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Small Molecule Probes of ABA Biosynthesis and Signaling

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The phytohormone ABA mediates many physiological and developmental responses, and its key role in plant water relations has fueled efforts to improve crop water productivity by manipulating ABA responses. ABA's core signaling components are encoded by large gene families, which has hampered functional studies using classical genetic approaches due to redundancy. Chemical approaches can complement genetic approaches and have the advantage of delivering both biological probes and potential agrochemical leads; these benefits have spawned the discovery and design of new chemical modulators of ABA signaling and biosynthesis, which have contributed to the identification of ABA receptors and helped to define PYR1 and related subfamily III receptors as key cellular targets for chemically manipulating water productivity. In this review, we provide an overview of small molecules that have helped dissect both ABA signaling and metabolic pathways. We further discuss how the insights gleaned using ABA probe molecules might be translated to improvements in crop water productivity and future opportunities for development of small molecules that affect ABA metabolism and signaling.

Keywords: ABA • Agonist • Antagonist • Biosynthesis • Signaling • Small molecule.

Abbreviations: ABF, ABRE-binding factor; ABI, ABA INSENSITIVE; ABRE, ABA-responsive element; AHG, ABA HYPERSENSITIVE AT GERMINATION; AHI4, ABA 8′-hydroxylase inhibitor 4; AREB, ABRE-binding protein; CCD, carotenoid cleavage dioxygenase; HAB1, HYPERSENSITIVE TO ABA 1; NCED, 9-cis-EPOXYCAROTENOID DIOXYGENASE; NDGA, nordihydroguaiaretic acid; OST1, OPEN STOMATA1; PA, phaseic acid; PP2C, group-A type 2 C of protein phosphatases; PYR/PYL/RCAR, Pyrabactin Resistance 1/PYR1-Like/Regulatory Component of ABA Receptor; SnRK2, subclass III Snf1-related protein kinases2.

General Introduction

The phytohormone ABA has multiple roles in physiological processes including embryo maturation, induction of seed dormancy, plant growth regulation, stomatal closure, and biotic and abiotic stress responses. ABA levels fluctuate in response to physiological and environmental cues and act through a land-plant conserved signal transduction pathway to alter physiological outputs. ABA acts through a negative regulatory pathway to regulate the activity of subclass III Snf1-related protein kinases2 (SnRK2s), which in turn control the activities of numerous downstream factors. Genetic studies uncovered several key components in both the ABA metabolic pathway and signal transduction, but have been complemented tremendously through the use of diverse chemical probes (Nambara and Marion-Poll 2005, Cutler et al. 2010). Forward genetic approaches in Arabidopsis identified *ABA insensitive* (*abi1–abi5*) mutants and *ABA hypersensitive at germination* (*ahg1* and *ahg3*) mutants which helped define multiple core ABA signaling components including group-A type 2 C of protein phosphatases (PP2Cs) (ABI1 and 2, AHG1 and 3) and transcription factors (ABI3, 4 and 5) (Cutler et al. 2010, Finkelstein 2013). Similarly, *open stomata1* (*ost1*) mutants, which were identified because of increased transpiration and decreased leaf temperature, helped define SnRK2s as key mediators of ABA signaling. However, early genetic studies failed to identify ABA receptors which are encoded by a large gene family. For the most part, loss-of-function ABA receptor mutants show either no or weak ABA-related phenotypes, making them essentially invisible to the lens of genetic analysis. The selective ABA agonist pyrabactin was isolated as an ABA mimic using forward chemical genetic screens and bypassed this redundancy because it selectively activates PYR1 and a small number of other receptors. This selectivity enabled loss-of-function PYR1 alleles to display strong pyrabactin resistance phenotypes which facilitated the identification of soluble ABA receptors (Park et al. 2009). In this review, we provide a non-exhaustive overview of key small molecules which have helped to better understand both ABA biosynthesis and signaling, and discuss how we can further improve and utilize small molecules in ABA research and agriculture.

The ABA Metabolic Pathway

Both ABA biosynthesis and catabolism determine cellular ABA levels and, as a consequence, the degree of ABA signaling (Nambara and Marion-Poll 2005, Finkelstein 2013). In land plants ABA is synthesized via C40 epoxycarotenoids that are synthesized from isopentenyl diphosphate (IPP), derived from the methylerythritol phosphate (MEP) pathway in plastids (**Fig. 1A**). Mutants in β-carotenoid biosynthetic genes show ABA deficiency phenotypes with pleiotropic phenotypes

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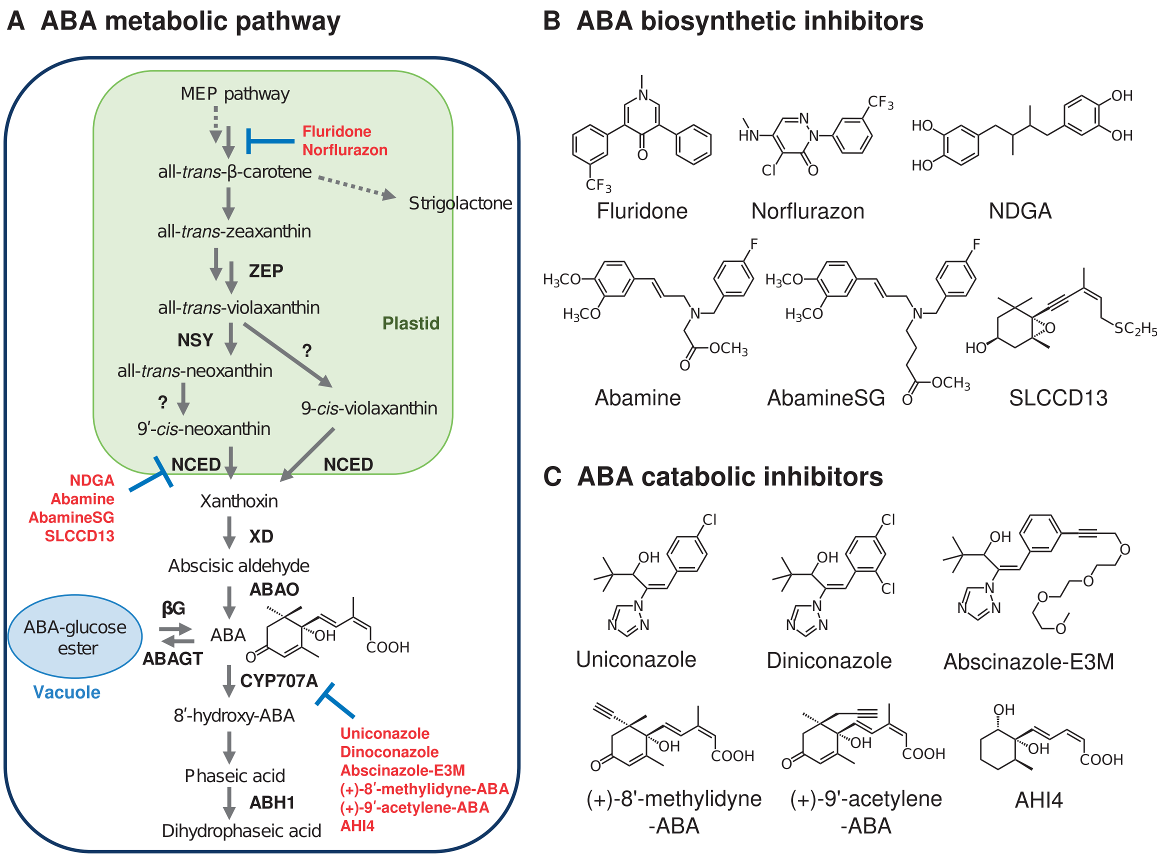

Fig. 1 (A) ABA biosynthetic and catabolic pathways. ABA precursor is synthesized from the MEP pathway. Enzymes are shown in bold. Abbreviations: ZEP, zeaxanthin epoxidase; NSY, neoxanthin synthase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; XD, xanthoxin dehydrogenase; ABAO, abscisic aldehyde oxidase; CYP707A, ABA 8′-hydroxylase; ABH1, phaseic acid reductase; ABAGT, ABA glucosyltransferase; BG, β-glucosidase. ABA biosynthetic inhibitors (B) and catabolic inhibitors (C).

including seedling lethality and photobleaching due to a lack of Chl. Downstream of the metabolite all-*trans*-zeaxanthin, conversions are more specific towards ABA biosynthesis. A two-step epoxidation of all-*trans*-zeaxanthin to all-*trans*-violaxanthin is catalyzed by ZEAXANTHIN EPOXIDASE (ZEP) (Finkelstein 2013). All-*trans*-violaxanthin is converted to either 9-*cis*-violaxanthin or all-*trans*-neoxanthin. Arabidopsis ABA4 is thought to catalyze the conversion of all-*trans*-violaxanthin into all-*trans*-neoxanthin (North et al. 2007); however, corresponding isomerases for all-*trans*-epoxycarotenoids, violaxanthin and neoxanthin to form their 9-*cis*-isomers are still unknown. Although there are two possible substrates for 9-*cis*-EPOXYCAROTENOID DIOXYGENASE (NCED), which are 9-*cis*-isomers of epoxycarotenoids and violaxanthin, 9′-*cis*-neoxanthin is believed to be the major substrate for NCED (North et al. 2007). The oxidative cleavage of 9-*cis*-epoxycarotenoids into xanthoxin by NCED is a key regulatory step for ABA biosynthesis (Finkelstein 2013). As such, changes of endogenous ABA levels are well correlated to those of NCED gene expression. Drought-induced ABA accumulation is preceded by increases in NCED gene expression, whereas rehydration after dehydration causes a rapid decrease in both endogenous ABA and NCED expression levels (Finkelstein 2013). NCED enzymes are encoded by multigene families across land plants, with distinct family members playing unique roles in developmental processes and stress responses (Iuchi et al. 2001, Tan et al. 2003). Conversion of xanthoxin to abscisic aldehyde is catalyzed by AtABA2, which encodes a short chain dehydrogenase/reductase (Cheng et al. 2002, González-Guzmán et al. 2002) (**Fig. 1A**). The last step of ABA biosynthesis is the conversion of abscisic aldehyde to ABA by abscisic aldehyde oxidase (Finkelstein 2013). Aldehyde oxidase requires a molybdenum cofactor (Moco) for enzyme activity. Therefore, mutants defective in Moco biosynthesis also exhibit ABA-deficient phenotypes (Xiong et al. 2001).

Catabolism of ABA can occur through hydroxylation and conjugation (**Fig. 1A**). Among them, 8’-hydroxylation is a key step in the major ABA catabolic route. Hydroxylation at the C-8’ position of ABA is catalyzed by the CYP707A family, which belongs to the Cyt P450 monooxygenases. The product, 8’-hydroxy-ABA, is unstable, and is isomerized spontaneously to phaseic acid (PA) (Kushiro et al. 2004, Saito et al. 2004). CYP707As are encoded by multigene families across higher plant species (Hanada et al. 2011), and each member of those families plays different physiological or developmental roles (Okamoto et al. 2006, Okamoto et al. 2009). CYP707A genes are transcriptionally induced in response to rehydration, submergence and in developmental processes such as germination

(Kushiro et al. 2004). PA has been described as a weak ABA agonist (Kepka et al. 2011, Weng et al. 2016). Conversion of PA to dihydrophaseic acid (DPA), which is biologically inactive, is catalyzed by PA reductase, encoded by Arabidopsis *ABA HYPERSENSITIVE 2* (*ABH2*) (Weng et al. 2016). ABA's hydroxylated catabolites are further conjugated with glucose [ABA glucosyl ester (ABA-GE) is the most common conjugate]. ABA-GE is thought to be a storage or long-distance transport form of ABA, which functions as a mobile stress signal from root to shoot (Nambara and Marion-Poll 2005, Finkelstein 2013); however, the function of long-distance ABA transport is debated. Glucosylation of ABA's carboxy group is catalyzed by glucosyltransferases. In order to release ABA, β-glucosidases hydrolyze ABA-GE, and thus can regulate the local ABA concentration in the cell. Enzyme activity of endoplasmic reticulum-localized β-glucosidases is activated by their polymerization in response to dehydration, whereas vacuole-localized β-glucosidase is protected from degradation under dehydration stress (Finkelstein 2013).

Small Molecules for Regulating ABA Metabolism

Inhibitors of ABA metabolism have been utilized not only for analyzing the physiological effect of ABA in non-model plant species, but also for chemical genetic studies in model plant species (**Fig. 1B, C**). Since ABA is synthesized from carotenoids, carotenoid biosynthetic inhibitors can reduce endogenous ABA levels. Fluridone and norflurazon are phytoene desaturase inhibitors. Thus, treatment with these compounds not only reduces endogenous ABA, but also causes concurrent bleaching of plants due to the photo-oxidation of Chl (Gamble and Mullet 1986). Due to these pleiotropic effects, fluridone and norflurazon are not favored as ABA biosynthesis inhibitors. Carotenoid cleavage enzymes involved in ABA biosynthesis were initially predicted to be lipoxygenase-like enzymes before NCEDs were identified (Creelman et al. 1992). Nordihydroguaiaretic acid (NDGA) was known as an inhibitor of lipoxygenase, which catalyzes deoxygenation of polyunsaturated fatty acids, and inhibited the accumulation of ABA in soybean suspension cells subject to osmotic stress (Creelman et al. 1992). However, NDGA also inhibits lipid synthesis and plant growth (Mérigout et al. 2002). Therefore, a more specific inhibitor of NCED was developed, abamine, inspired by the structure of NDGA (Han et al. 2004). In addition, abamine analogs have also been designed. For example, abamineSG, which possess increased specificity against NCED without the negative effects on plant growth compared with NDGA, is more potent in suppressing ABA accumulation and repression of ABA-inducible gene expression during osmotic stress treatment in Arabidopsis compared with abamine (Kitahata et al. 2006). Hydroxamates similar to abamine have been described yielding antagonists of both NCEDs and closely related carotenoid cleavage dioxygenases (CCDs) involved in strigolactone biosynthesis (Sergeant et al. 2009). An alternative strategy for the development of a more specific NCED inhibitor entails sesquiterpene-like carotenoid cleavage dioxygenase (SLCCD) inhibitors, which were developed based on the chemical structure of the substrate and product of NCED (Boyd et al. 2009). This resulted in a compound which suppresses osmotic stress-inducible ABA accumulation and ABA-responsive genes in Arabidopsis (Boyd et al. 2009). A high-resolution crystal structure of NCED with bound substrate or inhibitors would greatly assist in the development of improved NCED inhibitors, since the current structure is a relatively low-resolution structure of the apo form of maize VP14 (Messing et al. 2010).

Treatment with ABA biosynthetic inhibitors can overcome dormancy of seeds, buds and tubers in non-model plant species. Consequently, it is widely known that ABA is a key small molecule for inducing and maintaining dormancy. These insights might contribute to yield improvements in agriculture using ABA biosynthetic inhibitors. For example, constitutive *NCED*-overexpressing plants show drought tolerance and dormant seed phenotypes. However, an increase in seed dormancy will negatively affect yield. Application of ABA biosynthetic inhibitors as seed coating can overcome the negative effect of increased seed dormancy.

The treatment of ABA biosynthetic inhibitors can break dormant states of seeds, buds and tubers in non-model plant species. Consequently, it is widely known that ABA is a key small molecule for the induction and maintenance of dormancy. These insights will contribute to the improvement of agricultural productivity using ABA biosynthetic inhibitors. For example, constitutive *NCED*-overexpressing plants show drought tolerance and dormant seed phenotypes. However, a low efficiency of seed germination reduces final harvest in agriculture. It is possible to overcome only the negative aspect of ABA on agriculture by coating ABA biosynthetic inhibitors on the seeds.

Small molecules affecting ABA catabolism (**Fig. 1C**) primarily target the Cyt P450 monooxygenase CYP707A. Among these small molecules, two groups can be distinguished: azole-type inhibitors and ABA analogs. Uniconazole and diniconazole are examples of azole-type inhibitors. Uniconazole inhibits the activity of CYP707A and increases endogenous ABA, and as a result uniconazole treatment confers drought stress tolerance (Kitahata et al. 2005, Saito et al. 2006). However, uniconazole was originally developed as a gibberellin biosynthesis inhibitor and consequently induces dwarfism (Saito et al. 2006). Diniconazole, whose structure is similar to that of uniconazole, was developed as a fungicide. Like uniconazole, diniconazole inhibits CYP707A and induces the expression of ABA-responsive genes (Kitahata et al. 2005). However, azole inhibitors can cause non-specific inhibition of Cyt P450 monooxygenases as a consequence of their co-ordination between azoles and P450 heme–iron complexes. Although both uniconazole and diniconazole inhibit CYP707A, they also inhibit other Cyt P450 monooxygenases and, as a consequence, negatively affect plant growth (Rademacher 2000). The structure of uniconazole has been used as a scaffold to develop more specific inhibitors for CYP707A. For example, abscinazole-E3M selectively inhibits CYP707A, but not the related CYP701A which is required for gibberellin biosynthesis (Takeuchi et al. 2016). As a result, abscinazole-E3M increases endogenous ABA levels and confers drought stress tolerance with less growth arrest (Takeuchi et al. 2016).

In contrast to azole-type inhibitors, ABA analogs can function as specific ABA catabolic inhibitors. The first step in ABA catabolism is the hydroxylation of 8′ and 9′ ring methyl groups by CYP707A enzymes. Suicide inhibitors have been designed by synthesizing 8′- and 9′-modified ABA analogs. Examples include (+)-8′-methylidyne-ABA, (+)-9′-acetylene-ABA and (+)-9′-vinyl-ABA, which irreversibly inhibit CYP707A activity (Cutler et al. 2000). Interestingly, (+)-9′-acetylene-ABA is most potent in inhibiting CYP707A (Cutler et al. 2000), despite CYP707A's preference for ABA's 8′ methyl substituent, suggesting that CYP707A recognizes both C-8′ and C-9′ methyl groups of ABA. While interesting, these compounds retain ABA agonist activity (Benson et al. 2015); to eliminate this activity, several analogs were designed, synthesized and tested which lacked structural features important for ABA agonist activity (Ueno et al. 2005, Araki et al. 2006). Among them, ABA 8′-hydroxylase inhibitor 4 (AHI4) did not show ABA activity such as growth arrest and inhibition of seed germination, but it strongly inhibited the activity of CYP707A (Araki et al. 2006). Therefore, AHI4 modulates ABA levels (and associated drought stress tolerance) without strong growth inhibition. These probe molecules can contribute to a better understanding of ABA metabolic regulation in addition to being potentially of value in agriculture.

The ABA Signaling Pathway

Signal transduction triggered by ABA is initiated by perception by soluble receptors called Pyrabactin Resistance 1/PYR1-Like/Regulatory Component of ABA Receptor (PYR/PYL/RCAR, or PYLs for simplicity) proteins, which are members of the START-domain superfamily ligand-binding proteins (Ma et al. 2009, Park et al. 2009). The key function of PYL receptors is indirectly to control the activity of subclass III SnRK2s, which phosphorylate numerous stress-activated targets in response to ABA (Fujii and Zhu 2009, Soon et al. 2012). This is accomplished through a negative regulatory pathway, involving PYL, PP2C and SnRK2 proteins (**Fig. 2A**). When ABA accumulates in cells in response to environmental stress or developmental cues, ABA binds to PYL receptors and triggers a conformational change that allows the receptor–ABA complex to bind to and inhibit clade A PP2C phosphatase activity. Consequently, subclass III SnRK2s are released to phosphorylate and control the activity of downstream factors in order to trigger physiological responses. Two important classes of SnRK2s targets include membrane channel proteins and transcription factors. For example, SLOW ANION CHANNEL 1 (SLAC1), POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1 (KAT1) and NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOG F (RBOHF), which are plasma membrane proteins, are phosphorylated by SnRK2 and control stomatal movement (Cutler et al. 2010, Finkelstein 2013). Transcription factor targets include the basic leucine zipper (bZIP) domain transcription factors including ABRE-binding proteins (AREBs), ABRE-binding factors (ABFs) and ABI5, which can bind to ABA-responsive elements (ABREs) in the promoters of ABA-inducible genes (Cutler et al. 2010, Finkelstein 2013). In turn, ABA-inducible genes control expression of genes involved in compatible solute biosynthesis, late embryogenesis abundant (LEA) proteins and heat shock proteins, all of which probably contribute to desiccation tolerance. ABI5 binds to plant-specific VP1/ABI3 transcription factors and the complex controls seed dormancy (Nakamura et al. 2001).

Core ABA signaling components, which consist of PYLs, PP2Cs, SnRK2s and ABF/AREB transcription factors, form gene families in land plants (Hanada et al. 2011, Hauser et al. 2011). Recent genome sequences have revealed that PP2Cs, SnRK2s and ABF/AREBs are present throughout Viridiplantae (green algae and land plants). An ABA receptor has recently been described in the liverwort *Marchantia polymorpha*, which complements an Arabidopsis quadruple receptor mutant strain (Bowman et al. 2017). Interestingly, the *Marchantia* genome sequence revealed a small family of PYL paralogs of unknown function; these sequences are divergent in highly conserved regions critical for ABA recognition (such as the -SGLPA- 'gate', described below). It appears that PYL receptors arose in embryophytes in association with the transition of plants to land (Umezawa et al. 2010, Hauser et al. 2011).

Angiosperm PYL receptors are grouped into three subfamilies (I, II and III) (Ma et al. 2009, Hauser et al. 2011) (**Fig. 2B**). Subfamilies I and II are present in all land plants (with the exception of liverworts, which appear to have only clade I receptors); subfamily III receptors appear to be restricted to angiosperms (Hauser et al. 2011, Bowman et al. 2017). For the most part, subfamily III receptors form homodimers that are less sensitive to ABA than subfamily I/II receptors, which have higher affinity and are monomeric. A key function of ABA in angiosperms is to regulate stomatal aperture and gas exchange, and co-ordinate growth and photosynthesis with available water. Although stomata are present in all embryophytes (with the exception of liverworts), sensitive ABA-mediated control of stomata is primarily a feature of angiosperms (Sussmilch et al. 2017) and correlates with the emergence of subfamily III receptors. Analyses using selective ABA agonists in combination with Arabidopsis mutants lacking different classes of receptors suggest that activation of subfamily III receptors, and in particular PYR1 and PYL1 (subfamily IIIA), is sufficient to induce stomatal closure and inhibit seed germination and ABA-like transcriptional responses in Arabidopsis (Okamoto et al. 2013, Vaidya et al. 2017), defining PYR1 and its close relatives as key chemical targets for manipulating transpiration (Helander et al. 2016). Recent experiments expressing different PYL receptors in Arabidopsis protoplasts suggest that subfamily I and II receptors mediate responses to the low basal ABA levels found in unstressed plants, while subfamily III receptors require higher ABA concentrations to activate signaling, trends that are consistent with their different intrinsic ABA affinities (Tischer et al. 2017). Genetic analyses have, however, shown extensive redundancy between receptor subfamilies in Arabidopsis, and genetic removal of receptors from all three families is required to mimic the severe phenotypes of ABA-deficient mutants (Park et al. 2009, Gonzalez-Guzman et al. 2012).

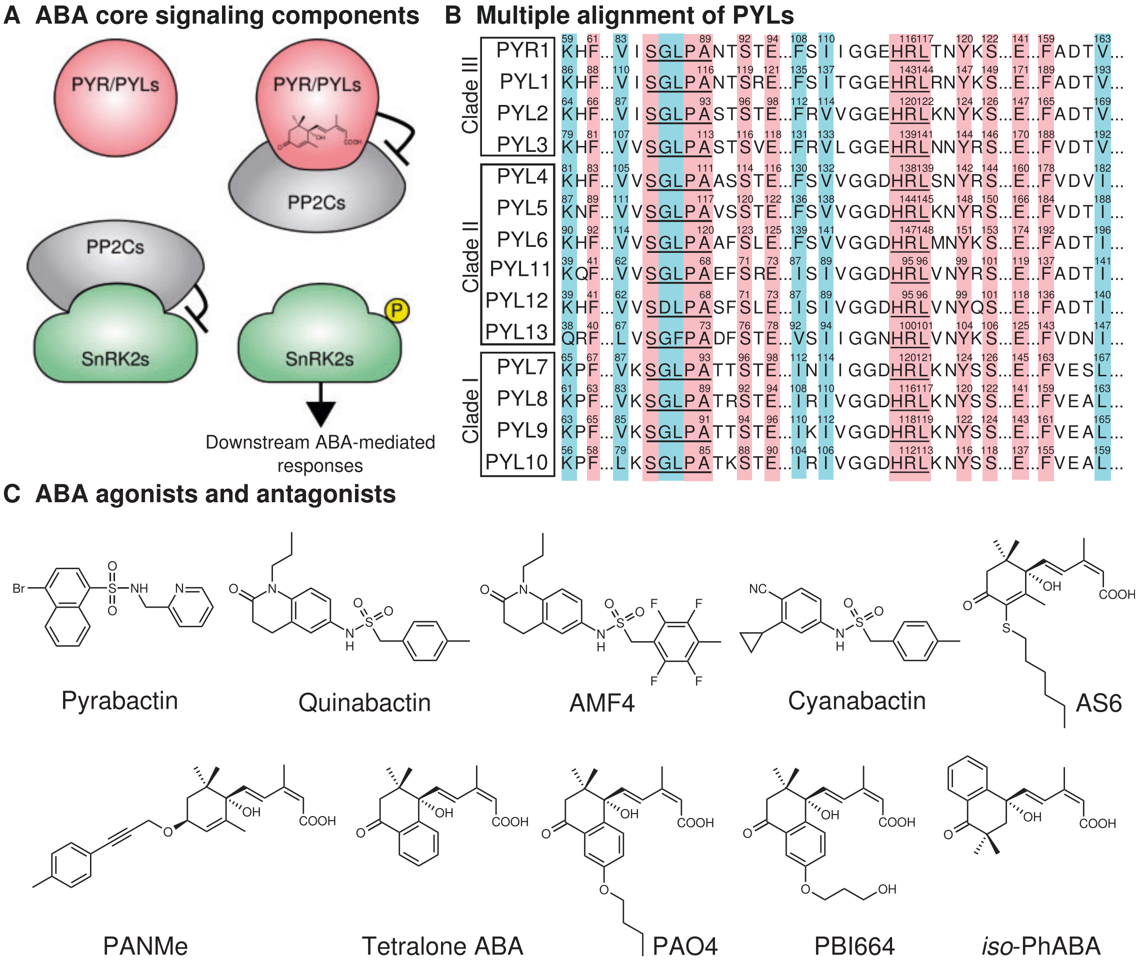

Fig. 2 (A) Schematic representation of the core ABA signaling components. Pyrabactin Resistance 1/PYR1-Like (PYR/PYLs), group-A type 2 C of protein phosphatases (PP2Cs), subclass III Snf1-related protein kinases2 (SnRK2s) (B) Fragments of a COBALT multiple alignment for all the PYL receptors in *A. thaliana*. Numbered residues are reported to interact with ABA (Santiago et al. 2009a), underscored residues constitute the gate (SGLPA) and latch (HRL) loops. Residues highlighted in salmon are highly conserved among PYLs; cyan indicates variability. Sequences are grouped according to the different clades. (C) ABA agonists and antagonists.

In *Brachypodium*, genetic removal of PYR1 leads to increased transpiration (decreased leaf temperature) and decreased sensitivity to exogenous ABA, perhaps suggesting less redundancy between grass receptor subfamilies (Pri-Tal et al. 2017).

In Arabidopsis, nine clade A PP2Cs participate in ABA signaling. Several PP2C mutants in Arabidopsis show ABA-hypersensitive phenotypes. PP2C activities are selectively inhibited by ABA receptors (Antoni et al. 2012, Bhaskara et al. 2012, Tischer et al. 2017). Interestingly, among clade A PP2Cs, only AHG1 lacks the conserved tryptophan lock residue (see below), which is required to form a water-mediated hydrogen bond with ABA's cyclohexenone oxygen in the receptor–ligand–PP2C complex (Antoni et al. 2012, Tischer et al. 2017). Although AHG1 is resistant to receptor-mediated inhibition, *ahg1* mutant seeds are ABA hypersensitive in seed germination assays, indicating that AHG1 participates in ABA responses (Nishimura et al. 2007, Antoni et al. 2012).

The SnRK2 kinase activities are activated by osmotic stress. Of the nine Arabidopsis SnRK2s, three that form subclass III are induced by ABA (SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3). A triple SnRK2 mutant shows nearly complete ABA insensitivity with regard to seed germination, plant growth, stomatal closure and ABA-responsive gene expression (Fujii and Zhu 2009, Fujita et al. 2009, NakashiMa et al. 2009, Umezawa et al. 2009). Thus, ABA action is triggered by subclass III SnRK2-mediated phosphorylation of substrate proteins, although only a small number of direct SnRK2 substrates have been identified. Phosphoproteomics analyses of the wild type or SnRK2 triple mutants treated with ABA has identified new candidate substrates for SnRK2, including proteins involved in flowering, nucleotide binding, transcriptional regulation, signal transduction and chloroplast processes (Kline et al. 2010, Umezawa et al. 2013, Wang et al. 2013). In addition, the SnRK2s appear to be a signaling hub that can be regulated by multiple pathways; for example, Arabidopsis OST1 (SnRK2.6) is activated by cold stress in an ABA-independent manner and regulates cold-induced gene expression (Ding et al. 2015). In addition, regulated proteolysis of receptors and PP2Cs as well as interactions between receptors and diverse targets have been reported, suggesting complex regulation of the core pathway (Belda-Palazon et al. 2016, Wu et al. 2016, Yu et al. 2016a, Yu, et al. 2016b, Zhao et al. 2017).

The core negative regulatory circuit comprised of PYL, PP2C and SnRK2 proteins is understood at the atomic level. Briefly, ABA binds into the hydrophobic ligand-binding pocket of ABA receptors via direct and water-mediated contacts involving highly conserved amino acids. The binding pocket is defined by a seven-sheet β-sheet resembling a folded hand, and one large and two smaller α-helices (Melcher et al. 2009, Santiago et al. 2009a). Ligand binding promotes a conformational change that involves a gate–loop between β3 and β4, which closes over the binding pocket and forms contacts with ABA. The gate–loop contains the sequence -SGLPA- in all PYLs except PYL12 and PYL13, which contain the sequence -SDLPA- and -SGFPA-, respectively (**Fig. 2B**). A second 'latch' loop between β5 and β6 (containing the invariant sequence -HRL-) also changes conformation. This, in turn, allows the receptor–ligand complex to dock into and inhibit clade A PP2Cs, which contain a highly conserved tryptophan residue located in a clade A-specific recognition loop, which inserts into a small pocket created by the closure of the gate–loop and makes a water-mediated contact to ABA's ketone (Melcher et al. 2009, Yin et al. 2009). This water molecule is at the center of an a H-bond network between ABA, and the gate proline (-SGL<u>P</u>A-), latch arginine (-H<u>R</u>L-) and PP2C tryptophan lock. ABA binding is stabilized by direct or water-mediated contacts to 21 residues in the PYL receptors; in addition, binding of the PP2C to the closed receptor conformer greatly increases ABA affinity (>10-fold), presumably by reducing K_{off}.

The inhibitory interaction of PP2Cs with SnRK2s has been reported based on crystal structures (Zhang et al. 2015). Crystal structures of SnRK2.3 and SnRK2.6 (Ng et al. 2011) indicated that both SnRK2s possess a canonical kinase fold. They display a low basal activity that is strongly increased via autophosphorylation of their activation loops (Ng et al. 2011, Zhang et al. 2015). The crystal structure of SnRK2.6 HYPERSENSITIVE TO ABA 1 (HAB1) (Soon et al. 2012) indicates that SnRK2s and PYLs bind to overlapping surfaces that encompass the PP2C catalytic site. SnRK2s are inhibited by PP2Cs through a catalytic mechanism (via dephosphorylation of the SnRK2 activation loop) and stoichiometrically via the formation of a stable complex.

Small Molecule Modulators of ABA Signaling

Small molecules have played an important role in identification and characterization of different ABA receptors as a consequence of the extensive redundancy in ABA perception. Pyrabactin (**Fig. 2C**), the first synthetic ABA mimic, was identified in a forward chemical genetic screen through its activity as a seed germination inhibitor (Zhao et al. 2007, Park et al. 2009). A screen for pyrabactin-resistant mutants defined PYR1 as a genetic factor necessary for pyrabactin's effects (Park et al. 2009) and helped to establish that PYR1 is a bona fide ABA receptor. Pyrabactin is a selective ABA agonist—it preferentially activates PYR1 and PYL1, of which PYR1 is highly expressed in seeds (Park et al. 2009). The ligand-binding pockets of PYR1 and PYL2 are highly similar, yet PYL2 is not activated by pyrabactin. This is due to subtle differences between the PYR1 and PYL2 ligand-binding pockets, which enable pyrabactin to bind PYL2 in a non-productive orientation. This non-productive orientation prevents gate closure and weakly antagonizes ABA binding (Melcher et al. 2010, Peterson et al. 2010, Yuan et al. 2010), although it does not measurably antagonize ABA effects in vivo (Takeuchi et al. 2014). Analogs of pyrabactin have been identified by virtual screening (Melcher et al. 2010) and targeted syntheses (Frackenpohl et al. 2011, Huang et al. 2015). Phosphonate or phosphonamide analogs have also been reported to retain activity, although they have not be validated in in vitro receptor activation assays (Van Overtveldt et al. 2015).

Pyrabactin is a useful probe molecule but its seed selectivity limits its uses. Okamoto *et al.* hypothesized that an agonist that activated more receptors would be required for ABA effects in vegetative tissues and developed a yeast two-hybrid-based receptor activation assay and screened large numbers of compounds for agonists, which led to the discovery of quinabactin (**Fig. 2C**) (Okamoto et al. 2013). Quinabactin is more potent than ABA and pyrabactin when tested against PYR1, and activates more receptors. It mimics many aspects of exogenous ABA treatment: it inhibits seed germination, elicits ABA transcriptional responses, provides protection against water deficit and mimics ABA responses to the extent that it can rescue phenotypes arising from the ABA-deficient mutant *aba2* (Cao et al. 2013, Okamoto et al. 2013). Although PYR1 is highly expressed in seeds, it is also expressed in leaves and guard cells; systematic genetic analyses have shown that quinabactin exerts its effects predominantly through the dimeric subfamily III receptors PYR1 and PYL1 (Vaidya et al. 2017), demonstrating that pan-agonism is not a prerequisite for activity in Arabidopsis vegetative tissues. Quinabactin's broad-spectrum activity in crops (Okamoto et al. 2013, González-Guzmán et al. 2014) has spawned the development of many similar compounds reported in the patent literature, some of which are being developed as potential tools for regulating crop water use (S.R. Cutler et al. 2016, Frackenpohl et al. 2016, Frackenpohl et al. 2017, Lachia et al. 2018). A tetraflouro derivative of quinabactin (AMF4, **Fig. 2C**), was recently described with improved potency and persistence (Cao et al. 2017), and analogs of quinabactin have emerged from recent ABA receptor agonist screens (Nemoto et al. 2018); quinabactin has therefore proven to be an excellent scaffold for the development of ABA receptor agonists.

X-ray crystallographic analysis of a quinabactin–PYL2–HAB1 complex revealed that quinabactin's bicyclic quinolinone ring occupies a position that is normally occupied by ABA's cyclohexenone ring and that its quinolinone oxygen is as a hydrogen bond acceptor that interacts with the tryptophan lock water and its interconnected hydrogen bond network (Okamoto et al. 2013). Vaidya et al. (2017) hypothesized that quinabactin's quinolinone ring could be simplified by replacement with a substituted cyanophenyl ring in which a nitrile would function as an H-bond acceptor in place of quinabactin's quinolinone oxygen; this general strategy yielded a potent cyanophenyl sulfonamide agonist called cyanabactin (**Fig. 2C**). Cyanabactin has approximately 10-fold greater selectivity than quinabactin for PYR1/PYL1 relative to PYL2, but still retains strong ABA activity in vivo, suggesting that high potency activation of PYR1/PYL1 is critical for synthetic agonist activity in vegetative tissues. Consistent with this, the physiological effects of both quinabactin and cyanabactin can be greatly reduced by genetic removal of PYR1 and PYL1, which belong to subfamily IIIA (Vaidya et al. 2017). PYR1 and PYL1 are, therefore, key targets that can be leveraged to control water use chemically. The development of agonists selective for subfamilies I and II will help address whether other receptor subfamilies can be similarly leveraged to control transpiration.

Rational design approaches have also been used with the ABA structure as a scaffold, in order to design novel agonists or antagonists, and to improve on the poor stability of ABA in vivo and to light. For example, AS6 (**Fig. 2C**) was described in an effort to design ABA antagonists. Variable length alkyl chains were attached to the ring structure of ABA, resulting in different 3'-alkylsulfanyl ABAs, or AS*n*. The *n* denotes the alkyl chain length. Analogs for $n = 2–12$ were synthesized and tested. Analogs with short alkyl chains ($n < 4$) were found to act as agonists, while analogs with alkyl chains for which $n > 5$ acted as antagonists, based on seed germination assays and ABA-responsive gene expression (Takeuchi et al. 2014). While AS6 was found to bind PYLs with comparable affinity for ABA, its antagonistic characteristics were explained by the *S*-hexyl chain protruding into a small solvent-exposed tunnel above ABA's 3' carbon (named the 3' tunnel) and thus interfering with PP2C/PYR1 interactions by steric hindrance (Takeuchi et al. 2014). Similarly, PANMe, an ABA analog with a 4'-*O*-phenylpropynyl moiety at the C4' position, proved to be a more potent antagonist compared with AS6 (**Fig. 2C**) (Takeuchi et al. 2018).

Tetralone ABA analogs expand ABA's single cyclohexenone ring into a bicyclic system that extends into a region of the ligand-binding pocket normally occupied by ABA's 7' methyl group (**Fig. 2C**); the initial motivation for this design was to block ABA catabolism by modifying ABA's ring to prevent cyclization of 8'-hydroxylated ABA into PA (Nyangulu et al. 2006). The initial tetralone analogs synthesized by Abrams and colleagues displayed increased bioactivity, and this scaffold has proven fruitful for the design of both ABA agonists (Nyangulu et al. 2006, Han et al. 2017) and antagonists (Takeuchi et al. 2015, Rajagopalan et al. 2016) (**Fig. 2C**). Han et al. report crystal structures for an tetralone ABA isomer (*iso*-PhABA) in complex with PYL10 and confirm agonist activity across multiple receptors (Han et al. 2017). PAO4 and PBI664 are tetralone antagonists with alkyl extensions placed adjacent to the 3' tunnel (Takeuchi et al. 2015, Rajagopalan et al. 2016). PBI664 and PAO4 antagonize PYL–PP2C interaction across all three receptor clades, although less so on subfamily III receptors, and antagonize ABA effects in seed germination and stomatal closure assays (Takeuchi et al. 2015, Rajagopalan et al. 2016).

Exploiting ABA Receptors for Improved Water Use

The identification of ABA receptors has created new opportunities for tuning plant ABA responses and modulating transpiration and water use, which numerous PYL overexpression experiments have validated (Ma et al. 2009, Santiago et al. 2009b, Pizzio et al. 2013). Systematic overexpression of all 14 Arabidopsis receptors (Yang et al. 2016, Zhao et al. 2016)) revealed differing improvements in water productivity depending on the specific receptor overexpressed, with improvements of up to 40% reported for PYLs 4 and 12 (RCAR10 and 6; subfamily II). These experiments show that overexpressing any PYL receptor increases ABA sensitivity (as indicated by seed germination assays) and that there is probably an optimal sensitivity window that allows transpiration to be reduced without adverse impacts on biomass accumulation. Recent examples in tomato (González-Guzmán et al. 2014), poplar (Yu et al. 2016, Yu et al. 2017) and cotton (Liang et al. 2017) suggest that receptor overexpression effects also translate to crop plants. Although these examples all use transgenic plants, genome editing technologies will allow investigators to introduce point mutations into endogenous receptors that increase ABA affinity and whole-plant ABA sensitivity (S. Cutler et al. 2016).

The use of drought-inducible promoters to control receptor overexpression may have some advantages for increasing survival against terminal drought stress (Zhao et al. 2016); however, for agricultural applications, terminal drought stress is not a useful metric for drought tolerance (Van Breusegem et al. 2011, Nuccio et al. 2018). 'Water banking' or 'water saving' strategies are likely to be more useful than inducing protective responses after water deficit has occurred (Nemali et al. 2015, Helander et al. 2016, Nuccio et al. 2018). Water banking aims to maximize the available soil water during critical reproductive stages by reducing crop water use early in the season when water is usually more plentiful; crops with constitutively reduced transpiration are, therefore, desirable if they can be achieved without yield penalties, which may be challenging across multiple environments. In this context, ABA receptor agonists are advantageous because they allow, in principle, on-demand tuning of transpiration in any germplasm in response to anticipated water stress (Helander et al. 2016).

A parallel strategy for developing 'on-demand' chemical control of ABA signaling and transpiration can be achieved by engineering receptors to recognize new ligands through the design of orthogonal receptor–ligand interactions (Bishop et al. 2000, Park et al. 2015). Park *et al.* developed PYR1$^{\mathrm{MANDI}}$, a PYR1-derived receptor with six mutations that reprograms its ligand binding specificity to yield nanomolar sensitivity to the agrochemical mandipropamid, which is used to control oomycete pathogens. Plants overexpressing PYR1$^{\mathrm{MANDI}}$ induce ABA-like responses upon application of mandipropamid, including inhibition of seed germination and increased survival after water deficit (Park et al. 2015).

Conclusions and Perspectives

The development of ABA agonists and antagonists has provided valuable insights into ABA signaling mechanisms and receptor specificity, and may ultimately lead to the development of new agrochemical tools for managing water use.

The recent example of the orthogonal receptor–ligand approach using mandipropamid (Park et al. 2015) provides one example of a route towards precision agriculture that would allow a particular trait (water savings) to be activated in response to environmental data. New tools to enable generic agrochemical control of gene expression or gene function could broaden the palette of traits accessible to this idea. There is also a large target space provided by the core ABA response, biosynthetic and transport pathways that can probably be manipulated chemically to gain new insights into plant biology and develop new probes for modulating diverse aspects of ABA biology. Taken together, small molecules have proven critical in the quest to understand ABA action in plants and will proabably continue to do so in the future. Moreover, these probes can be leveraged to create chemical tools for managing water use and abiotic stress in agriculture.

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Disclosures

The author has no conflicts of interest to declare.

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