論文の内容の要旨

応用生命化学　専攻
令和元年度博士課程　入学
氏 名　王 建文
指導教員名　浅見 忠男

論文題目

Studies on the interaction of α/β-hydrolase-type plant hormone receptors with their regulators
(α/β-加水分解酵素型植物ホルモン受容体と制御剤の相互作用解析研究)

Chapter 1. Introduction

α/β-hydrolase (ABH) fold superfamily is ubiquitously distributed throughout all living organisms in three kingdoms of life. Sharing the canonical framework of the α-turn-β supersecondary geometry with high plasticity in substructure, ABH proteins have served as diverse roles to participate in various biological pathways, including biosynthesis, signal communication and transduction, and individual growth and development. Therefore, an increasing number of studies were paid on the drugs discovery for therapeutics and the chemical tools development for probing biological processes through modulation of ABH proteins activity. Plant hormones are endogenously signal and regulatory small molecules metabolically derived to regulate all aspects of plant lifestyle. Plants possess three plant hormone receptors belonging to the ABH superfamily, two of which are the receptor of gibberellin (GID1) and karrikin (KAI2). The chemical regulation of their function and signal is of great importance and benefits to boost our agricultural system, as well as basic research.

**Chapter 2. Development of orthogonal AtGID1a variant-AC94377 pairs**

Gibberellins (GAs) are a large family of tetracyclic diterpenoid plant hormones, and well acknowledged as the essential roles that regulate many developmental processes through the entire life cycle of the plant, including seed germination, cell division and elongation, leaf expansion, pollen maturation, root growth, flowering induction, fruit enlargement, etc. Previously, we have found that chemical AC94377 shows GA agonistic activity in Arabidopsis and rice. Despite that its mode of action has been clarified, the accurate binding mode of AC94377 with GID1s still remains unknown. What' more, GA-activated GID1s proteins have engaged in regulating diverse aspects of plant growth and development, but delineation of the precise biological events of a specific GID1 is of great challenge, especially in Arabidopsis because the pleiotropic effects of various receptor-ligand-repressor combinations (AtGID1s-GAs/AC94377-DELLA) lead to the complicated regulatory networks that difficult to be artificially and specifically manipulated. With these in mind, I firstly made attempts to uncover the mode of interaction between GID1 and AC94377 at atomic level, with the expectation of developing more potent GA regulators based on the chemical structure of AC94377 skeleton. Secondly, I tried to design orthogonal ligand-receptor pairs between GID1-AC94377 for probing specific GA response **(Figure 1)**.

In this study, I firstly applied X-ray crystallography to get insight into the binding mode of AC94377 in GID1. However, even though we had paid substantial efforts on crystal screening and optimization of GID1-AC94377, I was unable to get quality-sufficient crystals for X-ray diffraction. Therefore, to understand the perception mechanism of AC94377 by GID1 protein, I performed the

structure-activity relationship studies on AC94377 by using a series of its derivatives containing different substituent on chloro and carboxamide groups of AC94377. Combining the results of *in vivo* (Y2H and physiological assessment) and *in vitro* (ITF and DSF) assays, I realized that both carboxamide and chloro groups are important to retain the high GA-like activity for AC94377. Moreover, I found that AC94377 also favors the Ile-subtype OsGID1 (V319I), which is particularly consistent with our previous work. In order to support the results from SAR studies, I performed the molecular docking simulation of AC94377 with OsGID1 and AtGID1a. Superposition of simulated structures of both OsGID1-AC94377 and AtGID1a-AC94377 complexes shows good agreement in the binding pose of AC94377 in the pocket of GID1s. Notably, based on this simulation, I thought that the reinforced hydrophobic interaction between the aromatic plane of AC94377 and the extended alkyl group of isoleucine (compared with valine) may account for the improved affinity of AC94377 for the Ile-subtype GID1. However, it is difficult to fully explain the results using current simulating models because two AC94377 derivatives with the modifications of carboxamide group in AC94377 by changing amine into hydroxyl or isopropoxy (ACOH and ACOIp) did not show the preference to Ile-subtype GID1. Here, I proposed that the ligand-induced conformational changes may occur to accommodate the occupation of AC94377 in the pocket of GID1.

Since the absence of a precise structure complex of GID1-AC94377, I performed the AC94377-directed laboratory evolution on AtGID1a by Y2H-based screening system to generate the orthogonal ligand-protein variant pair of GID1-AC94377. To efficiently achieve this goal, I firstly adopted the combinatorial saturation mutagenesis on AtGID1a (CSM) by grouping the 24 pocket-lining residues into 9 sites/sublibraries. This has produced several beneficial AtGID1a variants from 3 sites with 10-folds higher AC94377-binding affinity than wild type. Next, I performed the iterative random mutagenesis (IRM) on these positive variants to maximize the cooperative effects of mutagenesis. Fortunately, these efforts have enabled to generate the AtGID1a variants with greatly enhanced affinity and selectivity to AC94377. Specifically, Y2H assay reveals that the strength of AC94377-induced PPI between these AtGID1a variants and GAI is increased about 16,000-folds than that between AtGID1a wild-type and GAI (at 50 µM AC94377), and the sensitivity of AtGID1a variants to AC94377 is increased 10-30 folds than that of AtGID1a wild-type to GA_4.

To explore the direct binding event between ligands and variant receptors, I performed the ITF and DSF assay to evaluate the binding affinity. Notably, some of the AtGID1a variants show greatly improved affinity to AC94377 with about 500-
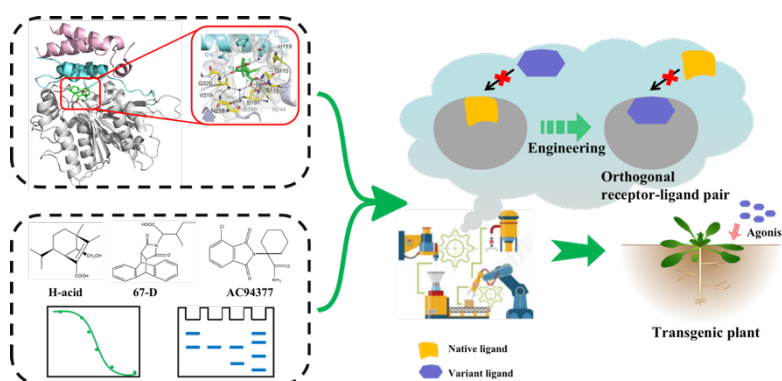
Figure 1. Generation of an orthogonal ligand-receptor pair

folds higher than wild type, whereas they totally lost the GA_4-binding affinity. The DSF assay also supports these results that AC94377 induces the enhancement of thermostability for AtGID1a variants in the same manner as GA_4 did for wild type. However, GA_4 cannot increase the structural stability of AtGID1a variants. Interestingly, even without the treatment of ligand, thermostability of AtGID1a variants is also 10-12 ℃ higher than that of AtGID1a wild type, suggesting the more stable

interactions between these mutated residues.

My works on the establishment of orthogonal AtGID1a variant-AC94377 pairs could be served as a powerful tool to delineate specific GID1-mediated responses through bypassing the complexity of genetic redundancies, further advancing our understanding of GA biology.

Chapter 3. Structural basis for the perception of a Karrikin antagonist KK181N1 by receptor AtKAI2

Karrikins (KARs) are a family of butenolides derived from smoke and ash of burning materials. KARs are identified as the seed germination stimulant in the early stage of plant development, and also enable to shape seedling photomorphogenesis. Through the recent works in our laboratory, a compound (named KK181N1) that shows antagonistic effect on karrikin induced suppression of hypocotyl growth in Arabidopsis has been screened. This chemical binds directly and alleviate the hydrolysis activity of karrikin receptor AtKAI2. However, the precise binding mode of KK181N1 in AtKAI2 still remains unknow. In this study, the structure of AtKAI2-KK181N1 complex was solved at 1.90 Å resolution with a clear electron-density map of KK181N1. KK181N1 belongs to N-heterocyclic ureas and designed to covalently modify α/β hydrolase fold-containing proteins through the catalytic nucleophile. However, unexpectedly, I found that this binding was caused by noncovalent interaction and KK181N1 was firmly embedded in the pocket of AtKAI2. This binding mode of KK181N1 is totally different from that of OsD14-KK094, although OsD14 is highly homologous to AtKAI2 and KK094 only differs from KK181N1 in one methyl group. To thoroughly understand the structural basis of KK181N1's binding mode in AtKAI2, we divide the binding pocket into three regions: the bottom, the middle region, and the entrance of the pocket.

At the bottom of the pocket, I found a four water molecules-mediated hydrogen-bonding network connecting the 1,2,3-triazole moiety of KK181N1 to the residues surrounded, including the catalytic nucleophile S95, catalytic base H247, Y124, S119, and the backbone of F26 and V96. Our structure and activity relationship studies have indicated that this hydrogen-bonding network is of great importance for KK181N1-binding affinity in AtKAI2. It is notable that the variants S95C and S95D respectively retain 97% and 65% of wild type's affinity to this chemical, suggesting the broad-spectrum applicability of our in-house chemical library for α/β hydrolase fold superfamily proteins. Intriguingly, the structure comparison between OsD14-KK094 and AtKAI2-KK181N1 complexes has shown that, even though these two chemicals have different binding mode in the pocket, they all participate in this water molecules-mediated hydrogen-bonding network with greatly similar topology, suggesting this hydrogen-bonding network, which seems to be conserved among KAI2/D14 family proteins, would be favored for the binding of 1,2,3-triazole compounds.

At the middle region of the pocket, we found extensive hydrophobic interactions between the aromatic plane of KK181N1 with polar residues shaping the active pocket. Alanine scanning experiment reveals that the hydrophobic interactions of these residues are essential for the KK181N1-binding in the pocket. It's interesting to notice that a variant A219S shows two-folds higher activity than wild type for the binding to KK181N1. Previously, A219V was revealed to narrow the entrance gate for the ligand entry. However, the structure comparison between AtKAI2-KK181N1 complex and *apo*-OsD14 (corresponding residue: S270) indicates that the side chain of serine at this site would not cause the steric clash with ligands, including KAR₁ and KK181N1. Moreover, I compared the structures of AtKAI2-KAR₁ and *apo*-AtKAI2 with that of AtKAI2-KK181N1 complex and found that F134, F157, L142, F194 and L218 have undergone the

conformational changes for the perception of KK181N1.

At the entrance of pocket, I notice that three residues, including F134, L142 and L218, account for the recognition of ligand KK181N1. It is interesting to find that each of the alanine substituent at these positions all possesses increased affinity to KK181N1, which may suggest that the releasing of steric effect at the entrance will promote the recognition of KK181N1. This is particularly verifying my structural information where all of the residues (F134, L142 and L218) occur conformational shift to accommodate the KK181N1.

To study the ligand selectivity, I place my focus on the three residues (F157, L218, and A219), which interact with the methyl group of KK181N1 by hydrophobic interaction but are different from the residues at the corresponding location in OsD14. The structural comparison and affinity assay suggested that although S270 (A219 in AtKAI2) and V269 (L218 in AtKAI2) in OsD14 may elevate the binding affinity of OsD14 to KK181N1, the Y209 (F157 in AtKAI2) would cause sever steric clash with carbonyl and the methyl group of KK181N1. This may partially explain the binding preference of KK181N1 to AtKAI2 but not OsD14 and is consistent with our physiological result that KK181N1 exhibit the inhibitory effect on hypocotyl growth through karrikin signaling pathway, but not SL. On the other hand, to further confirm the significance of this methyl group for ligand recognition by AtKAI2, we prepared an array of derivatives with different substitution at this group, including hydrogen, nitro, chloro, and fluoro group. However, all of them shows decreased affinity to AtKAI2, suggesting that methyl group is most favored by this hydrophobic environment.

The chemical synthesis of KK compounds produces two forms of isomers. In one compound, carbonyl group is connected to nitrogen atom at 1-position of 1,2,3- triazole moiety (annotated as N1), whereas in another carbonyl group is connected to nitrogen atom at 2-positon of 1,2,3-triazole moiety (annotated as N2). Since I have found the great contribution of hydrogen-bonding network to anchoring the KK181N1 in the deep site of pocket, I am wondering whether this network also contributes to the specificity of KK compounds in AtKAI2. The affinity comparison between KK181N1 and KK181N2 or KK094N1 and KK094N2 suggested that AtKAI2 only shows higher affinity toN1-type ureas, which is consistent with the structural details with respect to the topology of hydrogen-bonding network where N1-type 1,2,3-triazole ureas form three hydrogen bonds to join but N2-type 1,2,3-triazole ureas only have one. This result was further examined by applying the chemical KK122 for affinity assay. Even in the absence of one nitrogen atom (indicated by N5) forming one hydrogen bond, the presence of another nitrogen (indicated by N6) harboring two hydrogen bonds could reinforce the KK122-binding affinity to about 3 folds higher than KK094N2, or 2 folds than KK181N2, stating that N6 atom-formed hydrogen bonds are essential for the binding affinity of 1,2,3-triazole-containing chemicals toward AtKAI2. What's more, by using other N-heterocyclic ureas with divergent moieties, I also found that N1-type1,2,3-triazole is greatly favored by AtKAI2 proteins, which is contributed by the unique hydrogen-bonding network. Taken together, the structural analysis and SAR studies suggested that the water molecules-mediated hydrogen-bonding network not only promotes the tight binding of KK181N1, but also confers the ligand selectivity of AtKAI2 for different isomers, and the residues involved in the recognition of the methyl group of KK181N1 greatly account for the selectivity of KK181N1 to AtKAI2 but not D14.

The study of uncovering the interaction mechanism of AtKAI2-KK181N1 will not only enlighten us to perform structure-aided optimization of KK181N1 to generate a more robust inhibitor but also encourage us to apply this antagonist in plants for basic research use, such as for the exploration of the native ligand of KAI2 and the solo establishment of SL function that bypass KAI2 signaling.