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REVIEW PAPER

ABA receptors: the START of a new paradigm in phytohormone signalling

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Abstract

The phytohormone abscisic acid (ABA) plays a central role in plant development and in plant adaptation to both biotic and abiotic stressors. In recent years, knowledge of ABA metabolism and signal transduction has advanced rapidly to provide detailed glimpses of the hormone's activities at the molecular level. Despite this progress, many gaps in understanding have remained, particularly at the early stages of ABA perception by the plant cell. The search for an ABA receptor protein has produced multiple candidates, including GCR2, GTG1, and GTG2, and CHLH. In addition to these candidates, in 2009 several research groups converged on a novel family of *Arabidopsis* proteins that bind ABA, and thereby interact directly with a class of protein phosphatases that are well known as critical players in ABA signal transduction. The PYR/PYL/RCAR receptor family is homologous to the Bet v 1-fold and START domain proteins. It consists of 14 members, nearly all of which appear capable of participating in an ABA receptor–signal complex that responds to the hormone by activating the transcription of ABA-responsive genes. Evidence is provided here that PYR/PYL/RCAR receptors can also drive the phosphorylation of the slow anion channel SLAC1 to provide a fast and timely response to the ABA signal. Crystallographic studies have vividly shown the mechanics of ABA binding to PYR/PYL/RCAR receptors, presenting a model that bears some resemblance to the binding of gibberellins to GID1 receptors. Since this ABA receptor family is highly conserved in crop species, its discovery is likely to usher a new wave of progress in the elucidation and manipulation of plant stress responses in agricultural settings.

Key words: Abiotic stress, abscisic acid, Bet v 1-fold, drought, PP2C, PYR/PYL/RCAR, salinity, SnRK, signal–receptor, START domain.

Introduction

Abscisic acid (ABA) is a sesquiterpenoid molecule made by organisms across kingdoms, including fungi, plants, and metazoans (Oritani and Kiyota, 2003; Bruzzone *et al.*, 2007). It was first discovered and is best understood in angiosperms, where it acts as a hormone to regulate diverse processes including seed development, dormancy, germination, and seedling growth (Finkelstein *et al.*, 2002). ABA is also a central regulator of plant adaptation to biotic (Fujita *et al.*, 2006) and abiotic stressors (Zhu, 2002). ABA protects plants under abiotic stress, particularly dehydration and salinity, through mechanisms including the production of osmoprotective proteins and metabolites, and the regulation of stomatal conductance (Zhu, 2002). Many details of ABA biosynthesis, transport, catabolism, and ABA-mediated effects on global transcription and metabolism have been elucidated (Nambara and Marion-Poll, 2005; Christmann *et al.*, 2006; Verslues and Zhu, 2007; Urano *et al.*, 2009). Genomic resources of *Arabidopsis*, in particular, have enabled the identification of key components of ABA signal transduction, including protein kinases, phosphatases, transcription factors, RNA processing factors, proteasome components, chromatin remodelling proteins, and histone

deacetylases that mediate epigenetic regulation (Hirayama and Shinozaki, 2007; Chinnusamy and Zhu, 2009; Cutler *et al.*, 2010). However, many significant gaps in our understanding of ABA signal transduction still exist.

One active area of ABA research has been the quest for protein receptors that directly bind the hormone and trigger signalling events leading to ABA's distinctive effects on plant physiology. Genetic redundancy has complicated the search for ABA receptors. Moreover, receptor searches have led to multiple—in some cases controversial—candidate proteins (McCourt and Creelman, 2008). Recently, a combination of chemical genetic and proteomic approaches led to significant breakthroughs in the receptor hunt. In this review, we focus on the convergence of separate research groups upon a novel class of ABA-binding proteins in *Arabidopsis*—the PYR/PYL/RCAR family—that bear unequivocal hallmarks of ABA receptors. These rapid developments have opened a wide doorway for exploring the structural basis of ABA perception and signal transduction in great detail. The emerging picture reveals a remarkable, surprisingly simple, assemblage of key proteins that form an ABA signal–receptor complex, with an arrangement that bears some resemblance to perception mechanisms for other plant hormones, but that also presents a distinctive model likely to establish new paths of research. The PYR/PYL/RCAR family is highly conserved in other plant species, and its discovery could lead to the development of novel means for manipulating stress tolerance in crops.

Dodging redundancy

Independent, complementary approaches led to the first published reports that identified PYR/PYL/RCAR proteins as ABA receptors. One strategy employed yeast two hybrid (Y2H) screening to search for interacting partners of *Arabidopsis* protein phosphatases that are negative regulators of ABA signalling (Ma *et al.*, 2009). In an alternative, innovative approach, a chemical genetic screen of small synthetic molecules that perturb seed germination in *Arabidopsis* identified the receptor family through a forward genetic analysis (Park *et al.*, 2009).

In the latter approach, the Cutler group identified one small molecule, termed pyrabactin, that specifically inhibits seed germination and cell expansion (Park *et al.*, 2009). Pyrabactin's effects on global gene transcription in the seed are highly correlated with those of ABA but not with other germination inhibitors. This correlation is much weaker in seedlings, indicating that the chemical is a highly selective ABA agonist that mimics only a subset of the hormone's activities during plant development. A forward genetic screen for mutants resistant to pyrabactin identified the *PYRABACTIN RESISTANCE 1* (*PYR1*) locus; map-based cloning identified several mutations in PYR1, which is a founding member of the PYR/PYL/RCAR protein family. Thirteen PYR1-LIKE (PYL) proteins encoded in the *Arabidopsis* genome were identified by sequence analysis and designated PYL1 through PYL13. The specificity of pyrabactin for a seed germination phenotype and the relatively high expression level of *PYR1* in the seed, compared with other family members, may explain why a pyrabactin-insensitive mutant could be detected, bypassing the functional redundancy that has hindered other forward genetic screens for ABA receptors.

In a paper that was published simultaneously with that of the Cutler group, the Grill group used the Y2H method to screen the *Arabidopsis* proteome for interactors with ABI2, a type 2C serine threonine protein phosphatase (PP2C) (Ma *et al.*, 2009). PP2Cs are found across kingdoms, but comprise an exceptionally large and diverse family in plants (Schweighofer *et al.*, 2004). Some members of clade A of this family are known for their central roles in negative regulation of ABA signalling, including the well-studied proteins ABI1 and ABI2 (ABA INSENSITIVE 1 and 2), HAB1 (HOMOLOGY TO ABA INSENSITIVE 1), and AHG1 and AHG3 (ABA-HYPERSENSITIVE GERMINATION 1 and 3) (Koornneef *et al.*, 1984; Leung *et al.*, 1994, 1997; Meyer *et al.*, 1994; Saez *et al.*, 2004; Yoshida *et al.*, 2006; Nishimura *et al.*, 2007). By using the Y2H method with ABI2 as bait, the Grill group discovered a protein called REGULATORY COMPONENT OF ABA RESPONSE 1 (RCAR1), identical to PYL9, leading to the identification of the other 13 members of the PYR/PYL/RCAR family (Ma *et al.*, 2009). The simultaneous reports of PYR/PYL and RCAR proteins have created two sets of nomenclature for this family (Table 1). This report from the Grill group was followed by other publications in which researchers used similar approaches and added details about the PYR/PYL/RCAR receptor family.

The Rodriguez group performed a Y2H screen for interacting partners of HAB1, and identified PYL5, PYL6,

Table 1. Nomenclature and corresponding *Arabidopsis* accession numbers for the 14 members of the PYR/PYL/RCAR family of ABA receptors

Nomenclature		Locus accession no.
PYR/PYL[a]	RCAR	
PYR1 (1,2)	RCAR11	At4g17870
PYL1 (3,4,5)	RCAR12	At5g46790
PYL2 (4,5)	RCAR14	At2g26040
PYL3	RCAR13	At1g73000
PYL4	RCAR10	At2g38310
PYL5	RCAR8	At5g05440
PYL6	RCAR9	At2g40330
PYL7	RCAR2	At4g01026
PYL8	RCAR3	At5g53160
PYL9	RCAR1	At1g01360
PYL10	RCAR4	At4g27920
PYL11	RCAR5	At5g45860
PYL12	RCAR6	At5g45870
PYL13	RCAR7	At4g18620

[a] Crystal structure solved by: (1) Nishimura *et al.*, 2009; (2) Santiago *et al.*, 2009*a*; (3) Miyazono *et al.*, 2009; (4) Melcher *et al.*, 2009; (5) Yin *et al.*, 2009.

and PYL8 proteins (Santiago *et al.*, 2009*b*). In another protein-based approach, the Schroeder group screened for ABI1-interacting proteins *in planta* by affinity purification and mass-spectrometry, using YFP-tagged ABI1 expression lines of *Arabidopsis*. This method identified nine of the 14 PYR/PYL/RCAR proteins (Nishimura *et al.*, 2010). After identifying *PYR1* in the forward genetic screen, the Cutler group conducted a Y2H screen of protein interactors with PYR1, and identified HAB1 (Park *et al.*, 2009). This study also detected physical interactions between multiple PP2Cs and several members of the PYR/PYL/RCAR family. Physical interactions of PYR/PYL/RCAR with PP2Cs have been further supported *in planta* with other co-immunoprecipitation experiments and with bimolecular fluorescence complementation (Ma *et al.*, 2009; Park *et al.*, 2009; Santiago *et al.*, 2009*b*). These lines of evidence add weight to the *in vitro* reconstitution assays and crystallographic studies described later in this review.

Manipulations of PYR/PYL/RCAR expression have confirmed their central role in ABA signal transduction and adaptation to abiotic stress. Since the *pyr1* mutant responds normally to ABA, it appeared possible that functional redundancy from other family members could mask PYR1's role in ABA signal transduction. Triple and quadruple mutants with genotypes *pyr1pyl1pyl4* and *pyr1pyl1pyl2pyl4*, respectively, showed markedly reduced ABA sensitivity in seed germination and seedling growth (Park *et al.*, 2009). The quadruple mutant also showed reduced sensitivity to ABA-mediated stomatal closure (Nishimura *et al.*, 2010) and ABA-mediated transcriptional responses (Park *et al.*, 2009). Transgenic over-expression of either *PYL1* or *PYL4* restored ABA sensitivity in the triple mutant (Park *et al.*, 2009). Suppression of *RCAR1* through RNA interference in protoplasts lowered ABA-induced transcriptional responses, while over-expression of *RCAR1* in stably transformed lines enhanced ABA responses at the level of germination, seedling growth and stomatal aperture (Ma *et al.*, 2009). Similarly, over-expression of *PYL5* and *PYL8* enhanced ABA responses and resistance to water-deficit stress (Santiago *et al.*, 2009*b*; Saavedra *et al.*, 2010).

Helix-grip fold receptors

The 14-member PYR/PYL/RCAR family is part of the ancient, ubiquitous Bet v 1-fold superfamily, named for a major allergen in pollen of white birch (*Betula verrucosa*) (Iyer *et al.*, 2001; Radauer *et al.*, 2008). A central feature of this superfamily is the presence of a seven-stranded β-sheet and two α-helices enfolding a long, carboxy-terminal α-helix, which collectively form a helix-grip fold structural motif. The helix-grip fold creates a large cavity that can bind hydrophobic ligands including lipids and hormones (Iyer *et al.*, 2001; Radauer *et al.*, 2008). Some members of the Bet v 1-fold superfamily have been shown to bind cytokinin and brassinosteroid hormones *in vitro* (Markovic-Housley *et al.*, 2003; Pasternak *et al.*, 2006; Fernandes *et al.*, 2008), so it is not surprising that PYR/PYL/RCAR proteins bind ABA, as described below. Other Bet v 1-fold members are known as pathogenesis-related proteins (class PR-10) that play roles in defence against microbes and in abiotic stress tolerance (Liu and Ekramoddoullah, 2006). Bet v 1-fold proteins have been classed together with the large, ubiquitous START (steroidogenic acute regulatory—StAR—related lipid transfer) domain superfamily (Ponting and Aravind, 1999; Iyer *et al.*, 2001). START domain-containing proteins have also been implicated in plant adaptation to both biotic and abiotic stressors (Yu *et al.*, 2008; Cao *et al.*, 2009; Fu *et al.*, 2009). However, in a recent phylogenetic analysis many Bet v 1-fold proteins were found to differ from START domain proteins with respect to the numbers and relative positions of β-strands and α-helices that create the helix-grip fold; some Bet v 1-fold proteins of plants, including PYR/PYL/RCAR members from *Arabidopsis*, were segregated into a subfamily of polyketide cyclase-like proteins (Radauer *et al.*, 2008).

PP2C inhibition linked to ABA-receptor binding

The significance of PYR/PYL/RCAR interactions with PP2Cs is supported by multiple lines of evidence that link the ABA-binding properties of these receptors with their suppression of phosphatase activity and the release of ABA signal transduction from PP2C-mediated inhibition. PYR/PYL/RCAR proteins strongly inhibit the phosphatase activity of PP2Cs *in vitro*, an effect that is ABA dose-dependent (Ma *et al.*, 2009; Park *et al.*, 2009; Santiago *et al.*, 2009*b*; Szostkiewicz *et al.*, 2010). Site-directed mutations identify critical residues for these interactions. For example, PYR1 protein containing either of two of the amino acid substitutions ($PYR1^{S152L}$ or $PYR1^{P88S}$) that gave rise to pyrabactin resistance *in planta* failed to inhibit HAB1 phosphatase activity, and displayed a reduced ABA-dependent interaction with HAB1 in Y2H tests (Park *et al.*, 2009). Conversely, the $ABI2^{G168D}$ mutation, which confers the dominant *abi2* phenotype, abolished the inhibitory effect of PYR1 on phosphatase activity (Park *et al.*, 2009), and also blocked the physical interaction between ABI2 and RCAR1 (Ma *et al.*, 2009). A similar result was observed with the interaction between RCAR1 and $ABI1^{G180D}$, the mutation causing the dominant *abi1* phenotype (Ma *et al.*, 2009). Likewise, the $HAB1^{G246D}$ mutation abolished its physical interaction with PYL5 *in vivo* (Santiago *et al.*, 2009*b*). Furthermore, this same study reported that over-expression of *PYL5* and *HAB1* in the same plant removed the ABA-insensitivity of *HAB1*-overexpressing plants, consistent with PYL5 antagonism of HAB1 phosphatase activity.

The dependence of PYR/PYL/RCAR proteins on ABA to inhibit PP2Cs is paralleled by observations that the binding of ABA to PYR/PYL/RCAR proteins is greatly enhanced by the presence of PP2Cs (Ma *et al.*, 2009; Santiago *et al.*, 2009*b*). Interestingly, the evidence for PP2C-mediated enhancement of ABA binding comes from

experiments in which a structural variant of ABA was compared with the natural form; these comparisons provide insights into the stereoselectivity of plant responses to the molecule. Synthetic ABA can exist as two stereoisomers, S-(+) and R-(–), although only the S-(+) form occurs naturally. In some experimental contexts, the non-natural R-(–) form has been shown to be bioactive (Lin *et al.*, 2005; Nambara *et al.*, 2002; Sondheim *et al.*, 1971; Walker-Simmons *et al.*, 1992). These and other studies, including transcriptional profiling in response to ABA structural analogues (Huang *et al.*, 2007), indicated that multiple ABA receptors with differing specificities may exist.

Strong inhibition of ABI1 and ABI2 phosphatase activities was observed with the addition of both RCAR1 and S-ABA while R-ABA, the non-natural form, was relatively ineffective at inhibition (Ma *et al.*, 2009). Similarly, Santiago *et al.* (2009*b*) measured an 8-fold stronger inhibition of HAB1 phosphatase activity by PYL5 in the presence of S-ABA, compared with the R form. Isothermal titration calorimetry experiments found that the apparent binding affinity of RCAR1 to S-ABA was enhanced approximately 10-fold by the addition of ABI2, from a K_D of 660 nM to 64 nM (Ma *et al.*, 2009). Santiago *et al.* (2009*b*) report a similar result with this same technique, observing an apparent K_D of 38 nM S-ABA for PYL5 in the presence of the catalytic core of HAB1 phosphatase. These results show that ABA binding to PYR/PYL/RCAR proteins is intimately tied to PP2C inhibition, raising the possibility that the two proteins could serve as co-receptors. Interestingly, Park *et al.* (2009) found differences in ABA-dependence and in stereoselectivity among PYR/PYL/RCAR family members with respect to interactions with PP2C proteins. In Y2H experiments, PYL2, PYL3, and PYL4 required the presence of either ABA stereoisomer for their interaction with HAB1 in yeast, while PYL5 through PYL12 (excluding PYL8) were shown to interact constitutively with HAB1. Twelve out of the 14 members (excluding PYL8 and PYL13) interacted with HAB1 in the presence of the natural S-ABA, whereas all these 12 except PYR1 and PYL1 could interact in the presence of the non-natural R-ABA.

Defining core components of ABA signalling

The discovery of PYR/PYL/RCAR proteins has coincided with recent insights on the regulation of ABA-responsive genes by serine-threonine kinases and PP2Cs, providing tools that define a minimal set of factors required for ABA signalling *in vitro*. Three members of a plant-specific kinase group, called subfamily 2 SNF1-related kinases (SnRK2s), play central roles in Arabidopsis as positive regulators in ABA signal transduction (Mustilli *et al.*, 2002; Yoshida *et al.*, 2002; Fujii *et al.*, 2007). This was demonstrated recently by the extreme ABA-insensitivity of the *snrk2.2/2.3/2.6* triple mutant (Fujii and Zhu, 2009; Fujita *et al.*, 2009). The phenotype of this mutant suggests that phosphorylation of transcription factors and other substrates by SnRK2s may be a general requirement for ABA function at all stages of plant development. Transcription factors that are activated through phosphorylation by SnRK2s, and thereby promote ABA signalling, include basic leucine zipper (bZIP) proteins called ABFs/AREBs (Furihata *et al.*, 2006; Johnson *et al.*, 2002).

A transfection assay of protoplasts of the *snrk2.2/2.3/2.6* triple mutant background was developed to test the roles of several factors involved in ABA signal transduction, including an ABA-dependent ABF (ABF2), SnRK2s, PP2Cs, and PYR/PYL/RCARs (Fujii *et al.*, 2009). In these protoplasts, the co-transfection of DNAs encoding ABF2 and any of the three SnRK2s induced the transient expression of a luciferase (*LUC*) reporter driven by the ABA-responsive *RD29B* promoter, in an ABA-dependent manner. *LUC* expression was blocked by the co-transfection of DNA encoding PP2C proteins ABI1 or HAB1, while the additional co-transfection with almost any of the PYR/PYL/RCAR members restored *RD29B-LUC* expression. In this same study, bimolecular fluorescence complementation analysis demonstrated physical interactions between ABI1 and SnRK2s *in vivo*, which were localized in the cytoplasm and in the nucleus of tobacco cells; these results were supported by Y2H experiments. *In vitro* kinase assays indicated that SnRK2.6 (also named OST1; Mustilli *et al.*, 2002) undergoes autophosphorylation and can phosphorylate an ABF2 fragment. In these assays, PP2Cs were shown to inhibit both SnRK2-mediated phosphorylation of ABF2 and SnRK2 auto-phosphorylation, effects that could be blocked by the addition of different PYR/PYL/RCAR proteins. Yeast three hybrid assays established that PYR/PYL/RCAR protein PYL8 or PYL5 can disrupt physical interactions between SnRK2.6 and any of the PP2C proteins ABI1, ABI2 or HAB1, in an ABA-dependent manner.

The isolation, reassembly and manipulation of these signalling components thus demonstrated their interactions *in vitro,* in yeast and in protoplasts, showing that this minimal set of factors can reconstitute an ABA signal transduction pathway. Collectively, these results point to a model of a signal–receptor complex, in which PYR/PYL/RCAR proteins bind to ABA and thereby disrupt the physical interaction between PP2Cs and their target SnRK2s. In this way, the target kinases are released from intermolecular inhibition and are able to phosphorylate transcription factors such as ABF2, leading to the activation of ABA-responsive gene expression (Fig. 1).

Receptor activity involved in a rapid ABA response

Several lines of evidence suggest that SnRK2s, in particular SnRK2.6, also directly regulate the most immediate responses to ABA, such as the production of reactive oxygen species (ROS) and the regulation of plasma membrane anion channels. SnRK2.6 has been shown to interact with and phosphorylate AtRBOHF (RESPIRATORY BURST OXIDASE HOMOLOG F), a NADPH oxidase involved in the ABA-triggered production of second messenger ROS (Kwak *et al.*, 2003; Sirichandra *et al.*, 2009).

SLAC1 (SLOW ANION CHANNEL-ASSOCIATED 1) is a recently identified S-type anion channel on the plasma membrane of guard cells; its anion efflux activity causes membrane depolarization leading to stomatal closure (Vahisalu *et al.*, 2008; Negi *et al.*, 2008). These studies reported that *Arabidopsis* mutants deficient in SLAC1 fail to close stomata in response to CO_2 and ABA. Geiger *et al.* (2009) recorded currents on whole *Xenopus* oocytes expressing SLAC1, SnRK2.6 and/or PP2Cs to show that S-anion channel activity, driven by SLAC1, could be activated only in the presence of SnRK2.6. Moreover, the co-expression of ABI1 or ABI2 inhibited the activation of SLAC1 by SnRK2.6. Furthermore, it was shown that SnRK2.6 can interact with SLAC1 and phosphorylate the N-terminal, cytoplasmic region of this channel protein. No phosphorylation could be detected when ABI1 protein was added to the reaction mixture, indicating that ABI1 inhibits SnRK2.6-mediated channel activation (Geiger *et al.*, 2009). Similar results were obtained with the protein phosphatase PP2CA in place of ABI1 (Lee *et al.*, 2009).

It is therefore conceivable that PYR/PYL/RCAR proteins control, through SnRK2.6 and related SnRK2s, not only the activation of gene expression but also the activity of proximate effectors of an ABA response. To test this hypothesis, the *in vitro* reconstitution assay developed by Fujii *et al.* (2009), was used but with the N-terminal fragment of SLAC1 (SLAC N) as a final substrate instead of ABF2 (Fig. 2). As expected from previous reports (Geiger *et al.*, 2009; Lee *et al.*, 2009), when SnRK2.6 was incubated with SLAC N the kinase was capable of phosphorylating the substrate. When SnRK2.6 was pretreated with ABI1, phosphorylation of the substrate was significantly reduced, and the addition of His-PYR1 in the absence of ABA could not restore SLAC N phosphorylation

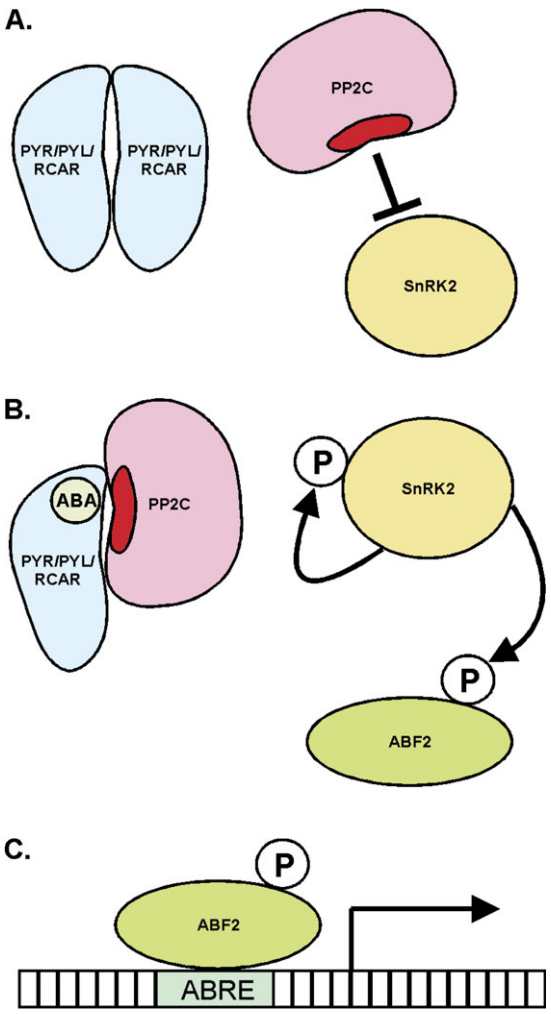

Fig. 1. Schematic view of the ABA signal–receptor complex, including a PYR/PYL/RCAR homodimer (A) and hormone-bound protomer (B); a PP2C phosphatase, with the active site indicated in dark red; a SnRK2 kinase; and the ABF2 transcription factor. In the absence of ABA (A), the receptor forms a homodimer, while the PP2C inhibits both autophosphorylation of the SnRK2 and phosphorylation of ABF2. In the presence of ABA (B), a receptor protomer engulfs the hormone within a pocket, allowing the receptor to bind the PP2C and cover the phosphatase active site. This permits the autophosphorylation of the SnRK2 and phosphorylation of its ABF2 substrate. In its phosphorylated, active state (C), ABF2 binds to an ABA-responsive element (ABRE) in the promoter of ABA-responsive genes, activating transcription.

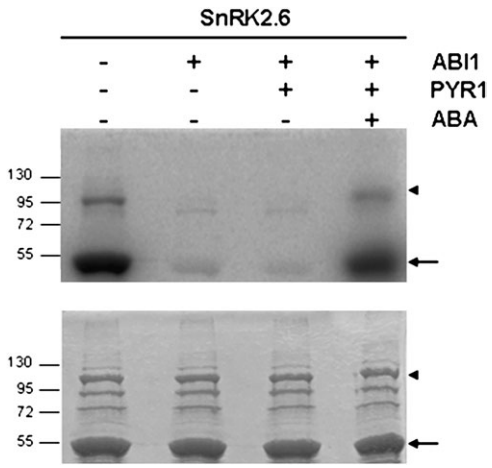

Fig. 2. Regulation of SLAC1 phosphorylation status by the ABA-dependent PYR1, ABI1, SnRK2.6 signalling cascade. GST-SLAC1 N-terminal fragment (SLAC N, Met1 to Phe188) was incubated in the presence of [γ-^{32}P]ATP with MBP-SnRK2.6 pretreated without (–) or with (+) GST-ABI1, His-PYR1, and 100 μM (±)-ABA. Bands of SLAC N fragment and MBP–SnRK2.6 are indicated by an arrow and an arrowhead, respectively. Coomassie-stained SDS-PAGE (bottom) and autoradiogram of the gel (top) are shown. Recombinant proteins and reaction conditions were as described previously (Fujii *et al.*, 2009; Park *et al.*, 2009). When SLAC N was incubated with SnRK2.6 not treated with GST-ABI1, both SnRK2.6 autophosphorylation and SLAC N phosphorylation bands were visualized (first lane). SnRK2.6 pre-treated with GST-ABI1 was unable to phosphorylate SLAC N (second lane). In the absence of ABA, the addition of His-PYR1 to the pretreatment of SnRK2.6 with GST-ABI could not restore SnRK2.6 phosphorylation activity on SLAC N (third lane). However, when ABA was added to the pretreatment reaction, SLAC N phosphorylation was recovered (fourth lane).

by SnRK2.6. However, the addition of 100 μM (±)-ABA to the pretreatment of SnRK2.6 with ABI1 and PYR1 could restore the ability of SnRK2.6 to phosphorylate SLAC N (Fig. 2). Our results suggest that the PYR/PYL/RCAR family of proteins controls not only the transcriptional response to ABA but also the transport activity of channels, thereby regulating the most immediate responses to stress signals mediated by ABA. Interestingly, a similar result was recently reported by Geiger *et al.* (2010), using RCAR1 (PYL9) instead of PYR1.

Crystal structures: pockets, gates, and latches

The discovery of PYR/PYL/RCAR proteins led to five crystallographic studies, published in a wave of reports in late 2009 (Melcher *et al.*, 2009; Miyazono *et al.*, 2009; Nishimura *et al.*, 2009; Santiago *et al.*, 2009*a*; Yin *et al.*, 2009). Collectively, these groups derived crystal structures for three proteins of the 14-member family (Table 1). These studies support the role of PP2Cs as intimate players, if not co-receptors, in ABA perception. All five reports confirm the importance of the helix-grip fold in ABA binding, as anticipated from previous bioinformatic analyses. This structure forms a large pocket that, in the absence of ABA, remains open to the solvent. Bound ABA is completely contained within the pocket, with the carboxylic acid portion of the hormone in contact with a Lys residue at the innermost region. Upon binding, the PYR/PYL/RCAR protein undergoes a conformational shift that covers the hormone from the solvent. In all proteins studied, the β-strands of the helix-grip fold, the three α-helices, and two loops joining β-strands β3–β4 and β5–β6 were deemed critical for the formation of the binding pocket and the structures covering bound ABA from the exterior. ABA is buried by the folding over of the two loops that connect the two pairs of β-strands. These loops consist of highly conserved residues that serve as 'lids' (Nishimura *et al.*, 2009; Miyazono *et al.*, 2009; Santiago *et al.*, 2009*a*; Yin *et al.*, 2009) or, more descriptively, as a 'gate' and 'latch' corresponding to the β3–β4 and β5–β6 loops, respectively (Melcher *et al.*, 2009; Fig. 1). The functional importance of residues within the binding pocket and within the gate and latch structures was confirmed by various *in vitro* experiments, and by transfecting *snrk2.2/2.3/2.6* triple mutant protoplasts with PYL2 containing site-directed mutations in these domains (Melcher *et al.*, 2009).

The folding of the β3–β4 loop forms a hydrophobic surface with which an individual PP2C (in these cases, ABI1 or HAB1) can interact. A highly conserved Trp residue in the PP2C protein inserts between the β3–β4 'gate' and the β5–β6 'latch' loops to interact, through a water-mediated H-bond, with the ketone group of ABA's cyclohexene ring, causing the PP2C protein to serve as a molecular 'lock' that stabilizes the closed position of the PYR/PYL/RCAR protein loops (Melcher *et al.*, 2009). This interaction is observed in four of the five structural studies, but is interpreted somewhat differently by different groups. Melcher *et al.* (2009) consider this interaction as evidence that PP2Cs serve as 'co-receptors' that sense binding of ABA with PYR/PYL/RCAR proteins, while Yin *et al.* (2009) consider the PYR/PYL/RCAR protein as the sole receptor and eschew the co-receptor terminology, presumably because the hormone is buried completely by PYR/PYL/RCAR residues and interacts only indirectly, through a water molecule, with the PP2C. The binding of a PP2C protein to the ABA-bound PYR/PYL/RCAR covers the active site of the phosphatase, which explains the mechanism by which the PYR/PYL/RCAR acts as a competitive inhibitor of PP2C activity.

Another finding reported in four of the five crystal structure papers (Melcher *et al.*, 2009; Nishimura *et al.*, 2009; Santiago *et al.*, 2009*a*; Yin *et al.*, 2009) is the formation of a homodimer between PYR/PYL/RCAR proteins in the absence of a PP2C protein. The homodimer interface encompasses the same region that participates in the interaction with PP2Cs (Fig. 1). The model arising from these observations is that the homodimer can exist with zero or one protomer binding ABA and, upon binding of an additional ABA molecule to the other protomer, the homodimer dissociates and allows the binding of a PP2C protein to an ABA-bound PYR/PYL/RCAR protein, forming a hormone–heterodimer complex.

Since all members of the PYR/PYL/RCAR family have highly conserved amino acid sequences in critical parts of the helix-grip fold structure, it appears likely that they generally share properties of ABA-binding and interaction with PP2Cs. One interesting exception may be PYL13, which has a Gln residue instead of the Lys common to all the other family members at a critical position. It is this Lys residue that contacts the hormone's carboxylic acid group at the pocket interior. Mutations that replace the Lys residue (K59 in PYR1; K64 in PYL2) eliminate ABA and PP2C binding to recombinant proteins (Melcher *et al.*, 2009; Nishimura *et al.*, 2009; Yin *et al.*, 2009), consistent with the lack of evidence for ABA or PP2C binding with PYL13. The relation between PYL13 and its other family members remains unclear. At least two other residues in PYL13 differ at important locations, compared with all other paralogues: the Leu residue within the SGLPA 'gate' is replaced with Asp; and Glu/Asp in the GG(E/D)HRL 'latch' is replaced with Asn. Minor structural differences among PYR/PYL/RCAR proteins, within the binding pocket and at other critical domains, are likely to play roles in the different strengths of interaction that have been observed between the receptors and ABA stereoisomers, and between the receptors and different PP2C proteins. If one considers the family diversity of the PYR/PYL/RCARs, the PP2Cs and the SnRK2s, it is clear that the ABA signal–receptor complex could be subjected to a highly sophisticated level of combinatorial control. This diversity of structure might enable the functional plasticity that plants require throughout their development, and across the environmental conditions they encounter.

Ma *et al.* (2009) grouped the PYR/PYL/RCAR family into three subfamilies, based on amino acid sequence

similarity. It is presently unclear whether these groupings relate to any functional specialization. Gene expression patterns vary among family members, according to the Arabidopsis eFP Brower (Winter *et al.*, 2007; http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi), but these patterns do not appear to correlate, in any obvious way, with phylogenetic groupings within the family, nor do they correlate with the ABA-dependency of their interactions with PP2C proteins in yeast (Park *et al.*, 2009). It remains to be seen whether patterns can be identified for the differences in properties among individual family members—at the level of ABA binding affinity, interaction with other components of the ABA signal–receptor complex(s), transcriptional control, protein turnover, subcellular localization, or cell-type specificity. It is possible that some of the PYR/PYL/RCARs bind to ABA biosynthetic intermediates and/or ABA metabolites.

Comparisons with receptors for other hormones

The discovery of ABA receptors coincides with other exciting developments in the field of phytohormone perception. Protein receptors for other phytohormones have been identified in recent years (Santner and Estelle, 2009), and interesting similarities and differences can be seen between these and the PYR/PYL/RCAR proteins that perceive ABA. The binding pocket and the gate–latch–lock structures of the PYR/PYL/RCAR-PP2C complex bear some resemblance to the binding features the gibberellin (GA) receptors GID1A and GID1 (gibberellin insensitive dwarf 1) of *Arabidopsis* and rice (*Oryza sativa*), respectively (Murase *et al.*, 2008; Shimada *et al.*, 2008). In both studies, the hydrophilic carboxylate portion of both the GA_3 and the GA_4 structural variants was shown to be directed at the inner, or bottom, part of the binding pocket of either receptor, while the hydrophobic, aliphatic rings are positioned near the pocket entrance. An N-terminal extension of GID1A (Murase *et al.*, 2008) or GID1 (Shimada *et al.*, 2008), which in each case contains α-helices, serves as a 'lid' that encloses the GA molecule when it enters the receptor pocket. In both studies, this movement of the lid appeared to alter the surface of the receptor, allowing it to interact with the Arabidopsis DELLA protein GAI (Murase *et al.*, 2008), or with the rice DELLA homologue SLR1 (Shimada *et al.*, 2008), which are negative regulators of GA hormone signalling (Peng *et al.*, 1997; Ueguchi-Tanaka *et al.*, 2007). These negative regulators are, therefore, analogous to the PP2Cs that inhibit ABA signalling. As with the PP2C proteins, the DELLA protein in the *Arabidopsis* GA-GID1A–GAI complex does not directly contact the receptor-bound hormone (Murase *et al.*, 2008). Whether an analogous situation exists in rice was not explicitly reported by Shimada *et al.* (2008).

Aside from these similarities with GA receptors, in other respects, the PYR/PYL/RCAR receptor model of competitive inhibition of an inhibitor is unique. For example, this model diverges from a common theme for other hormone receptors, wherein hormone binding triggers the ubiquitination and proteolysis—via the 26S proteasome—of negative regulators of the hormones' signal transduction pathways. This is the case for the GA-sensing GID1 family proteins that promote the proteolysis of DELLA transcriptional repressors (Griffiths *et al.*, 2006), the auxin-sensing TIR1 and AFBs, F-box proteins that promotes the proteolysis of Aux/IAA transcriptional repressors (Dharmasiri *et al.*, 2005), and the jasmonate-sensing COI1, another F-box protein that promotes the proteolysis of JAZ transcriptional repressors (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2009). Despite this distinction, ABA sensitivity in plants is known to be regulated by the 26S proteasome (Stone *et al.*, 2006; Zhang *et al.*, 2005). It is likely that one or more proteins of the ABA receptor–signal complex and its downstream targets are regulated by polyubiquitination and proteolysis.

Other putative ABA receptors

Lines of evidence for different sites of ABA perception, at the plasma membrane and within the cytoplasm, have been reported (Hamilton *et al.*, 2000; Levchenko *et al.*, 2005). Two separate groups have published reports of *Arabidopsis* proteins, localized at the cell periphery, that bind to ABA and meet other criteria consistent with roles as ABA receptors. The first report (Liu *et al.*, 2007) identified a G-protein-coupled receptor (GPCR) homologue, GCR2, as a protein that interacts with GPA1. GPA1 is the sole, canonical G-protein α subunit encoded by the *Arabidopsis* genome (Jones and Assmann, 2004). Liu *et al.* (2007) reported that this association was disrupted specifically by the naturally occurring S-ABA, and that the binding properties of the hormone to GCR2 suggested a single binding site, with a K_D (dissociation constant) value of 20.1 nM, consistent with a supposed physiological range for ABA. The authors presented a model in which the binding of ABA to GCR2 disrupts the Gαβγ heterotrimeric complex, leading to the activation of downstream ABA effectors. Consistent with this model, they reported ABA-related phenotypes for loss-of-function and overexpression of *GCR2*, at the level of seed germination, seedling growth, and stomatal aperture.

However, GCR2 has been controversial with respect to the reproducibility of ABA-related phenotypes (Gao *et al.*, 2007; Guo *et al.*, 2008), the unexpected ABA hyposensitivity of a GPCR mutant, based on previous studies of G-protein signalling during ABA responses (Chen *et al.*, 2008), ABA binding properties (Risk *et al.*, 2009), and, on the grounds that GCR2 lacks the prototypical seven transmembrane domains of GPCRs, and is, instead, homologous to mammalian lanthionine synthetase C (LanC)-like proteins (Johnston *et al.*, 2007; Illingworth *et al.*, 2008). Moreover, the apparent ease with which recombinant GCR2 was solubilized for *in vitro* analysis was one basis for questioning its identity as a transmembrane protein (Chen,

2008). Interestingly, the human LanC-like protein LANCL2 was recently shown to be required for ABA binding to the membranes of human granulocytes and rat insulinoma cells, where endogenous ABA is implicated in the control of inflammatory and diabetic responses (Sturla *et al.*, 2009). Nevertheless, no published reports to date have independently reproduced the results of Liu *et al.* (2007) in *Arabidopsis*, nor in any other plant species.

In a more recent study (Pandey *et al.*, 2009), a bioinformatic analysis of the *Arabidopsis* genome led the authors to focus on a pair of genes encoding unusual products with predicted features of both GPCRs and G-proteins. These authors reported that the *G*PCR-*t*ype *G* proteins GTG1 and GTG2 have nine predicted transmembrane domains, are localized at the plasma membrane, have specific GTP-binding and GTPase activities that are altered through interactions with GPA1, and can both bind specifically to the natural S-ABA stereoisomer in a saturable manner with K_D values of approximately 20 nM, similar to the value reported for GCR2 by Liu *et al.* (2007). Pandey *et al.* (2009) also reported that an ABA hyposensitive phenotype in seed germination and seedling growth required a *gtg1gtg2* double mutant, which could be complemented by introducing either of the wild-type genes, suggesting redundancy of the GTG function. The double mutant was also hyposensitive to ABA with respect to stomatal closure, although it displayed a normal phenotype with respect to ABA inhibition of stomatal opening. The model presented by Pandey *et al.* (2009) portrays an unusual type of G-protein signalling, in which the GTP-bound Gα protein 'turns off' signalling while the GDP-bound form allows ABA binding to the GTG receptor and the initiation of a signalling cascade. Curiously, this mode of guanine nucleotide regulation is the opposite of the conventional model for signalling by G-proteins. These authors were unable to show evidence for at least one binding site per GTG protein molecule, a result they attributed to non-optimal conditions for isolating pure and intact proteins for binding assays.

Another putative ABA receptor protein in *Arabidopsis* is CHLH (Mg-chelatase H subunit), a chloroplast protein involved in chlorophyll biosynthesis that has also been termed ABAR, for ABA receptor (Shen *et al.*, 2006; Wu *et al.*, 2009). This protein's role in the cell had previously been shown to extend beyond chlorophyll biosynthesis, since CHLH is the same as GUN5 (GENOMES UNCOUPLED 5), a regulator of plastid-to-nucleus retrograde signalling (Mochizuki *et al.*, 2001). ABA binding by CHLH has been demonstrated by more than one technique, with K_D values in the order of 30–40 nM (Shen *et al.*, 2006; Wu *et al.*, 2009). Experiments in which CHLH levels were modulated, through over-expression, RNAi, or through insertional and point mutations, showed ABA-related phenotypes at the levels of seed germination, seedling growth, and stomatal movements (Shen *et al.*, 2006; Wu *et al.*, 2009). Binding experiments with potential agonists and antagonists of the hormone showed that the affinity of CHLH is specific to S-ABA (Wu *et al.*, 2009). The chloroplast is the site of initial steps in ABA biosynthesis, and it is possible that one branch of ABA perception and signal transduction is localized within this source organelle. Like GCR2, GUN5/CHLH has been controversial, in this case because a barley homologue failed to bind ABA or show ABA-related phenotypes when mutated (Muller and Hansson, 2009). Moreover, a major unanswered question, acknowledged by Wu *et al.* (2009), is the mechanism by which CHLH—a wholly unexpected type of receptor for ABA—transmits a signal upon ABA binding.

A critical difference between the reports of these putative ABA receptors—GCR2, GTG1/GTG2, CHLH—and those of the PYR/PYL/RCAR family is that the evidence for the former candidates has not been independently corroborated. Jones and Sussman (2009) proposed the application of strict biochemical criteria when evaluating the candidacy of a protein as a hormone receptor. It may be difficult for one or even two publications by a single research group to meet all of the desirable criteria these authors list. Perhaps the strongest case for the PYR/PYL/RCAR family is the sheer volume of data, comprising multiple lines of evidence, that has emerged from multiple groups in rapid succession. The crystallographic studies, the site-specific mutations, and the integration of PYR/PYL/RCAR function with other components of ABA signalling, through ligand binding and *in vitro* reconstitution assays, are the types of evidence that may need to accumulate for other candidate ABA receptors before they are widely accepted by the scientific community.

Applications in cultivated species

The challenges of genetic redundancy and multiple levels of regulation have previously hindered progress in understanding ABA perception mechanisms. Although a great number of questions about the integration of signals remain unanswered, the discovery of PYR/PYL/RCAR proteins has created a major front of progress in the elucidation of ABA function in higher plants. The complexity of this family, and of the families encoding its interacting proteins, point to a high capacity for combinatorial control by the plant to effect fine-tuning of ABA signal transduction in response to developmental and environmental cues. The great diversity among plant species in their environmental adaptations may result, to some degree, from the diversity of ABA receptor–signal complexes. Considering the abundant evidence of ABA-mediated cross-talk between biotic and abiotic stress responses (Fujita *et al.*, 2006) it is intriguing that the Bet v 1-fold superfamily contains proteins involved in pathogen defence (e.g. PR-10) as well as ABA receptors. Future studies of this receptor family may clarify the connections between ABA-mediated responses to abiotic and biotic stressors, and lead to progress in both areas of plant stress research.

The PYR/PYL/RCAR family is well-conserved in crop species (Fig. 3), so it is likely that the modulation of these

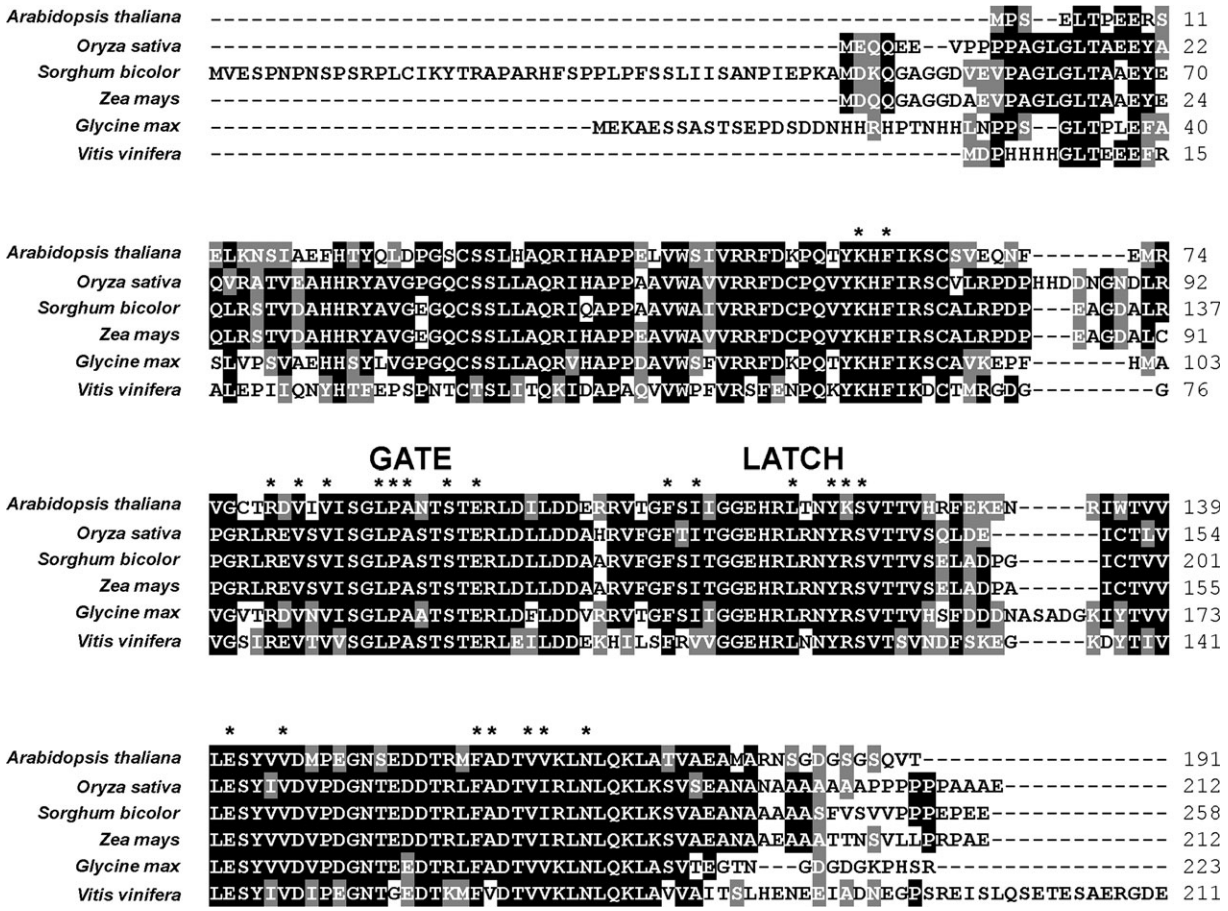

Fig. 3. Amino acid sequence alignment of the *Arabidopsis* PYR1 protein with the most similar homologues in five cultivated species. The alignment was performed with the CLUSTALW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) using the default settings. Asterisks indicate residues in contact with ABA hormone, according to the PYL2 crystal structure of Melcher *et al.* (2009). The locations of the gate and latch domains are indicated.

receptors and their interacting partners will enable new methods of enhancing crop tolerance to multiple types of stress. Research on these ABA receptors is already underway in non-model plant species. For example, a PP2C homologue from the beechnut tree (*Fagus sylvatica* L.) was shown to interact, in an ABA-dependent manner, with *Arabidopsis* PYR7 and PYR8, based on Y2H assays and bimolecular fluorescence complementation experiments in tobacco cells (Saavedra *et al.*, 2010). The available genome sequences of several crops indicate levels of family diversity for the PYR/PYL/RCAR proteins similar to that of *Arabidopsis* (Table 2). The discovery of pyrabactin, a selective ABA agonist of PYR/PYL/RCAR proteins (Park *et al.*, 2009), points to a potential chemical strategy for modulating ABA receptor activity in crops on a commercial scale (Cutler *et al.*, 2010). Genetic methods of manipulating ABA perception, through conventional breeding or transgenic approaches, may also provide greater control of the hormone's function in the field. In conclusion, it appears likely that the ABA signal transduction model, as developed in *Arabidopsis*, will continue to create and facilitate new approaches for enhancing stress tolerance in crops.

Table 2. Numbers of genes homologous to PYR1 protein in seven cultivated species

Genes were identified by the BLAST algorithm with PYR1 protein as query, using the BLOSUM62 matrix and a word length equal to three.

Species	Number of genes	Range of *E*-values	Highest % ID with PYR1
Glycine max	23	–74 to –34	70.2
Zea may	20	–56 to –37	61.5
Populus trichocarpa	14	–78 to –37	75.1
Oryza sativa	11	–57 to –33	58.7
Vitis vinifera	8	–75 to –39	54.3
Sorghum bicolor	8	–55 to –38	62.0
Medicago truncatula	6	–51 to –39	54.1

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