論 文 の 内 容 の 要 旨

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氏名　劉　華

論文題目 Functional analysis of two novel effector candidates of Arabidopsis heterotrimeric G protein β subunit, AGB1
（シロイヌナズナのヘテロ三量体 G タンパク質 β サブユニット（AGB1）の新奇エフェクター候補の機能解析）

The Arabidopsis and rice genomes contain a simple heterotrimeric G protein system, which function in various signal transductions. Some signaling functions are cell-type dependent, which could result from interaction with different receptors/effectors expressed in different cell types.

The G-protein-activated enzymes were collectively called effectors. In this study, a conserved RNA-binding protein, AtRBM22, and a type-2C protein phosphatase, AtPP2C52 were identified as novel AGB1-interacting partners in a two-hybrid (Y2H) screen using AGB1 as the bait.

1. Translocation of a putative pre-mRNA splicing factor (RBM22) to nuclear speckles in *Arabidopsis thaliana*

The interaction between AGB1 and AtRBM22 was confirmed with a BiFC assay. The N- and C-terminal domains of mVenus were fused to the C-terminus AGB1 and AtRBM22, respectively, and were co-introduced into wild-type Arabidopsis protoplasts and onion epidermal cells. Fluorescence from the reconstituted mVenus was observed in nuclear speckles of Arabidopsis protoplasts and onion epidermal cells.

Coimmunoprecipitation (Co-IP) was used to determine whether AtRBM22 interacted with AGB1 *in vitro*. Myc-tagged AGB1 (Myc:AGB1), HA-tagged AtRBM22 (HA:AtRBM22) and HA epitope tag (HA) were synthesized *in vitro* in a rabbit reticulocyte lysate system. Either HA epitope tag or HA:AtRBM22 were mixed with Myc-AGB1, and then precipitated by anti-HA antibody. Subsequently, G Sepharose was added. After incubation, Myc-AGB1 in the elutant from the G Sepharose was analyzed by immunoblotting using anti-Myc antibody. Specific signals of Myc-AGB1 were detected only when AtRBM22 was present, indicating that AtRBM22 interacts with AGB1 *in vitro*.

Gβ and Gγ subunits form a Gβγ dimer to activate effector molecules. To determine the role of Gγ subunit, one of the Gγ subunit (AGG2) as the third protein was cloned into the pBridge-AGB1 construct for Y3H analysis. Co-expression of AGG2 impaired the interaction between AGB1 and AtRBM22. These results imply that AtRBM22 interacts with AGB1 monomer, but not the Gβγ dimmer. The specific interaction between AtRBM22 and AGB1 was further confirmed by using another AGB1-interacting protein, AtPP2C52. In contrast, co-expression of AGG2 did not

impair the interaction between AGB1 and AtPP2C52.

To check the subnuclear localization of AtRBM22, Green Fluorescent Protein (GFP)-tagged AtRBM22 was transiently expressed under control of the constitutive Cauliflower Mosaic Virus 35S (CaMV35S) promoter in Arabidopsis protoplasts. Consistent with a previous report, AtRBM22-GFP fusion protein was predominantly located in nuclei, while GFP alone displayed fluorescence throughout the cytoplasm and nucleus. Interestingly, two patterns of fluorescence were observed in protoplasts carrying the *AtRBM22-GFP* fusion gene: (i) evenly in the nucleus and (ii) in nuclear speckles. The nuclear size of protoplasts with fluorescence pattern ii (N/C ratio: up to 0.12) was significantly larger than that of protoplasts with fluorescence pattern i (N/C ratio: around 0.06). When the *AGB1* gene and the *AtRBM22-GFP* fusion gene were co-introduced into the protoplasts, nearly 60% of the protoplasts had fluorescence pattern ii, while when only *AtRBM22-GFP* was introduced, only 38% of the protoplasts had fluorescence pattern ii. These results suggest that AGB1 has a role in the translocation of AtRBM22 into the nuclear speckles.

In onion epidermal cells, the fluorescence of AtRBM22-GFP fusion protein was evenly diffused in the nucleus. AGB1-GFP fusion protein was located in the plasma membrane and nucleus as described previously. When *AGB1* and *AtRBM22-GFP* fusion gene were co-introduced into onion epidermal cells, the fluorescence of AtRBM22-GFP fusion protein was diffused in the nucleus, and speckle-like structures were visible in the nuclei of almost all cells. These results indicate that the localization of AtRBM22 in nuclear speckles in Arabidopsis protoplasts is not due to the high expression level of AtRBM22 and suggest that the translocation of AtRBM22 to nuclear speckles requires AGB1.

To confirm whether the WT protoplasts with fluorescence pattern ii depended on the presence of endogenous AGB1, *agb1* null mutant (*agb1-2*) was used to check the localization of AtRBM22-GFP fusion protein. Similarly, both patterns of fluorescence were observed from *agb1-2* protoplasts carrying *AtRBM22-GFP* fusion gene only. This result raises the possibility that proteins other than AGB1 also mediated the translocation of AtRBM22 to nuclear speckles.

In another Y2H screening using full-length AtRBM22 as the bait, Ntf2 and RBE were identified as AtRBM22 targets. Both Ntf2 and RBE interacted with AtRBM22 in the nucleus speckles, suggesting that translocation of AtRBM22 into nuclear speckles is mediated by multiple proteins.

Two RBM22 homologues of Arabidopsis, F16P2.4 and MOJ9.23 (GenBank Accession Nos. NP_180518 and NP_196323) share 86.8% and 71.3% identity with AtRBM22, respectively. F16P2.4, which shares higher similarity with AtRBM22, interacted with AGB1 in nuclear speckles and was located in nuclear speckles. Fluorescence from BiFC of AGB1 and F16P2.4 was observed in nuclear speckles in onion epidermal cells. On the other hand, BiFC fluorescence of AGB1 and MOJ9.23 was observed in the plasma membrane and nucleus, which was possibly a result of both proteins being in close proximity but not close enough to interact directly. Consistently, MOJ9.23 failed to interact with AGB1 in Y2H, while F16P2.4 had Y2H interaction with AGB1. MOJ9.23:GFP fusion protein was evenly diffused in the

nucleus.

2. The Arabidopsis RNA-binding motif protein RBM22 (AtRBM22) affects seed germination, floral transition and response to ABA and salt

Transgenic plants expressing a promoter-reporter fusion gene ($P_{AtRBM22}$::*GUS*) were used to examine the temporal-spatial expression pattern of *AtRBM22*. In young seedlings, $P_{AtRBM22}$::*GUS* was specifically expressed in root tips. In adult plants, GUS signal was appeared in pollen but not in pistil before flower stage 12. *AtRBM22* was found in stigma and ovary during pollination and subsequently *AtRBM22* disappeared from anther. *AtRBM22* was predominantly expressed in the sporangia of your silique and disappeared in mature silique. $P_{AtRBM22}$::*GUS* was not found in juvenile leaves and stem. It was conclude that *AtRBM22* expressing in stigma and ovary results from the pollinating process and AtRBM22 is expressed in a tissue-specific manner during plant development.

Salk_132881 (*atrbm22*) carried an insertion in the second exon of AtRBM22. It was confirmed as genuine *atrbm22* knockout mutants, and *AtRBM22* transcript level in *atrbm22* mutants was lower than that WT. Under long day photoperiods (LDs), *atrbm22* mutants were early flowering, and the leaves of *atrbm22* mutants were curled and smaller than WT leaves. Early flowering of *atrbm22* mutants was associated with a reduction in the length of juvenile phase of the plant vegetative development. A MADS box transcription factor *FLOWERING LOCUS C* (*FLC*) acts as an inhibitor of flowering and functions in the leaf and meristem to delay flowering by repressing floral promoters *FLOWERING LOCUS T* (*FT*). In 8-day-old plants grown under LDs, *FLC* transcript level in *atrbm22* mutants were decreased compared with WT, while the floral promoter *FT* was upregulated in the *atrbm22* mutants. In *atrbm22* mutants during flowering, the expression of *FLC* was still lower than that in WT. In addition, *atrbm22* mutants generated shorter silliques. These results suggest that AtRBM22, a putative pre-mRNA splicing factor, plays critical roles in plant development by directly or indirectly modulating genes expression.

In Arabidopsis overexpressing AtRBM22 (OE), the vegetative growth of almost all the transgenic plants was normal. The phenotype of inflorescence, flower and sillique of OE plants were obviously different from these of WT plants. Compact inflorescence of OE plants resulted from the shorter peduncle. Floral organs including petal, stamen and carpel of OE plants were shorter than these of WT, while the sepal of OE was similar with that of WT in size. More serious phenotype observed from OE plants was that a great mount of flowers with withered petal, stamen and carpel which failed to produce seeds. The siliques of OE plants were approximately 50% shorter than that from WT. The OE plants rarely produced mature seed.

The expression of AtRBM22 is more or less regulated by ABA. In the absence of exogenous ABA, OE and *atrbm22* mutant seeds germinated as well as WT seeds. In the presence of 1uM ABA, the germination of OE and *atrbm22* seeds was earlier than WT seeds. The greening rates of OE plants and *atrbm22* mutants were higher than that of WT. The primary roots of OE pants and *atrbm22* mutants were longer than that of WT. Salt stress and ABA signaling pathways constitute a complex network. I also

determined the NaCl sensitivity at seed germination stage. OE and *atrbm22* seeds germinated as well as WT seeds on the medium without NaCl. In the presence of 100 or 150mM NaCl, the germination of OE plants were late. When 3-day-old OE and *atrbm22* seedlings cultured on medium without NaCl were transferred to medium containing NaCl (200mM), OE seedlings were more sensitive than WT and *atrbm22* mutants.

3. A type-2C protein phosphatase (AtPP2C52) interacts with heterotrimeric G protein beta subunit (AGB1) in *Arabidopsis thaliana*

It was identified that AtPP2C52 was a plasma membrane protein and interacted with AGB1 on the plasma membrane. In addition, AtPP2C52 interacted with AGB1 *in vitro*.

To check the temporal-spatial expression pattern of *AtPP2C52*, transgenic plants expressing a promoter-reporter fusion gene ($P_{AtPP2C52}$::*GUS*) were used. $P_{AtPP2C22}$::*GUS* was expressed in almost all the plant organs. In 4-day-old seedlings, $P_{AtPP2C52}$::*GUS* was predominantly expressed in vascular, root tip and apical meristem. In 3-week-old plants, A*tPP2C52* was evident in the whole plant. Obviously, *AtPP2C52* transcript level was still higher in vascular and apical meristem. In adult plant, *AtPP2C52* was found in all the organs of flower, excepting the anther. The expression level of *AtPP2C52* was lower in the sporangia.

Three AtPP2C52 mutations ($AtPP2C52^{G99D}$, $AtPP2C52^{G105D}$ and $AtPP2C52^{DGH102-104ERN}$) were generated because their high conservation in PP2Cs and their involvement in the PP2C active site. The mutated sequences encoded unrelated amino acids. None of these mutations affected the expression and molecular weight of these proteins. However, all of these mutations abolished the interaction between AGB1 and AtPP2C52.

To further identify signaling compartments of the signaling pathway mediated by AtPP2C52, full-length AtPP2C52 was used as the bait in the Y2H screening. Some AtPP2C52-interacting proteins were identified. Among them, a proteasome maturation factor, UMP1 (At5G38650), and a cysteine proteinase, RD21A (At1G47128), were used for further analysis. Y2H interaction of AtPP2C52 with either UMP1 or RD21A was confirmed. $AtPP2C52^{G99D}$ and $AtPP2C52^{DGH102-104ERN}$ failed to interact with UMP1 in Y2H. G105D mutations did not affect the Y2H interaction between AtPP2C52 and UMP1. All of these mutations abolished the Y2H interaction between AtPP2C52 and RD21A. UMP1 interacted with AtPP2C52 in the plasma membrane. AtPP2C52 interacted with RD21A not only in the plasma membrane but also in the nucleus. These results suggest that RD21A and UMP1 may be the substrates of AtPP2C52.

Taken together, AtRBM22 was expressed in a highly tissue-specific manner, and play roles in multiple developmental processes by modulating gene expression. On the other hand, AtPP2C52 was identified as an AGB1-interacting protein. It was found that not only G99D and DGH102-104ERN mutations, but also G105D abolished the ability of AtPP2C52 to interact with AGB1, suggesting that AGB1 may be specific substrate of AtPP2C52.