

論文の内容の要旨

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論文題目 植物の機械的ストレスへの応答の分子機構に関する研究

Plants are exposed to mechanical stress caused by various factors such as rain, animals, pathogens, and plants themselves. Mechanical stress limits plant growth and productivity. Plants have adaptive responses to alleviate mechanical stress. For example, when roots touch an object, they bend to avoid the object. Moderate mechanical stress can enhance pathogen resistance if imposed on plant shoots. Characterizing mechanisms underlying plant mechanical stress responses is important because it can provide ways to improve crop development and yield under stressed conditions.

VIP1 is an *Arabidopsis thaliana* basic leucine zipper (bZIP) transcription factor that has a transcriptional activation potential and affinity to DNA with either AGCTGG or AGCTGT (AGCTG[GT]) and that is involved in regulating mechanical stress responses. VIP1 is phosphorylated by CALCIUM-DEPENDENT PROTEIN KINASE 21 (CPK21) and retained in the cytoplasm by 14-3-3 proteins under stable conditions but is dephosphorylated and localized to the nucleus when *Arabidopsis* cells are exposed to mechanical stress. The VIP1 dephosphorylation is likely mediated by protein phosphatase 2A (PP2A) because VIP1 interacts with PP2A B''-family B subunits. Overexpression of a repression domain-fused form of VIP1 (VIP1-SRDX) causes enhanced touch-induced root bending, and this is suppressed by the expression of GFP-fused VIP1 (VIP1-GFP). Thus, VIP1 is thought to be a suppressor of the touch-induced root bending. *Arabidopsis* has 11 close VIP1 homologs, and at least six of them (bZIP29, bZIP30, PosF21, bZIP69, bZIP52, and bZIP18) share most of the above functions of VIP1. The aim of this study is to further characterize factors involved in the VIP1-mediated mechanical stress signaling and thereby to better understand plant mechanical stress responses.

1. NDR/LATS-family protein kinases phosphorylate VIP1

Serine residues at positions 35, 115, and 151 (S35, S115, and S151, respectively) of VIP1 are putative phosphorylation sites that interact with 14-3-3 proteins and that are responsible for cytoplasmic retention of VIP1. All of these three residues were found to be a part of the consensus sequence for NDR/LATS-family protein kinase phosphorylation. NDR/LATS-family protein kinases are conserved among eukaryotes and regulate the cell cycle in yeast and animals. However, the functions of these protein kinases in plants are unclear. This led us to determine that not only CPK21 but also NDR/LATS-family protein kinases phosphorylate VIP1, and to characterize physiological functions of these protein kinases in Arabidopsis. Arabidopsis has eight NDR/LATS-family protein kinase genes (*NDR1-8*). Reverse transcription-PCR detected the expression of all of these genes in seedlings, rosette leaves, roots, flower stalks and flowers. NDR2, NDR3, and NDR8 were expressed as recombinant (maltose-binding protein (MBP)-fused) proteins in *Escherichia coli*, and purified by affinity chromatography. All of these proteins phosphorylated GST-fused VIP1 (GST-VIP1) *in vitro* but did not phosphorylate a GST-fused VIP1 variant with S → A substitutions at S35 and S115. These results suggest that S35 and S115 of VIP1 are phosphorylated by Arabidopsis NDR/LATS-family protein kinases *in vitro*. Arabidopsis mutant lines lacking one, two, or three of *NDR4*, *NDR6*, *NDR7* and *NDR8* could be obtained, and their phenotypes were similar to the wild-type phenotype under a normal growth condition. However, a line lacking all of these four genes could never be obtained. Based on genetic analysis, this was shown to be because an *NDR4 NDR6 NDR7 NDR8* quadruple knockout causes embryonic (yet not gametophytic) lethality.

2. PP2A B''-family B subunits are indispensable for dephosphorylation of VIP1 and its close homologs *in vitro*

PP2A consists of the scaffolding A subunit, the regulatory B subunit, and the catalytic C subunit. PP2A B subunits bind substrates of PP2A, and are classified into B, B', B'', and B''' families. PP2A B''-family B subunits have Ca²⁺-binding EF-hand motifs. Arabidopsis has six genes encoding the PP2A B''-family subunits (ATB'' α - ϵ and FASS). Of these proteins, ATB'' δ and FASS bind VIP1 in the presence of Ca²⁺. However, it is unclear whether PP2A B''-family subunits mediate the VIP1

dephosphorylation. We further characterized the interactions between these proteins and VIP1. By yeast two-hybrid and in-vitro pull-down assays, interactions between two Arabidopsis PP2A A subunits (RCN1 and PP2A-A2), two PP2A C subunits (PP2A-C3 and PP2A-C5) and all the six PP2A B''-family subunits were confirmed. Pull-down assays also showed that His-tagged forms of ATB'' α - γ and ATB'' ϵ as well as ATB'' δ and FASS interact with GST-VIP1 in the presence of Ca^{2+} *in vitro*. GST-VIP1 and GST-fused bZIP29 were both dephosphorylated *in vitro* when mixed with protein solutions containing Myc-tagged ATB'' α , Myc-tagged ATB'' δ or Myc tagged FASS in addition to PP2A A and C subunits. This result suggests that the PP2A B''-family subunits are indispensable for dephosphorylation of VIP1 and its close homologs *in vitro*. On the other hand, VIP1-GFP shuttled between the cytoplasm and the nucleus in mechanically stressed *ATB'' β ATB'' δ* double knockout plants as it did in wild-type plants. A mutant that lacks four (*ATB'' α - δ*) of the six PP2A B''-family subunit genes exhibited phenotypes similar to the phenotypes of the wild type under various growth conditions. These results support the idea that those PP2A B''-family subunits have functional redundancy. Further studies are necessary to elucidate the physiological functions of these proteins.

3. VIP1 and its close homologs confer the mechanical stress tolerance in Arabidopsis leaves

To further examine physiological functions of VIP1 and its close homologs, QM1 and QM2, two lines with mutations in *VIP1*, *PosF21*, *bZIP29* and *bZIP30*, were generated. In both QM1 and QM2 plants, *PosF21*, *bZIP29*, and *bZIP30* are knocked out by transfer DNA (T-DNA) insertions. QM1 plants have T-DNA in *VIP1* as well but exhibits expression of *VIP1* 5' region that corresponds to the N-terminal 244 amino acids of the 341 amino acids of VIP1. QM2 plants have a two-base pair deletion in *VIP1* 5' region and should exhibit expression of N-terminal 140 amino acids of VIP1. Both QM1 and QM2 plants exhibited phenotypes similar to the phenotypes of the wild type under various growth conditions. However, although leaves of wild-type and VIP1-GFP-overexpressing plants survived mechanical stress induced by repeated brushing, leaves of QM1, QM2 and VIP1-SRDX-overexpressing plants did not. A high level of reactive oxygen species (ROS) was detected in wild-type leaves one minute after they were brushed, but not five minutes after. In contrast, a high level of ROS was detected in QM1 and QM2 leaves both one minute and five minutes after they were brushed. DAPI-stained areas and electrolyte leakage of VIP1-SRDXox #7, QM1, and QM2 plants after a brushing treatment were significantly higher

than wild-type and VIP1-GFP-overexpressing plants. These results suggest that VIP1 and its close homologs confer the mechanical stress tolerance in Arabidopsis leaves. Transcriptomes of the brushed leaves of the wild-type and QM2 plants were analyzed by RNA sequencing. Expression (i.e., transcripts per million (TPM)) values obtained for *VIP1*, *PosF21*, *bZIP29* and *bZIP30* were all lower in the QM2 plants than in the wild-type plants. Expression values of previously identified putative VIP1 target genes, *XTH23* and *EXLAI*, were also lower in QM2 plants than in the wild-type plants. Consistent results were obtained in quantitative reverse transcription-PCR for *XTH23* and *EXLAI*. These results confirm the validity of our RNA sequencing. Many genes that were either upregulated or downregulated by the leaf brushing were shared between the wild-type and QM2 plants. *De novo* motif scanning identified AGCTG[GT], which is bound by VIP1 and its close homologs (see above), as a part of a sequence motif enriched in the promoters of the genes that are more weakly expressed in the stressed QM2 leaves than in the stressed wild-type leaves. This suggests that AGCTG[GT], VIP1 and its close homologs are relevant to the mechanical stress-responsive gene expression. Such motif scanning also identified a CAMTA (calmodulin-binding transcription activator)-binding sequence as a part of a motif enriched in the promoters of the genes that are upregulated by brushing in either the QM2 or wild-type plants. This raises the possibility that CAMTAs are also important for the mechanical stress-dependent induction of genes in Arabidopsis.

In conclusion, here we show (1) that NDR/LATS protein kinases as well as CPK21 can phosphorylate VIP1 and are essential for embryogenesis, (2) that Arabidopsis PP2A B''-family subunits are indispensable for the *in-vitro* dephosphorylation of VIP1 and bZIP29, (3) that VIP1 and its close homologs are relevant to the mechanical stress-induced changes in gene expression and to the resistance of leaves to mechanical stress, and (4) that CAMTAs are also important for the mechanical stress-dependent induction of genes. Our data can help to further dissect plant mechanical stress responses.