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SHORT COMMUNICATION

VIP1, a bZIP protein, interacts with the catalytic subunit of protein phosphatase 2A in Arabidopsis thaliana

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ABSTRACT

VirE2-INTERACTING PROTEIN1 (VIP1) is a basic leucine zipper protein in *Arabidopsis thaliana*. VIP1 changes its subcellular localization from the cytoplasm to the nucleus when cells are exposed to mechanical or hypo-osmotic stress. The nuclear localization of VIP1 is inhibited either by inhibitors of calcium signaling or by inhibitors of protein phosphatases 1, 2A and 4 (PP1, PP2A and PP4, respectively). VIP1 binds to the PP2A B"-family subunits, which have calcium-binding EF-hand motifs and which act as the regulatory, substrate-recruiting B subunit of PP2A. The VIP1 de-phosphorylation can therefore be mediated by PP2A. However, details of the PP2A-mediated de-phosphorylation of VIP1 are unclear. Here, with yeast two-hybrid assays and in-vitro pull-down assays, we show that VIP1 does not interact with the scaffolding A subunit of PP2A, but that VIP1 does interact with the catalytic C subunits. Our data raise the possibility that not only the B"-family B subunit of PP2A but also its C subunit contributes to the PP2A-mediated de-phosphorylation of VIP1.

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VIP1 (VirE2-INTERACTING PROTEIN 1) is a basic leucine zipper (bZIP) protein in *Arabidopsis thaliana* and one of the plant group I bZIP proteins.[1] VIP1 plays roles in plant development and responses to various stimuli such as hypo-osmotic stress and mechanical stress.[2–9] VIP1 is present in the cytoplasm under a stable condition but is transiently localized to the nucleus when Arabidopsis cells are either hypo-osmotically or mechanically stressed.[8–10] The nuclear localization of VIP1 requires calcium signaling, VIP1 de-phosphorylation, and dissociation from 14-3-3 proteins.[11–13] Inhibitors of protein phosphatases 1, 2A and 4 (PP1, PP2A and PP4, respectively) inhibit both the de-phosphorylation and the nuclear localization of VIP1.[13] VIP1 does not bind either B-family or B'-family B subunits of PP2A. However, VIP1 does bind its B"-family B subunits, which have calcium-binding EF-hand motifs.[13] These findings raise the possibility that the VIP1 de-phosphorylation is mediated by PP2A.[13] However, mechanisms of the PP2A-mediated de-phosphorylation of VIP1 remain to be elucidated.

PP2A consists of the scaffolding A subunit, the regulatory, substrate-recruiting B subunit, and the catalytic C subunit.[14–17] To further characterize the interactions between VIP1 and PP2A, one of the Arabidopsis PP2A A subunit isoforms, PP2A-A2, and two of the PP2A C subunit isoforms, PP2A-C3 and PP2A-C5, were subjected to yeast two-hybrid (Y2H) and in-vitro pull-down assays. In the Y2H assays, GAL4 DNA-binding domain (BD)-fused PP2A-A2 did not enable yeast cells to survive on the highly stringent quadruple-dropout (QDO) selection medium, which lacks leucine, tryptophan, histidine and adenine, when co-transformed with GAL4 activation domain (AD)-fused VIP1, but BD-fused PP2A-C3 did enable it (Figure 1a, left panels). Co-expression of AD-fused VIP1 and BD-fused PP2A-C5 did not enable yeast cells to survive on the QDO medium, but did enable them to survive on the moderately stringent triple-dropout (TDO, leucine-, tryptophan- and histidine-deficient) medium supplemented with 3-amino-1,2,4-triazole (3-AT, a histidine biosynthesis inhibitor that can suppress false-positive results in Y2H assays) (Figure 1a, right panels). In similar Y2H assays, co-expression of BD-fused PP2A-C3 with an AD-fused truncated version of VIP1 (either AD-fused VIP1 N (1–186 amino acids of VIP1, which contain putative phosphorylation sites) or AD-fused VIP1 C (165–341 amino acids of VIP1, which contain the bZIP domain)) enabled yeast cells to survive on the QDO medium (Figure 1b, left panel). Neither AD-fused VIP1 N nor AD-fused VIP1 C enabled yeast cells to survive on the QDO medium when co-expressed with BD-fused PP2A-C5 (Figure 1b, middle). However, co-expression of BD-fused PP2A-C5 and one of the AD-fused VIP1 variants did enable yeast cells to survive on the TDO medium supplemented with 3-AT (Figure 1b, right).

Glutathione S-transferase (GST)-fused VIP1 and His-tagged forms of PP2A-A2 and PP2A-C3 were expressed in *Escherichia coli* cells and used for in-vitro pull-down assays. GST-fused VIP1 did not pull down His-tagged PP2A-A2 (Figure 2a, left panel), but did pull down His-tagged PP2A-C3 (Figure 2a, middle). His-tagged PP2A-C5 could not be expressed well in *Escherichia coli* but could be expressed as Myc-tagged protein with in-vitro transcription and translation. GST-fused VIP1 also pulled down Myc-tagged PP2A-C5

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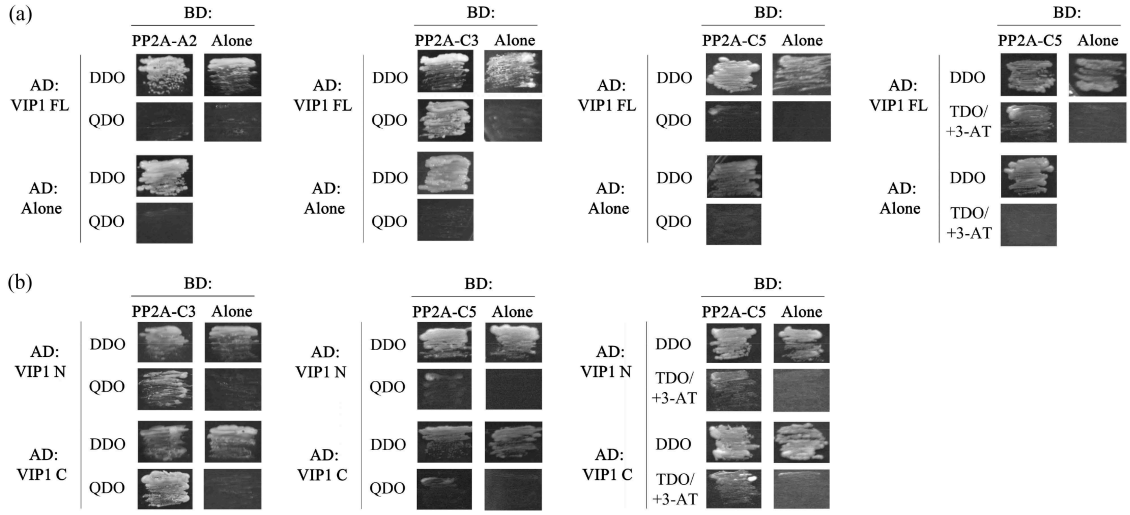

Figure 1. VIP1 interacts with PP2A-C subunits in a yeast two-hybrid system.

(a) The pGBKT7 vector (TaKaRa Bio, Kusatsu, Japan) containing no insert (BD: Alone), *PP2A-A2* (BD: PP2A-A2), *PP2A-C3* (BD: PP2A-C3), or *PP2A-C5* (BD-PP2A-C5) was co-introduced with the pGADT7-Rec vector (TaKaRa Bio) containing no insert (AD: Alone) or *VIP1* (AD: VIP1 FL) into the yeast strain AH109. (B) The pGBKT7 vector containing no insert (BD: Alone), *PP2A-C3* (BD: PP2A-C3), or *PP2A-C5* (BD-PP2A-C5) was co-introduced with the pGADT7-Rec vector containing no insert (AD: Alone) or truncated forms of *VIP1*, VIP1 N (amino acids 1–186; AD: VIP1 N) and VIP1C (amino acids: 165–341; AD: VIP1 C) into the yeast strain AH109. The transformed yeast cells were cultured on the double-dropout medium (DDO: synthetic dextrose (SD) medium lacking tryptophan and leucine), the quadruple-dropout medium (QDO: SD medium lacking tryptophan, leucine, histidine, and adenine), and the triple-dropout medium supplemented with 3-amino-1,2,4-triazole (3-AT) (TDO/+3-AT: SD medium that lacks tryptophan, leucine, and histidine, and that contains 5mM 3-AT) to examine activation of the reporter genes *HIS3* and *ADE2*. Experiments were performed four times and a representative result is presented.

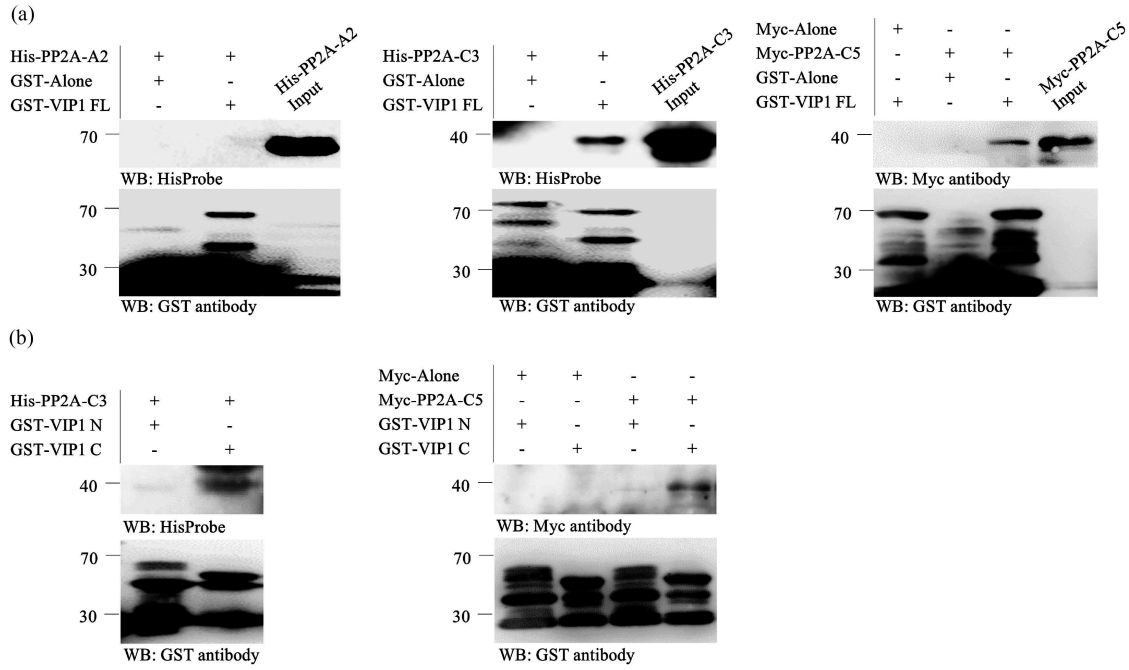

Figure 2. VIP1 interacts with PP2A-C subunits *in vitro*.

(a) Pull-down assay with full-length VIP1 (VIP1 FL), His-tagged PP2A-A2 (His-PP2A-A2), His-tagged PP2A-C3 (His-PP2A-C3), and Myc-tagged PP2A-C5 (Myc-PP2A-C5). Either GST alone (GST-Alone) or the GST-VIP1 FL fusion protein was bound to the Glutathione Sepharose 4B resin (GE Healthcare, Little Chalfont, UK), reacted with His-PP2A-A2, His-PP2A-C3, Myc-Alone, or Myc-PP2A-C5, and then eluted from the resin. GST-fused proteins, His-tagged proteins and Myc-tagged proteins in the resulting solutions were detected by western blotting with a horseradish peroxidase (HRP)-conjugated GST antibody (Fujifilm Wako Pure Chemical Co., Osaka, Japan), HisProbe-HRP (Thermo Fisher Scientific Inc., Waltham, MA, USA), and anti-Myc-tag pAb (Medical & Biological Laboratories CO., Nagoya, Japan), respectively. Experiments were performed three times and a representative result is presented. Protein masses (kDa) are indicated on the left. (B) Pull-down assay with the N-terminal region (1–186 amino acids) of VIP1 (VIP1 N) and its C-terminal region (165–341 amino acids, VIP1 C). Either GST-fused VIP1 N (GST-VIP1 N) or GST-fused VIP1 C (GST-VIP1 C) was bound to the resin and reacted with either His-PP2A-C3 (left panel) or Myc-PP2A-C5 (right). These proteins were then detected as in the panel A. Experiments were performed three times and a representative result is presented. Protein masses (kDa) are indicated on the left.

(Figure 2a, right). GST-fused VIP1 C pulled down His-tagged PP2A-C3 and Myc-tagged PP2A-C5 more strongly than GST-fused VIP1 N (Figure 2b).

Together, these results support the idea that VIP1 binds not only the B"-family B subunit of PP2A but also its C subunit. The PP2A C subunit may contribute to stabilizing the interaction between VIP1 and the PP2A holoenzyme containing the B"-family B subunit. PP2A-C3 and PP2A-C4 regulate auxin responses,[18] whereas PP2A-C5 binds Arabidopsis chloride channel proteins, AtCLCc and AtCLCb, and regulates salt stress responses.[19] VIP1 also regulates auxin responses and salt stress responses.[7,9] It should be interesting to examine the physiological relevance of the interaction between VIP1 and the PP2A C subunit.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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