論 文 の 内 容 の 要 旨

生産・環境生物学専攻

平成 23 年度博士課程　入学

氏名　ガンサップ　ジラーポン

指導教員名　高野　哲夫

論文題目　Studies on two novel interaction partners of Arabidopsis heterotrimeric G protein β subunit (AGB1): Adaptor protein 3μ (AP-3μ) and Nonphototropic hypocotyl 3 (NPH3)

（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)の新規相互作用因子：細胞内小胞輸送因子 AP-3μ と青色光信号伝達因子 NPH3 に関する研究）

Heterotrimeric G proteins (G proteins) have been implicated in ubiquitous signaling mechanisms in eukaryotes. G proteins consist of three subunits, Gα, Gβ and Gγ. In animals, G proteins transmit the ligand-binding signals from G protein-coupled receptors (GPCRs) to downstream pathways to affect numerous cellular behaviours. In plants, G proteins have structural similarities to the corresponding molecules in animals but transmit signals by atypical mechanisms and effector proteins to modulate hormonal and stress responses, and regulate diverse developmental processes. However, the molecular mechanisms of their functions are largely unknown.

Gβ of Arabidopsis is named AGB1. *agb1-1* and *agb1-2*, which are the loss-of-function mutants of AGB1, show morphological aberrations, an etiolated and light-grown phenotype under dark conditions, increased stomatal density, altered phytohormone responsiveness and reduced responsiveness to pathogens. The multiple phenotypes of *agb1* mutants suggest that AGB1 is a key factor of several signaling pathways. Although the roles of AGB1 in plants are becoming clearer, the downstream effectors of AGB1 and other components of the AGB1 signaling pathways remain largely unknown. To identify interacting partners of AGB1, we performed a yeast two-hybrid screen. Some novel interacting partners of AGB1 were identified, for example, a plasma membrane 2C-type protein phosphatase PP2C52, a U-box E3 ubiquitin ligase PUB20, and a bZIP protein VIP1, which is a regulator of osmosensory signaling.

In this study, two novel AGB1-interacting proteins, an adaptor protein 3μ (AP-3μ, At1g56590) and a phototropin-interacting protein nonphototropic hypocotyl 3 (NPH3, At5g64330), were functional characterized. Furthermore, the physiological roles of the interaction between AGB1 and AP-3μ or NPH3 were examined.

1. The Arabidopsis adaptor protein AP-3μ interacts with the G protein β subunit AGB1 and is involved in abscisic acid regulation of germination and post-germination development.

AP-3μ (At1g56590) is an adaptor protein. Adaptor proteins (APs) are key regulators of endocytosis and secretory pathways. AP complexes (AP-1, AP-2, AP-3, AP-4, and AP-5) have been characterized so far in eukaryotes. The AP-3 complex consists of two large subunits (δ and β3), a medium subunit (μ3), and a small subunit (σ3). In Arabidopsis, each subunit of the AP-3 complex is encoded by a single-copy gene. Loss-of function mutants of several subunits of the AP-3 complex have been shown to be the suppressors of *zigzag1* (*zig1*), which is abnormal in both shoot gravitropism and morphology due to the lack of a vesicle trafficking regulator, SNARE VTI11. The AP-3 complex also plays a role in vacuolar function in Arabidopsis, including mediation of the transition between storage and lytic vacuolar identity. However, it is unclear whether the AP-3 complex also has roles in stress and hormonal responses.

The interaction between AGB1 and AP-3μ was confirmed by an *in vitro* pull-down assay and bimolecular fluorescence complementation (BiFC) assay. When co-expressed in onion epidermal cells, GFP-fused AP-3μ (AP-3μ-GFP) was detected in the cytoplasm and nucleus, while mCherry-fused AGB1 (AGB1-mCherry) was detected in the cytoplasm, nucleus, and the plasma membrane, suggesting the possibility that AP-3μ and AGB1 are co-localized in the cytoplasm and nucleus.

Two *ap-3μ* T-DNA insertional mutants were obtained. The abscisic acid (ABA) sensitivities of *ap-3μ* mutants were examined. *ap-3μ* mutants were hyposensitive to ABA during germination and post-germination growth, whereas *agb1* mutants were hypersensitive to ABA. To investigate the interaction between *AP-3μ* and *AGB1* at the genetic level, *agb1/ap-3μ* double mutants (DMs) were generated and the ABA sensitivities of them were examined. During seed germination, *agb1/ap-3μ* double mutants were more sensitive to ABA than the wild type but less sensitive than *agb1* mutants. However, in post-germination growth, the double mutants were as sensitive to ABA as *agb1* mutants. These data suggest that AP-3μ positively regulates the ABA responses independently of AGB1 in seed germination, while AP-3μ does require AGB1 to regulate ABA responses during post-germination growth. Furthermore, mutants of AP-3δ subunit and clathrin heavy chain (CHC) were obtained. *ap-3δ* and *chc1* mutants showed ABA-hyposensitive phenotypes in post-germination growth, suggesting that AP-3δ and CHC, as well as AP-3μ, function in the ABA response during post-germination growth.

2. Studies on Nonphototropic hypocotyl 3 (NPH3)

2.1 Arabidopsis G protein β subunit AGB1 interacts with Nonphototropic hypocotyl 3 (NPH3) and is involved in phototropism.

(NPH3, At5g64330) is a phototropin-interacting protein and is required for the phototropic response, leaf positioning and leaf flattening. NPH3 is a member of a plant-specific family of proteins, designated the NRL family. Three characteristics define members of the NRL family of proteins: 1) an N-terminal BTB (*broad complex, tramtrack,* and *bric a `brac*) domain, 2) a centrally located NPH3 domain, designated domain I-IV, and 3) a C-terminal coiled coil domain. Although the functional role(s) of these domains are not fully understood, the coiled-coil region of NPH3 is necessary and sufficient for interaction with phot1, and the BTB domain interacts with CULLIN3 (CUL3), which is a hydrophobic protein that provides a scaffold for ubiquitin ligases. NPH3 has two splicing variants, NPH3.1 and NPH3.2. NPH3.1 is the full-length NPH3, while NPH3.2 is the C-terminus-truncated NPH3, lacking the latter half of NPH3 domain IV and coiled coil domain.

The interaction between AGB1 and NPH3 was confirmed by an *in vitro* pull-down assay, an *in vivo* co-immunoprecipitation using *Brassica rapa* var. *perviridis* leaves, and BiFC assay in onion epidermal cells. The open reading frame (ORF) of AGB1 was fused downstream of the ORF of nYFP (yellow fluorescence protein) and the ORF of full length NPH3 (NPH3.1) was fused upstream of the ORF of cYFP. YFP fluorescence was recovered in the plasma membrane when nYFP-fused AGB1 (nYFP-AGB1) and cYFP-fused NPH3.1 (NPH3.1-cYFP) were expressed together in onion epidermal cells, suggesting that they interact in the plasma membrane in plant cells. Although AGB1 was localized to the cytoplasm, nucleus, and the plasma membrane, the site of the interaction between AGB1 and NPH3 was limited to the plasma membrane. This was probably because the subcellular localization of NPH3 was limited to the plasma membrane.

Two novel *nph3* T-DNA insertional mutants were obtained and their non-phototropic phenotypes were confirmed by exposure of unilateral blue light. On the contrary, *agb1* mutants showed a smaller phototropic response than the wild type. To investigate the interaction between *NPH3* and *AGB1* at the genetic level, *agb1/nph3* double mutants were generated. *agb1/nph3* double mutants displayed non-phototropic phenotypes similar to those of *nph3* mutants. These data suggest that AGB1 function is required for normal phototropism and that AGB1 and NPH3 function in the same pathway during the phototropic response. The expression level of *AGB1* gene was lower in the *nph3* mutants than in the wild type, raising the possibility that NPH3 positively regulates the expression of *AGB1* gene. It is known that in dark-grown seedlings, NPH3 exists as a phosphorylated protein and blue light stimulates its dephosphorylation. This post-translational modification represents a crucial event in phot1-dependent phototropism. Further studies are needed to understand how AGB1 is involved in the phototropism and to elucidate whether AGB1 is involved in the phosphorylation state change of NPH3.

2.2 Functional characterization of NPH3

In our study, NPH3 was found to be an interacting partner of Arabidopsis G protein β subunit AGB1, which plays roles in many hormonal signalings, such as auxin, abscisic acid (ABA) and brassinosteroid (BR). Arabidopsis seedlings lacking AGB1 show BR hyposensitivities and BRZ (brassinazole, a BR biosynthesis inhibitor) hypersensitivities in hypocotyl elongation. Recently, AGB1 was found to regulate BR responses via interaction with a glycogen synthase kinase 3/SHAGGY-like protein kinase (GSK), which is a component of BR signaling. A reduction in blue light (low blue) induces a set of phenotypic traits, such as shoot elongation, to consolidate light capture; these are called shade avoidance responses. A well-known phenotypic response to blue light depletion is elongation of hypocotyls. BR plays an important role in the regulation of enhanced hypocotyl elongation in response to blue light depletion. Mutants that have defects in BR biosynthesis or BR signaling had a strongly reduced low-blue response. Furthermore, inhibition of BR biosynthesis via the application of BRZ led to impaired low-blue-induced hypocotyl elongation. NPH3 interacts with AGB1 and is involved in blue light signaling, such as phototropism. However, the involvement of NPH3 in BR signaling and hypocotyl elongation is unknown. Furthermore, there is no report about the involvement of NPH3 in other hormone signaling except auxin. In this part, the subcellular localization and the function of NPH3 in hormone responses were characterized.

Transient and stable subcellular localization analysis confirmed the plasma membrane localization of NPH3. *nph3* mutants exhibit BR hyposensitivities and BRZ hypersensitivities similar to *agb1* mutants. However, *agb1/nph3* double mutants show additive phenotypes in the BR hyposensitivities and the BRZ hypersensitivities, suggesting that NPH3 is involved in brassinosteroid (BR) signaling independently of AGB1. NPH3 overexpression lines show ABA hyposensitivity during seed germination. Furthermore, the expression of NPH3 is induced by ABA. These data suggest that NPH3 is involved in the ABA regulation of seed germination. The functional roles of NPH3 in BR signaling and ABA signaling are discussed.

This study revealed that AGB1 specifically interacts with AP-3μ or NPH3 depending on the cues from the environment, and regulates many processes in plant, including ABA signaling, BR signaling and phototropism. In this study, AP-3μ and NPH3 were identified as two novel AGB1-interacting proteins that are involved in ABA signaling. NPH3 is also involved in BR signaling. So far, only two G protein-interacting proteins, namely AtPIRIN1 and PLDα1, have been reported to function in ABA signaling. AtPIRIN1, which is a member of the cupin protein superfamily, binds AtGPA1 and negatively regulates ABA signaling in seed germination and early seedling development. PLDα1, which is a major isoform of phospholipase D, binds AtGPA1 and positively regulates ABA-inhibited stomate opening. Furthermore, to our knowledge, BIN2 is the only one protein which has been reported to interact with AGB1 and function in BR signaling.