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Abscisic acid analogs as chemical probes for dissection of abscisic acid responses in *Arabidopsis thaliana*

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ABSTRACT

Abscisic acid (ABA) is a phytohormone known to mediate numerous plant developmental processes and responses to environmental stress. In *Arabidopsis thaliana*, ABA acts, through a genetically redundant family of ABA receptors entitled Regulatory Component of ABA Receptor (RCAR)/Pyrabactin Resistant 1 (PYR1)/Pyrabactin Resistant-Like (PYL) receptors comprised of thirteen homologues acting in concert with a seven-member set of phosphatases. The individual contributions of *A. thaliana* RCARs and their binding partners with respect to specific physiological functions are as yet poorly understood. Towards developing efficacious plant growth regulators selective for specific ABA functions and tools for elucidating ABA perception, a panel of ABA analogs altered specifically on positions around the ABA ring was assembled. These analogs have been used to probe thirteen RCARs and four type 2C protein phosphatases (PP2Cs) and were also screened against representative physiological assays in the model plant *Arabidopsis*. The 1′-O methyl ether of (*S*)-ABA was identified as selective in that, at physiologically relevant levels, it regulates stomatal aperture and improves drought tolerance, but does not inhibit germination or root growth. Analogs with the 7′- and 8′-methyl groups of the ABA ring replaced with bulkier groups generally retained the activity and stereoselectivity of (*S*)- and (*R*)-ABA, while alteration of the 9′-methyl group afforded an analog that substituted for ABA in inhibiting germination but neither root growth nor stomatal closure. Further *in vitro* testing indicated differences in binding of analogs to individual RCARs, as well as differences in the enzyme activity resulting from specific PP2Cs bound to RCAR-analog complexes. Ultimately, these findings highlight the potential of a broader chemical genetics approach for dissection of the complex network mediating ABA-perception, signaling and functionality within a given species and modifications in the future design of ABA agonists.

Introduction

The plant hormone (*S*)-abscisic acid (**1**, (*S*)-ABA, (+)-ABA; Fig. 1) is a key signaling molecule employed by all plants for both amelioration of responses to abiotic stress and modulation of general plant growth and development (Wasilewska et al., 2008). Although the complete mechanism of ABA signal transduction mediating this breadth of physiological functions remains unclear, recent advances in the understanding of ABA perception have helped clarify some of the earlier steps (Cutler et al., 2010; Raghavendra et al., 2010). In particular, two proteins, RCAR1 (Regulatory Component of ABA Receptor 1)/PYL9 (Pyrabactin Resistant 1-Like 9) and RCAR11/PYR1 (Pyarabactin Resistant 1) were identified independently, using protein interaction analyses and chemical genetics approaches respectively, to be members of a family of fourteen homologues in *Arabidopsis thaliana*(*A. thaliana*), forming the RCAR/PYR1/PYL family of ABA receptors (Ma et al., 2009; Park et al., 2009).

Abbreviations: ABA, abscisic acid; ABI, ABA insensitive; *A. thaliana*, *Arabidopsis thaliana*; HAB, homology to ABA insensitive; ITC, isothermal titration calorimetry; RCAR, Regulatory Component of ABA Receptor; PP2C, type 2C protein phosphatases; PYL, Pyrabactin Resistant-Like; PYR1, Pyrabactin Resistant 1.

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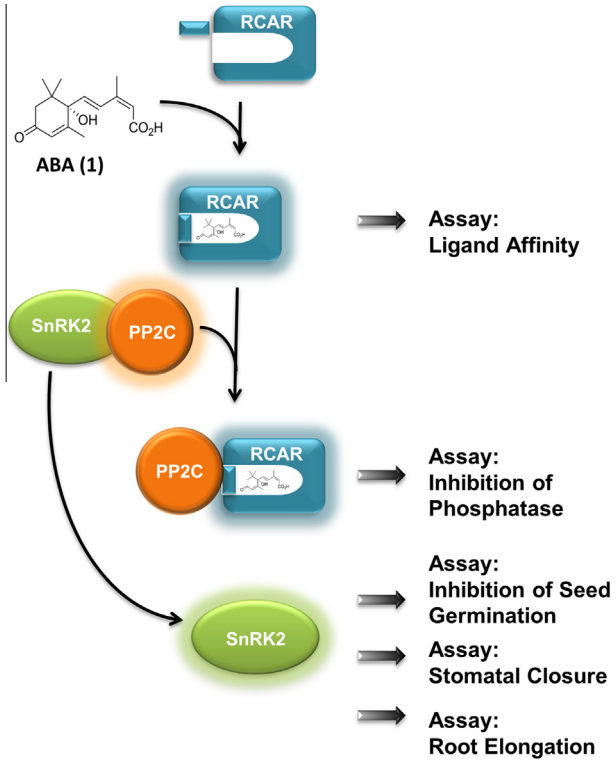

Fig. 1. Signal transduction by ABA (1) and associated assays. ABA (1) binding to the RCAR receptor, leads to closure of the gate and latch lid over the active site. The affinity of the receptor–ABA or receptor–analog interaction is measured by isothermal titration calorimetery herein. The complex presents a surface that includes the gate and latch regions, that has high affinity for the PP2C, sequestering it away from SnRK2 and inactivating it. The degree of sequestration of the PP2c can be assessed by measuring its phosphatase activity in the presence of the ligand-stimulated RCAR. Once released from the inhibitory effect of the PP2C, SnRK2 stimulates downstream signalling, leading to well document physiological effects. Some these include, inhibition of seed germination, stomatal closure and inhibition of root elongation, all which can be measured.

The genetic and functional redundancy of the members of this family of receptors, which made initial identification by classical genetics impossible, was further highlighted later by the need to knock out at least three of the family members at once to elicit a change in phenotype (Park et al., 2009). This redundancy has made further functional characterization of the individual family members difficult. Indeed to date, only a few targeted studies have linked particular members of this family to specific physiological effects. For example, RCAR1 was recently shown to modulate downstream phosphorylation of the guard cell linked anion channel SLAH3, but this was only demonstrated *in vitro* to date (Geiger et al., 2011). Other reports link RCAR10 (PYL4) over-expression to regulation of jasmonic acid signaling (Lackman et al., 2011) and RCARs 8 and 10 (PYLs 4 and 5) over-expression to increased drought resistance (Santiago et al., 2009b; Pizzio et al., 2013)). At the same time RCAR 8 has been linked to modulation of root growth (Antoni et al., 2013). Additionally, RCAR7 (PYL13) was shown to modulate classic ABA-sensitive physiological effects, through interactions with PP2Cs, but independently of any interaction with ABA itself (Zhao et al., 2012). However, another report documents the characterization of triple, quadruple, quintuple and even sextuple RCAR mutants, targeting RCARs 3, 8, 10, 11, 12 and 14, and concluded that the family members contribute additively to roles in regulation of seed germination, plant growth and reproduction, stomatal aperture, and transcriptional response (Gonzalez-Guzman et al., 2012). Finally orthologs of the *A. thaliana* receptors have been reported in a variety of other plant species including rice (Kim et al., 2012), strawberry (Chai et al., 2012; Jia et al., 2011; Li et al., 2011), grape (Boneh et al., 2012; Li et al., 2012), citrus (Romero et al., 2012), cucumber (Wang et al., 2012) and soy bean (Bai et al., 2013), with roles for these receptors broadly correlated to ABA sensitivity, ripening and stress perception processes. In general, the functional roles of individual ABA receptor family members remain to be deciphered.

The regulation of ABA-mediated RCAR signaling downstream of perception appears to be very complex. On the one hand, as many as seven different members of the clade A PP2C family in *A. thaliana* have been implicated in ABA responses, each with independent and overlapping functions (Merlot et al., 2001; Kuhn et al., 2006; Robert et al., 2006; Saez et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007; Antoni et al., 2012). While some of these PP2Cs have been shown to interact with multiple RCAR receptors, they, like the receptors themselves, are also differentially expressed throughout plant tissues during different developmental stages (Nishimura et al., 2010; Szostkiewicz et al., 2010). On the other hand, recent reports suggest that the same receptor surface that binds to PP2Cs also mediates homodimerization of a subset of the RCAR receptor family (Dupeux et al., 2011; Hao et al., 2011). While such receptor dimerization has been linked to inhibition of basal receptor activity against the PP2Cs as well as a decreased sensitivity to ABA (**1**) in general, a more recent report questions the biological relevance of this interaction (Antoni et al., 2012). Additionally, in contrast to inactive ABA metabolites, specific hydroxylated catabolites of ABA have been shown to interact with the receptors and inhibit the activity of associated PP2Cs, introducing the possibility of a role for ABA catabolites in regulation of signaling (Kepka et al., 2011). Together these findings suggest a complex network of interplay mediating ABA-perception and signaling that relies on spatially and temporally regulated gene expression of the genetically redundant receptors and PP2Cs, as well as regulation of signaling through protein–ligand and protein–protein interactions.

Mechanistically, structural analyses of RCAR receptors have demonstrated conformational differences in ABA-bound and ABA-free receptor forms, highlighting open access of the ligand to an internal binding cavity in the unbound form (Melcher et al., 2009; Melcher et al., 2010; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009a; Yin et al., 2009; Shibata et al., 2010; Soon et al., 2012; Miyakawa et al., 2012). However, once ABA (**1**) has entered, and docked with its side-chain carboxyl group deepest into the cavity, two loops that are located at the entrance of the protein's ABA binding pocket (termed the gate and latch), close over the 2,6,6-trimethylcyclohexenone ABA ring to form a 'lid' on the cavity, thus encapsulating ABA (**1**) within the receptor. The resulting hydrophobic area on the receptor surface formed by the 'lid' binds to a specific group of type 2C protein phosphatases (PP2Cs) including a direct interaction between a PP2C tryptophan residue and ABA (**1**). This tight interaction causes inactivation of the PP2C co-receptor, effectively removing the brake on ABA signal transduction and leading to well documented ABA responses (Raghavendra et al., 2010; Miyakawa et al., 2012; Fig. 1).

Studies using small molecule ligands are shedding light on the structural requirements of the binding site in the cavities of the RCAR ABA receptors. Screening of large chemical libraries has led to the identification of several synthetic aromatic sulfonamides, non-ABA-like, small molecules selective for groups of receptors and physiological effects (Okamoto et al., 2013; Cao et al., 2013). One of these non-ABA related chemicals, pyrabactin, activates two of the RCAR receptors, while another, quinabactin, activates an additional three RCARs. Pyrabactin affects seed ABA processes while quinabactin has effects on stomatal closure in a number of plant species.

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