insertion in the fourth exon corresponding to the middle of the coding sequence of ATB''δ (Fig. 7A). When these plants were incubated in a hypotonic solution, VIP1–GFP signals were detected in the nucleus, and then in the cytoplasm, just as they were in the wild-type background (Fig. 7B), suggesting that neither the ATB''δ single knockout nor the MLO4 MLO11 MLO14 triple knockout affects the subcellular localization of VIP1. It is possible that ATB''α–ε are functionally redundant and can mediate dephosphorylation of VIP1 and other group I bZIP proteins. Further studies such as characterization of a mutant lacking multiple PP2A B''-family subunit genes are necessary to elucidate their physiological roles. MLO4, MLO11, and MLO14 are likely to function independently of VIP1 in Arabidopsis mechanical stress responses. There should be a sensor acting upstream of VIP1 that increases the cytoplasmic calcium concentration in response to mechanical stress and that is inhibited by streptomycin (Tsugama et al., 2018), but further studies are needed to identify it.

In conclusion, our results show that PP2A- or PP4-mediated dephosphorylation is involved in the nuclear accumulation of VIP1 and its close homologs. The dephosphorylation of VIP1 can be mediated by direct interactions between VIP1 and PP2A B''-family subunits, and can suppress root waving. The current model for the hypo-osmotic stress- and/or touch-responsive pathway mediated by group I bZIP proteins is presented in Fig. 8.

FASS interacts with a microtubule-associated protein, TONNEAU 1 (TON1), and regulates the dynamics of cortical microtubules (Camilleri et al., 2002; Spinner et al., 2013). ATB''α and ATB''β regulate the activity of the enzyme 3-hydroxy–3-methylglutaryl-CoA reductase (HMGR), which is involved in the mevalonate pathway for isoprenoid biosynthesis and thereby plays a pleiotropic role in plants (Leivar et al., 2011). Further studies on the interactions between group I bZIP proteins, PP2A B''-family subunits, and their interacting partners such as TON1 and HMGR will help to better understand the mechanisms underlying mechanical stress sensing in plants.

Fig. 7. Neither the ATB''δ single knockout nor the MLO4 MLO11 MLO14 triple knockout affects the subcellular localization of VIP1. (A) Genomic PCR analysis of T-DNA insertion in ATB''δ, MLO4, MLO11, and MLO14. For the left panel, genomic DNA was extracted from the wild type and VIP1-GFPox/atb''δ, which expresses VIP1–GFP in the atb''δ background. For the right panel, genomic DNA was extracted from the wild type and VIP1-GFPox/mlo4 11 14, which expresses VIP1–GFP in the mlo4 11 14 background. The signal of VIP1-GFP is presented as a positive control. (B) Patterns of VIP1–GFP signals in the wild-type background (‘GFP/WT’ and ‘VIP1–GFP/WT’, respectively) are presented as controls. The experiment was performed three times with at least five individual plants for each genotype, and representative results are presented. Scale bars=100 µm. (This figure is available in colour at JXB online.)
Fig. 8. Hypo-osmotic stress- and/or touch-responsive pathway mediated by VIP1 and its close homologs. CAM, calmodulin; VIP1h, close VIP1 homolog.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Primers used for genomic PCR.

Table S2. Primers used to generate constructs.

Table S3. Primers used to introduce mutations into VIP1 and bZIP52.

Fig. S1. Signals of GFP–VIP1AAA. Fig. S2. A rooted phylogenetic tree of Arabidopsis PP2A B*-family subunits.

Fig. S3. GAL4 DNA-binding domain (BD)–ATBδ and BD–FASS fusion proteins both activate reporter genes in the Y2H system.

Fig. S4. VIP1 does not exhibit Y2H interactions with PP1 and PP2A components other than PP2A B*-family subunits.

Fig. S5. Root vertical growth indices (VGI) and primary root length of the transgenic plants expressing GFP or GFP–VIP1AAA in the VIP1–SRDX-overexpressing background.

Fig. S6. Primary root length of the transgenic plants expressing GFP, VIP1–SRDX, or GFP–VIP1AAA in the wild-type background.

Fig. S7. Multiple alignment of amino acid sequences of Arabidopsis group I bZIP proteins that transiently accumulate in the nucleus in response to hypo-osmotic stress.

Fig. S8. Y2H interactions between GRF8 and variants of VIP1 and bZIP52.

Fig. S9. Images of signals of GFP-fused VIP1 variants transiently expressed in lettuce epidermal cells.

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