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The structure of ligand-bound intermediates of crop ABA receptors highlights the role of the PP2C as necessary ABA co-receptor

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Short title: Insight into ABA signaling in crops

Dear Editor,

Abscisic acid (ABA) is the main phytohormone involved in the adaptive crop responses to drought. ABA signaling relies on the family of pyrabactin resistance 1/PYR1-like/regulatory components of ABA receptors (PYR/PYL/RCAR) receptors, which upon ABA binding form high-affinity ternary complexes with clade A protein phosphatases type 2C (PP2Cs) and inhibit them (Cutler et al., 2010). It results in the activation of a SnRK2-dependent phosphorylation cascade affecting a high number of targets in the plant cell, regulating stomatal aperture and stress-responsive gene expression (Cutler et al., 2010).

Our current understanding on the structural mechanism of ABA signaling relies exclusively on crystallographic analyses performed with *Arabidopsis thaliana* ABA receptors (reviewed by Santiago et al., 2012). Although mechanistic insights obtained in *Arabidopsis* are usually translated to other plant species, studies in crops might overturn or update the dogma established in *Arabidopsis*. As a result from structural studies performed with *Citrus sinensis* (sweet orange) and *Solanum lycopersicum* (tomato) ABA receptors, we have identified a novel latch-closed gate-open ABA-bound intermediate that provides novel mechanistic insight on ABA signaling. We propose an updated model that highlights the role of the PP2C as necessary co-receptor to increase the ABA binding affinity by facilitating the interconversion among the different conformations adopted by the receptor toward the formation of the Receptor-ABA-PP2C complex.

To understand the structural basis of ABA recognition by ABA receptors in crop species, we have solved the crystal structures of the apo form of CsPYL1 and SlPYL1, their binary complexes with ABA and the ternary complex CsPYL1-ABA-AtHAB1ΔN (amino acids 179-511). (Figure 1, Supplemental Figures 1 and 2, Supplemental Table 1 and Supplemental Methods). The comparison of the apo forms and the ternary complex reveals the well reported conformational changes of the latch and gate loops surrounding the ABA binding pocket that

are required for productive PP2C binding (Figure 1A and 1C) (Melcher et al., 2009; Yin et al., 2009). ABA binding in the presence of the PP2C induces a structural reorganization of these loops that generates a solvent accessible surface complementary to the PP2C active site. Intriguingly, unlike other *Arabidopsis* ABA-bound receptor complexes that display identical closed conformation in the binary ABA-receptor form and in the ternary complex with PP2C (Melcher et al., 2009), the crystal structures of the ABA-bound CsPYL1 and SlPYL1 complexes show a closed conformation of the latch while the gate displays a nonproductive open conformation incompatible with PP2C binding (Figure 1C and Supplemental Figure 3).

In addition to these structural features, the binding of ABA in the binary complex does not induce the intermonomer swiveling that is reported to favor dissociation of homodimeric receptors by a reduction of the dimeric interface buried area (Zhang et al., 2012) (Supplemental Figure 4). The fact that the apo and ABA-bound forms of SlPYL1 and CsPYL1 display largely identical structures, although they were crystallized in different space groups with different crystal packing contacts, indicates that their conformations and oligomeric state are unlikely to be affected by crystal packing (Table S1). To provide an additional biophysical support to this statement, we have characterized the oligomeric state of SlPYL1 in solution in absence of ABA and under ABA saturating conditions (0.2mM ABA). Using dynamic light scattering, we monitored the hydration radius (Rh) of SlPYL1 and AtPYL1 (both known to be dimers in the apo form) and compared them with those of the monomeric AtPYL6 (Ma et al. 2009; Dupeux et al. 2011). Our data show that, in absence of ABA, SlPYL1 and AtPYL1 display an Rh of 3.6nm and AtPYL6 2.6nm (Supplemental Figure 5). ABA addition induces a reduction of the Rh of AtPYL1 to 2.9nm while the Rh on SlPYL1 remains constant. This indicates that while ABA binding promotes the dissociation of AtPYL1, the dimeric nature of SlPYL1 remains unaltered despite ABA binds into the pocket as it is observed in the crystal structures of SlPYL1 and CsPYL1 (Supplemental Figure 5). Hence, all these data suggest that the crystallization of the ABA bound SlPYL1 and CsPYL1 has selected a stable intermediate between the apo and the ternary receptor complex with ABA and PP2C.

The open conformation of the latch is incompetent for ABA binding

The isolation of a gate-open latch-closed ABA-bound intermediate makes it possible a deeper structural analysis of how receptors interconvert between different folded states to develop their function. Previous structural analyses assumed that ABA binding, gate and latch closure and dimer dissociation were coupled events (Melcher et al., 2009; Weiner et al., 2010). However, our crystallographic data clearly show that closure of the latch and the gate are independent events that could be studied separately.

To investigate the bases of the latch closure, we compared the apo and ABA-bound SlPYL1 structures. The analysis highlights the role of the conserved Glu/Asp-His-Arg motif at the latch, as it changes drastically its conformation (Figure 1D). In particular, the transition from the open to the closed structure involves the coordinated movement of the Glu150 and Arg152 pair to make the binding site accessible and the His151 reorientation to define the ABA binding pocket. This means that the so-called open and closed conformations of the latch (Melcher et al., 2009) should not be referred to as accessibility of the ABA binding site, but rather open is an incompetent conformation for ABA binding and closed is the conformation of the latch in the ternary complex, competent for ABA and PP2C binding. The analysis also shows that the His151 side chain should move to a closed conformation before ABA enters into the pocket, otherwise, the hormone would hinder the structural rearrangement required for receptor activation (Figure 1D). To explore further whether these rearrangements precede or follow the ABA binding step, we analyzed the crystal structures of the apo forms of the ABA receptors available in the literature (Figure 2E and Supplemental Methods). These data show that while the *Arabidopsis* structures of PYR1, PYL1 and PYL2 display a variable latch-open conformation incompetent for ABA binding, the structures of PYL3, PYL5 and PYL10 adopt a latch-closed conformation competent for ABA binding. This indicates that ABA receptors are in an equilibrium state between the latch-open and -closed in the absence of ABA in the pocket and consequently, the hormone would either induce the shift of this equilibrium to the closed form or

will selectively bind to the closed conformation (Agafonov et al., 2015; Vogt et al., 2014) (Figure 1I).

Proposed mechanism for ABA perception in crop receptors

Once ABA is in the binding pocket, the receptor should undergo a structural rearrangement leading to gate closing for a successful PP2C interaction. However, the analyses of the crystal structures of the binary complexes of CsPYL1 and SlPYL1 with ABA show that this is not always the case, rather it indicates that ABA receptors are able to adopt several stable conformations including a gate-open latch-closed or a gate-closed latch-closed. To understand the bases of this behavior, we compared the gate loop structures of CsPYL1 and SlPYL1 receptor in complex with ABA with that of the active form of CsPYL1 in complex with the PP2C. This analysis shows that the conserved Pro117/124 (for orange/tomato, respectively) undergoes a cis to trans isomerization upon gate closure (Figure 1F and 1G). Proline cis to trans isomerization has been reported to occur from the apo to the ABA bound forms of AtPYR1 (Nishimura et al., 2009) and AtPYL1 (Melcher et al., 2009). This transition occurs in the slow microsecond-to-milisecond time scales and constitutes a rate determining step in protein folding (Wedemeyer et al., 2002). Indeed, NMR-HSQC experiments suggest that, under ABA saturated conditions, the PYR1 receptor displays equilibrium between the open and closed forms of the gate within these time scale (Peterson et al., 2010). Hence, this suggests that this equilibrium has been shifted to the open conformation in the ABA-bound SlPYL1 and CsPYL1 crystal structures (Figure 1I).

Despite the ABA-bound forms of CsPYL1 and SlPYL1 are not compatible with PP2C binding, it has been shown that these receptors are able to interact and inhibit PP2C activity under physiological conditions (Arbona et al., 2017; Gonzalez-Guzman et al., 2014). Moreover, we provide structural data characterizing this interaction (Figure 1C) and biochemical data showing that CsPYL1 and SlPYL1 are able to interact and inhibit PP2C activity with similar IC_{50} values of those measured for AtPYL1 (Supplemental Figure 6A). This highlights the role of the PP2C as ABA co-receptor by selecting the gate-closed

latch-closed receptor form. The phosphatase will solely interact with the ABA-bound gate-closed form of the receptor stabilizing its conformation by the insertion of the gate loop into the PP2C active site and the formation of a water-mediated conserved network of hydrogen bonds that links the carbonyl of Arg 145/152 at the latch, the carbonyl of Pro 117/124 at the gate, the ketone group of ABA and the side chain of Trp385 from AtHAB1ΔN (Figure 1H). Moreover, the HAB1^{W385A} phoshatase was markedly refractory to inhibition by either Arabidopsis or crop receptors (Dupeux et al., 2011a) (Supplemental Figure 6B-C), highlighting the crucial role played by the hydrogen bond network that connects the PP2C with both ABA and the gate/latch loops of the receptor. To provide additional biochemical evidence for the role of PP2C as co-receptor we have quantified the ABA binding properties of SlPYL1 in presence or absence of phosphatase using a fluorescence emission assay (Supplemental Figure 7). According to our data, the PP2C can be considered as co-receptor in the ternary complex as the equimolar presence of phosphatase and SlPYL1 results in an apparent ABA Kd (dissociation constant) of 158 ± 60 nM whereas the ABA Kd for SlPYL1 in absence of phosphatase is approximately three orders of magnitude higher (90± 10 μM). Thus, the receptor-ABA-phosphatase complex behaves as a high-affinity system for ABA binding able to detect physiological changes in ABA concentration whereas the dimeric receptor alone could not perform a biologically relevant function. Therefore, both biochemical and structural evidences support the role of the PP2C as ABA co-receptor. In our model the PP2C has a proactive role by shifting the gate-open gate-closed equilibrium of the binary complex toward the formation of the ternary complex (Figure 1I). This provides an updated model to explain why is the receptor-ABA-phosphatase complex which behaves as a high-affinity sensor for ABA binding rather than the receptor itself (Ma et al., 2009; Santiago et al., 2009). Altogether, our data suggest that ABA perception conveys three steps (Figure 1I). First, the physical binding of the hormone to a latch-closed competent state; second, a conformational transition to activate the receptor and third, the binding of PP2C to the receptor in the latch and gate closed conformation.

As it has been observed for the *Arabidopsis* dimeric receptors (Dupeux et al., 2011b), our structural data show that the dimerization interface of CsPYL1 and SlPYL1 overlaps with that formed between CsPYL1 and the PP2C (Figure 1). Therefore, for PP2C binding of dimeric receptors, the ABA perception mechanism would include an additional dissociation step between the gate-open ABA-bound state and the latch and gate closed state (Supplemental Figure 4).

Accession Numbers

The coordinates and structure factors of the apo forms of CsPYL1 and SlPYL1, the ABA bound CsPYL1 and SlPYL1 and the ternary complex AtHAB1ΔN have been deposited in the Protein Data Bank under accession codes 5MMQ, 5MOB, 5MN0, 5MOA and 5MMX respectively.

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AUTHOR CONTRIBUTIONS

M.M.-A., C.Y., L.I., M.M.-R. and A. A. purified proteins, performed crystallography and analyzed the crystallographic data; J.L.B, M.M., M.G.-G., V.A., A.G.-C. and J.L.-J performed biochemical analyses; P.L.R and A.A analyzed the data, conceived experiments and wrote the paper.

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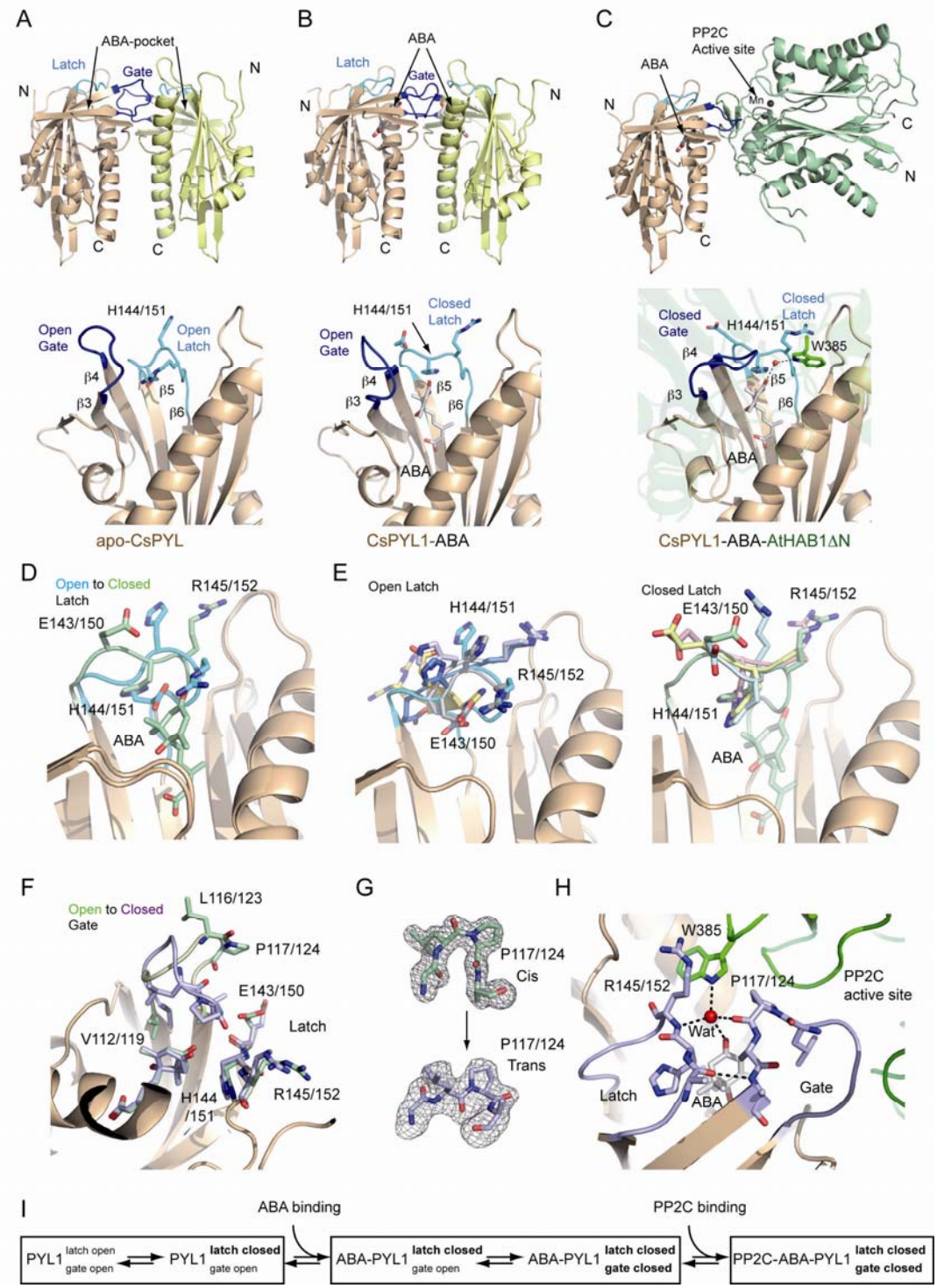

Figure 1. An updated model for ABA perception that highlights the role of the PP2C as necessary co-receptor. Structures of the apo- and ABA-bound receptors as well as ternary receptor-ABA-PP2C complex. Ribbon representations of the overall structure together with a section of the ABA binding site of (A) the apo form of CsPYL1,(B) the complex of CsPYL1 with ABA and (C) the ternary complex CsPYL1-ABA-AtHAB1ΔN. The CsPYL1 protomers are colored wheat and yellow and AtHAB1ΔN

in green. The gate and the latch loops are highlighted in blue and cyan colors respectively. The two Mn^{2+} ions at the phosphatase catalytic site are depicted as gray spheres. The ABA molecule is depicted in stick mode. Relevant secondary structural elements are labeled. (D) Overlaid of the ABA binding pocket of the apo (cyan, latch open) and ABA bound (pale green, latch closed) forms of SlPYL1. The residues of the Glu/Asp-His-Arg motif of the latch and the ABA molecule are highlighted as sticks. We selected the SlPYL1 structures for the analysis because both were solved to similar high resolution but similar conclusions could be obtained from CsPYL1 structures (E) (Left) Overlaid of the apo forms of AtPYR1 (yellow, 3K3K), AtPYL1 (grey, 3KAY), AtPYL2 (light blue, 3KAZ) and AtPYL2 (dark blue, 3KL1) onto apo-SlPYL1 (cyan) and (Right) the apo forms of PYL3 (yellow, 3KLX), PYL5 (pink, 4JDL) and PYL10 (blue, 3UQH) onto ABA-SlPYL1 (green). The PDB codes are indicated in brackets. (F) A detail comparing the conformation of the gate and latch loops of SlPYL1 in complex with ABA (pale green) and in complex with ABA and PP2C (light blue). (G) A section of the simulated annealed omit Fo-Fc maps contoured at 2.5 σ on the area of the gate loop of SlPYL1 in complex with ABA (pale green) and in complex with ABA and PP2C (light blue). Labels correspond to the CsPYL1/SlPYL1 receptor respectively. (H) A section of the CsPYL1-ABA-PP2C complex showing the water mediated hydrogen bond network linking ABA, latch, gate and PP2C. (I) The biochemical scheme for ABA perception in three steps that explains the available structural and biochemical data. The presence of PP2C drives the coupled equilibrium to the formation of the ternary complexes and increases the overall ABA binding affinity.