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Chemical manipulation of plant water use

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ABSTRACT

Agricultural productivity is dictated by water availability and consequently drought is the major source of crop losses worldwide. The phytohormone abscisic acid (ABA) is elevated in response to water deficit and modulates drought tolerance by reducing water consumption and inducing other drought-protective responses. The recent identification of ABA receptors, elucidation of their structures and understanding of the core ABA signaling network has created new opportunities for agrochemical development. An unusually large gene family encodes ABA receptors and, until recently, it was unclear if selective or pan-agonists would be necessary for modulating water use. The recent identification of the selective agonist quinabactin has resolved this issue and defined *Pyrabactin Resistance 1* (PYR1) and its close relatives as key targets for water use control. This review provides an overview of the structure and function of ABA receptors, progress in the development of synthetic agonists, and the use of orthogonal receptors to enable agrochemical control in transgenic plants.

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1. Introduction

Drought is the major source of crop losses worldwide and major improvements to agricultural productivity may be realized by improving water use and drought tolerance.[1] There are many strategies for mitigating the effects of drought on yield, including the development of drought tolerant crops through breeding or transgenic approaches;[2–4] here we focus on abscisic acid (ABA **1**, Fig. 1) receptor agonists, which afford direct control of plant transpiration by targeting a highly conserved family of receptors that control a negative regulatory response pathway (Fig. 1). ABA controls plant water use primarily through modulating ion transport in guard cells, pairs of specialized epidermal cells that form a stomatal pore that opens and closes in response to environmental signals (Fig. 1). The accumulation of new biomass through photosynthesis depends on entry of atmospheric CO_2 to inner leaf mesophyll cells through stomata, but this comes at the cost of H_2O escape driven by the large difference in water vapor pressure between the inner leaf and atmosphere. Plants therefore face an intrinsic tradeoff between water conservation and growth, and consequently perturbations that reduce water consumption typically come at the cost of reduced growth. Conversely, selection for high yielding crop varieties has been associated with increased stomatal conductance in some crops.[5,6]

Although the water/growth tradeoff may appear to create an insurmountable dilemma from the perspective of increasing yields during drought, the effects of drought vary throughout a plant's life cycle. In maize, for example, drought during the early juvenile growth phases or late growth phases is less detrimental to final grain yield than during flowering, where drought can cause reproductive failure.[7] Monsanto's recently introduced DroughtGard™ trait achieves ∼6% yield increases under conditions of moderate drought by overexpression of a *Bacillus subtilis* cold-stress induced RNA chaperone protein.[4] This trait reduces the water consumption of juvenile plants during water deficit, which in turn increases soil water content at flowering relative to non-GMO controls.[4] The molecular mechanism of the trait's physiological action is unclear, but it nonetheless illustrates the potential of 'water banking' to improve yield during drought. Synthetic ABA agonists, such as quinabactin (**2**) and pyrabactin (**3**),[8,9] are attractive because agonists can, in principle, enable an agrochemical strategy for water banking in any crop of interest.

2. Molecular aspects of ABA perception and action

S-(+)-ABA is a chiral sesquiterpenoid, with a decorated cyclohexenone ring appended to a dienoic acid sidechain. ABA is derived from β-carotene and was discovered in the 1960s by activity guided identification of plant growth regulators.[10–12] In addition to its role in guard cell physiology, ABA mediates other abiotic stress responses (for example freezing tolerance) and plays a central role in inducing seed dormancy, controlling root architecture,

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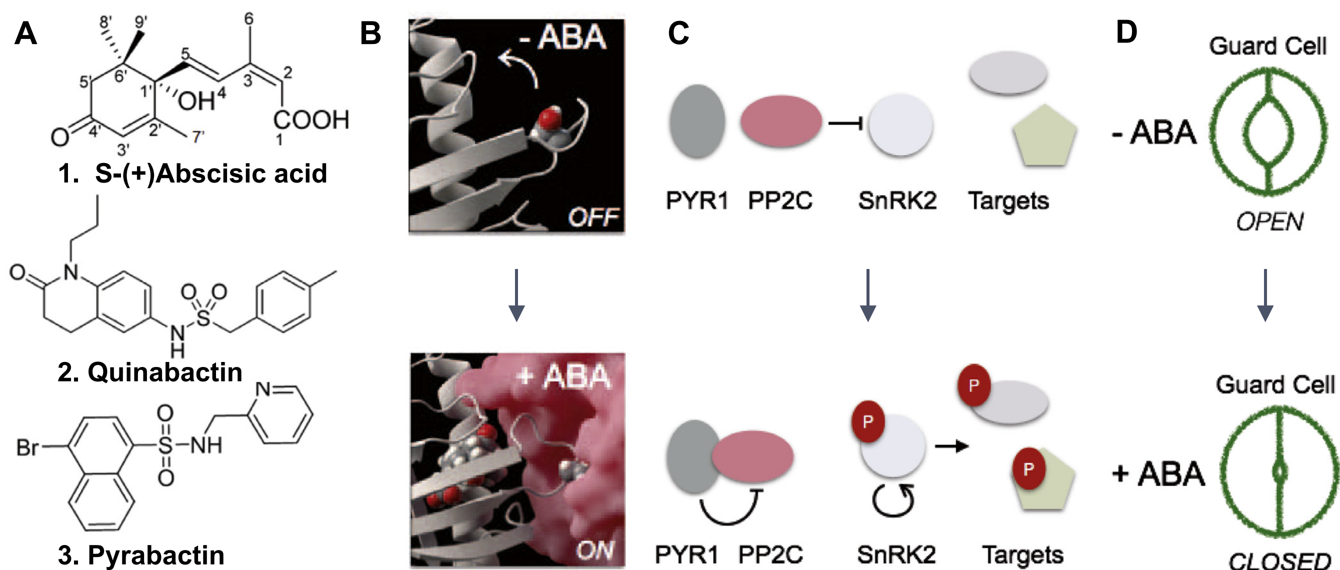


Figure 1. (A) Structures of *S*-(+) abscisic acid (**1**), quinabactin (**2**) and pyrabactin (**3**). (B) The gate-latch-lock structural mechanism for ABA recognition and biochemical activation. The receptor is depicted as a gray cartoon, ABA is depicted in a gray CPK model, and the PP2C is depicted as a pink surface. (C) Biochemical pathway downstream of activation of ABA receptors. (D) The physiological response of guard cell closure in the presence of ABA or other receptor agonists.

and influences several biotic interactions.[10] ABA biosynthesis is tied to water status and cellular osmotic pressure; reductions in osmotic pressure lead to rapid transcriptional induction of ABA biosynthetic enzymes, in particular nine-*cis* epoxycarotenoid dioxygenases (NCEDs), which act at the first committed step in ABA biosynthesis.[12] ABA levels rise greater than 25-fold under mild drought conditions due to de novo ABA biosynthesis and hydrolysis of inactive glucose-esters.[12,13] Mutants deficient in NCEDs or other biosynthetic enzymes lose leaf turgor (i.e., wilt) more rapidly than wild type plants.[12,14] Conversely, treatment of plants with exogenous ABA or synthetic agonists causes guard cell closure, reduces transpiration, and prolongs the time before wilting occurs relative to untreated plants.[8,9,13,15] ABA also induces the transcription of genes encoding enzymes that increase cellular osmolytes levels, and has other drought-protective effects.[16]

ABA responses are mediated by a negative regulatory signaling module that involves soluble *Pyrabactin Resistance 1/PYR1-Like/Regulatory Component of ABA Receptor* (PYR/PYL/RCAR) ABA receptors, clade A type 2C protein phosphatases (PP2Cs) and subfamily 3 Snf1-related kinases (SnRK2s; Fig. 1). The SnRK2s directly phosphorylate and control the activity of several downstream effectors such as transcription factors, and anion channels that are required for guard cell closure.[17] The SnRK2s autoactivate by *cis*- and *trans*-autophosphorylation, but their activity is suppressed by the PP2Cs, which dephosphorylate and inactivate the kinases.[18,19] When ABA binds to soluble PYR/PYL/RCAR ABA receptors, the receptors bind stably within PP2C active sites and inhibit PP2C activity, this in turn enables accumulation of activated SnRK2 kinases, which regulate downstream factors by direct phosphorylation.[20–22]

ABA receptors are members of the START/Bet v 1 superfamily,[5] an ancient family characterized by an α-β-α_2-β_6-α topology that forms a helix-grip fold in which 7 anti-parallel beta-sheets (and intervening short loops and helices) enclose a long C-terminal helix to form a central ligand binding pocket.[23–25] The structures of several ligand–receptor complexes have been elucidated by X-ray crystallography and depict the conformation of receptor-bound ABA as a half-chair with a pseudoaxial sidechain[26–32] (Fig. 2). ABA binding induces a conformational change that enables the receptors to dock into and inhibit PP2C activity. The largest conformational change occurs in a 'gate'-loop that flanks the ligand binding pocket, which adopts a closed conformation via direct hydrophobic contacts to ABA.[26,27,29,30,33] A second 'latch'-loop also changes conformation and encloses the bound ligand (Fig. 1). The sidechain of an invariant serine in the –SGLPA– gate-loop points in towards the ligand binding pocket in apo-receptor structures but becomes solvent exposed after agonist binding/gate closure, which enables the closed conformer to bind and competitively inhibit PP2C enzymatic activity. The majority of ABA recognition occurs inside the receptors and involves 25 highly conserved residues that make direct or water-mediated contacts to ABA. Additionally, a critical PP2C tryptophan located in a recognition loop, which is, specific to ABA regulated PP2Cs, called the 'lock', inserts into a small pore directly above ABA's 4′-carbon and makes a water

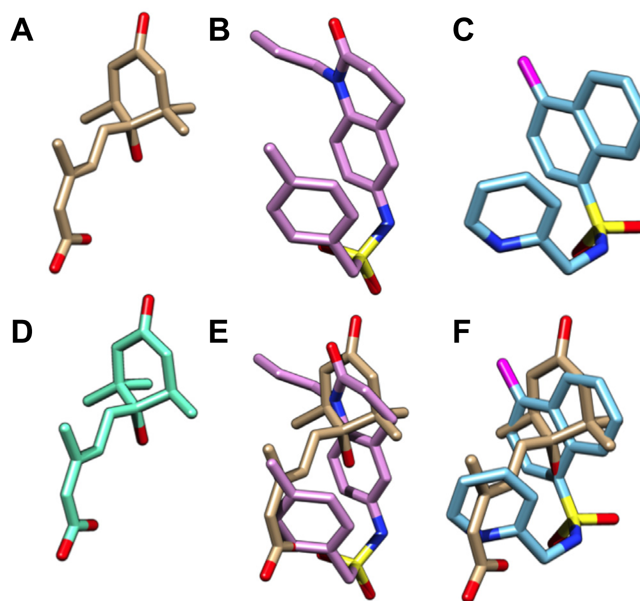
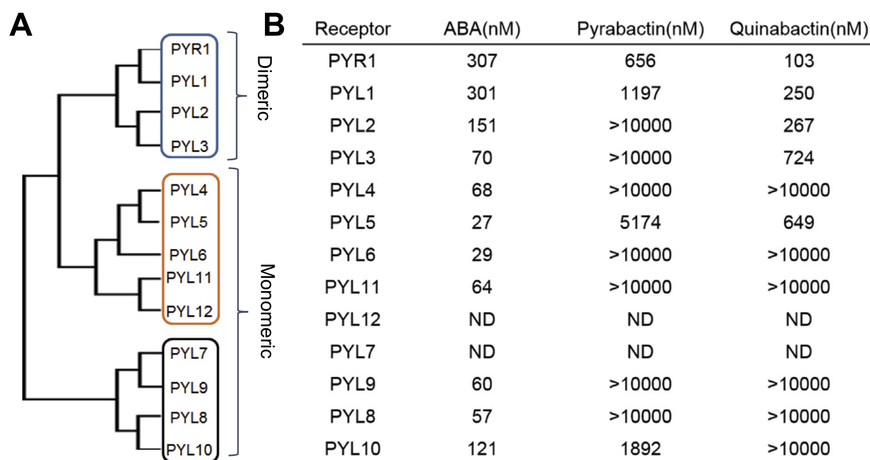


Figure 2. 3-D aligned structures of receptor-bound ABA receptor agonists. (A) ABA (3QN1), (B) quinabactin (4LA7), (C) pyrabactin (3NMN), (D) R-(−)-ABA, (E) overlay of ABA and quinabactin, and (F) overlay of ABA and pyrabactin. The PDB accessions used for the structures are listed in parentheses.



Receptor	ABA(nM)	Pyrabactin(nM)	Quinabactin(nM)
PYR1	307	656	103
PYL1	301	1197	250
PYL2	151	>10000	267
PYL3	70	>10000	724
PYL4	68	>10000	>10000
PYL5	27	5174	649
PYL6	29	>10000	>10000
PYL11	64	>10000	>10000
PYL12	ND	ND	ND
PYL7	ND	ND	ND
PYL9	60	>10000	>10000
PYL8	57	>10000	>10000
PYL10	121	1892	>10000

Figure 3. Arabidopsis ABA receptor sub-families. The left panel shows a phylogeny of the *Arabidopsis thaliana* PYR/PYL/RCAR receptors and the right panel shows IC_{50} values for receptor-mediated inhibition of HAB1 by ABA, pyrabactin and quinabactin. (adapted from Ref. 8). PYL13 has been omitted, but it is a member of the PYL5 subfamily.

mediated contact to ABA's ketone.[29,30,33,34] ABA binding is stabilized by both direct and water-mediated hydrogen bonds, in addition to multiple hydrophobic interactions. Notably, a conserved lysine in the PYL protein family (K59 in PYR1) forms a direct hydrogen bond to the carboxylate of ABA. A water-mediated H-bond network interacts with both carboxylate oxygen atoms of ABA to produce hydrogen bonding to residues homologous to PYR1's *Y120*, *S122*, and *E141*, which are part of a hydrophilic region located deep within the pocket.[26] The gate-latch interface facilitates hydrophobic interactions with the cyclohexanone ring and the 7′-, 8′- and 9′-methyl constituents of ABA,[26,30] forming a hydrophobic region of the top of the pocket.

The ABA receptor gene family is unusually large, for example, there are 14 members in *Arabidopsis thaliana*, 15 members in tomato, and 21 members in soybean.[35,36] The receptors form three phylogenetically distinct subfamilies that can be found in all flowering plant genomes sequenced (Fig. 3).[37–39] The biological roles of the different subfamilies are still being established, but as we describe below, activation of the PYR1-subfamily of dimeric receptors is sufficient to elicit guard cell closure, reduce water consumption and induce ABA-transcriptional responses in many species, which demonstrates its centrality in plant water relations.[8]

In Arabidopsis, there are nine clade A PP2Cs.[40] One of the nine PP2Cs (*ABA Hypersensitive Germination 1*, AHG1) is recalcitrant to PP2C mediated inhibition due to a naturally occurring mutation of the Trp lock residue.[41] Genetic evidence indicates redundant/overlapping functions for the PP2Cs,[42] but biochemical evidence demonstrates differences in ABA sensitivity for different PP2C–receptor complexes,[41,43] and at least one receptor (PYL13/RCAR7) selectively interacts with a subset of clade A PP2Cs.[44] The values measured in receptor-mediated PP2C inactivation assays are thus influenced by the specific PP2C utilized, and direct biophysical measurements, such as isothermal titration calorimetry (ITC), are valuable for inferring intrinsic agonist–receptor binding affinities. ITC measurements have shown low micromolar K_d values,[21,33,43,45,46] and that the inclusion of PP2Cs in binding reactions lowers apparent K_ds by ~10- to 25-fold,[21,45] presumably by reducing K_{off}. For example, PYL5 displays an apparent K_d of 1 μM for ABA binding, which is lowered to 38 nM by the addition of HAB1 to binding reactions.[45] In vivo potency, receptor–agonist K_d, and PP2C–inhibitory activity generally correlate and IC_{50} values can be used to rank compound potency and selectivity relative to ABA.[8] Typical IC_{50} values for ABA-mediated PP2C inhibition range from ~20 to 300 nM (Fig. 3) when assayed using the PP2Cs ABA Insensitive 1 (ABI1), ABA Insensitive 2 (ABI2) or Homolog of ABI1 (HAB1).[8,20,21,45]

Members of the PYR1 subfamily (PYR1, PYL1–3) form homodimers in solution, while the members of the other subfamilies are monomeric and display higher ABA affinity.[26,27,33,31] The PYR1 homodimerization interface overlaps substantially with the PP2C binding interface of the ligand-bound complex, which necessitates dimer dissociation prior to ligand-induced PP2C inhibition.[33,47] ABA shifts dimeric PYR1 towards a monomeric state in vitro, as measured using size exclusion chromatography[47] and analytical ultracentrifugation, the latter of which has shown that ABA increases the homodimerization K_ds of PYL2 and PYL3 ~3- to 6-fold.[46] The decreased ABA affinity of dimeric receptors has been proposed to result from a thermodynamic penalty imposed by dimer dissociation,[47] however it is also likely that an additional hydrogen bond to ABA in monomeric receptors, as observed with PYL9,[32] contributes to differences in sensitivity as well. PYL10 was initially described as a constitutively active receptor,[31] however this erroneous conclusion was due to an in vitro artifact caused by the spurious ability of BSA (utilized in PP2C assays) to weakly activate PYL10.[48] Additionally, backbone dynamics measurements indicate that PYL10's conformational entropy increases upon ABA binding, which may function to stimulate receptor–PP2C interactions.[48]

Thus extensive biochemical analyses of Arabidopsis receptors show that the PYR1 subfamily encodes dimeric receptors of lower intrinsic affinity and sensitivity to ABA, while the PYL5- and PYL8-subfamilies (Fig. 3) are monomeric, higher affinity receptors, a pattern conserved in tomato and rice receptor subfamilies as well.[36,49] It has been hypothesized that the different subfamilies function in different regions of the ABA dose response curve, with the PYR1-subfamily being activated by the high ABA levels produced during abiotic stress.[47] Genetic studies, however, have shown additive contributions of several Arabidopsis receptors for the control of stomatal conductance, implying overlapping functions within guard cells.[50] The PYL8 subfamily may contribute differentially to root responses to ABA, as Arabidopsis the roots of plants harboring *pyl8* mutations are less sensitive to ABA in multiple assays.[51,52] Given the extensive genetic redundancy observed for ABA receptors, selective agonists will undoubtedly help probe receptor function in vivo.

3. Synthetic ABA receptor agonists

The identification of ABA receptors and elucidation of their structures has created new opportunities for discovering and designing modulators of receptor function. Although ABA could,

in principle, be used directly as an agrochemical, it has been limited by its molecular complexity, photolability,[53–55] moderate chemical stability,[20,21] and rapid catabolic inactivation[56,57] by plant cytochrome P450s in the CYP707A subfamily.[57] Synthetic agonists with improved properties are therefore of interest and multiple synthetic agonists have been described and characterized, as have numerous ABA analogs (see below). These compounds have revealed functional differences between receptors and defined the dimeric ABA receptors as targets that can be used to chemically control transpiration.

The identification and characterization of the selective agonist pyrabactin (**3**) played a critical role in the identification of ABA receptors. Pyrabactin was identified as a molecule that phenocopies ABA's inhibitory effect on seed germination using a phenotype-based screen of a small molecule diversity library.[20,58] Molecular and physiological characterization demonstrated that pyrabactin activates seed ABA responses without strongly activating vegetative tissue ABA responses.[20] Genetic mechanism-of-action studies established that *PYR1* is required for pyrabactin's bioactivity, that pyrabactin and ABA both directly bind to PYR1, and that the key biochemical function of ABA agonists is to promote the formation of a receptor/PP2C complex that inhibits PP2C activity.[20] This work converged with the identification of RCAR1 (PYL9), which was identified by characterization of PP2C binding proteins and subsequent determination that RCARs bind ABA to mediate PP2C inhibition.[21] Additional studies identified multiple members of the receptor family as binding partners of ABI1 and HAB1.[45,59] Collectively, these studies defined the PYR/PYL/RCAR gene family as encoding ABA receptors. Numerous groups had previously attempted to identify ABA receptors using genetic approaches in Arabidopsis and in hindsight these attempts failed because of the extensive genetic redundancy between the 14-members of the receptor gene family. PYR1 loss-of-function mutants do not show pronounced defects in ABA responses, and higher-order mutants are required to observe strong reductions of ABA responses. Pyrabactin's selective effects on seeds is a result of both its preferential activation of PYR1 and the high level expression of *PYR1* mRNA in seeds; features which allowed a single loss of function mutation to reveal *PYR1*'s role in ABA signaling. Thus, the selectivity of pyrabactin enabled it to bypass the functional redundancy that had hampered discovery of ABA receptors by forward genetic approaches.

Pyrabactin is a selective agonist that can activate PYR1, PYL1, PYL5, and PYL10 (IC_{50} values of 660, 1200, 5200, and 1900 nM, respectively, ~2 to 60× that of ABA measured on the same receptors). Since mutations in *PYR1* block pyrabactin's bioactivity, it is likely that PYR1 is pyrabactin's key cellular target. Why does pyrabactin not activate all ABA receptors, like ABA does? This has been addressed with genetic and structural studies dissecting the differential pyrabactin sensitivity of PYR1 and PYL2,[60–62] which pinpointed two residues, PYR1 *I110* and to a lesser degree *I62*, as determinants of pyrabactin selectivity. PYL2 possesses smaller valine residues at its corresponding residues (*V67* and *V114*). Swapping these residues to create the mutants $PYR1^{I62V,I110V}$ and $PYL2^{V67I,V114I}$ converts PYL2 into a pyrabactin responsive receptor and removes responsiveness from PYR1.[60–62] X-ray crystallographic and HSQC-binding studies unexpectedly revealed that pyrabactin can bind to, but not activate, PYL2 because it binds in an unproductive orientation that prevents gate closure.[60–63] This property makes pyrabactin an antagonist of PYL2, however it is too weak to measurably antagonize ABA action in vivo.[60,64] Thus, PYR1 and PYL2 contain subtle sequence differences in ligand contacting residues (Fig. 4) that can be exploited to tune agonist selectivity.

Several analogs of pyrabactin (Fig. 5) have been characterized and provide insight into structure–activity relationships. Apyrabactin (**4**), a biologically inactive analog lacking the pyridyl nitrogen, is unable to form a water-mediated hydrogen bond to the highly conserved lysine (*K59* in PYR1) that normally forms a hydrogen bond to ABA's carboxylate.[60,62] Changing the positioning of the nitrogen on the pyridyl ring (compound **5**) or replacement of the bromine atom on the naphthyl ring with hydrogen or a methyl group (compounds **6** and **7**) reduce activity. Non-polar alterations to the naphthyl moiety are also detrimental, but to a lesser effect (compounds **8** and **9**),[20] suggesting that the gate-latch-agonist interface can tolerate subtle modifications in this region of the pyrabactin scaffold.[62] Replacement of pyrabactin's pyridyl methylamino substructure with a methionine-derivative (**10**), a modification suggested from virtual screening experiments, yields an equipotent compound in PYR1/PP2C binding assays.[60] Bioisosteres of pyrabactin's sulfonamide moiety have recently been described: phosphonamide (**11**) and phosphonate substitutions of the sulfonamide linker have yielded compounds with similar or stronger effects than pyrabactin, however these analogs have not been characterized biochemically.[65] Pyrabactin's napthyl ring can be replaced by a naptholactam moiety (**12**), which can likely form a hydrogen bond to the conserved gate-latch water, however the activity of **12** is reduced in comparison to pyrabactin. Other naptholactams that replace the pyridyl ring with a tetrahydrofuranyl moiety (**13**) or an ethyl ester (**14**) display modest in vitro

PYR1	H_{60}	I_{62}	V_{83}	F_{108}	I_{110}	A_{160}	V_{163}	V_{164}
PYL1	H_{87}	I_{89}	V_{110}	F_{135}	I_{137}	A_{190}	V_{193}	I_{194}
PYL2	H_{65}	V_{67}	V_{87}	F_{112}	V_{114}	V_{166}	V_{169}	V_{170}
PYL3	H_{80}	I_{82}	V_{107}	F_{132}	V_{134}	V_{189}	V_{192}	V_{193}
PYL4	H_{82}	L_{84}	V_{105}	F_{130}	V_{132}	V_{179}	I_{182}	V_{183}
PYL5	N_{88}	I_{90}	V_{111}	F_{136}	V_{138}	V_{185}	I_{188}	V_{189}
PYL6	H_{91}	V_{93}	V_{114}	F_{139}	V_{141}	A_{193}	I_{196}	V_{197}
PYL7	P_{66}	I_{68}	V_{87}	I_{112}	I_{114}	V_{164}	L_{167}	I_{168}
PYL8	P_{62}	I_{64}	V_{83}	I_{108}	I_{110}	V_{160}	L_{163}	I_{164}
PYL9	P_{64}	V_{66}	V_{85}	I_{110}	I_{112}	V_{162}	L_{165}	I_{166}
PYL10	P_{57}	I_{59}	L_{79}	I_{104}	I_{106}	V_{156}	L_{159}	I_{160}
PYL11	Q_{40}	V_{42}	V_{62}	I_{87}	I_{89}	A_{138}	I_{141}	V_{142}
PYL12	H_{40}	V_{42}	V_{62}	I_{87}	I_{89}	A_{137}	I_{140}	V_{141}
PYL13	R_{39}	V_{41}	L_{67}	V_{92}	I_{94}	V_{144}	I_{147}	I_{148}

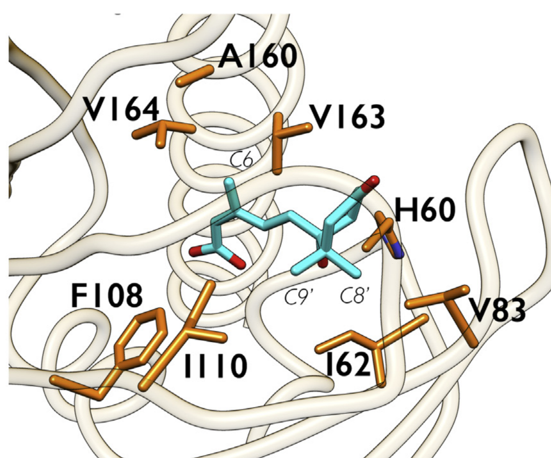


Figure 4. Variable amino acid residues within the ligand-binding pockets of *Arabidopsis thaliana* PYR/PYL/RCAR receptors. Shown at right are a representation of the residues in PYR1.

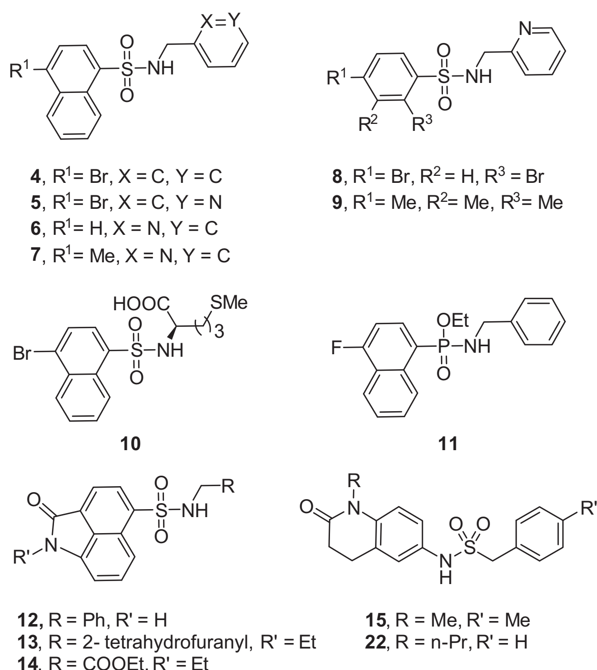


Figure 5. Structures of pyrabactin and quinabactin analogs.

activity.[60] Arylsulfonamides related to pyrabactin have been described in the patent literature as compounds for controlling abiotic stress tolerance,[66] however pyrabactin's limited activity in vegetative ABA responses will likely need to be circumvented in order to develop agriculturally useful agents from this scaffold. Nonetheless, pyrabactin has proven a valuable probe molecule for both genetic and functional dissection of receptor function.

Given pyrabactin's limitations, Okamoto et al. conducted small molecule screens for compounds capable of activating multiple receptors (PYR1–PYL4) using heterologous yeast two hybrid reporter strains, which report agonist activity by the ligand-induced physical interaction between receptors and PP2C.[8,20,61] This screening effort identified quinabactin (**2**), a dihydroquinolinone sulfonamide that, like pyrabactin, contains two aromatic rings separated by a sulfonamide-containing linker. Remarkably, quinabactin (also named AM1) was independently isolated in a screen for small molecules that induce PYR1-HAB1 binding using a direct biophysical assay.[9] Quinabactin possesses activity on PYR1, PYL1, PYL2, PYL3 and PYL5 in phosphatase inhibition assays, although its IC_{50} values for PYL3 and PYL5 are >10-fold higher than ABA's.[8,9] Quinabactin also preferentially activates tomato dimeric subfamily receptors.[36]

The biological effects of quinabactin are highly similar to those of ABA, despite its inability to activate the full complement of ABA receptors. Quinabactin inhibits Arabidopsis seed germination and induces a genome-wide ABA-like transcriptional response in seedlings.[8,9] Quinabactin also induces guard cell closure and reduces transpiration in multiple species and, like ABA, increases survival after extended water deficit in Arabidopsis.[8] Collectively, these observations demonstrate that a pan-agonist is not necessary for controlling guard cell and other vegetative ABA responses.

Although quinabactin most strongly activates the dimeric subfamily of receptors, it also weakly activates PYL5. Mutational analyses using a *pyr1/pyl1/pyl2/pyl4* quadruple mutant genotype have shown that PYL5 does not make a substantial contribution to quinabactin action. The *pyr1/pyl1/pyl2/pyl4* quadruple mutant is ABA hyposensitive in many assays, but still retains some ABA sensitivity due to redundancy between receptors. For example, ABA-induced transcription of marker genes is reduced by only ~80% in the quadruple mutant strain. Subsequent genetic removal of *PYL5* and *PYL8* strongly enhances the ABA insensitivity of the quadruple mutant, indicating that the residual ABA-response in the quadruple is mediated by other PYL receptors.[8,50] Unlike ABA however, quinabactin does not induce a residual transcriptional response in the quadruple mutant, which is also highly insensitive to quinabactin's inhibition of seed germination.[8] Collectively, these data show that the dimeric receptors are targets that can be used to chemically control transpiration, however the relative importance of specific subfamily members is unclear. Quinabactin and pyrabactin differ in that pyrabactin is unable to activate PYL2. This could imply that PYL2 activation or co-activation is important for quinabactin's unique bioactivity. The development of agonists with greater selectivity will help resolve this point. Additionally, the development of agonists selective for the other subfamilies will help establish if the other receptor subfamilies can be used to control transpiration.

Shortening quinabactin's *N*-alkyl chain from *N*-propyl to *N*-methyl (**15**) substantially reduces in vitro activity[9], which can be rationalized by reduced hydrophobic interactions with the 3′-tunnel, a small hydrophobic pore directly above ABA's 3′-carbon that forms after gate closure (Fig. 6A).[64] The importance of this region for modulating agonist interactions is supported by the improved activity of ABA analogs (Fig. 7) that expand into this pocket. For example, a series of 3′-alkylsulfanyl ABA analogs (**16** & **17**) were synthesized to probe this region.[64] The analog AS2 (ethylsulfanyl-ABA, **16**) possesses increased agonist activity both in vitro and in vivo and shows increased selectivity for the dimeric subfamily receptors relative to ABA. AS2, like quinabactin, activates vegetative ABA responses, which provides independent support for the conclusion that activating the dimeric receptors is sufficient to reduce water use.[64] The analog AS6 (hexylsulfanyl-ABA, **17**), which has a sufficiently long carbon chain to protrude through the 3′-tunnel, blocks PYR1–PP2C interactions and antagonizes endogenous ABA responses in multiple species.[64]

Manipulating hydrophobic interactions within the vicinity of the 3′-tunnel and gate may provide a general strategy for improving dimeric receptor agonist activity, as hydrophobic expansion into the 3′-tunnel improves activity of other ABA analogs. For example, bicyclic tetralone ABAs (**18**), which incorporate ABA's 3′, 4′ and 7′ carbons into a phenyl ring, have improved activity in vitro and in vivo.[67–69] Tetralone-ABA derivatives with 11′-alkyl ether chains (**19**) that are predicted to extend into the 3′-tunnel gain favorable activity with short alkyl chains ($n = 1$), but sufficiently long alkyl-chains ($n = 4$) yield antagonists.[70] 7′-nor-ABA (**20**), which replaces the 7′-methyl with an H, which should reduce hydrophobic interactions with the gate, has greatly reduced in vitro and in vivo activity, indicating interactions in this region are necessary with the ABA scaffold.[71] Thus, modifications of ligands in positions proximal to the 3′ tunnel and gate can be used to improve agonist activity or to engineer antagonists.[64]

Gate closure creates a second pore above ABA's 4′-carbonyl that enables a water mediated contact between ABA's ketone oxygen and the PP2C Trp lock. Quinabactin's quinolinone carbonyl oxygen, which can be superimposed on ABA's cyclohexenone oxygen when comparing receptor–ligand complexes (Fig. 2F), participates in a water mediated contact to the Trp lock.[8,9] Quinabactin's ability to H-bond to the Trp lock likely underlies its improved activity relative to pyrabactin, as pyrabactin does not possess a hydrogen bond acceptor positioned to interact with the Trp lock. Consistent with the general importance of this interaction, 4′-deoxo-ABA (**21**) displays greatly reduced bioactivity.[72]

Quinabactin's 4-methylbenzyl methyl substituent occupies a small cleft, which is normally occupied by ABA's C6-methyl (Fig. 6B). An analog of quinabactin that replaces the 4-methyl substituent with a hydrogen atom (**22**) has greatly reduced activity

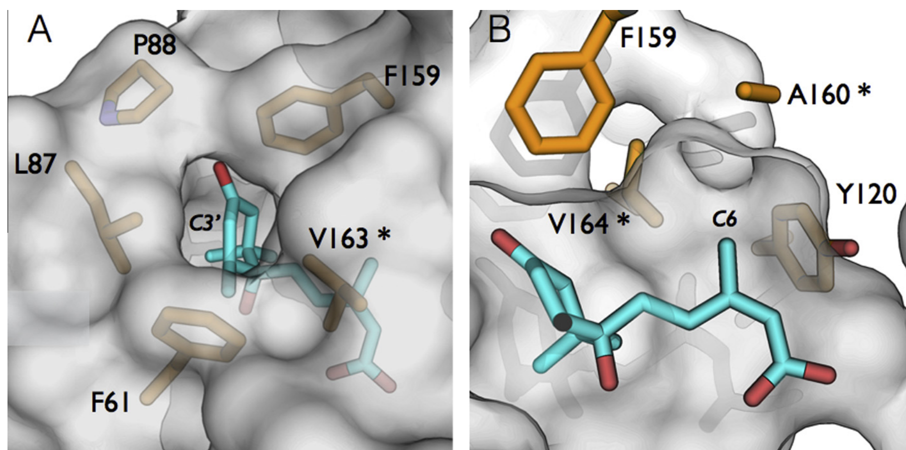


Figure 6. The 3′-tunnel (A) and C6-cleft (B) are variable regions of the receptors' ligand-binding pockets. Amino acids that are variable between receptors are labeled with an asterisk. V163 and F159, which are also part of the C6-cleft, have been omitted for clarity. Made from PDB 3QN1.

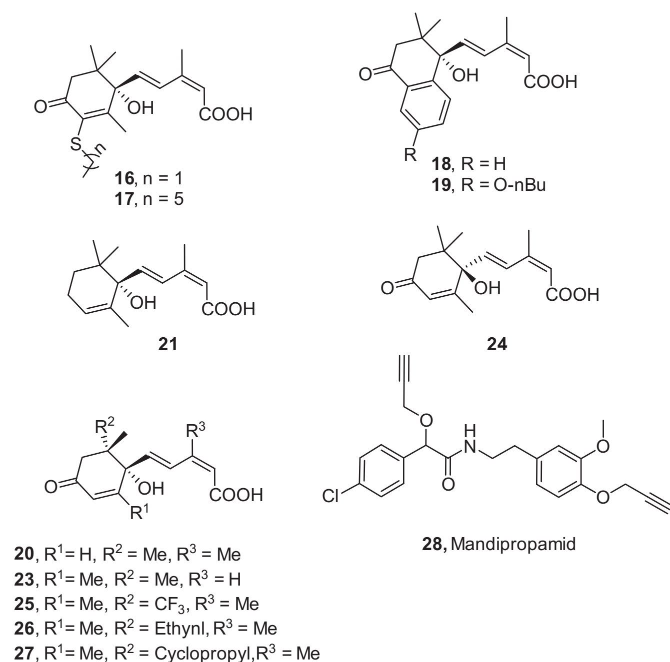


Figure 7. Structures of ABA analogs.

in vitro and in vivo,[9,73] implying that hydrophobic contacts inside the cleft are critical for activity. Consistent with this hypothesis, the ABA analog 6-nor-ABA (**23**), which converts ABA's C6 methyl to an H, also shows greatly reduced activity.[71] These studies demonstrate the importance of agonist interactions with the C6-cleft for activity. In addition, three of the 6 residues that form the C6-cleft are variable between receptors (Figs. 4 and 6B), suggesting that the C6-cleft might be exploited for tuning agonist selectivity.

Pyrabactin and quinabactin are both constructed from two aromatic ring systems connected by a short sulfonamide linker and both compounds adopt a similar U-shaped conformation (Fig. 2) in the ligand binding pocket, positioning their hydrophobic rings adjacent to the hydrophobic gate (3′-and 4′-tunnels) and C6-cleft, which normally contact ABA's hydrophobic cyclohexenone head group and its C6-methyl, respectively. The sulfonamide linkage in both molecules is positioned towards the bottom of the ligand binding pocket and interacts with residues that would normally contact ABA's carboxylate and ring hydroxyl. In both agonists, their sulfonamide NH forms a direct hydrogen bond to *E94* (*E98* in PYL2), which normally participates in water-mediated H-bonds to ABA's carboxylate and ring hydroxyl groups. The *pyr1–6* allele, which encodes *E94K*, disrupts this residue and possesses strong pyrabactin insensitivity in vivo, consistent with the critical importance of this interaction to agonist activity.[20] Across published ABA structures, ABA binding is stabilized by 4 water molecules that form an H-bond network; quinabactin binding displaces 3 of these waters, which may contribute favorably to the thermodynamics of quinabactin binding.

4. Stereoselectivity, ABA affinity and ABA metabolism

S-(+)-ABA is the naturally occurring ABA, however some ABA receptors can accommodate the *R*-(−)-isomer (**24**) if its ring rotates 180°;[11,26,28] *R*-(−)-ABA therefore possesses varying degrees of bioactivity.[74] The 180° rotation of the cyclohexenone ring needed for (−)-ABA binding causes the 8′- and 9′-methyl substituents to occupy the space normally occupied by ABA's 7′-methyl. Not all receptors can accommodate (−)-ABA due to sequence variation in residues homologous to *I62* and *I110* in PYR1 (which also influence pyrabactin sensitivity, as described above).[28] For example, wild type Arabidopsis PYL9 does not efficiently bind (−)-ABA, but PYL5 does. The PYL9 mutants *V66I* and *I112V* make their ligand binding pockets more PYL5-like and imbue partial *R*-(−)-ABA responsiveness on the mutant receptors.[28] In addition to stereoselectivity, ABA receptors also display differences in their intrinsic ABA binding affinities due to variable ligand-binding pocket architectures. For example, sequence variation in residues lining the C6-cleft (Fig. 6) orient ABA in the monomeric receptor PYL9 so that an additional H-bond between ABA's carboxylate and *N169* can form. This H-bond is not observed in dimeric receptor/ABA complexes and contributes to PYL9's higher intrinsic ABA affinity.[32] These data further illustrate the impact of relatively subtle sequence variation between receptors on agonist selectivity and affinity.

Manipulation of endogenous ABA levels provides an alternate path to controlling plant water relations. The concentration of free ABA is determined by rates of biosynthesis and catabolism and perturbations of either can change ABA levels.[12] ABA is catabolized primarily by oxidation of its ring methyl groups and glucosylation of its carboxylate. The CYP707A subfamily of cytochrome P450 enzymes catalyze the formation of 8′- and 9′-OH ABA, which spontaneously cyclize to form phaseic acid (PA) and neo-phaseic

acid (neoPA), respectively.[12,75] PA can be subsequently reduced to dihydroPA (DPA). The major ABA metabolites are unlikely to activate ABA receptors in vivo as PA, neoPA, 7′-OH ABA, and ABA-glucoside display IC_{50} values much lower than (+)-ABA when tested with representative members of each receptor subfamily.[68] Fluorine substitutions on the 8′-methyl group, in particular 8′,8′ difluoroabscicic acid and 8′,8′,8′ trifluoroabscicic acid (**25**), yields compounds with delayed metabolism in vivo and increased bioactivity.[76] Similarly, alkynyl derivatives at the 8′ (**26**) and 9′ position have yielded CYP707A suicide inhibitors that are of interest because they have more persistent biological effects than ABA.[77] Both 8′- and 9′-acetylene ABA analogs display agonist activity comparable to that of ABA in in vitro PP2C assays with multiple receptors, but a bulkier 8′-cyclopropyl analog (**27**) shows reduced activity[78] consistent with the limited space available adjacent to the 8′/9′ methyls.

5. Engineered agrochemical control of transpiration

ABA agonists hold potential as future agrochemicals for controlling plant water consumption and improving yield under drought conditions. A parallel strategy to gaining agrochemical control is to engineer ABA receptors so that they can respond to an existing agrochemical, a strategy based on orthogonal ligand/receptor systems, which have enabled selective chemical control of diverse targets.[79,80] For example, several kinases have been engineered to respond to kinase inhibitor analogs that are too bulky to inhibit wild type kinases, but can inhibit mutant kinases with enlarged ATP-binding pockets.[80] The orthogonal/ligand receptor strategy has been widely employed in chemical biology and used, for example, to engineer receptors activated by ‘near drugs’, inactive drug metabolites and other agonist/antagonist relatives.[81–84]

The orthogonal receptor/ligand design strategy commonly involves the modification of existing ligands concomitant with mutations to their receptors, however repurposing an existing agrochemical is a different task. To identifying suitable ligands, Park et al. used site-saturation mutagenesis to create a collection of mutant PYR1 receptors that contain all possible 475 single amino acid substitution mutations in the receptor's 25 ligand-contacting residues.[85] This library of receptor variants was constructed in combination with the PYR1-K59R mutation, which removes intrinsic ABA responsiveness by disrupting a highly conserved H-bond to ABA's carboxylate.[85,86] Park et al. screened a panel of commonly used non-herbicidal agrochemical for agonists of any of the 475 receptors using the yeast-two hybrid assay described earlier. These screens were conducted at high concentrations (100 μM) so that weakly interacting ligands could be identified and subsequently optimized. Four weakly activating ligands were identified and subsequent mutageneses and selections were utilized to improve the sensitivity of each ligand–receptor pair identified. This process worked most successfully with mandipropamid (**28**), sold as Revus™, which is used to control *Phytophthora infestans* (late blight) in vegetable crops. Multiple rounds of mutagenesis and functional selections yielded a hextuple mutant receptor, $PYR1^{MANDI}$, with nanomolar sensitivity to mandipropamid. X-ray crystallographic data show that two key mutations that replace bulky residues (*F108A* and *F159L*) are crucial for preventing steric clashes between mandipropamid's two *O*-propargyl substituents that would otherwise occur with the native receptor, and that mandipropamid's amide forms direct and water mediated H-bonds to polar residues in the mutant's binding pocket, functioning analogous to the interactions of sulfonamides with wild type receptors.[85]

Constitutive expression of $PYR1^{MANDI}$ in transgenic Arabidopsis and tomato enables mandipropamid triggered activation of multiple ABA responses, such as reduced transpiration and genome-wide ABA-like transcriptional responses. Importantly, overexpression of the mutant receptor, which does not possess intrinsic ABA responsiveness, is not associated with growth defects or substantial alterations of basal transcriptomes in the absence of mandipropamid treatment. Mandipropamid applications are able to improve Arabidopsis survival after water deficit with efficacy similar to that of ABA, suggesting that mandipropamid could be used as an agrochemical agent to control transpiration when used in combination with $PYR1^{MANDI}$.[85] Additionally, the data demonstrate that selectively activating a single receptor (when expressed at high enough levels) is sufficient to elicit guard cell closure and other important ABA responses. Thus, although ABA normally co-activates multiple ABA receptors in vivo, co-activation is not a prerequisite for ABA action.

6. Looking forward

Growing demands on the freshwater needs of agriculture, due to population growth and increasing weather extremes, are motivating efforts to improve crop yields under conditions of drought. Important improvements have already been achieved through classical breeding and new transgenes, and these strategies will likely continue to deliver improvements. Agrochemical modulators of stress tolerance and water use have the advantage that they can, in principle, be used with any genotype across multiple species. ABA receptors are validated targets and it is anticipated that agonists suitable for agriculture can be developed from existing scaffolds. The isolation and characterization of quinabactin has demonstrated the importance of dimeric ABA receptors as targets for controlling transpiration, but our understanding of the underlying biological functions of the multiple receptor subfamilies is still incomplete. Structure–activity relationships of ABA-receptor agonists will facilitate agonist and antagonist design efforts, which will undoubtedly help probe receptor function and facilitate agrochemical development efforts. Ultimately, field studies will be required to establish the efficacy of ABA agonists for improving yield during drought.

The development of $PYR1^{MANDI}$ has demonstrated that an existing agrochemical can be repurposed to control a defined trait (transpiration via the ABA response pathway). The future of plant biotechnology will undoubtedly involve greater use of engineered proteins and pathways to specifically modulate defined traits. Orthogonal control systems using agrochemical ligands offer the advantage that the traits can remain silent in the absence of chemical intervention.

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