Studies on Development of ABA Receptor Antagonists

（課程博士・様式７）（Doctoral qualification by coursework,Form 7）

学 位 論 文 要 旨

Abstract of Doctoral Thesis

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Abstract：
The plant hormone ABA is critical for plant growth and several abiotic stress responses. ABA signaling is normally repressed by group-A PP2Cs, which are negative regulators of the ABA response. A stress-induced ABA binds PYL receptors, which triggers the conformational change associated with gate closure to create an interaction surface with the PP2Cs. ABA-bound PYLs bind and inhibit PP2Cs, including HAB1, ABI1, and ABI2, which directly inactivate SnRK2s, to allow the activation of SnRK2s via autophosphorylation. Activated SnRK2s phosphorylate transcription factors and S-type anion channels to elicit ABA responses such as seed dormancy and stomatal closing. By focusing on the structural properties of ABA, which acts as a positive allosteric regulator of PYLs, we have developed a negative allosteric regulator that retains the high selectivity for PYLs but does not activate PYLs.

In the PYL-ABA complexes, ABA intrudes deeply into the ligand binding pocket to be tightly packed by PYL's residues. The C2′, C3′ and C4′ positions of ABA's ring were found to participate in gate closure via hydrophobic contacts with the gate loop, and furthermore, the X-ray structures of several PYL-ABA complexes revealed a small tunnel above ABA's 3′ ring that opens at the PP2C binding interface. Here, ABA analogs with sufficiently long 3′ alkyl chains were predicted to traverse this tunnel and block PYL-PP2C interactions. To test this, a series of 3′-alkylsulfanyl ABAs were synthesized with different alkyl chain lengths by nucleophilic addition of alkyl thiolate to the 2′,3′-epoxide, prepared from ABA. For simplicity, there are called the AS*n* compound series, where *n* denotes the alkyl chain length. Before preparing these compounds, a model of a PYR1-AS6 complex was constructed on the basis of the crystal structures of PYR1-ABA complexes. This model suggested that alkyl chains of compounds with $n > 4$ protrude through the PYR surface and block the interactions with PP2C, whereas the compounds with $n < 4$ might stabilize gate closure and act as agonists.

In seed germination and a seedling growth assay using *Arabidopsis* and lettuce, AS2 and AS3 acted as ABA mimics, where AS5–AS12 suppressed ABA's effect in coapplications with ABA. Radish seedlings treated with AS2 enhanced drought tolerance, whereas AS6-treated seedlings wilted more quickly than non-treated controls. In addition, AS2 induced the expression of ABA-responsive genes and the β-glucuronidase gene on an ABA-responsive, transcriptional, reporter line, as expected of an agonist. Conversely, AS4 and AS6 lessened the expression levels of ABA-responsive genes and the

β-glucuronidase gene in cotreatments with ABA. This antagonist effect was stronger for AS6 than AS4. Notably, AS6 also repressed the expression levels of stress-induced ABA-responsive genes in response to treatments with mannitol, which induced ABA synthesis by mimicking the water loss caused by drought. These physiological data suggested that AS2 and AS6 functioned as a PYL agonist and antagonist, respectively, whereas AS4 exhibited an intermediate effect.

The effects of AS*n* on 11 of the 13 *Arabidopsis* receptors were examined using receptor-mediated phosphatase assays. AS2 activated PYR1 and PYL1–3 with potencies comparable to that of ABA, but did not show the activation of PYL8–10; thus, AS2 was not a broad-spectrum agonist. On the other hand, AS6 antagonized the ABA-dependent PP2C inhibition for all of the receptors tested in a cotreatment assay, indicating a broad-spectrum antagonist activity. The mechanism of the AS6 antagonist activity was further characterized by a pull-down assay. In cotreatment experiments, AS6 blocked ABA-induced PYL-PP2C interactions in a dose-dependent manner. These biochemical results indicated that AS6 acts as an antagonist by blocking ABA-induced PYL-PP2C interactions.

Isothermal titration calorimetry was next used to characterize AS6 binding with PYL5 and PYL10. These analyses revealed apparent dissociation constants of AS6 (0.4–1.3 μM) with negative enthalpies, indicating an exothermic binding process. These values were comparable to those of ABA (0.8 μM). The molecular mechanism of AS6 activity was then clarified by determining the crystal structure of recombinant PYR1 bound to AS6 (Protein Data Bank code 3K90). The PYR1-AS6 complex was found to very closely resemble the PYR1-ABA complex. Consistent with the present model-based predictions, the gate loop adapted a closed conformation, and the 3′ tunnel accommodated the AS6 *S*-hexyl chain, which also protruded out onto PYR1's PP2C-interaction surface. These observations provide direct evidence that AS6 induces the gate-closed conformer but the *S*-hexyl chain interferes with the PYL-PP2C interactions.

To examine the functional selectivity of AS*n*, the effects of AS2 and AS6 on two well-characterized, non-receptor ABA binding sites were characterized. Both compounds were found not to be potent inhibitors of the ABA metabolic CYP707A enzyme. No significant differences were observed in endogenous ABA concentrations between AS*n* and non-treated *Arabidopsis* seedlings. In addition, AS6 did not substantially inhibit the ABA transport activity of NPF4.6/AIT1. These data suggest that AS*n* has little effect on ABA metabolism and transportation.

This study provides a new approach for the design of ABA analogs, and the results validated structure-based designs of this target class.