論文内容の要旨

論文題目：AGF1, an AT-hook protein, is involved in GA-negative feedback regulation of *AtGA3ox1* encoding GA 3-oxidase in *Arabidopsis*
(シロイヌナズナにおける GA 3 酸化酵素遺伝子 *AtGA3ox1* のフィードバック制御に関わる因子の探索と解析)

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Gibberellic Acid (GA) is involved in various aspects of plant developmental programs such as seed germination, stem elongation, leaf expansion, floral induction. Therefore both the biosynthesis and the signal transduction of GA should be under rigid controls. Recently, almost all the enzymes participating in GA biosynthesis have been identified in *Arabidopsis thaliana*, and molecular genetic approaches have unraveled several components responsible for GA signal transduction including a soluble GA receptor, GID1. In spite of these progresses, however, the relation between the GA biosynthesis and the GA signal transduction still remains elusive. One of interesting phenomenon is GA-negative feedback regulation of the genes encoding GA biosynthetic enzymes. Namely, increase in endogenous GA levels represses these genes, and decrease in endogenous GA levels activates them. Thus it is hypothesized that the biosynthesis of GA is regulated by GA signal transduction in response to the amount of endogenous GA. Furthermore, it is indicated that plants maintain the homeostasis of endogenous GA through the feedback. In this study, I tried to reveal the molecular mechanism underlying GA-negative feedback regulation.

Based on the analysis of several plant species, the genes regulated by GA-negative feedback are those of GA 20-oxidases and GA 3 oxidases. Because GA 3-oxidase catalyzes the last step to generate active GAs, GA-negative feedback regulation of GA

3-oxidase gene seemed relatively more important than that of GA 20-oxidase. In *Arabidopsis* genome, there are at least four genes encoding GA 3-oxidase, *AtGA3ox1-4*. Among them, *AtGA3ox1* and *2* had been investigated about their GA-negative feedback regulation, and it had been shown that the expression of *AtGA3ox1* is under GA-negative feedback but that of *AtGA3ox2* are not. To determine whether other *AtGA3ox*es are under GA-negative feedback regulation, I performed RT-PCR DNA gel blot analysis. In results, neither *AtGA3ox3* nor *4* were not under GA-negative feedback, thus only *AtGA3ox1* is under GA-negative feedback. Moreover, although *AtGA3ox2*, *3* and *4* showed organ specific expression pattern, *AtGA3ox1* was expressed ubiquitously and highly in every organ examined. In view of GA-negative feedback regulation and the expression pattern, it was suggested that *AtGA3ox1* is a major GA 3-oxidase gene in *Arabidopsis*.

Since GA-negative feedback regulation seemedr4 transcriptional regulation, it was suggested that *AtGA3ox1* promoter region contains *cis*-elements necessary for GA-negative feedback. To identify the *cis*-elements, we generated several 5'-deleted variants of *AtGA3ox1* promoter. These promoter regions were transcriptionally fused to a reporter gene of β -glucronidase, and introduced into *Arabidopsis* for stable transformation. Transformants obtained were subjected to the treatment with GA or GA biosynthetic inhibitor uniconazole P, and GUS activity was measured. In result, although both -2008 bp and -1016 bp promoter regions from translation initiation site showed GA-negative feedback response in GUS activity, deletion of 208 bp from -1016 bp to -809 bp abolished GA-negative feedback. Consistently, five tandem copies of the 208-bp DNA fragment endowed the reporter with a remarkable property of GA-negative feedback regulation by GA, suggesting that the sequences are sufficient for GA-negative

feedback. To further define *cis*-elements for GA-negative feedback of *AtGA3ox1* promoter, 5' deletion analysis of the 208-bp sequence between -1016 bp and -809 bp was performed. Deletion of 56 bp from -1016 bp to -961 bp resulted in a loss of feedback regulation by GA. Furthermore, addition of 43-bp sequence between -1003 bp to -961 bp into -918 bp, which did not show GA-negative feedback response by itself, recovered the GA-negative feedback. These results substantiate the importance of the 43-bp sequence in feedback regulation of GA. In summary, my results show that the 208-bp sequence between -1016 bp and -809 bp is sufficient for GA-negative feedback, and that the 43-bp sequence from -1003 bp to -961 bp is indispensable. I named the 43-bp sequence GNFEI (for GA-negative feedback element I) and focused on the *trans*-acting factor that binds to it.

Next, I proceeded to isolate trans-factor(s) which binds to GNFEI and regulates the expression of *AtGA3ox1*. By using 3 × GNFEI, yeast one hybrid screening was performed. In result, about 3×10^6 colony yielded several positive clones. Sequence analysis revealed that they encode proteins containing AT-Hook. Thus, I named these proteins AGF1 and 2 (for AT-Hook protein for GA-negative Feedback). AGF1 and 2 are member of a gene family consisting of 30 genes, and characterized by DNA-binding motif AT-Hook and function unknown PPC (plants and prokaryotes conserved) domain. I chose AGF1 for further analysis. For confirmation, I performed gel mobility shift assay and detected the binding of AGF1 to GNFEI. In addition, nucleotide substitution into specific AAAT repeat sequences in GNFEI abolished the binding of AGF1 to GNFEI. Therefore, AGF1 binds to GNFEI through the repeat sequence at least *in vitro*. Furthermore, I demonstrated that GUS gene fused with -1016 bp promoter region of *AtGA3ox1* with mutation in the AGF1 binding repeat sequences could not respond to

GA *in vivo*. These results strongly suggested that AGF1 binds to GNFEI *in vivo*, and involved in GA-negative feedback.

If AGF1 is involved in GA-negative feedback, function of AGF1 might be regulated by GA. To know how GA controls AGF1, we investigated whether the expression and the intracellular localization of AGF1 is affected by GA. RT-PCR DNA gel blot revealed that gene expression of AGF1 is not regulated transcriptionally by GA. Microscopic analysis of transgenic *Arabidopsis* expressing AGF1-GFP fusion protein showed that AGF1 is located in nucleus but its localization and amount were not influenced by GA. These observations indicated that functional regulation of AGF1 by GA does not depend on transcriptional control and intracellular localization, rather depend on other mechanisms including covalent modification and protein-protein interaction. Finally, to determine whether AGF1 could affect the expression of endogenous *AtGA3ox1 in vivo*, we generated AGF1 constitutive expressor transformant. Compared to wild type, the expression of *AtGA3ox1* in AGF1 constitutive expressor was not affected in normal growth condition; however, it showed hyper sensitive to decrease of GA levels. Consistently, in AGF1 constitutive expressor, the expression of *AtGA3ox1* was more resistant to the repression by application of GA than wild type. Thus we propose that AGF1 is a transcriptional activator, specific to GA-negative feedback.

Here in this study, I found AGF1, a transcriptional activator involved in GA-negative feedback regulation of *AtGA3ox1*. To identify additional factors which bind and/or modify AGF1 will lead to understanding of the mechanism underlying GA-negative feedback. Moreover, such analysis of GA-negative feedback will pave the way to the comprehensive picture of GA action and GA homeostasis.