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Possible function of VIPP1 in thylakoids

Protection but not formation?

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VIPP1 protein in photosynthetic organisms is homologous to bacterial PspA, which protects plasma membrane integrity upon stresses. Despite the proposed role of VIPP1 in thylakoid biogenesis, its precise function remains unclear. Recently, our in-depth analysis of Arabidopsis *vipp1* mutants revealed VIPP1's involvement in the maintenance of chloroplast envelopes. Chloroplasts in intact *vipp1* leaves exhibited spherical balloon-like morphology, which resulted from osmotic stress across envelopes. In fact, observation of VIPP1 fused to green fluorescence protein in vivo revealed that most VIPP1 is localized as a lattice-like macro complex attached along with the envelope. Because of the proposed function in thylakoids, we examined whether *vipp1* also exhibited altered morphologies in thylakoids. Results show that thylakoid morphologies were detected irregularly, but *vipp1* chloroplasts retained normal-appearing grana stacks. We infer that VIPP1 might influence thylakoids as well as envelopes, but that it is not involved directly in thylakoid membrane formation.

Keywords: *Arabidopsis*, chloroplast, envelope membrane, grana, osmotic stress, PspA, thylakoid biogenesis

Abbreviation: Col, wild-type Columbia; DGDG, digalactosyldiacylglycerol; GFP, green fluorescent protein; LHCII, light harvesting complexes of PSII; MGDG, monogalactosyldiacylglycerol; PspA, phage-shock protein A; VIPP1, vesicle-inducing protein in plastids1; *vipp1-kd*, *vipp1* knock down mutant

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Biomembranes of chloroplasts have been proposed as a prime site of vulnerability to heat and cold stresses in plants.[1,2] In general, such abiotic stresses induce damage in any biomembrane and ultimately engender disturbance in the membrane potential.[3,4] Because of the presence of multiple membrane systems (outer and inner envelopes and thylakoid membrane), maintenance of membrane integrity within chloroplasts is critically important for plant growth.[5,6] However, little has been documented about the mechanism(s) controlling plastid membrane integrity. Chloroplastic biomembranes, when ruptured, are presumably resealed spontaneously. Membrane maintenance has therefore been regarded as a physical process rather than a biochemical reaction. Nevertheless, accumulated evidence in various organisms implies that resealing of the plasma membrane is conducted via a dynamic and complex mechanism that requires novel cytoplasmic factors.[7-9] It is reasonable to assume that organelles have similar mechanisms to protect biomembranes from damage.

Our recent work provided insight into a mechanism of plastid envelope maintenance in which vesicle-inducing protein in plastids1 (VIPP1) plays a pivotal role.[10] Although VIPP1 has been originally identified as a factor involved in thylakoid biogenesis, its precise role remains unclear.[11,12] VIPP1 is homologous to phage-shock protein A (PspA) in *Escherichia coli*, which is involved in plasma membrane integrity,[11-14] and which was shown to recover the defective proton leakage when expressed in *E. coli pspA* mutant.[10] Results showed that the Arabidopsis *vipp1* mutants have globular, swollen, balloon-like chloroplasts representing increased osmotic stress of plastid envelope. A series of experiments, including the expression of VIPP1 fused to green fluorescent protein (VIPP1-GFP) in *vipp1* mutants, demonstrated that the lattice-like macro complex of VIPP1 acts as scaffold to facilitate the resealing of osmotically damaged chloroplast envelope.[10] It is particularly interesting that the dynamic movement of VIPP1-GFP along

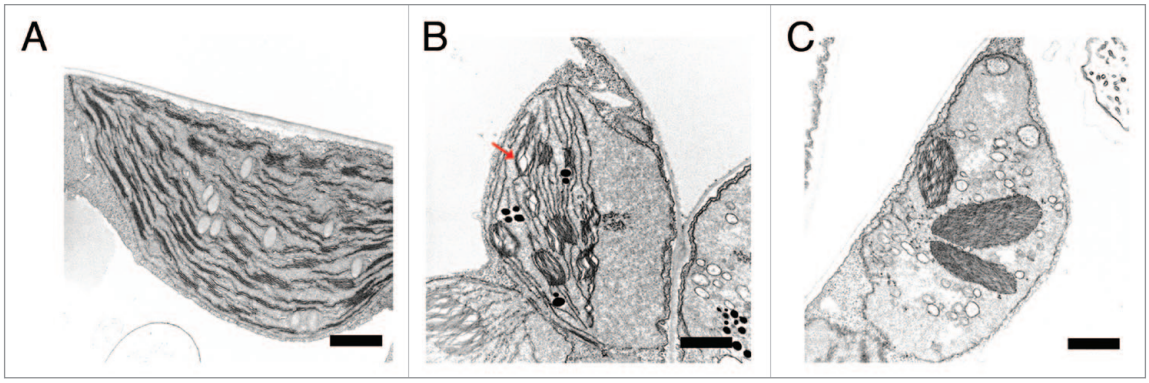

Figure 1. Chloroplasts in wild-type (Col) and *vipp1-kd* mutant examined using transmission electron microscopy. Chloroplast ultrastructures of Col (**A**) and *vipp1-kd* (**B** and **C**) mutants were observed using transmission electron microscopy. Extra lumen space (**B**, red arrow) was detected using electron micrography in *vipp1-kd* mutants. Furthermore, some larger blocks of mutlilayer membrane structures were found in *vipp1-kd* chloroplast (**C**). Bars show 1.0 μm.

with envelopes was detectable, which was correlated with osmotic stress (presented as Supplemental Movies). Based on these observations, we concluded that VIPP1 is necessary for plastid envelope maintenance. The question remains as to whether VIPP1 is also involved in thylakoid membrane as originally proposed. Here, we characterize the morphological alteration of thylakoids in *vipp1* knockdown mutants (*vipp1-kd*).

First, the thylakoid ultrastructure in fixed leaf tissues from wild-type Columbia (Col) and *vipp1-kd* was examined using electron microscopy (**Fig. 1**). In addition to the balloon-like structure, *vipp1-kd* chloroplasts exhibited irregular distorted thylakoids. Although thylakoids in Col were abundant in granal stacks that developed uniformly within the chloroplast (**Fig. 1A**), *vipp1-kd* showed distorted thylakoids that appeared to segregate into either well-stacked grana or single-membrane regions such as stroma thylakoid (**Fig. 1B**). In an extreme case, some *vipp1-kd* chloroplasts contained only several stacks that were tightly clustered (**Fig. 1C**). It is noteworthy that the clustered granal stacks frequently contained a large lumenal area (**Fig. 1B**, indicated by an arrow) that was similar to swollen stroma in the balloon-like chloroplasts and which might represent osmotic stress of thylakoid membranes. A similar fluffy structure of thylakoids has also been observed in chloroplasts suffered from water-osmotic stress or high/low temperature,[3,15,16] implying that VIPP1 is involved in the maintenance of thylakoid membranes as well as of envelopes.

In addition to these data obtained from electron microscopy, observation of chlorophyll fluorescence with fresh tissue supported the existence of altered grana-like structures (**Fig. 2A**). Protoplasts prepared from *vipp1-kd* contained balloon-like chloroplasts in which chlorophyll fluorescence signals tended to form a larger cluster. To test whether the grana stacks observed in *vipp1-kd* indeed retained functional thylakoid membranes, we attempted to detect light-harvesting complexes of PSII (LHCII). Blue-native gel electrophoresis of solubilized thylakoid membranes indicated that LHCII trimer had accumulated significantly in both Col and *vipp1-kd* mutant (**Fig. 2B**). Accumulation of LHCII was confirmed further by SDS-PAGE and subsequent western blot analysis (**Fig. 2C**). We concluded that the reduction of VIPP1 engenders altered thylakoid morphology, raising the possibility that VIPP1 is a multifunctional protein that affects both envelope and thylakoid membranes. However, the morphological alteration in thylakoids was not a simple reduction but rather a reorganization of the functional thylakoid membranes. Although further analysis is needed, experimental data obtained from our laboratory do not support the model that VIPP1 is involved in the direct formation of thylakoid membranes. Consistent with this inference, recent observations from other laboratories suggest that VIPP1 affects thylakoid formation indirectly through protein complex formation and maintenance, or through protein import across thylakoid membranes.[17,18] These data are favorable to a scenario in which VIPP1 acts to protect membranes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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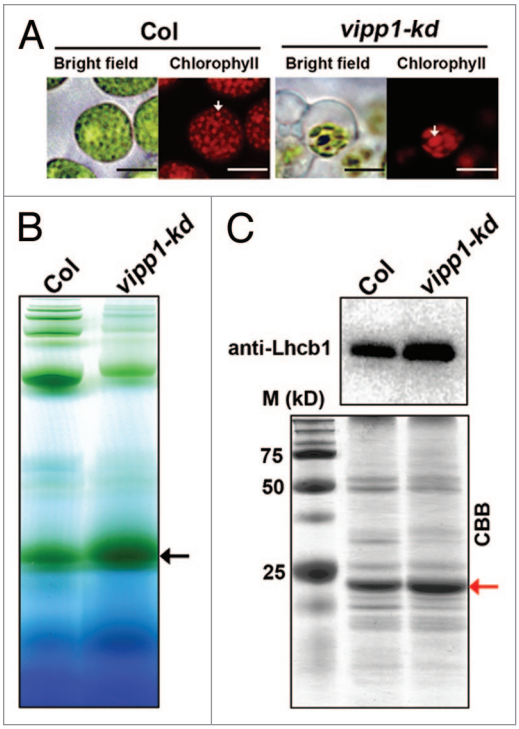

Figure 2. Accumulation of LHCII in *vipp-kd* mutant. (**A**) Chloroplast images of Col and *vipp-kd* protoplast photographed using fluorescence microscopy. White arrows indicate grana thylakoids. Bars show 10 μm. (**B**) Photosynthetic supercomplexes located on thylakoid membrane were analyzed using blue native gel. The thylakoid was extracted from the leaves of Col and *vipp-kd* mutant and incubated with 1% n-dodecyl-β-maltoside (DM) for 1.0 h on ice. Samples were normalized by chlorophyll content and were loaded to 0.75-mm-thick 5–13.5% acrylamide gradient gels. The black arrow indicates the LHCII trimer location. (**C**) The level of Lhcb1 protein in Col and *vipp-kd*. Upper panel, western blot probed with the antibody against Lhcb1. Lower panel, a Coomassie-stained gel image is shown as loading control. The red arrow indicates the Lhcb1 position in SDS PAGE.