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Chapter 19

# Abscisic Acid Signalling as a Target for Enhancing Drought Tolerance

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**Abstract**

Abscisic acid (ABA) is a vital hormone that confers abiotic stress tolerance in plants. The identification of PYR/PYL/RCAR proteins as bona fide ABA receptors and the subsequent elucidation of the structural mechanisms of the core ABA signalling pathway in recent years has provided new and powerful insights in targeting ABA signalling to enhance abiotic stress tolerance in agriculture. This chapter reviews the components and molecular mechanisms of the core ABA signalling pathway, as revealed by X-ray crystallography studies, and how these knowledge led to preliminary efforts in novel biotechnological developments to improve stress tolerance in plants.

**Keywords:** Abscisic acid signalling, ABA receptors, PYL/PYR/RCAR, abiotic stress tolerance

## 1. Introduction

The persistence of drought and climate change continues to cause heavy crop losses worldwide and pose a threat to the global food security. To meet the demands of a booming global population, the World Bank estimates that food production must increase by at least 50% in the year 2050 [1]. Increasing agricultural productivity is one practical solution to the food crisis, as has been demonstrated by the Green Revolution, which was estimated to have saved more than one billion people from famine [2]. However, such intensive farming can leave harmful impacts on the environment, such as land degradation and freshwater depletion. Agriculture

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is the largest consumer of global freshwater resources [3], and it is predicted that in the year 2030, the world will be in a 40% water shortage [4]. Therefore, in view of the water and food crises, solutions that aim to increase crop productivity while limiting agricultural water consumption are the most valuable. Such solutions may potentially arise from the fine understanding of how plants perceive and respond to abiotic stress signals.

The plant hormone abscisic acid (ABA) was first discovered in the 1960s and shortly after, its role as the central and critical regulator of abiotic stress response has become clear. Under environmental stress such as drought, ABA levels rise strongly and rapidly, triggering stress tolerant effects, such as stomatal closure, to enable the plants to conserve water and survive through the harsh condition [5]. Exogenous ABA treatment has been shown to maintain the survival and quality of plants subjected to drought stress [6]. However, the agricultural use of ABA has been limited by its short-lived bioactivity, which is due to its chemical instability and rapid catabolism in plants [7]. Therefore, there has been much interests in the development of synthetic compounds that functionally mimic ABA but exhibit longer periods of bioactivity. While earlier attempts of designing ABA analogues guided by the hormone's chemical structure and catabolic pathway had not led to any desirable candidates [8], recent approaches based on the molecular mechanisms of ABA receptor signalling has shown more promising results. Such developments have made been possible by advances in the structural mechanisms of ABA signalling components that have emerged in the past few years, as reviewed in the following sections.

## 2. ABA is a regulator of abiotic stress tolerance

Stress signals, such as drought, salinity, and temperature extremes, trigger the biosynthesis of ABA from carotenoid precursors [9]. ABA is synthesised in vascular tissues and transported to the roots and leaves where the actions occur [10]. In leaves, ABA induces stomata closure to prevent transpirational water loss and promotes the accumulation of osmocompatible solutes to retain water [11]. ABA inhibits root and shoot growth and promotes seed dormancy. Such inhibitory effects help plants to pull through adverse conditions and germinate only when the conditions are favourable for growth. ABA also confers tolerance to freezing through the induction of dehydration-tolerance genes [12].

The effects of ABA has immense agricultural and economic value. Massive amounts of crops are lost to drought every year and the situation may worsen as climate change persists. The ability to manipulate ABA responses offers an innovative solution to alleviate crop loss and sustain agricultural yield in the face of the inevitable climatic change. Moreover, the activation of ABA responses may enhance agricultural water use efficiency, addressing concerns of the global water scarcity issue. A sound understanding of ABA signalling is critical to the successful development of approaches to manipulate ABA responses.

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## 3. Components of the core ABA signalling pathway

### 3.1. ABA receptors

"There are things known and there are things unknown, and in between are the doors of perception." — Aldous Huxley

ABA is a messenger that carries the abiotic stress signal. The first step for the plant cells to be aware of the stress is to perceive the stress signal. The perception and transmission of the signal is carried out by ABA receptors, which recognise the ABA molecule and convey the message to downstream effectors.

Although the importance of ABA and many aspects of its signalling has been established following the discovery of the hormone in the 1960s, the identity of ABA receptors has remained elusive for almost half a century. Early efforts to identify ABA receptors employ forward genetics screens of ABA-insensitive mutants, which have instead identified several mediators of ABA signalling downstream of the receptors, such as PP2Cs and transcription factors [13, 14]. The use of alternative approaches by virtue of ABA binding has led to a number of putative candidates (FCA, CHLH, GCR2, GTG1, and GTG2) but none has been further substantiated [15]. In retrospect, these approaches had been futile for two reasons. First, the true identity of ABA receptors had been masked by genetic redundancy, and had thus eluded identification by classical forward genetic screens. Second, the identification of ABA-binding proteins had not addressed the links of the putative candidates to well-established components of ABA signalling.

The discovery of the PYR/PYL/RCAR family of proteins as bona fide ABA receptors had been different from the earlier attempts. Several groups had independently discovered this family of novel candidates, which fit elegantly into a model that connected the core components of the ABA signal transduction pathway. The first landmark studies were two reports published in Science in May 2009 [16, 17]. To overcome genetic redundancy issues, Park et al. [17] used a selective ABA agonist, pyrabactin, in a chemical genetic screen of mutagenised Arabidopsis seeds and identified *PYR1* (for pyrabactin resistance 1). PYR1 belongs to the START domain superfamily of proteins that includes the mammalian STARD proteins. By homology, 13 other Arabidopsis PYR1-like (PYL) members were identified and named PYL1–13 (Table 1). In a different approach, Ma et al. [16] screened for interactors with the type 2C protein phosphatase (PP2C) ABI2, which is a known component of ABA signalling, and identified the same 14 START domain family members, naming them as RCAR1–14 (Regulatory Component of ABA Response) (Table 1). For simplicity, the PYR/PYL/RCAR members are referred to in this chapter as PYL proteins. Other similar studies have also identified PYL members by their interactions with PP2Cs [18, 19] and collectively, these studies showed that PYL proteins are able to bind to ABA and in the presence of ABA, PYL proteins interact with and inhibit the ABA-signalling PP2Cs.

| | Protein Name | | Solved Structures | | | |
|---|---|---|---|---|---|---|
| | PYR/PYL | RCAR | Mutation(s) | Complex with | | PDB ID |
| | | | | Ligand | PP2C | |
| Members of the PYR/PYL/RCAR family | PYR1 | RCAR11 | | | | 3K3K |
| | | | | ABA | | 3K3K, 3K90 |
| | | | | Pyrabactin | | 3NJO |
| | | | | AS6 | | 3WG8 |
| | | | | ABA | HAB1 | 3QN1 |
| | | | H60P | ABA | HAB1 | 3ZVU |
| | | | K59R, V81I, F108A, F159L | Mandipropamid | HAB1 | 4WVO |
| | PYL1 | RCAR12 | | | | 3KAY |
| | | | | ABA | | 3JRS |
| | | | | Pyrabactin | | 3NEF, 3NEG |
| | | | | ABA | ABI1 | 3KDJ, 3JRQ |
| | | | | Pyrabactin | ABI1 | 3NMN |
| | PYL2 | RCAR14 | | | | 3KDH, 3KL1, 3KAZ |
| | | | | ABA | | 3KB0, 3KDI |
| | | | | Pyrabactin | | 3NJ0, 3NMH, 3NR4, 3NS2 |
| | | | V114I | Pyrabactin | | 3NJ1 |
| | | | A93F | Pyrabactin | | 3NMP |
| | | | A93F | Pyrabactin | HAB1 | 3NMT |
| | | | A93F | Pyrabactin | ABI2 | 3NMV |
| | | | | ABA | HAB1 | 3KB3 |
| | | | | ABA | ABI2 | 3UJL |
| | | | | Quinabactin (AM1) | HAB1 | 4LG5, 4LA7 |
| | | | | AM2 | HAB1 | 4LGA |
| | | | | AM3 | HAB1 | 4LGB |
| | PYL3 | RCAR13 | | | | 3KLX |
| | | | | ABA | | 4DSB, 4DSC |
| | | | | Pyrabactin | | 3OJI |
| | | | | (-)-ABA | | 4JDA |

| | | | | |
|---|---|---|---|---|
| | | ABA | HAB1 | 4DS8 |
| **PYL4** | **RCAR10** | | | |
| **PYL5** | **RCAR8** | | | 4JDL |
| **PYL6** | **RCAR9** | | | |
| **PYL7** | **RCAR2** | | | |
| **PYL8** | **RCAR3** | | | |
| **PYL9** | **RCAR1** | ABA | | 3OQU, 3W9R |
| | | | | 3UQH, 3RT2 |
| **PYL10** | **RCAR4** | ABA | | 3R6P |
| | | | HAB1 | 3RT0 |
| **PYL11** | **RCAR5** | | | |
| **PYL12** | **RCAR6** | | | |
| **PYL13** | **RCAR7** | | PP2CA | 4N0G |

| | **Protein Name** | **Solved Structures** | | |
|---|---|---|---|---|
| | | **Complex with** | | **PDB ID** |
| | | PYR/PYL/RCAR | SnRK2 | |
| **Subset of Group A PP2CS** | ABI1 | PYL1-ABA | | 3KDJ, 3JRQ |
| | | PYL1-Pyrabactin | | 3NMN |
| | ABI2 | | | 3UJK |
| | | PYL2-ABA | | 3UJL |
| | | PYL2(A93F)-Pyrabactin | | 3NMV |
| | HAB1 | PYR1-ABA | | 3QN1 |
| | | PYR1(H60P)-ABA | | 3ZVU |
| | | PYR1(K59R, V81I, F108A, F159L)-Mandipropamid | | 4WVO |
| | | PYL2-ABA | | 3KB3 |
| | | PYL2(A93F)-Pyrabactin | | 3NMT |
| | | PYL2-Quinabactin (AM1) | | 4LG5, 4LA7 |
| | | PYL2-AM2 | | 4LGA |
| | | PYL2-AM3 | | 4LGB |
| | | PYL3-ABA | | 4DS8 |
| | | PYL10 | | 3RT0 |
| | | | SnRK2.6 | 3UJG |
| | PP2CA | PYL13 | | 4N0G |

| | Protein Name | Solved Structures | | |
|---|---|---|---|---|
| | | Mutation(s) | Complex with | PDB ID |
| Subclass III SnRK2s | SnRK2.2 | | | |
| | SnRK2.3 | D57A, K58A | | 3UC3 |
| | SnRK2.6 | D59A, E60A | | 3UC4 |
| | | D160A | | 3ZUT |
| | | D160A, S175D | | 3ZUU |
| | | S7A,S29A,S43A,C131A,C137A,C159A,S166A,T176A | | 3UDB |
| | | | HAB1 | 3UJG |

**Table 1.** List of members belonging to the components of the core ABA signalling pathway and their solved structures.

### 3.2. Group 2C Protein Phosphatases (PP2Cs)

PP2Cs are a group of monomeric $Mg^{2+}/Mn^{2+}$-dependent serine/threonine phosphatases found in virtually all organisms and are known for their roles in the regulation of cell growth and cellular stress signalling. In Arabidopsis, there are 76 known PP2Cs that are genetically clustered into 10 groups (A-J), with the exception of 6 genes that could not be clustered [20]. At least 6 of the 9 members of group A PP2Cs have been shown to be involved in ABA signalling. Among these, ABI1, ABI2, and HAB1 are the most well-studied members and are known to be the negative regulators of ABA signalling.

The *abi1* and *abi2* (ABA insensitive) mutants were isolated from genetic screens of ABA insensitive mutants [21-24]. HAB1 was subsequently identified by homology to ABI1 and ABI2 [25]. The *abi1-1* (ABI1 G180D), *abi2-1* (ABI2 G168D), and *hab1* (HAB1 G246D) mutants displayed dominant ABA insensitive phenotypes, which are later known to be attributed to the loss of regulation by PYL proteins. The isolation of additional recessive loss-of-function mutations that resulted in ABA hypersensitive phenotype provided the early evidence that PP2Cs are negative regulators of ABA signalling [26-28]. This was further supported by the observations that double- or triple-PP2C-knockout mutants displayed enhanced ABA responses while constitutive expression of HAB1 led to reduced ABA sensitivity [28-30].

### 3.3. Snf1-related protein kinases 2 (SnRK2s)

While members of PP2Cs are known to play a negative regulatory role in ABA signalling, it is not surprising that a group of protein kinases are conversely the positive effectors. The Arabidopsis Snf1-related protein kinase (SnRK) group of kinases share a high degree of homology with the yeast Snf1 and catalytic subunits of mammalian AMPK. There are three classes of SnRKs in Arabidopsis, namely SnRK1, SnRK2, and SnRK3, which comprises of 3, 10, and 25 members, respectively [31]. SnRK2s are further divided into subclasses I, II, and III. While members of subclass I are not responsive to ABA, subclass II members are weakly

activated by ABA. All three members of subclass III, namely SnRK2.2/SRK2D, SnRK2.3/SRK2I, and SnRK2.6/SRK2E/OST1, are strongly activated by ABA and are known to be the main positive regulators of ABA signalling. SnRK2.6 is known for its role in stomata regulation in guard cells [32, 33], while SnRK2.2 and SnRK2.3 are important in the inhibition of seed germination and root growth [34]. Consequently, triple mutants lacking SnRK2.2, SnRK2.3, and SnRK2.6 are deficient in almost all ABA responses, indicating that class III SnRK2s play a central role in the core ABA signalling pathway [35-37].

Active SnRK2s are autophosphorylated and are able to directly phosphorylate target proteins such as ion channels and transcription factors to elicit the ABA response. ABA-induced stomata closure is mediated by the effects of SnRK2.6 on ion channels. Under ABA stimulation, SnRK2.6 activates the slow-type anion channel SLAC1 and inhibits the inward-rectifying potassium channel, KAT1, by the phosphorylation of these ion channels, resulting in stomata closure [38-40].

ABA induction of target gene expression is mediated by the SnRK2 phosphorylation of transcription factors known as ABRE-binding (AREB) proteins or ABRE-binding factors (ABFs). The AREB/ABFs are basic domain leucine zipper (bZIP) transcription factors that recognises the ABA-responsive elements (ABREs) located in the promoters of ABA-responsive genes. Of the nine AREB/ABF homologs found in Arabidopsis, the AREB1/ABF2, AREB2/ABF4, and ABF3 were found to be master transcription factors responsible for regulating the ABRE-dependent expression of stress-responsive genes [41-44]. Such genes include Late Embryogenesis Abundant (LEA)-class genes, transcription factors, and mediators of ABA signalling.

## 4. Structural mechanisms of ABA receptor signalling

The core ABA receptor signalling cascade is comprised of the PYL ABA receptors, PP2Cs and SnRK2s (Figure 1). In the absence of ABA, PP2Cs such as ABI1, ABI2, and HAB1 inhibit the activities of SnRK2s, thus silencing the ABA response. During abiotic stress, ABA is generated rapidly and binds to the PYL proteins. ABA binding induces conformational changes to the PYL proteins, allowing the activated receptors to interact with and inhibit the PP2Cs. Consequently, the SnRK2s are relieved of inhibition by PP2Cs and are autoactivated by autophosphorylation. Active SnRK2s are able to phosphorylate their targets such as ion channels and AREB/ABF transcription factors to activate the ABA responses. Reconstitution of the core ABA signalling pathway has been demonstrated by co-expression of the core components (PYL, PP2C, SnRK2, and ABF) in plant protoplasts [45]. The following subsections will explain in detail the molecular mechanisms of each step of the core ABA signalling pathway.

### 4.1. Structure and properties of ABA receptors

To date, the structures of eight out of 14 PYL members have been solved either in their apo- or ligand-bound forms or in complexes with PP2C (Table 1). The overall PYL structures exhibit the helix-grip fold, a hallmark of START domain/Bet v 1-fold proteins, which is characterised

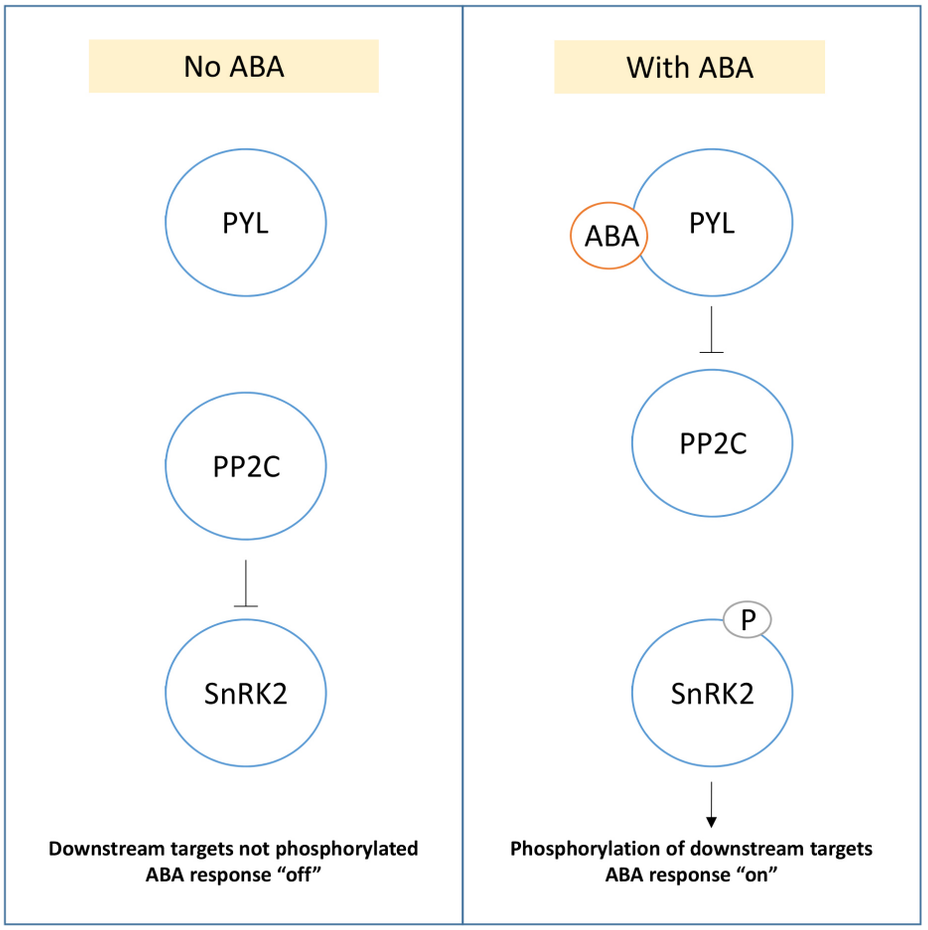


**Figure 1.** Summary model of the core ABA signalling pathway.

by the presence of a central β-sheet surrounded by N- and C-termini α-helices, with a long C-terminal α-helix packing tightly against the β-sheet (Figure 2). The helix-grip fold creates a large cavity constituting the ligand binding pocket.

Static light scattering and ultracentrifugation experiments showed that the apo PYR1, PYL1, and PYL2 are dimers in the solution, while PYL4–10, except for the untested PYL7, are monomers [46]. PYL3 exist in a monomer-dimer equilibrium. Consistently, the crystal structures of apo PYR1 and PYL1–3 revealed a cis-homodimer arrangement, with the two molecules associated in parallel orientation at their pocket entrance, thus hindering ligand entry [47-52]. Homodimeric receptors dissociate into monomers upon ABA binding. PYL3 has been shown to form a trans-homodimer intermediate that is able to bind ABA and dissociate into monomers more easily, consistent with its observed mixed monomer-dimer distribution [52]. While the dimeric receptors require ABA for their activity, the monomeric PYL members are able to inhibit PP2Cs constitutively in the absence of ABA, suggesting that receptor monomerisation is crucial for PP2C inhibition [46]. Despite the ABA-independent activity, it is important to note that the interactions of monomeric PYLs with PP2Cs are greatly enhanced

in the presence of ABA [16, 19, 46]. PYL13 is a divergent member that play a unique role in ABA signalling by its ability to heterodimerise with other PYL members and antagonise their activities [53]. While it was originally thought that PYL13 selectively inhibits PP2CA in an ABA independent manner [53, 54], recent evidence showed that PYL13 inhibits ABI1, ABI2, and PP2CA with the requirement of ABA [55].

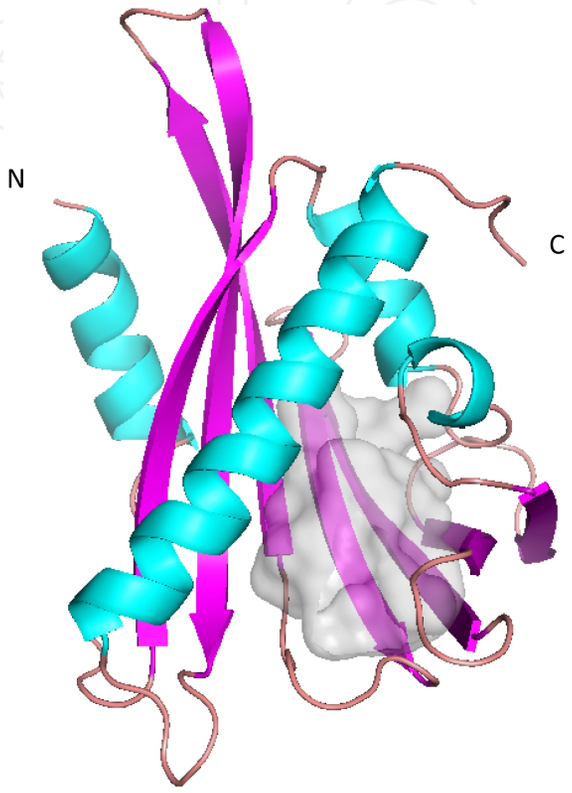


**Figure 2.** Structure of the ligand-free PYL2 ABA receptor (PDB code: 3KAZ) exhibiting the helix-grip fold. The ligand binding pocket is shown as surface presentation in grey.

### 4.2. ABA binding

The structures of PYL bound to ABA revealed a number of features in the ABA recognition. The entrance of the ligand pocket is surrounded by two functionally important β-loops that are known as the "gate" and "latch" loops [47] (alternatively named Pro-Cap and Leu-Lock [49], CL2, and CL3 [51], and the β3–β4 and β5–β6 lid loops [48, 50]), which contain the conserved amino acid sequences SGLPA and HRL, respectively. In the structure of the apo receptor, the gate loop appears to be in an open conformation to allow ligand access (Figure 3a). In the ABA-bound structure, the gate is in a closed conformation, making contact with the latch residues (Figure 3b).

Within the ligand pocket, ABA interacts with the receptor residues through a network of charged interactions, hydrogen bonds, and hydrophobic interactions [47-51]. ABA is anchored to the inner end of the ligand pocket by a direct charged interaction between its carboxylate group

and a conserved lysine residue (K59, K86, and K64 in PYR1, PYL1, and PYL2, respectively). Nearer to the outer end of the pocket, the cyclohexene ring of ABA interacts with the receptor gate and latch residues, thus pulling the gate loop into a closed conformation (Figure 6a).

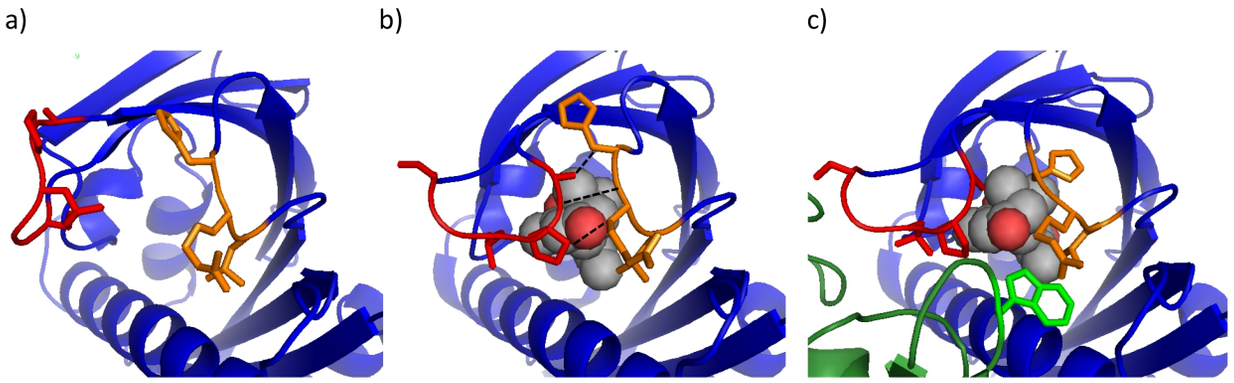


**Figure 3.** A gate-latch-lock mechanism of ABA sensing and signal relay by the PYL ABA receptor. a) The empty pocket of the ligand-free ABA receptor, represented by apo PYL2 (PDB code: 3KAZ), is guarded by a latch loop (shown in orange) and an open gate loop (shown in red). b) Structure of PYL2–ABA (PDB code: 3KB0) showing that ABA binding induces closure of the gate onto the latch loop. ABA is shown in sphere model. c) PP2C (shown in green) binding inserts a conserved tryptophan to "lock" the closed receptor gate and latch in a stable conformation, shown by the structure of the PYL2–ABA–HAB1 complex (PDB code: 3KB3). In all panels, PYL2 is shown in blue with its gate loop in red and latch loop in orange.

### 4.3. Signal relay to PP2Cs

The closure of the receptor gate upon ABA binding is functionally important for its signal transmission to the PP2Cs. While PP2Cs do not undergo obvious conformational changes in their interactions with PYLs, the ABA-induced receptor gate closure creates the necessary PYL conformation for PP2C interaction. The ABA-bound PYL protein interacts with PP2C at its closed gate and latch interface (Figure 4). In this interaction, the PP2C inserts a conserved tryptophan indole ring into the receptor pocket, forming a water-mediated network of hydrogen bonds with the receptor gate and latch residues, as well as with the ketone group of ABA (Figure 3b). The PP2C tryptophan thus acts as a molecular lock that further stabilises the receptor gate and latch interactions. This explains the observations of the marked increase in ABA binding affinities of PYLs in the presence of PP2Cs [16, 19, 48, 51]. The formation of the PYL–ABA–PP2C complex inhibits the PP2C activities in two ways. First, the PYL–ABA interacts with the PP2C at the phosphatase catalytic region (Figure 4a), thus competitively blocking substrate (SnRK2) access [56]. Second, a catalytic glutamate residue of the PP2C (E203 in HAB1) is bonded to the serine residue of the PYL gate loop (Figure 4b and 4c), thus impairing the phosphatase catalytic activity [57].

### 4.4. PP2C inhibition of SnRK2s

In the absence of ABA, PP2Cs bind to and inhibit SnRK2s. The solved structure of the SnRK2.6-HAB1 complex together with biochemical data has provided insights into the mechanisms of how PP2Cs inhibit SnRK2s [56]. In this structure, the phosphatase-kinase interaction occur

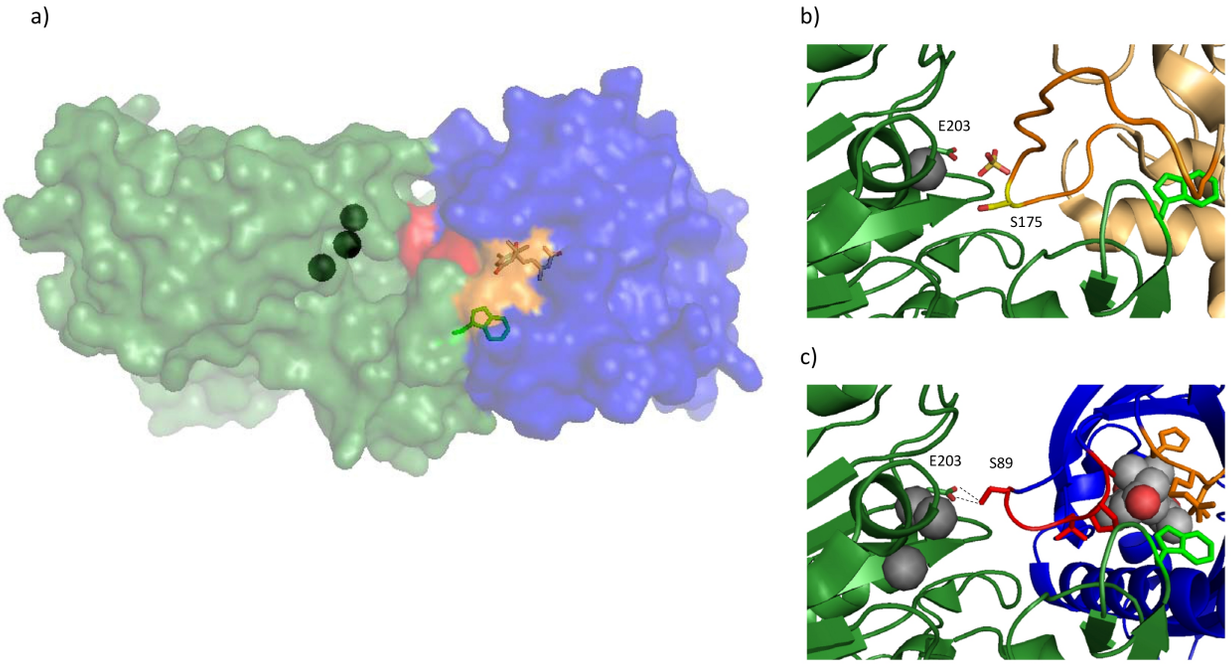


**Figure 4.** Dual mode of PP2C inhibition by the activated ABA receptor. a) Structure of PYL2–ABA–HAB1 complex (PDB code: 3KB3) in surface presentation showing the steric blocking of PP2C (shown in green, catalytic site marked by $Mg^{2+}$ ions in grey balls) by ABA-bound PYL2 (shown in blue, with its gate and latch in red and orange respectively). ABA is shown in stick model to indicate the ligand pocket. b) Partial structure of the HAB1–SnRK2.6 complex (PDB code: 3UJG), focusing on the catalytic sites of both components. In the phosphatase reaction, a serine residue (S175 of SnRK2.6) in the activation loop of the SnRK2 (SnRK2.6 shown in light orange, with its activation loop in darker orange) is dephosphorylated. A sulphate molecule, mimicking the cleaved phosphate, is shown in stick model. This catalysis requires the PP2C catalytic glutamate (E203 of HAB1) to polarise a water molecule, enabling its nucleophilic attack on the phosphorylated S175 [57]. c) Binding of PYL2–ABA to HAB1 results in the formation of a hydrogen bond between PYL2 S89 and HAB1 E203, thus catalytically inhibiting the PP2C's phosphatase activity. In all panels, the PP2C tryptophan "lock" is shown as stick model in light green.

through mutual packing of both catalytic sites (Figure 4b and 5a). HAB1 interacts with SnRK2.6 at its PYL-interaction interface and inserts its ABA-sensing tryptophan into the catalytic cleft of SnRK2.6. Unresolved in the crystal structure, extensive evidence indicated the presence of a second interaction interface formed by the highly negatively charged C-terminal ABA box of SnRK2 and a positively charged surface region of PP2C (Figure 5a). Biochemical data has shown that at low PP2C:SnRK2 molar ratio, the kinase activity is reduced but not completely abolished [56]. This partial inhibition occur by enzymatic dephosphorylation of a critical serine residue in the kinase activation loop. At stoichiometric levels, full inhibition is achieved through the mutual packing of catalytic sites as shown by the crystal structure.

### 4.5. Autoactivation of SnRK2s

The understanding of how PP2Cs inhibit SnRK2s has provided partial explanations to how SnRK2s gain catalytic activity. PP2C interact with both PYL and SnRK2 at its catalytic region, suggesting that the inhibition of SnRK2 is competed away by active PYL interacting at the same PP2C interface (Figure 5a and 5b). Further biochemical and structural analyses has furnished the complete mechanisms of SnRK2 activation [58-60]. Biochemical data showed

that phosphorylation of a serine residue in the SnRK2 activation loop (S177/176/175 in SnRK2.2/2.3/2.6, respectively) is critical for full kinase activity [61]. Kinases with this serine mutated to alanine are non-phosphorylatable at this position and showed marked decrease, but not complete abolishment of kinase activity, suggesting that unphosphorylated SnRK2s have basal kinase activity [58]. Crystal structures of unphosphorylated SnRK2s have been solved in their active and inactive states, providing structural insights into the mechanisms of the basal activity [58]. The SnRK2 structures revealed a canonical bilobal kinase fold, with a well-ordered SnRK2 box packing closely in parallel to the αC-helix in the N-terminal lobe (schematically illustrated in Figure 5). The structure of the active SnRK2 adopted a closed conformation, with its N-terminal lobe positioned closer to the C-terminal lobe (Figure 5d), whereas that of the inactive SnRK2 exhibited an open conformation (Figure 5c) resembling the structures of active and inactive Snf1 kinases, respectively. The basal kinase activity is attained by the closing of the catalytic cleft, which is regulated by the SnRK2 box interactions with the αC-helix. The SnRK2 box mediates positioning of the αC-helix to form a network of interaction that favours the binding of $Mg^{2+}$ and ATP in the catalytic cleft. Such a positioning enables full kinase activity to be achieved upon autophosphorylation of the activation loop, which can occur intramolecularly or intermolecularly (Figure 5e).

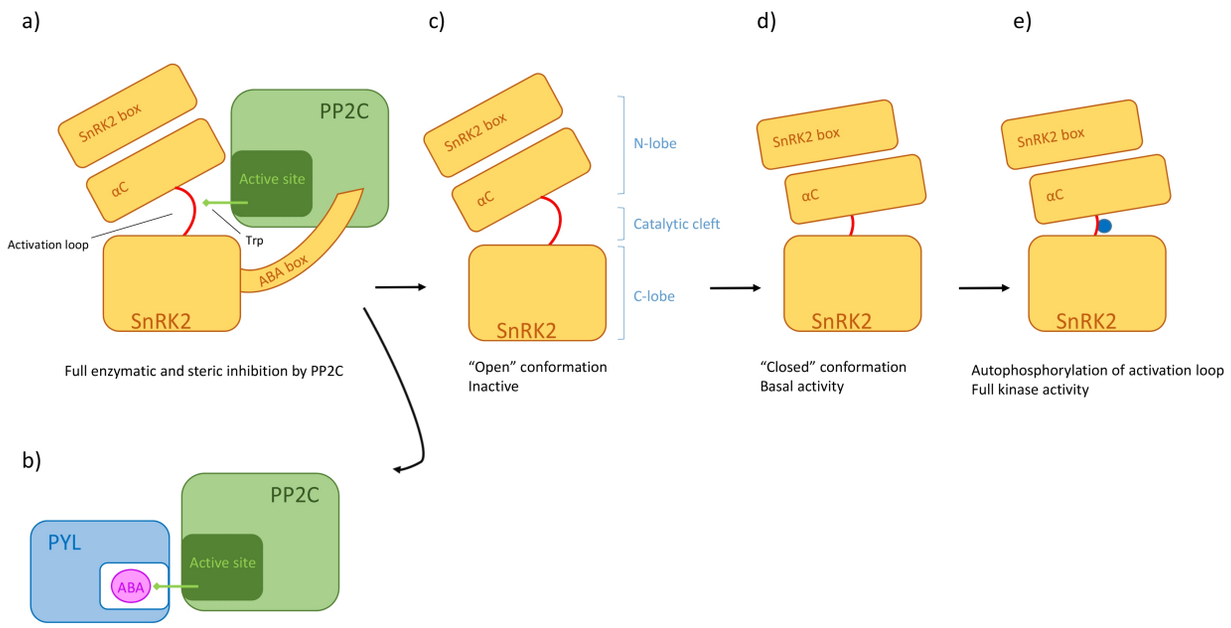


**Figure 5.** Mechanisms of kinase activation. a) In basal state, PP2C inhibit SnRK2 by enzymatic dephosphorylation of the kinase's activation loop serine, as well as steric inhibition by physically binding to the kinase's catalytic cleft. The two PP2C-SnRK2 interaction interfaces are shown. First is through the mutual packing of active sites as indicated by the kinase's activation loop and the phosphatase's ABA-sensing tryptophan "lock". Second is through the SnRK2's C-terminal ABA box region. b) When activated by ABA, PYL compete with SnRK2 for PP2C interaction at the same PP2C catalytic region. c) and d) When relieved of PP2C inhibition, unphosphorylated SnRK2s spontaneously adopt inactive and partially active states by the opening and closing of the catalytic cleft, mediated by contacts between the SnRK2 box and αC-helix. e) The closing of the catalytic cleft produces interactions that facilitates the autophosphorylation of the activation loop serine residue, which is required to attain full kinase activity.

## 5. Emerging agricultural biotechnology targeting ABA receptor signalling

The understanding of how plants perceive stress provides the opportunity to develop novel solutions to promote crop survival by boosting stress responses during adverse conditions such as water shortage. As water conservation is one of the physiological effects of ABA, the activation of ABA signalling may also promote water use efficiency in agriculture. The world is facing a crisis of freshwater shortage and the agricultural sector is the largest consumer of the global freshwater resources. Thus, the ability to manipulate ABA responses has immense value, promoting both water conservation and food productivity. ABA responses may be manipulated by transgenic approaches involving the overexpression of the effectors of ABA signalling, the structure-guided development of high efficacy agonists, or a combination of these methods.

### 5.1. Transgenic approach

Having understood the roles and mechanisms of the key players in the core ABA signalling, one approach to enhance ABA sensitivity is to generate transgenic plants overexpressing the effectors of ABA signalling. While the dimeric PYL members, PYR1, PYL1, and PYL2 require ABA for their activation, the monomeric receptors, PYL5-10, show ABA-independent constitutive activity which is greatly enhanced in the presence of ABA [46]. Thus, the constitutively active monomeric receptors are thought to be suitable candidates for transgenic overexpression to enhance ABA sensitivity. This idea is supported by the observation that transgenic Arabidopsis overexpressing PYL5 showed enhanced ABA sensitivity and increased drought stress tolerance [19]. Similar results have been shown in transgenic Arabidopsis overexpressing PYL13 [53], which has shown ABA independent inhibition of PP2CA [54]. In rice (*Oryza sativa*), the constitutive expression of OsPYL, the rice orthologue of Arabidopsis PYL, has also shown to improve drought and salt stress tolerance [62].

With the known structural mechanisms, genetic modifications of PYL may be incorporated to further enhance their activity. The constitutive expression of a PYL2 mutant designed to stabilise PYL-PP2C interactions has shown increased ABA signalling in transgenic Arabidopsis seeds [63]. However, the expression of the mutant receptor has not been detected in vegetative tissues, thus precluding further analyses in this study. In another study, overexpression of a mutant PYL4 receptor ($PYL4^{A194T}$), which showed ABA-independent inhibition of PP2CA in vitro, resulted in enhanced drought tolerance in the transgenic plants [64]. However, as ABA is a negative growth regulator, a drawback of constitutively activating ABA responses is the impairment of growth under normal conditions, thus affecting overall yield. Approaches that allow inducible activation of ABA responses only under stressful conditions may be useful to overcome this problem.

Alternatively, the heterologous expression of SnRK2s of wheat (*Triticum aestivum* L.), TaSnRK2.4, and TaSnRK2.8 in Arabidopsis has been tested and the transgenic plants showed enhanced tolerance to drought, salt, and cold stresses [65, 66]. Similarly, transgenic Arabidopsis overexpressing the maize SnRK2 orthologue, ZmSAPK8, exhibited increased tolerance to salt stress [67]. In these studies, the heterologous expression of members of the SnRK2s did

not seem to retard the normal plant growth under unstressed conditions, thus appearing to be a potential approach for the development of stress-tolerant transgenic crops.

### 5.2. Agonist design

The direct application of ABA in the fields has been shown to promote drought stress tolerance. However, the widespread use of ABA in agriculture is limited due to its chemical instability and difficulty to synthesise. Therefore, there has been much research focus on the development of ABA analogues that are able to elicit the effects of ABA and exhibit better chemical stability.

Pyrabactin is a synthetic seed germination inhibitor that mediates a selective part of ABA's activities. Being a selective ABA agonist, pyrabactin has been used to overcome the problems of genetic redundancy in the identification of ABA receptors [17]. Although the direct application of pyrabactin is not practical for agriculture use, as its effects are most strongly in seeds rather than in vegetative tissues, the study of ABA receptors bound to pyrabactin has provided insights into the design of ABA receptor agonists. While ABA is a pan-agonist of all PYL members, pyrabactin selectively activates few members, including PYR1 and PYL1. Conversely, pyrabactin acts as an antagonist in PYL2. Comparisons between the structures of ABA-bound PYL, pyrabactin-activated PYL and pyrabactin-antagonised PYL complexes have revealed the ligand-receptor interactions that are important to induce the closed gate conformation necessary for receptor activation [68-71]. In the pyrabactin-activated receptor (PYR1 or PYL1) structures, pyrabactin interacts with receptor pocket residues in a similar manner as ABA, with its sulphonamide group forming a water-mediated hydrogen bond with the conserved lysine residue and its naphthalene double ring interacting with the receptor gate residues, producing the close-gate conformation (Figure 6b). These features provided a framework for the development of novel ABA agonists. On this basis, Melcher et al. screened virtual chemical libraries for compounds containing the naphthalene-1-sulphonamide group and computationally docked them into the known PYL structures [69]. In vitro screening of top candidates identified four compounds with efficacies comparable to that of pyrabactin.

Examination of the structure of the pyrabactin-antagonised PYL2 receptor revealed a flip in the orientation of pyrabactin relative to that in the pyrabactin-activated receptor structures (Figure 6c). In the antagonist position, the naphthalene double ring and pyridine ring are in reversed order, placing the smaller pyridine ring towards the pocket entrance in a distance too far to interact with the receptor gate. Therefore, pyrabactin antagonises PYL2 by occupying the receptor pocket while being unable to produce the closed gate conformation. With this knowledge, an agonist termed AM1/quinabactin was designed with a similar scaffold as pyrabactin but with the sulphonamide group in reversed orientation [72, 73]. The structures of PYL2 bound to AM1/quinabactin showed that the AM1/quinabactin is oriented with its double ring facing the receptor gate, producing a closed gate conformation (Figure 6d). Furthermore, AM1/quinabactin showed potent in vitro efficacies in PP2C interaction, ability to promote drought tolerance when exogenously applied to plants and higher stability than ABA when exposed to mild UV [72, 73]. Therefore, AM1/quinabactin emerged as a highly promising candidate to be further evaluated for agricultural use.

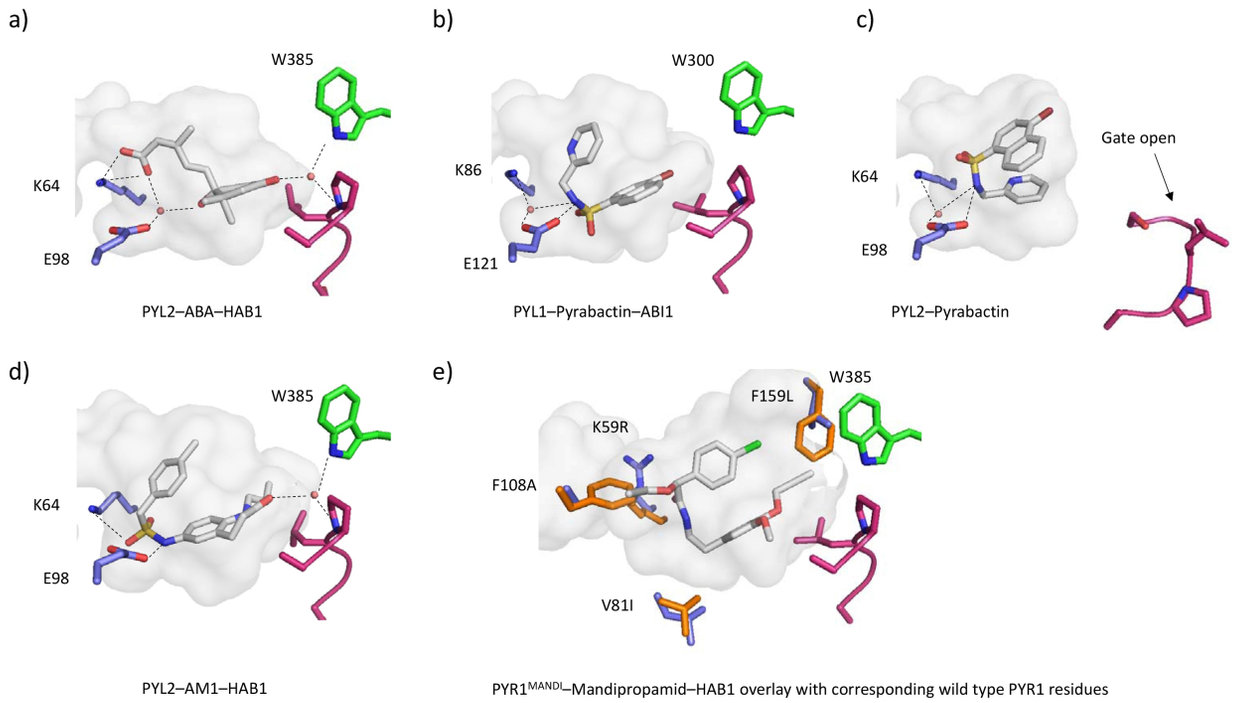


**Figure 6.** Mode of receptor binding by various ligands. Ligand binding pockets of a) PYL2–ABA–HAB1 structure (PDB code: 3KB3), b) PYL1–Pyrabactin–ABI1 (PDB code: 3NMN), c) PYL2–Pyrabactin (PDB code: 3NMH), d) PYL2–AM1–HAB1 (PDB code: 4LG5), and e) PYR1$^{MANDI}$–Mandipropamid–HAB1 (PDB code: 4WVO). PYL ligand pockets are shown as surface presentation in light grey. In (a) to (d), the conserved lysine and glutamate residues that anchor the ligands to the inner end of the receptor pocket are shown as stick models in blue. In all panels, receptor gate loop residues are shown in red, while the conserved PP2C tryptophan "lock" is shown as a stick model in green. In (c), pyrabactin antagonises the PYL2 receptor, leaving the receptor gate in an open conformation. In (d), the four mutant PYR1$^{MANDI}$ residues are shown in blue, while the corresponding wild type residues from the PYR1–ABA–HAB1 (PDB code: 3QN1) structure are shown in orange.

### 5.3. Orthogonal receptor-ligand approach

Although the discovery of AM1/quinabactin has shown promising results, pushing a new chemical into the market can be a long and costly process as the potential effects on human health and the environment need to be thoroughly assessed. To bypass such a process, an alternative solution is to make use of currently approved agrochemicals. Park et al. has demonstrated that the ABA receptor can be engineered to be activated by existing agrochemicals [74]. This effort has identified a hextuple mutant PYR1$^{MANDI}$ (PYR1(Y58H/K59R/V81I/F108A/S122G/F159L)) that showed strong PP2C inhibition with the agrochemical mandipropamid at nanomolar sensitivity. To understand the mode of the orthogonal receptor-ligand interactions, the crystal structure of a quadruple mutant PYR1(K59R/V81I/F108A/F159L), which contains 4 of the 6 mutations of PYR1$^{MANDI}$ and yielded higher quality crystals than PYR1$^{MANDI}$, has been solved in complex with mandipropamid and HAB1 (Figure 6e). The crystal structure revealed that the F108A/F159L mutations created more space in the receptor pocket to fit the larger ligand. The arginine of R59 forms a hydrogen bond with the amide carbonyl of mandipropamid, mimicking the interaction between the carboxylate group of ABA with K59 of wild type PYR1. In vivo studies of transgenic Arabidopsis constitutively express-

ing PYR1$^{MANDI}$ showed enhanced drought survival with mandipropamid treatment, thus demonstrating the feasibility of such an approach.

## 6. Conclusions

The identification of PYL proteins as ABA receptors has placed a crucial piece of the puzzle into the previous knowledge of the core ABA signalling pathway. As such, the early efforts in the discovery of PYL proteins and the elucidation of their structural mechanisms have been recognised as one of the breakthroughs of the year 2009 by *Science* and *Science Signaling* journals [75, 76]. Subsequently, further structural studies have rapidly emerged to provide a comprehensive understanding of the molecular mechanisms of the entire core ABA signalling pathway, which has led to preliminary developments of novel approaches targeting at ABA signalling for the agricultural enhancement of abiotic stress tolerance. Using several lines of approach, these studies have demonstrated the ability to target ABA signalling for improving stress tolerance. While several studies have shown promising data at the preliminary stage, further testing in crop plants will be necessary to evaluate their agricultural feasibility. Having progressed rapidly since the initial discovery of the PYL proteins, further breakthrough advances in the agricultural improvement of abiotic stress tolerance seems highly conceivable and is much awaited for.

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