

1 **Members of the abscisic acid co-receptor PP2C protein family mediate salicylic acid-**
2 **abscisic acid crosstalk**

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21 **Abstract**

22 The interplay between abscisic acid (ABA) and salicylic acid (SA) influences plant responses to
23 various (a)biotic stresses; however, the underlying mechanism(s) for this crosstalk is largely
24 unknown. Here we report that type 2C protein phosphatases (PP2Cs), some of which are negative
25 regulators of ABA signaling, bind SA. SA binding suppressed the ABA-enhanced interaction
26 between these PP2Cs and various ABA receptors belonging to the PYR/PYL/RCAR protein
27 family. Additionally, SA suppressed ABA-enhanced degradation of PP2Cs and ABA-induced
28 stabilization of SnRK2s. Supporting SA's role as a negative regulator of ABA signaling,
29 exogenous SA suppressed ABA-induced gene expression, whereas SA-deficient *sid2-1* mutants
30 displayed heightened PP2C degradation and hypersensitivity to ABA-induced suppression of seed
31 germination. Together, these results suggest a new molecular mechanism through which SA
32 antagonizes ABA signaling. A better understanding of the crosstalk between these hormones is
33 important for improving the sustainability of agriculture in the face of climate change.

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37 **Introduction**

38 Elaborate hormone signaling networks allow plants to perceive and respond adaptively to various
39 biotic and abiotic stresses (Tuteja, 2007; Raghavendra *et al.*, 2010). One of the vital hormones that
40 plays a central role in the adaptation to abiotic stresses, particularly drought and salt stresses, is
41 ABA. In addition, ABA is involved in regulating plant growth and developmental processes under
42 non-stress conditions (Raghavendra *et al.*, 2010) and modulating defense responses following
43 pathogen attack (Robert-Seilaniantz *et al.*, 2011; Denance *et al.*, 2013). Because of its essential
44 role in multiple physiological processes both under stressed and non-stressed conditions, the ABA
45 signaling pathway has been studied intensively during the last two decades. Initial attempts to
46 identify ABA receptors were met with controversy and frustration. Several proteins were proposed
47 to be ABA receptors, but their exact role in ABA response and their associated mechanisms were
48 never established (Hauser *et al.*, 2011).

49 The discovery that members of the pyrabactin resistance 1/PYR1-like/regulatory
50 component of ABA receptor (PYR/PYL/RCAR) protein family are ABA receptors, and that they
51 interact with members of the type 2C protein phosphatase (PP2C) protein subfamily, was a major
52 breakthrough in dissecting the ABA signaling pathway (Fujii *et al.*, 2009; Ma *et al.*, 2009;
53 Miyazono *et al.*, 2009; Park *et al.*, 2009; Santiago *et al.*, 2009; Soon *et al.*, 2012). In the absence
54 of ABA, PP2Cs are able to bind and dephosphorylate members of the sucrose non-fermenting 1-
55 related subfamily 2 protein kinase (SnRK2) family. This negatively regulates ABA signaling
56 because autophosphorylation is required for SnRK2 kinase activity, and thus their ability to
57 transduce the ABA signal by phosphorylating downstream targets. In the presence of ABA, the
58 ABA-receptor complex tightly binds to PP2Cs, thereby preventing PP2C-mediated
59 dephosphorylation of SnRK2. This, in turn, allows activated SnRK2s to relay the ABA signal.

60 The reversible phosphorylation of proteins by protein kinases and phosphatases is an
61 important mechanism for regulating many biological processes. In contrast to eukaryotic protein
62 kinases, whose primary and three-dimensional structures are very similar, protein phosphatases
63 are diverse. Depending on their substrate specificity, protein phosphatases can be divided into two
64 classes, serine/threonine (Ser/Thr) or tyrosine phosphatases (Schweighofer *et al.*, 2004; Fuchs *et al.*,
65 2013; Singh *et al.*, 2015). The Ser/Thr phosphatases have been further organized into the
66 phosphoprotein phosphatase (PPP) and metal-dependent protein phosphatase (PPM) families. In

67 plants, PP2Cs, which belong to the PPM family, represent a major portion of the phosphatase-
68 encoding gene family. To date, 80 or more genes have been identified in the Arabidopsis, tomato,
69 rice and hot pepper genomes. Phylogenetic analyses have further divided the PP2C families from
70 these plant species into ten or more subclades designated alphabetically from A onward (Fuchs *et al.*
71 *al.*, 2013; Singh *et al.*, 2015).

72 Of the PP2C subclades, members of “clade A” have been studied the most extensively, as
73 they negatively regulate ABA signaling in various plant species. In Arabidopsis, clade A proteins
74 such as ABA-insensitive 1 (ABI1), ABI2, Hypersensitive to ABA 1 (HAB1), and PP2CA/AHG3,
75 have been shown to mediate ABA-induced responses to abiotic and biotic stresses via their
76 interaction with SnRK2s and PYR/PYL/RCARs (de Torres-Zabala *et al.*, 2007; Fujii *et al.*, 2009;
77 Santiago *et al.*, 2012; Soon *et al.*, 2012; Lim *et al.*, 2014). Functional studies of PP2C proteins
78 from other clades are limited, but they suggest that some of these proteins are involved in
79 responding to (a)biotic stresses. For instance, the clade B member AP2C1 (*Arabidopsis*
80 phosphatase 2C1) and its ortholog MP2C from *Medicago sativa* regulate the activity of stress-
81 induced mitogen-activated protein kinases (MAPKs; (Meskiene *et al.*, 2003; Schweighofer *et al.*,
82 2004), and the clade F member PIA1 (PP2C induced by AvrRpm1) regulates immune responses
83 in Arabidopsis (Widjaja *et al.*, 2010). By contrast, clades C and D contain PP2Cs that regulate
84 developmental processes (Schweighofer *et al.*, 2004; Fuchs *et al.*, 2013; Singh *et al.*, 2015).
85 Members of clade C, including POL (Poltergeist) and PLL (POL-like), control shoot and root
86 meristem formation and embryo formation (Song & Clark, 2005), whereas members of clade D
87 negatively regulate the activity of plasma membrane H⁺-ATPases, and thus cell expansion in the
88 absence of auxin (Spartz *et al.*, 2014).

89 SA is another important plant hormone involved in diverse physiological and metabolic
90 processes, including plant responses to various abiotic stresses. In addition, SA is an essential
91 regulator of plant immune responses (Vlot *et al.*, 2009; Manohar *et al.*, 2015; Klessig *et al.*, 2016).
92 While several recent studies have identified components of SA signaling networks and revealed
93 some SA-mediated signaling mechanisms, a full picture of SA-based signaling in plants is far from
94 complete. Indeed, the identity of the SA receptor(s) remains unclear. It was recently proposed that
95 Non-expresser of PR1 (NPR1), which functions as a master regulator of SA-mediated immune
96 signaling, is an SA receptor (Wu *et al.*, 2012). In contrast, Fu and coworkers (2012) suggested that

97 NPR1's two homologs, NPR3 and NPR4, rather than NPR1, are SA receptors. Since NPR3 and
98 NPR4 are adaptors for Cullin 3 ubiquitin E3 ligase, they may regulate the SA signaling pathway
99 by fine-tuning NPR1 protein levels via degradation (Fu *et al.*, 2012). In addition, nearly 30 SA-
100 binding proteins (SABPs) have been identified (Klessig *et al.*, 2016). These proteins exhibit a wide
101 range of affinities for SA, and SA binding alters their activities. Given that SA levels vary
102 dramatically within a plant depending on the subcellular compartment, tissue type, developmental
103 stage and external cues, such as infection, these findings raise the possibility that SA exerts its
104 effects by interacting with multiple targets, rather than a small number of receptors.

105 Although SA's role in activating disease resistance and ABA's role in signaling abiotic
106 stress responses are well-known, it is only recently becoming apparent that ABA also influences
107 immune responses (Robert-Seilaniantz *et al.*, 2011; Denance *et al.*, 2013). ABA treatment
108 suppressed defense responses and enhanced plant susceptibility to certain bacterial and fungal
109 pathogens (Ward *et al.*, 1989; McDonald & Cahill, 1999; Mohr & Cahill, 2003; Thaler & Bostock,
110 2004; De Torres Zabala *et al.*, 2009; Robert-Seilaniantz *et al.*, 2011). Additionally, the virulence
111 of *Pseudomonas syringae* in Arabidopsis was dependent on manipulation of the ABA signaling
112 pathway by secreted bacterial effectors (de Torres-Zabala *et al.*, 2007). Growing evidence also
113 indicates that there is substantial crosstalk between the ABA and SA pathways during immune
114 signaling (de Torres-Zabala *et al.*, 2007; Yasuda *et al.*, 2008). Arabidopsis mutants deficient in
115 ABA synthesis or response not only exhibited reduced susceptibility to pathogen infection, but
116 also showed enhanced expression of SA-responsive genes, such as *Pathogenesis-Related Protein*
117 *-1 (PR-1)* and *PR-4* (Audenaert *et al.*, 2002; Thaler & Bostock, 2004; Sanchez-Vallet *et al.*, 2012).
118 Conversely, Arabidopsis overexpressing RCAR3, which confers increased ABA sensitivity,
119 displayed enhanced susceptibility to *P. syringae* DC3000 infection, which correlated with
120 decreased expression of *PR-1* and *NPR1* (Lim *et al.*, 2014). Further demonstrating the antagonistic
121 interaction between ABA and SA, exogenous ABA suppressed the ability of an SA functional
122 analog to enhance pathogen resistance in Arabidopsis, while pretreatment with this analog
123 suppressed NaCl-induced expression of several ABA biosynthetic or responsive genes (Yasuda *et*
124 *al.*, 2008). ABA appears to suppress immune responses by down-regulating SA biosynthesis (de
125 Torres-Zabala *et al.*, 2007; Yasuda *et al.*, 2008); however, the mechanism through which SA
126 inhibits ABA signaling is unknown.

127 In previous studies, we have identified several SABPs that are involved in various biotic
128 and abiotic stress responses (Tian *et al.*, 2012; Manohar *et al.*, 2015). Here, we identify PP2Cs
129 from clade A and D as novel SABPs and show that SA binding to these PP2Cs suppresses their
130 ABA-enhanced interaction with the ABA receptors. In addition, SA suppresses ABA-induced
131 degradation of PP2Cs and suppresses ABA-mediated stabilization of SnRK2s. Combined with the
132 demonstration that SA treatment antagonizes ABA-induced gene expression and SA deficient
133 *sid2-1* mutants are ABA hypersensitive, these results suggest that SA antagonizes ABA signaling
134 through multiple mechanisms.

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152 Results

153 Identification of PP2Cs as novel SA-binding proteins

154 To help define the SA signaling network in plants, we developed several high-throughput screens
155 capable of identifying SABPs on a genome-wide scale (Tian *et al.*, 2012; Choi *et al.*, 2015;
156 Manohar *et al.*, 2015). In one screen, protein extracts prepared from Arabidopsis leaves were
157 subjected to affinity chromatography on a Pharmalink column to which SA was attached. After
158 stringent washing with the biologically inactive SA analog 4-hydroxy benzoic acid (4-HBA), SA-
159 bound proteins on the column were eluted with excess SA. The eluted proteins were analyzed by
160 mass spectroscopy and a PP2C belonging to clade D (PP2C-D4; At3g55050) was identified along
161 with other putative SABPs. Recombinant histidine-tagged PP2C-D4 was produced in *Escherichia*
162 *coli* and the purified protein was further assessed for SA-binding activity using three different
163 assays, including surface plasmon resonance (SPR), photoaffinity crosslinking, and size-exclusion
164 chromatography. SPR analysis was performed with a CM5 sensor chip to which the SA derivative
165 3-aminoethyl SA (3AESA) was immobilized via an amide bond. Binding to 3AESA was detected
166 when purified PP2C-D4 was passed over the sensor chip (Figure 1A). In the presence of increasing
167 concentrations of SA, PP2C-D4 binding to the 3AESA-immobilized sensor chip was modestly
168 reduced (Figure 1A). Similar to these results, the photoaffinity labeling approach indicated that
169 PP2C-D4 bound and was crosslinked to 4-azido SA (4AzSA). This binding also was suppressed
170 by SA in a dose-dependent manner (Figure 1B), arguing that PP2C-D4 binding to both 3AESA
171 and 4AzSA represents authentic SA-binding activity. PP2C-D4's ability to bind SA also was
172 confirmed by size-exclusion chromatography using [³H]SA; binding to [³H]SA was partially
173 suppressed by excess unlabeled SA, but not by excess amount of 4-HBA (Figure 1C). An
174 independent, parallel screen using a SA-derived ligand in combination with the yeast three-hybrid
175 technology, which relies on the *in vivo* interaction between the ligand (small molecule) and its
176 protein target in the yeast nucleus, also identified PP2C-D4 (called PP2C6 in (Cottier *et al.*, 2011)).
177 Based on these five independent assays, we conclude that PP2C-D4 is a true SABP.

178 Several clade A PP2C family members, including ABI1, ABI2, and HAB1, have been
179 identified as core components of the ABA signaling network (Soon *et al.*, 2012). This prompted
180 us to test whether these proteins also bind SA. Like PP2C-D4, recombinant ABI1 and ABI2 bound
181 the 3AESA-immobilized sensor chip and crosslinked with 4AzSA (Figure 1D, 1E, 1G, 1H). The

182 ability of ABI1 and ABI2 to bind 3AESA and crosslink to 4AzSA also was partially suppressed
183 by SA in a dose-dependent manner. ABI2's binding to [³H]SA was comparable to that of PP2C-
184 D4 and this binding was partially suppressed by excess unlabeled SA but not by excess 4-HBA,
185 while ABI1 exhibited relatively weak binding to [³H]SA and suppression by excess unlabeled SA
186 (Figure 1F; 1I). Interestingly, SA suppressed the binding of these proteins to 3AESA more
187 effectively than that of PP2C-D4 (Figure 1D, 1G). In contrast, HAB1 displayed much weaker
188 binding to the 3AESA-immobilized sensor chip (Supporting Figure 1A). The ability of
189 phosphoprotein phosphatase 2A regulatory subunit A (PP2A), a component of phosphatases
190 belonging to the PPP family, also was tested for SA binding. This protein was previously identified
191 during our screens, but it failed to meet the criteria as an SABP (Manohar *et al.*, 2015). Consistent
192 with these results, PP2A exhibited very weak binding to the 3AESA-immobilized sensor chip
193 (Supporting Figure 1B). Whether other key components involved in ABA signaling also bind SA
194 was then assessed. Size-exclusion chromatography revealed little or no binding of [³H]SA by three
195 members of the PYR/PYL/RCAR family of ABA receptors (PYL1, PYL2, and PYR1) or by three
196 SnRK2s (SnRK2.2, 2.3, and 2.6; Supporting Figure 1C). Likewise, SnRK2.2 displayed only very
197 low-level binding to the 3AESA-immobilized sensor chip (Supporting Figure 1D). Together, these
198 results suggest that SA preferentially interacts with specific PP2C family members, but not with
199 other major components of the ABA signaling pathway.

200 Since clade A PP2Cs are negative regulators of the ABA signaling pathway, we tested
201 whether the presence of ABA affects PP2C-SA interactions by flowing the PP2Cs over 3AESA-
202 immobilized sensor chips in the absence or presence of ABA. Notably, binding of PP2C-D4, ABI1,
203 and ABI2 to 3AESA was significantly enhanced in the presence of ABA (Figure 2). This ABA-
204 induced enhancement was suppressed by excess SA, further arguing that 3AESA binding by these
205 PP2Cs represents authentic SA-binding activity. In contrast, ABA failed to enhance the weak
206 binding of HAB1 and PP2A or to enable SnRK2.2 to bind 3AESA (Supporting Figure 1A, 1B,
207 1D).

208 **SA suppresses the ABA-enhanced interaction between PP2Cs and ABA receptors**

209 To assess whether SA binding by PP2Cs alters their ability to interact with ABA receptors, SPR
210 was performed. Purified PYL1 was immobilized on the CM5 sensor chip via an amide bond and
211 interactions were detected by flowing purified PP2C-D4, ABI1, or ABI2 over the sensor chip.

212 Dose-dependent binding responses were obtained with all three PP2Cs tested, and this binding was
213 enhanced in the presence of ABA (Figure 3; Supporting Figure 2). Notably, SA partially
214 suppressed the ABA-enhanced interaction between PYL1 and all three PP2Cs (Figure 3). In the
215 absence of ABA, SA slightly enhanced the interaction between PYL1 and PP2C-D4, but modestly
216 suppressed binding by ABI1 and ABI2. SPR analysis with PYL2 and PYR1 revealed similarly
217 dosage-dependent binding to the PP2Cs, that was further enhanced in the presence of ABA
218 (Supporting Figures 3 & 4). Furthermore, this ABA-enhanced binding was suppressed by SA,
219 although the level of suppression varied depending on the identity of the interacting proteins. For
220 example, SA only weakly suppressed the ABA-enhanced interactions between PYR1 and all three
221 PP2Cs, while interactions between these PP2Cs and either PYL1 or PYL2 were more strongly
222 suppressed by SA (Figure 3, Supporting Figures 3 & 4). The ABA-enhanced interactions between
223 PP2C-D4 and PYL1 or PYL2 also were suppressed less effectively by SA than the interactions
224 between these ABA receptors and other PP2Cs (Figure 3, Supporting Figure 3). Interestingly, SA
225 alone consistently enhanced the interaction between PP2C-D4 and all three ABA receptors, in
226 some cases by a substantial amount (Figure 3, Supporting Figures 3 & 4).

227 **SA suppresses ABA-enhanced degradation of PP2Cs and ABA-induced stabilization of** 228 **SnRK2s**

229 Proteolysis plays an important role in regulating plant responses to various stresses by fine-tuning
230 the turnover of key signaling components (Vierstra, 2009). Recent studies in Arabidopsis and rice
231 have demonstrated that all three key components of ABA signaling, including PYR/PYL/RCARs,
232 PP2Cs, and SnRK2s, are regulated by controlled proteolysis (Irigoyen *et al.*, 2014; Kong *et al.*,
233 2015; Lin *et al.*, 2015). For example, ABA promotes degradation of ABI1, but it suppresses
234 degradation of certain PYR/PYL/RCARs and SnRK2s (Kong *et al.*, 2015; Lin *et al.*, 2015).
235 Furthermore, the plant hormone gibberellic acid (GA) antagonizes ABA signaling, in part, by
236 stimulating degradation of PYR/PYL/RCARs and SnRK2s (Lin *et al.*, 2015). To determine
237 whether SA affects protein turnover, we analyze the stability of purified recombinant PP2Cs and
238 SnRK2s in a cell-free degradation assay. Following incubation in protein extracts prepared from
239 Arabidopsis seedlings supplemented with ABA and/or SA, immunoblot analyses indicated that the
240 levels of His6-tagged PP2C-D4, ABI1, and ABI2 decreased in extracts supplemented with 10 μ M
241 ABA (Figure 4A). By contrast, the levels of these proteins remained fairly stable in extracts

242 supplemented with both ABA and SA. SA alone had little effect on ABI1 or ABI2 levels but a
243 modest decrease of PP2C-D4 levels was detected (Figure 4A). Together, these results suggest that
244 ABA enhances PP2C degradation, and that this heightened turnover is suppressed by SA.

245 The stability of three SnRK2s, SnRK2.2, SnRK2.3, and SnRK2.6, was then assessed using
246 the cell-free protein degradation assay. The levels of all three recombinant SnRK2s were slightly
247 greater in extracts supplemented with 10 μ M ABA as compared with unsupplemented extracts
248 (Figure 4B). By contrast, SnRK2 levels were reduced in extracts containing both ABA and SA,
249 with the greatest decrease detected after supplementation with ABA and 100 μ M SA. Thus, ABA
250 appears to stabilize SnRK2s, while SA suppresses ABA's effect. Analysis of PYL1 did not reveal
251 any change in protein levels regardless of supplementation with ABA and/or SA, suggesting that
252 these hormones do not affect PYL1 stability (Supporting Figure 5).

253 The above results raised the possibility that endogenous SA antagonizes ABA signaling,
254 at least in part, by stabilizing PP2Cs. To further assess this, the rate of ABI1 degradation was
255 compared in protein extracts prepared from wild-type (WT) plants and the SA biosynthesis-
256 deficient mutant *sid2-1*. ABI1 levels in the extract from *sid2-1* plants decreased substantially by
257 30 min and were barely detectable after 1 hour, whereas those in the WT extract decreased
258 gradually over time (Figure 4C). Surprisingly, the enhanced degradation observed in *sid2-1*
259 extracts was not reversed by i) adding SA to the extract, ii) spraying SA on *sid2-1* plants, or iii)
260 supplementing *sid2-1* growth media with SA (Supporting Figure 6). Thus, while these results
261 suggest that SA stabilizes ABI1, the failure of exogenous SA to slow ABI1 degradation in *sid2-1*
262 extracts suggests that another factor(s) might be involved in this process.

263 **SA antagonizes ABA-induced gene expression *in vivo***

264 Previous studies have demonstrated that exogenously supplied ABA induces the accumulation of
265 *ABI1* and *ABI2* transcripts (Hoth et al., 2002). To determine whether SA antagonizes the
266 expression of these ABA signaling components, transcript levels for *ABI1* and *ABI2*, as well as
267 *PP2C-D4*, were monitored in ABA- and/or SA- treated Arabidopsis. Quantitative reverse
268 transcriptase-PCR (qRT-PCR) analyses showed that transcripts for *ABI1* and *ABI2* accumulated
269 after ABA, but not SA, treatment (Figure 5A). An intermediate level of transcripts was detected
270 in plants treated with SA and ABA, suggesting that the ABA-induced expression of these genes

271 was partially suppressed by SA (Figure 5A). In comparison to the clade A PP2Cs, transcript
272 accumulation for *PP2C-D4* was reduced in plants treated with either ABA or SA; an even greater
273 reduction was observed in plants treated with both hormones. The expression of two well-known
274 ABA-responsive genes, Response to Desiccation 29A (*RD29A*) and ABA-Responsive Element
275 Binding Protein 2 (*AREB2*), also was analyzed. Consistent with previous studies, the expression
276 of *RD29A* and *AREB2* was induced by ABA (Figure 5B) (Uno *et al.*, 2000; Nakashima *et al.*,
277 2006). Importantly, plants treated with ABA and SA accumulated reduced levels of *RD29A* and
278 *AREB2* transcripts, indicating that the ABA-induced expression of these genes is partially
279 suppressed by SA. By contrast, SA alone did not affect the expression of either gene.

280 **An SA-deficient mutant is more sensitive to ABA-mediated seed dormancy**

281 In addition to (a)biotic stress responses, ABA is involved in growth and developmental processes,
282 including maintaining seed dormancy to prevent untimely germination (Kermode, 2005; Hubbard
283 *et al.*, 2010). To investigate whether SA antagonizes ABA's ability to suppress germination, we
284 monitored Arabidopsis seed germination on plates containing Murashige and Skoog (MS) medium
285 in the presence or absence of ABA and/or SA. In the presence of 1 μ M ABA, germination was
286 dramatically reduced at all times monitored (Figure 6A). By contrast, 10 μ M SA reduced
287 germination slightly at 36 hrs, but from 48 hrs onward, the germination percentage of SA-treated
288 and control seeds was comparable. Plates containing both SA and ABA displayed an intermediate
289 level of germination. Thus, SA appears to suppress ABA-mediated inhibition of germination.
290 Whether endogenous SA levels also affect ABA-mediated suppression of germination was then
291 tested by comparing the germination of WT and *sid2-1* seeds. The germination rate for *sid2-1*
292 seeds grown on ABA-containing plates was consistently lower than that of comparably grown WT
293 seeds; by 72 hours, 15% of the *sid2-1* seeds had germinated in the presence of 1 μ M ABA, in
294 contrast to 40% of WT seeds (Figure 6B). While SA completely overcame ABA suppression of
295 seed germination in WT at 96 hrs post plating, it only partially reversed ABA's effect in *sid2-1*.
296 Based on the ABA hypersensitive phenotype displayed by *sid2-1* seeds, endogenous SA appears
297 to play an important role in antagonizing ABA-mediated suppression of seed germination *in*
298 *planta*.

299

300 Discussion

301 Elucidating the crosstalk between biotic and abiotic stress signaling pathways in plants is a rapidly
302 expanding area of research. There is a growing recognition that ABA not only regulates abiotic
303 stress responses and developmental processes, but also impacts plant-pathogen interactions
304 (Robert-Seilaniantz *et al.*, 2011; Denance *et al.*, 2013). Likewise, SA not only signals plant
305 immunity (Vlot *et al.*, 2009; Manohar *et al.*, 2015; Klessig *et al.*, 2016), but also regulates
306 responses to abiotic stresses and various aspects of growth and development (Hayat *et al.*, 2010;
307 Khan *et al.*, 2015). To gain insights into how SA exerts its myriad effects, we previously developed
308 several high throughput screens for identifying SABPs (Tian *et al.*, 2012; Choi *et al.*, 2015;
309 Manohar *et al.*, 2015). Here we report that several PP2Cs, including PP2C-D4, a member of clade
310 D, and ABI1 and ABI2, members of clade A, are novel SABPs. By contrast, SA binding was not
311 detected for two other phosphatases, HAB1 or PP2A, or for other components of the ABA
312 signaling pathway, including various PYR/PYL/RCARs and SnRK2s. SPR analysis also revealed
313 that binding of PP2C-D4, ABI1 and ABI2 to the SA analog 3AESA was enhanced in the presence
314 of ABA. This finding suggests that PP2Cs from both clade A and D bind both ABA and SA, and
315 that they do so in a cooperative manner. Although a previous report failed to detect binding
316 between ABI1 and ABA (Ma *et al.*, 2009), this discrepancy may be due to the very high sensitivity
317 of SPR. Indeed, Ma *et al.* (2009) noted that ABI1 phosphatase activity was reduced up to 20% in
318 presence of ABA.

319 Since clade A PP2Cs are critical negative regulators of ABA signaling, the discovery that
320 they bind SA suggested that they play a role in modulating SA/ABA crosstalk. Several studies
321 have documented an antagonistic relationship between SA and the ABA signaling pathway. For
322 example, SA suppressed ABA-mediated inhibition of shoot growth and expression of cell cycle-
323 related genes in rice (Meguro & Sato, 2014). Likewise, pretreating Arabidopsis with a compound
324 that activates SA-dependent defense signaling antagonized the induction of ABA biosynthesis-
325 related and ABA-responsive genes after NaCl treatment (Yasuda *et al.*, 2008). Expanding on these
326 findings, we demonstrated that SA treatment suppresses ABA-induced expression of the ABA
327 signaling components *ABI1* and *ABI2* and the ABA-responsive genes *RD29A* and *AREB2*. In
328 addition, SA antagonized ABA's ability to suppress seed germination. The combined observations
329 that i) SA-deficient *sid2-1* seeds germinated more slowly than WT seeds, and ii) *sid2-1* seeds were

330 hypersensitive to exogenously supplied ABA, argue that endogenous SA plays an important role
331 in counteracting the effects of both endogenously and exogenously supplied ABA.

332 To investigate the mechanism through which SA antagonizes ABA signaling, we
333 monitored the interaction between several ABA receptors and PP2Cs. SPR analyses revealed that
334 the clade A PP2Cs, ABI1 and ABI2, bind PYL1, PYL2, and PYR1 even in the absence of ABA
335 (Figure 7A); however, these interactions were strongly enhanced in the presence of ABA (Figure
336 7B). Strikingly, SA suppressed the ABA-enhanced interaction between these proteins, albeit to
337 varying extents depending on the identity of the interacting partners (Figure 7C). Consistent with
338 these results, both *in vitro* and *in vivo* analyses have previously demonstrated that ABA strongly
339 enhances binding between clade A PP2Cs and certain ABA receptors, including PYL1, PYL2 and
340 PYR1 (Park *et al.*, 2009). Crystal structure analyses further demonstrated that this interaction
341 inhibits PP2C activity by occluding PP2C's active site (Melcher *et al.*, 2009; Miyazono *et al.*,
342 2009). Since PP2Cs repress ABA signaling by preventing autophosphorylation-dependent
343 activation of SnRK2s (Umezawa *et al.*, 2009; Soon *et al.*, 2012), ABA-induced binding of PP2Cs
344 by ABA receptors is a critical step in activating ABA signaling (Fujii *et al.*, 2009). SA's ability to
345 suppress the interaction between ABI1 or ABI2 and the ABA receptors therefore provides one
346 mechanism through which SA can antagonize ABA signaling. In addition, our cell-free
347 degradation assay revealed that SA suppresses the ABA-enhanced turnover of PP2Cs and
348 stabilization of SnRK2s. Given that ABI1 was degraded substantially more rapidly in extracts from
349 *sid2-1* mutants than from WT plants, endogenous SA appears to play an important role in
350 regulating cellular PP2C levels. Taken together, these results suggest that SA antagonizes ABA
351 signaling via multiple mechanisms that both promote the enzymatic activity and/or protein stability
352 of negative regulators and decrease the stability of downstream effectors.

353 Within this overall framework, differences among the binding specificities and affinities,
354 protein-protein interactions and/or stability of various ABA signaling components may further
355 influence SA/ABA crosstalk. For example, while ABI1 and ABI2 bound SA, another clade A
356 member, HAB1 did not. ABI1 differs from ABI2 as it displayed substantially greater affinity for
357 all three ABA receptors in the absence of ABA; it also was the most stable PP2C in our *in vitro*
358 degradation assay. SA's ability to disrupt the ABA-enhanced interactions between ABA receptors
359 and PP2Cs also varied, depending on the proteins involved. In particular, the interaction between

360 PYR1 and ABI1 or ABI2 was suppressed less effectively by SA than the interactions between
361 these PP2Cs and the other ABA receptors. Similar to these findings, reconstitution of the ABA
362 signaling pathway in Arabidopsis protoplasts using different combinations of ABA receptors,
363 PP2Cs and SnRK2s previously revealed that the intensity of interactions varied significantly
364 depending on which members of the protein families were involved (Fujii *et al.*, 2009). Combined
365 with our findings, these results suggest that while the various the members of the PP2C, ABA
366 receptor and SnRK2 families serve overlapping functions, differences in their temporal and/or
367 spatial expression patterns, as well as their affinity for specific interacting partners and/or SA and
368 ABA, could fine-tune ABA signaling and regulate crosstalk with the SA pathway.

369 In comparison to the clade A PP2Cs, members of clade D were recently shown to
370 negatively regulate cell expansion by dephosphorylating, and thereby inactivating plasma
371 membrane H⁺-ATPases (Spartz *et al.*, 2014). In the presence of auxin, this suppression is relieved
372 by members of the SAUR (small auxin up-regulated) protein family, which bind and inhibit PP2C-
373 Ds. Interestingly, while different SAURs inhibited the activity of several PP2C-D family members
374 (including PP2C-D4) to varying extents, they did not inhibit the activity of a clade A PP2Cs, ABI1,
375 a clade E member, or a phosphatase belonging to the PPP family. Our studies provide additional
376 insights into PP2C-D function, as we demonstrate that PP2C-D4 is an SABP, whose binding to
377 3AESA is enhanced by ABA. PP2C-D4 also bound several ABA receptors, and this interaction
378 was enhanced by the presence of ABA. In comparison to the clade A PP2C-ABA receptor
379 interactions, however, the binding between PP2C-D4 and the ABA receptors was substantially
380 weaker and its suppression by SA was less effective. In addition, SA consistently stimulated this
381 interaction in the absence of ABA. The expression pattern of *PP2C-D4* in the presence of ABA
382 and/or SA also differed significantly from that of the clade A PP2Cs. Thus, while PP2C-D4 and
383 the clade A PP2Cs share some common features, their many differences are consistent with the
384 previous demonstration that clade D and clade A PP2Cs serve distinct functions.

385 In response to stress, plants maximize their chances of survival and reproduction by
386 redistributing cellular resources from growth and developmental processes to defensive responses
387 (Asselbergh *et al.*, 2008; Atkinson & Urwin, 2012). Many studies have assessed plant responses
388 to individual stresses, but there is a growing recognition that plants in the field contend with
389 multiple stresses simultaneously, and that, depending on the specific stresses, the responses may

390 be additive or antagonistic. Thus, the hormones responsible for mediating developmental processes
391 and stress responses are involved in complex crosstalk that ultimately allows the plant to tailor its
392 response to the environmental conditions. A previous study demonstrated that ABA can interfere
393 with SA-mediated innate immune responses by down-regulating SA biosynthesis (De Torres
394 Zabala *et al.*, 2009). By contrast, our results provide a novel mechanism through which SA can
395 antagonize ABA by interfering with multiple aspects of the ABA signaling pathway. The
396 discovery that PP2C-D4 binds SA and several ABA receptors, and that this binding is enhanced
397 in the presence of ABA, suggests an additional mechanism through which SA and ABA can
398 negatively regulate auxin-mediated growth and developmental processes. Indeed, ABA was shown
399 to suppress hypocotyl elongation, and this correlated with dephosphorylation of H⁺-ATPases
400 (Hayashi *et al.*, 2014). These ABA-induced responses were suppressed in the *abil-1* mutant,
401 suggesting that clade A PP2Cs are involved in auxin-mediated physiological processes. Future
402 studies will be required to determine whether PP2C-D4 and/or other clade D PP2Cs also mediate
403 ABA antagonism of cell expansion, and whether SA binding by PP2C-D4 affects its ability to
404 interact with SAURs and thereby impact auxin signaling.

405 In summary, we have elucidated SA mechanisms of action in negatively regulating ABA
406 signaling, which likely serves to properly balance the plant response to multiple stresses. As plants
407 confront a changing climate, this balance and our understanding of how it is regulated and might
408 be beneficially altered, take on increase significance.

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417 **Materials and Methods**

418 **Plant materials and growth conditions**

419 The wild-type and *sid2-1 Arabidopsis thaliana* ecotype Col-0 plants were grown on standard
420 Murashige and Skoog (MS) media containing half-strength of MS with pH adjusted to 6.0 by KOH
421 and supplemented with 10 g /L sucrose. Arabidopsis seeds were first surface-sterilized by soaking
422 in a solution of 30% bleach with 0.1% triton X-100 for 5-10 min and then rinsed five times with
423 sterile water. The surface sterilized seeds were incubated at 4 °C for 2 days for stratification before
424 planting on the MS media. For the seed germination assay, (\pm) abscisic acid (Caisson labs) or
425 salicylic acid (Sigma) were added directly into the MS media. The plates with seed were placed
426 vertically in the growth chamber with 16/8h light/dark cycle, 22 °C, and 70% humidity. The
427 germination rate was measured. For spray treatment, one-week old seedlings were subjected to
428 water, ABA, SA, or ABA+SA spray treatment and whole seedlings were collected for RNA
429 analysis. For cell-free degradation assay, ten-day-old wild-type or *sid2-1* seedlings were subjected
430 to water, ABA, SA, or ABA+SA spray treatment to compare the effects of protein extracts on the
431 stability of ABI1.

432 **Cloning and plasmid constructs**

433 All oligonucleotides used for cloning and plasmid construction are listed in Table S1. ORFs of
434 PP2CD, ABI1, ABI2, and HAB1 were amplified from an Arabidopsis cDNA library. The resulting
435 PCR products were digested with *NdeI* and *BamHI* for ABI1, *NdeI* and *SacI* for ABI2, and HAB1
436 and cloned into the expression vector pET28a (EMD Millipore, MA, USA) for expression. PP2CD
437 was cloned into pET42a (EMD Millipore, MA, USA) using *NdeI* and *XhoI* cloning sites. Cloning
438 of PYL1, PYL2, PYR1, SnRK2.2, SnRK2.3, and SnRK2.6 into pSUMO- H6SUMO vector were
439 described previously (Soon *et al.*, 2012).

440 **Protein purifications**

441 Two-step protein purifications were performed as described previously (Manohar *et al.*, 2017).
442 Briefly, the Rosetta 2 (DE3) (EMD, Millipore, MA, USA) bacterial cells were grown at 37 °C in
443 LB medium containing 50 μ g/ml kanamycin and 34 μ g/ml chloramphenicol until the OD₆₀₀ of the
444 culture reached approximately 0.6 before addition of isopropyl- β -D-thiogalactoside (IPTG) to a
445 final concentration of 1 mM to induce expression. Induced culture was incubated overnight at 20

446 °C. The cells were then harvested by centrifugation and the pellet was resuspended in the lysis
447 buffer (50 mM Tris pH 7.5, 500 mM NaCl, 10% glycerol, 20 mM imidazole, 0.5% triton X-100
448 and 1 mM phenylmethylsulphonyl fluoride) and disrupted by sonication. The clarified supernatant
449 obtained after centrifugation was incubated with Ni-NTA His resin (Novagen, MA, USA) and the
450 bound protein was eluted in lysis buffer supplemented with 250 mM imidazole. The eluted proteins
451 were then subjected to gel filtration chromatography on a HiLoad 16/600 Superdex 200 prep grade
452 column (GE Healthcare, PA, USA), using gel filtration buffer (50 mM Tris pH 7.5, 150 mM NaCl,
453 and 10% glycerol). The two-step purified proteins were stored at -80 °C.

454 **Assessment of 3AESA-binding activities by Surface Plasmon Resonance (SPR)**

455 SPR analyses of 3AESA binding and competition by SA were performed with a Biacore 3000
456 instrument (GE Healthcare) as described previously (Manohar *et al.*, 2015). Immobilization of
457 3AESA on the CM5 sensor chip was performed as described previously (Tian *et al.*, 2012). To test
458 SA-binding activity, proteins were diluted in HBS-EP buffer (GE Healthcare) and passed over the
459 sensor surface of the 3AESA-immobilized and mock-coupled flow cells. The specific binding
460 signal was determined by subtracting the signal generated with the mock-coupled flow cell from
461 the signal generated by the 3AESA-immobilized cell. To re-use the sensor chips, bound proteins
462 were stripped off by injecting NaOH solution (pH 12).

463 **Assessment of protein-protein interactions by SPR**

464 Protein interaction analyses were performed by SPR using Biacore 3000 instrument. His-SUMO-
465 tagged PYL1, PYL2, and PYR1 were immobilized on a CM5 sensor chip by amine coupling (GE
466 healthcare), essentially by following the manufacturer's instructions. Briefly, proteins were diluted
467 in 10 mM sodium acetate, pH 5.0 buffer at a concentration of to 50µg/ml. CM5 sensor chip surface
468 was activated by injecting 85 µl of EDC/NHS solution with a flow rate of 10 µl/min. After
469 activation, protein solution was injected for 42 minutes with a flow rate of 10 µl/min. Finally, 85
470 µl of ethanolamine was flowed over the surface to deactivate remaining active groups and remove
471 non-covalently bound protein with a flow rate of 10 µl/min. The protein immobilization level was
472 stabilized for 12 hours by flowing HBS-EP buffer with a flow rate of 10 µl/min. To test protein-
473 protein interactions, protein analytes (PP2Cs and SnRK2s) were diluted to desirable concentration
474 in HBS-EP buffer in the presence or absence of various concentrations of ABA, SA, or ABA+SA,
475 and then passed over the protein-immobilized sensor surface and mock coupled flow cells with a

476 flow rate of 30 μ l/min. The higher flow rate was used to avoid mass-transfer as recommended by
477 the manufacturer. The binding signal was generated by subtracting the signal for mock-coupled
478 flow cells from that for the protein-immobilized flow cells. To re-use the chip, bound proteins
479 were stripped off by injecting 8 μ l of 10 mM glycine-HCl solution (pH 3) with a flow rate of 30
480 μ l/min.

481 **RNA analyses**

482 Unless stated otherwise, at least three biological replicates were used for all RNA analyses. For
483 each replicate, total RNA from one-week old Arabidopsis seedlings was isolated from a pool of
484 five seedlings. Total RNA was isolated from using Qiagen RNeasy plant mini kit (Qiagen)
485 according to the manufacturer instructions. DNase treatment was done using DNA-free kit
486 (Ambion) following the manufacturer's instructions. First strand cDNA was synthesized from 1
487 mg of RNA using M-MLV reverse transcriptase (Promega). For quantitative real-time PCR,
488 transcripts were amplified using SYBR premix Ex Taq II (Takara) with gene-specific primers
489 listed in table S1. Reactions were done using a CFX96 touch Biorad Real-time PCR system (Bio-
490 Rad). The PCR conditions were 95 $^{\circ}$ C for 3 min (initial denaturation) followed by 44 cycles of
491 amplifications (95 $^{\circ}$ C for 10s, 60 $^{\circ}$ C for 30s), followed by generation of a dissociation curve. The
492 relative fold change was calculated according to the $2^{-\Delta\Delta C_t}$ method (Manosalva *et al.*, 2015).
493 Ubiquitin was used as endogenous reference gene. The paired t-test with an α -level of 0.05 was
494 used to compare transcript level in the ABA, SA, ABA+SA, and mock-treated plant samples.

495 **Cell-free degradation assay**

496 The tissue samples were collected from 10 day old seedlings of wild-type and *sid2-1* and finely
497 grounded using liquid nitrogen. The total protein extracts were then prepared using protein
498 extraction buffer (25 mM Tris pH 7.5, 10 mM NaCl, 10 mM MgCl₂, and 4 mM PMSF). The
499 sample was vortexed to mix and centrifuged twice at 17,000 g for 10 min at 4 $^{\circ}$ C to remove debris.
500 The clear supernatant was pre-treated with 1 mM cycloheximide (MP Biomedicals) for 1 h to
501 inhibit *de novo* protein biosynthesis. The extracts were then adjusted to equal protein
502 concentrations in degradation buffer (25 mM Tris pH 7.5, 10 mM NaCl, 10 mM MgCl₂, and 4
503 mM PMSF, 5 mM DTT, and 10 mM ATP). For degradation assay, an equal amount (approximatly
504 500 ng) of purified PP2Cs, SnRK2s, and PYL1 were incubated in 50 μ l of Arabidopsis total protein
505 extract (containing approximatly 100 μ g total proteins) at 28 $^{\circ}$ C for 3 h, unless otherwise indicated.

506 For ABI1 and SnRK2.6 twice as much total protein extract (approximately 200 μ g) was used to
507 more clearly visualize the effect of SA (Figure 4A, 4B). Immunoblot analyses were performed to
508 detect protein levels by using an α -His₆-HRP polyclonal antibody (QED Biosciences).

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698

699 **Authors' contributions:**

700 M.M, P.M, and D.F.K conceived the research. M.M, D.W, E. K, and D.F.K designed the research.
701 M.M, D.W, P.M, and H.W.C performed the research. M.M, D.W, E.K, and D.F.K analyzed the
702 data. M.M and D.F.K wrote the paper. All authors have read and approved the final manuscript.

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707 **Figure Legends**

708 **Figure 1: Several PP2Cs bind SA.** (A, D, G) Sensorgrams for 1 μ M of His₆-tagged PP2C-D4
709 (At3g55050) (A), ABI1 (At4g26080) (D), and ABI2 (At5g57050) (G) in the absence (0 mM) or
710 presence of two concentrations of SA (5 or 10 mM) using a 3AESA-immobilized SPR sensor chip.
711 Signals detected from a mock-coupled control chip were subtracted. (B, E, H) Photo-activated
712 crosslinking of 50 ng of PP2C-D4 (B), ABI1 (E), and ABI2 (H) to 4AzSA (50 μ M) in the absence
713 or presence of increasing amounts of SA was detected by immunoblotting using an α -SA antibody.
714 Reactions without 4AzSA served as negative controls. Proteins stained with Coomassie Brilliant
715 Blue (CBB) served as the loading controls. (C, F, I) Binding of [³H]SA (200 nM) by 200 ng/ μ l
716 PP2C-D4 (C), ABI1 (F), and ABI2 (I) in the absence or presence of a 10,000-fold excess of
717 unlabeled SA was determined by size-exclusion chromatography. Chromatography with [³H]SA
718 in the absence of protein served as negative controls. Reactions with [³H]SA with excess of 4-
719 amino benzoic acid (4-HBA), an inactive SA analog, served as negative controls for SA-specific
720 competitive inhibition. The experiments was independently repeated at least twice.

721 **Figure 2: SA-binding activities of PP2Cs are enhanced by ABA and this binding is partially**
722 **suppressed by SA.** (A-C) Sensorgrams obtained with recombinant, purified 1 μ M of His₆-tagged
723 PP2C-D4 (A), ABI1 (B), and ABI2 (C) using a 3AESA-immobilized sensor chip in the absence
724 or in the presence of 2 mM ABA or 2 mM ABA plus 2.5 mM SA. Signals detected from a mock-
725 coupled control chip were subtracted. The experiments was independently repeated at least twice.

726 **Figure 3: SA disrupts the ABA-induced interaction between PP2Cs and PYL1.** (A-C)
727 Sensorgrams obtained with recombinant, purified 10 μ M of His₆-tagged PP2C-D4 (A), 1 μ M of
728 ABI1 (B) or 2.5 μ M of ABI2 (C) using a His₆-SUMO tagged PYL1-immobilized sensor chip in
729 the absence or presence of the indicated concentrations of ABA or SA. Signals detected from a
730 mock-coupled control chip were subtracted. The experiments was independently repeated at least
731 twice.

732 **Figure 4: SA alters ABA-mediated turnover of PP2Cs and SnRK2s.** (A) Cell-free degradation
733 assay using total protein extracts prepared from ten-day-old Arabidopsis seedlings supplemented
734 with 500 ng of His₆-tagged PP2Cs (PP2C-D4, ABI1, and ABI2) and indicated concentrations of
735 ABA, SA, or ABA+SA. (B) Cell-free degradation assay using total protein extracts prepared from

736 ten-day-old *Arabidopsis* seedlings supplemented with 500 ng of His₆-Sumo-tagged SnRK2s
737 (SnRK2.2, 2.3, and 2.6) and indicated concentrations of ABA or ABA+SA. For A & B, the
738 degradation assay was carried out at 30° C for 3 hrs. All lanes shown are from the same experiment;
739 some lanes unrelated to this study have been removed and lanes were then merged for clarity of
740 presentation. (C) Cell-free degradation assay using total protein extracts prepared from ten-day-
741 old wild-type or *sid2-1* *Arabidopsis* seedlings supplemented with 500 ng of His₆-tagged ABI1.
742 Samples were taken after 0, 0.5, 1, or 2 hrs of incubation; proteolysis was stopped by addition of
743 SDS-PAGE buffer. Proteins were detected by immunoblotting using an α -His₆-HRP antibody.
744 Staining with Coomassie brilliant blue (CBB) staining of the gel served as a loading control. The
745 experiments was independently repeated at least twice.

746 **Figure 5: SA suppresses ABA-induced gene expression.** (A) Transcript levels, as measured by
747 qRT-PCR in seedlings pretreated with either water, 100 μ M ABA, 100 μ M SA or 100 μ M ABA
748 plus 100 μ M SA. Transcript levels of *PP2C-D4*, *ABII*, and *ABI2* were determined at 3 hrs post
749 treatment (hpt). Data are averaged \pm s.d (n=3). (B) Transcript levels as measured by qRT-PCR of
750 ABA-responsive marker genes in seedlings pretreated with either water, 100 μ M ABA, 100 μ M
751 SA or 100 μ M ABA plus 100 μ M SA. Transcript levels of *RD29A* (RESPONSIVE TO
752 DESICCATION 29A) and *AREB2* (ABRE BINDING FACTOR 2) were determined at 0, 3, 6, 12
753 and 24 hpt. The relative expression levels were quantified by normalizing to ubiquitin expression
754 level. Data are averaged \pm s.d (n=4). *P \leq 0.05; **P \leq 0.005; ***P \leq 0.0005; ****P \leq 0.00005;
755 two-tailed *t*-test. The experiments was independently repeated at least twice.

756 **Figure 6: SA antagonizes ABA-mediated suppression of seed germination.** (A) Germination
757 rate of *Arabidopsis* wild-type seeds on MS medium containing no hormone or in the presence of
758 1 μ M ABA, 10 μ M SA or 1 μ M ABA plus 10 μ M SA. (B) Comparison of germination rate between
759 wild-type and *sid2-1* on MS medium containing no hormone or in the presence of 1 μ M ABA or
760 1 μ M ABA plus 10 μ M SA. The germination time course for wild-type seeds is shown with a solid
761 line, while for *sid2-1* it is shown with a broken line (n=40 seeds). The percent of germinated seeds
762 was determined at 36, 48, 60, 72, 84, 96, 108 and 120 hrs post plating. The experiments was
763 independently repeated at least twice.

764

765 **Figure 7: Schematic illustrating part of SA's antagonistic effects on the ABA signaling**
766 **module (A).** In the absence of ABA, free PP2Cs prevents autophosphorylation-dependent
767 activation of SnRK2s by dephosphorylating them. **(B)** In the presence of ABA, PYR/PYL
768 receptors tightly bind to PP2Cs, thereby preventing free PP2C-mediated dephosphorylation of
769 SnRK2s. Receptor-mediated occlusion of PP2Cs allows autophosphorylated-activated SnRK2s to
770 relay the ABA signaling by phosphorylating downstream targets such as abscisic acid responsive
771 elements-binding factor 2 (ABF2), which enables its binding to ABA-responsive elements
772 (ABRE) in the promoter region of ABA-responsive genes. **(C)** SA suppresses ABA's enhancement
773 of the interaction of PP2Cs with the PYR/PYL/RCAR receptor and the resulting
774 autophosphorylation-dependent activation of SnRK2s, which results in reduced ABA signaling.
775 The length and thickness of the arrows indicate the equilibrium between free and receptor-bound
776 PP2Cs and between inactive, nonphosphorylated and active, autophosphorylated SnRK2s.

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780

781 **Supplementary Figures**

782 **Supplementary Figure 1: HAB1, PP2A and members of the PYR/PYL/RCAR and SnRK2**
783 **families are not SABPs (A-C)** Sensorgrams obtained using a 3AESA-immobilized SPR sensor
784 chip with 5 μM of His₆-HAB1 (At1g72770), His₆-PP2A (At1g25490), or His₆-SUMO-SnRK2.2
785 (At3g50500) in the absence or presence of 2 mM ABA. Signals detected from a mock-coupled
786 control chip were subtracted. **(D)** Binding of [³H]SA (200 nM) by 200 ng/ μl SnRK2.2, 2.3, 2.6,
787 PYL1, PYL2, or PYR1 was determined by size-exclusion chromatography. Chromatography with
788 [³H]SA in the absence of protein served as negative control.

789 **Supplementary Figure 2: Effect of ABA on the interactions of PP2Cs with PYL1. (A, C, E)**
790 Sensorgrams obtained with the indicated concentrations of recombinant, purified His₆- tagged
791 PP2C-D4, ABI1, or ABI2 using a His₆- SUMO-tagged PYL1-immobilized sensor chip. **(B, D, F)**
792 ABA dose-dependent effect on the interactions between 10 μM PP2C-D4, 1 μM ABI1, or 2.5 μM
793 ABI2 and His₆- SUMO-tagged PYL1 immobilized on the sensor chip. Signals detected from a
794 mock-coupled control chip were subtracted. The experiments was independently repeated at least
795 twice.

796 **Supplementary Figure 3: SA disrupts the ABA-enhanced interactions between PP2Cs**
797 **(PP2C-D4, ABI1, and ABI2) and the ABA receptor PYL2. (A, D, G)** Sensorgrams obtained
798 using a His₆-SUMO-tagged PYL2-immobilized sensor chip and the indicated concentrations of
799 recombinant, purified His₆- tagged PP2C-D4, ABI1, or ABI2. **(B, E, H)** ABA dose-dependent
800 effect on the interactions of 10 μM PP2C-D4, 1 μM ABI1, or 2.5 μM ABI2 with His₆-SUMO-
801 tagged PYL2-immobilized sensor chip. **(C, F, I)** Sensorgrams obtained with 10 μM of the His₆-
802 tagged PP2C-D4, 1 μM ABI1, or 2.5 μM ABI2 using a His₆-SUMO-tagged PYL2-immobilized
803 sensor chip in the absence or presence of the indicated concentrations of ABA, SA or ABA plus
804 SA. Signals detected from a mock-coupled control chip were subtracted. The experiments was
805 independently repeated at least twice.

806 **Supplementary Figure 4: SA disrupts the ABA-enhanced interactions between PP2Cs**
807 **(PP2C-D4, ABI1, and ABI2) and ABA receptor PYR1. (A, D, G)** Sensorgrams obtained using
808 a His₆- SUMO-tagged PYR1-immobilized sensor chip and the indicated concentrations of
809 recombinant, purified His₆- tagged PP2C-D4, ABI1, or ABI2. **(B, E, H)** ABA dose-dependent
810 effect on the interactions of 10 μM PP2C-D4, 1 μM ABI1, or 2.5 μM ABI2 with His₆-SUMO-

811 tagged PYR1-immobilized sensor chip. **(C, F, I)** Sensorgrams obtained with recombinant 10 μ M
812 of the His₆- tagged PP2C-D4, 1 μ M ABI1 or 2.5 μ M ABI2 using a His₆- SUMO-tagged PYR1-
813 immobilized sensor chip in the absence or presence of the indicated concentrations of ABA, SA,
814 or ABA plus SA. Signals detected from a mock-coupled control chip were subtracted. The
815 experiments was independently repeated at least twice.

816 **Supplementary Figure 5: ABA and SA alone or in combination do not affect turnover of**
817 **PYL1.** Cell-free degradation assay using total protein extracts prepared from ten-day-old
818 Arabidopsis seedlings supplemented with 500 ng of His₆-Sumo-tagged PYL1 and indicated
819 concentrations of ABA, SA, or ABA+SA. The degradation assay was carried out at 30⁰ C for 3
820 hrs. Proteins were detected by immunoblotting using an α -His₆-HRP antibody and Coomassie
821 brilliant blue (CBB) staining of the gel served as a loading control. The experiments was
822 independently repeated twice.

823 **Supplementary Figure 6: SA does not affect the rate of ABI1 degradation in *sid2-1* plants.**
824 **(A)** Cell-free degradation assay using 500 ng of His₆- tagged ABI1 and Arabidopsis protein
825 extracts from Wt or *sid2-1* mutant plants in the absence or presence of 10 μ M SA that was added
826 directly to the reaction mix. His₆- tagged ABI1 was detected in samples harvested at the indicated
827 times by immunoblotting using a α -His₆-HRP antibody. **(B)** Cell-free degradation assay using 500
828 ng of His₆-tagged ABI1 and Arabidopsis protein extracts from ten-day-old Wt or *sid2-1* mutant
829 plants that were sprayed with water or 10 μ M SA three hours prior to extract preparation. His₆-
830 tagged ABI1 was detected in samples harvested at the indicated times by immunoblotting using a
831 α -His₆-HRP antibody. **(C)** Cell-free degradation assay using Arabidopsis protein extracts from
832 ten-day-old wild-type (Wt) or SA-deficient *sid2-1* mutant plants grown in MS medium in the
833 absence or presence of 10 μ M SA and supplemented with 500 ng of His₆-tagged ABI1. His₆-
834 tagged ABI1 was detected in samples harvested at the indicated times by immunoblotting using
835 an α -His₆-HRP antibody. For B & C, all lanes are from the same experiment; some lanes unrelated
836 to this study were removed and lanes were then merged for clarity of presentation. The experiments
837 was independently repeated twice.

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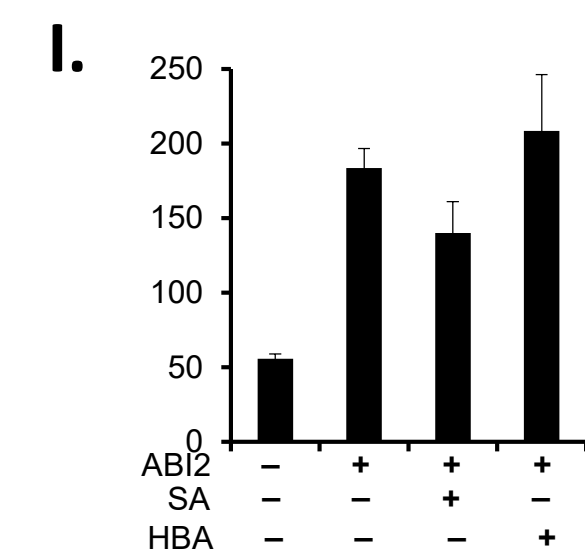
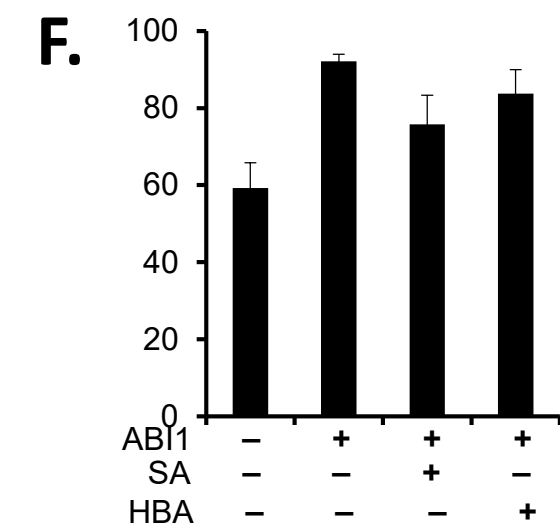
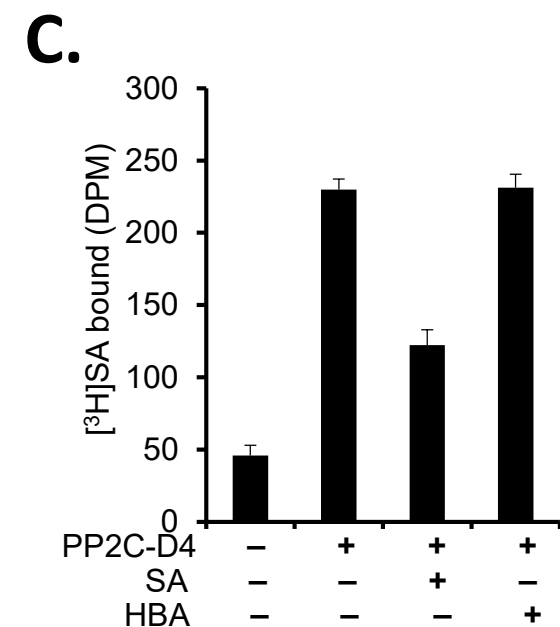
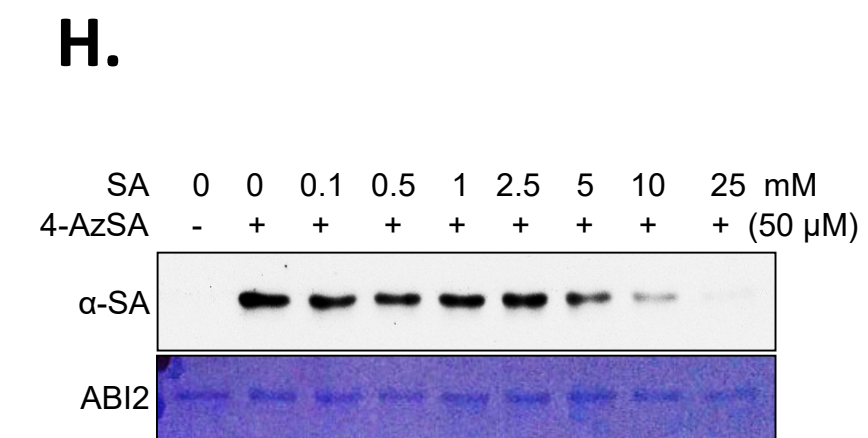
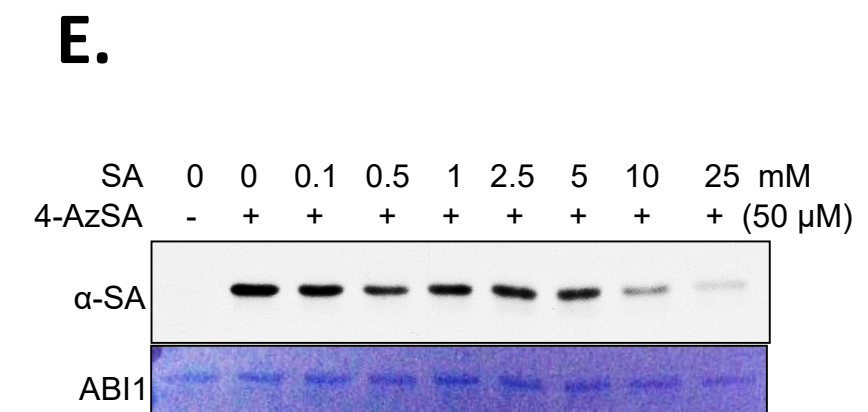
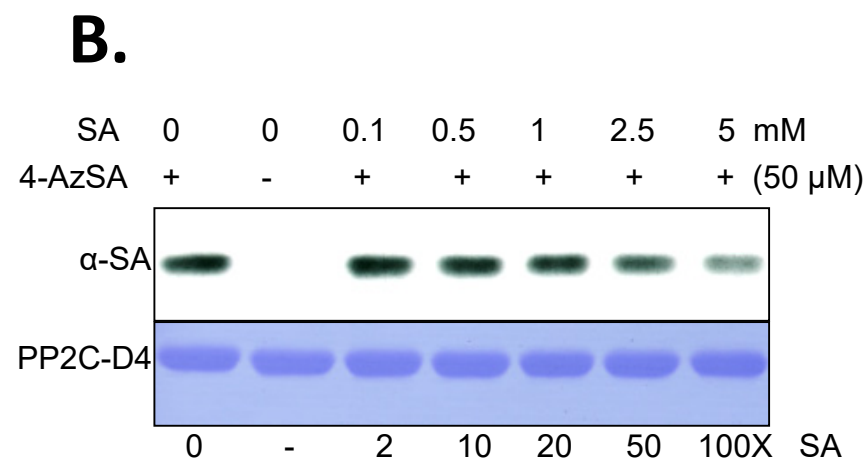
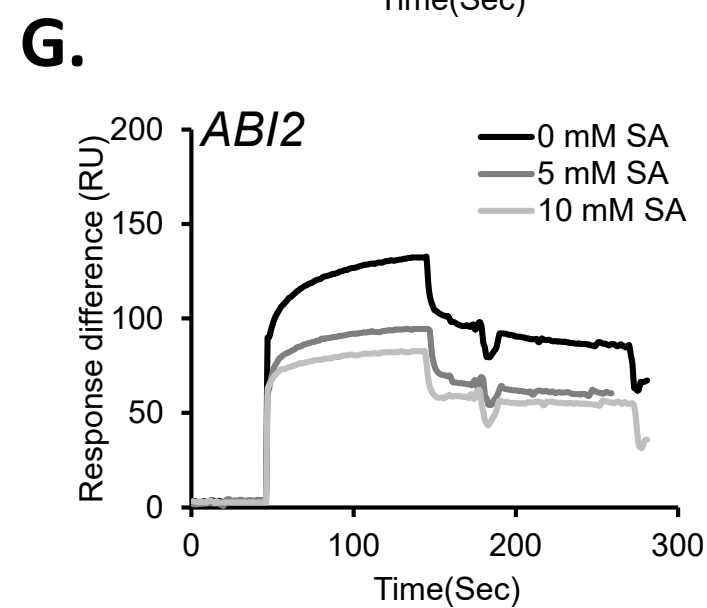
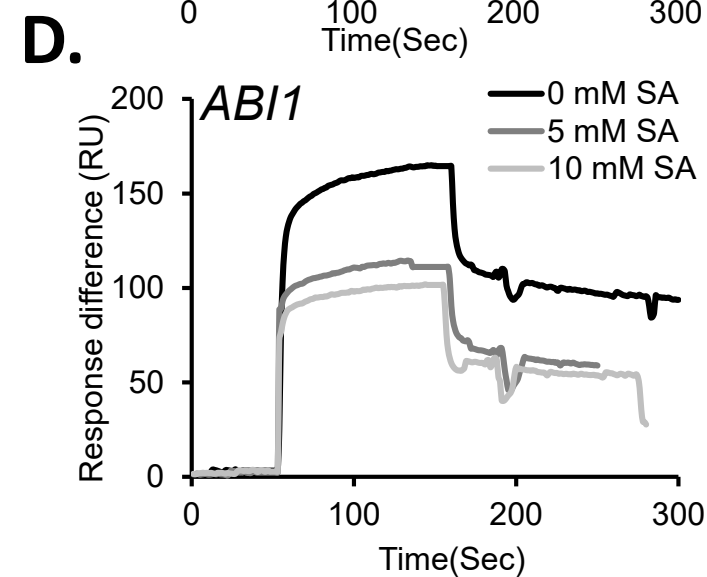
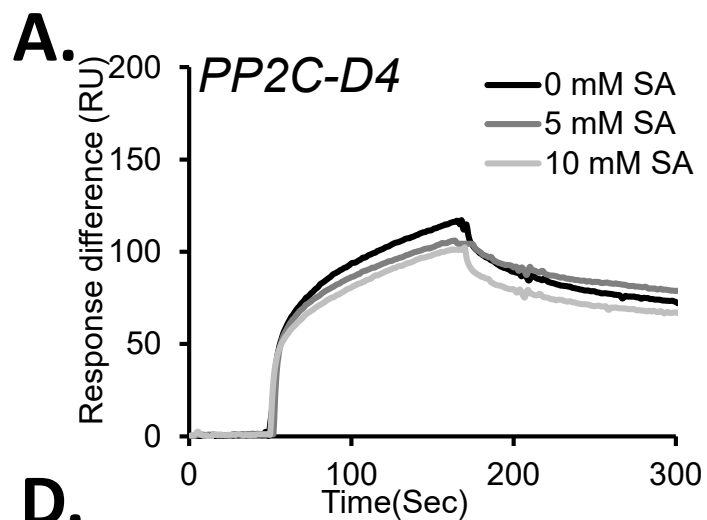
Figure 1

Figure 2

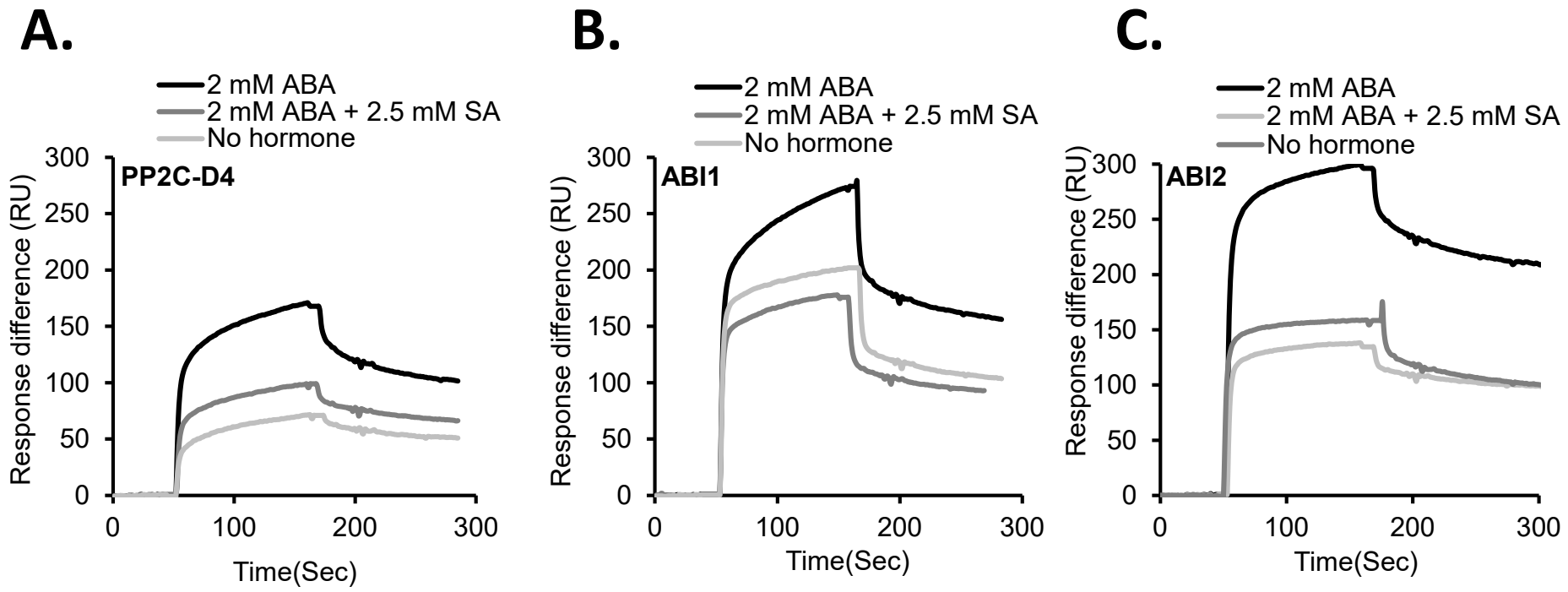
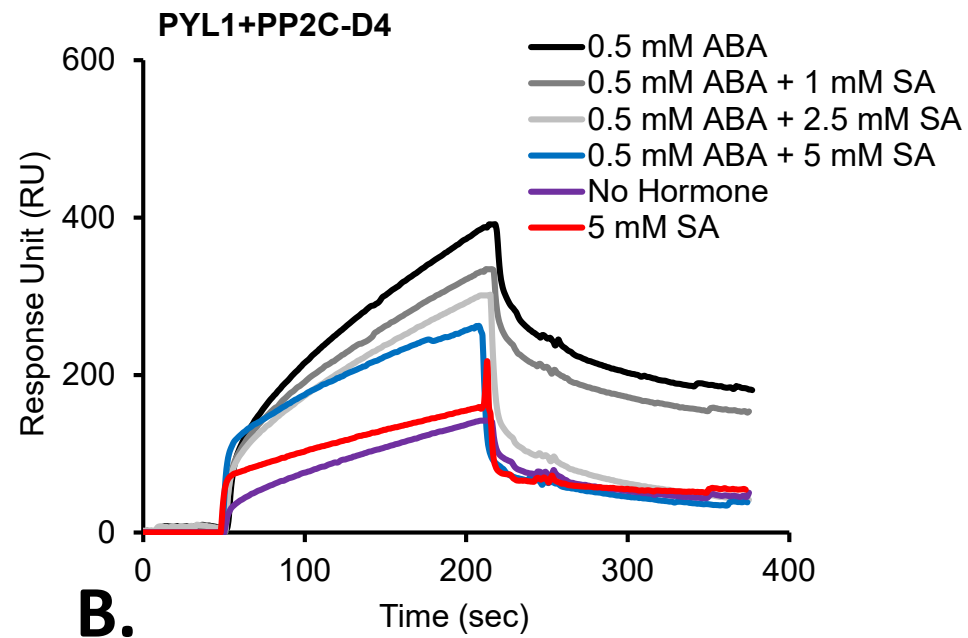
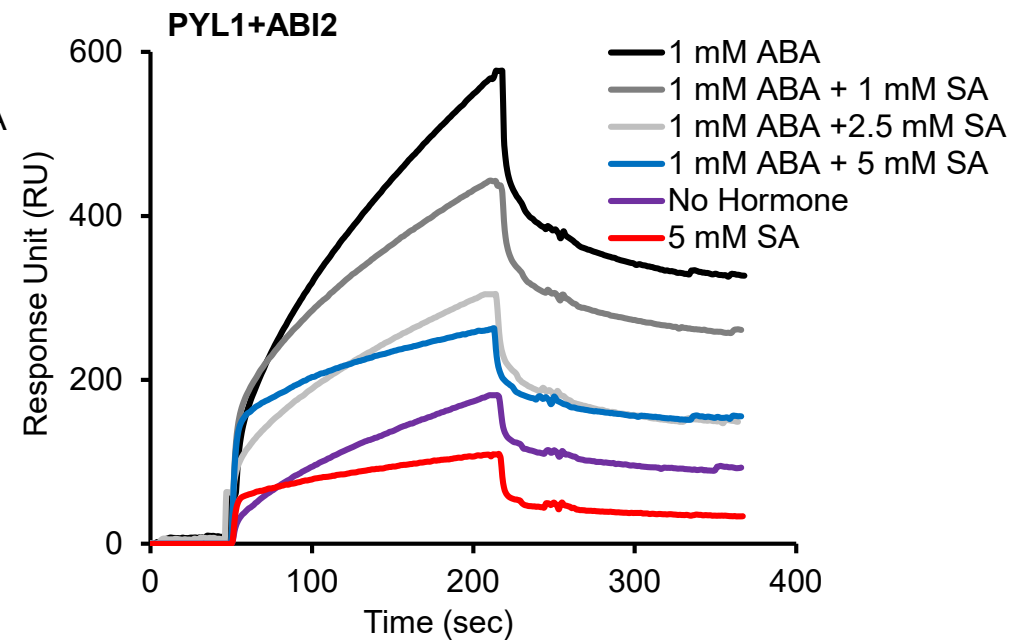


Figure 3

A.



C.



B.

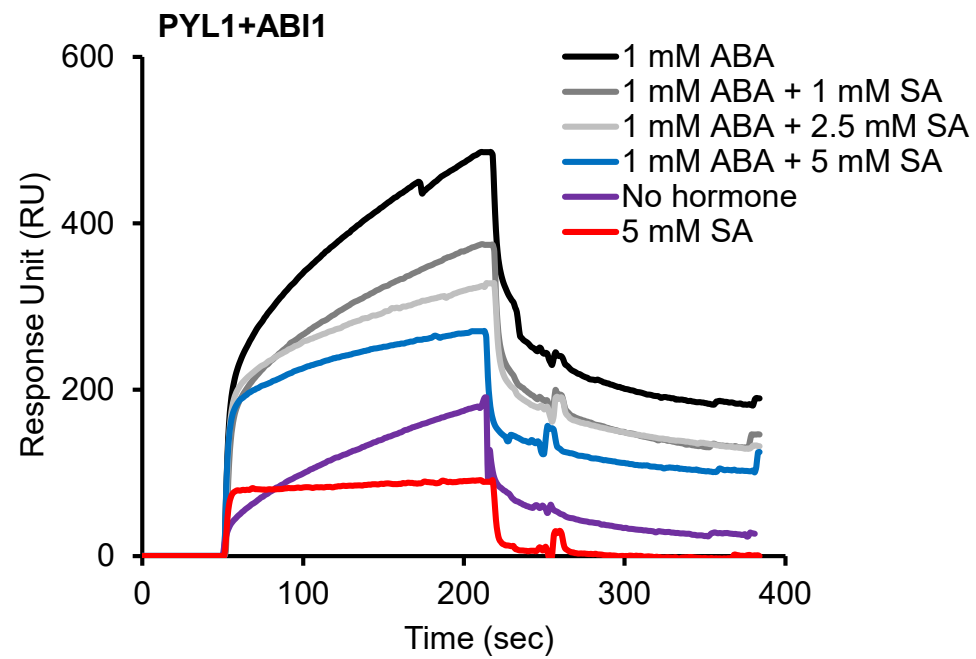
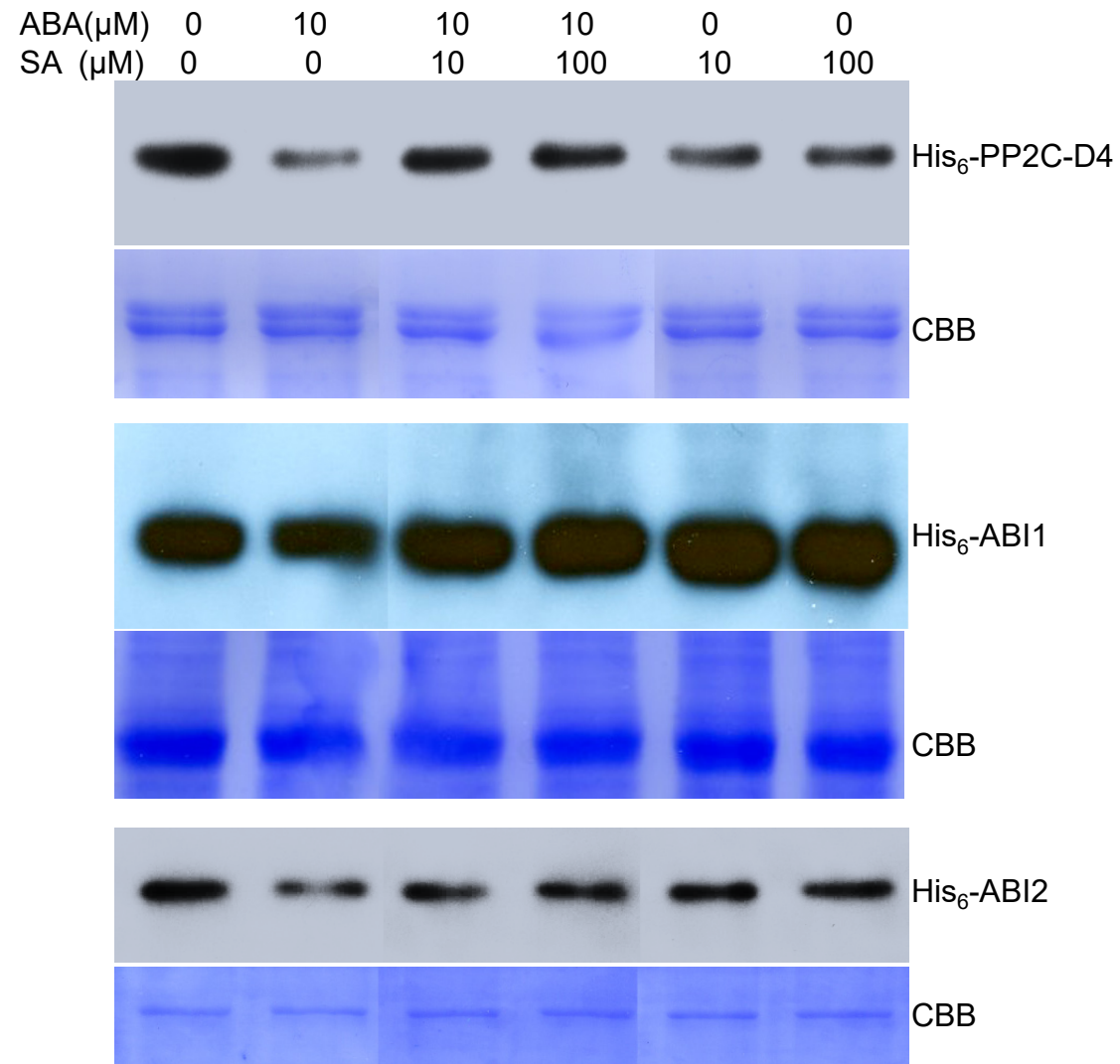
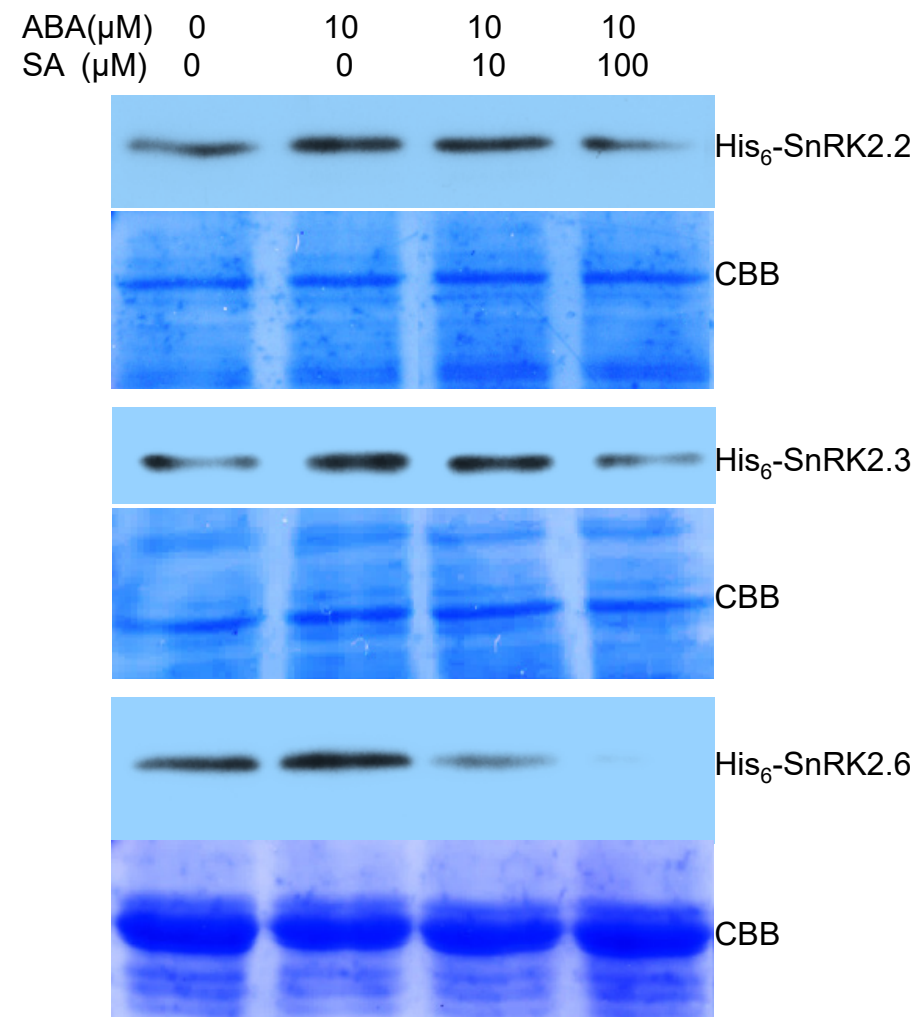


Figure 4

A.



B.



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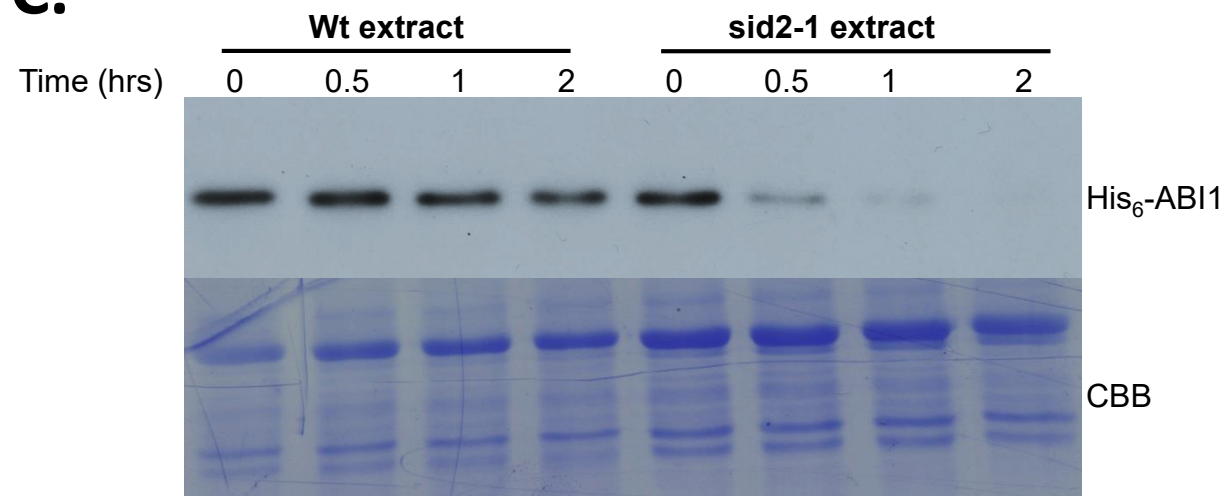
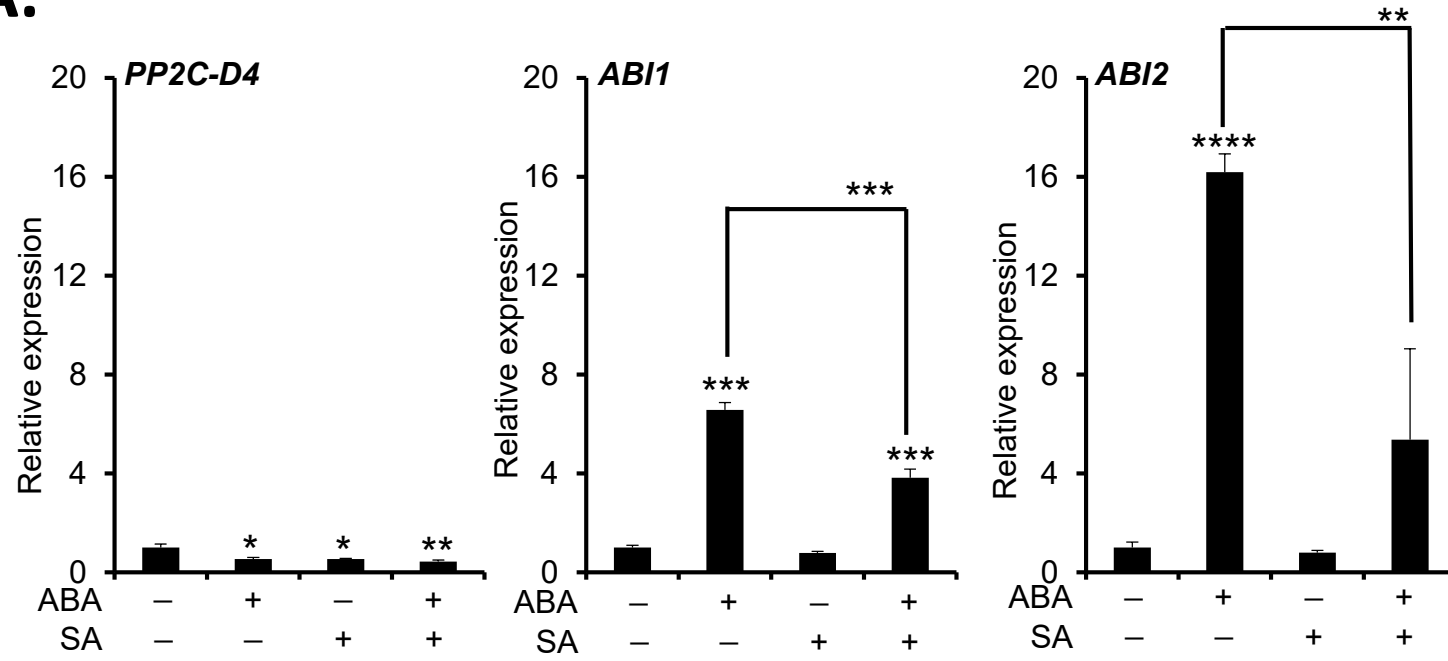


Figure 5

A.



B.

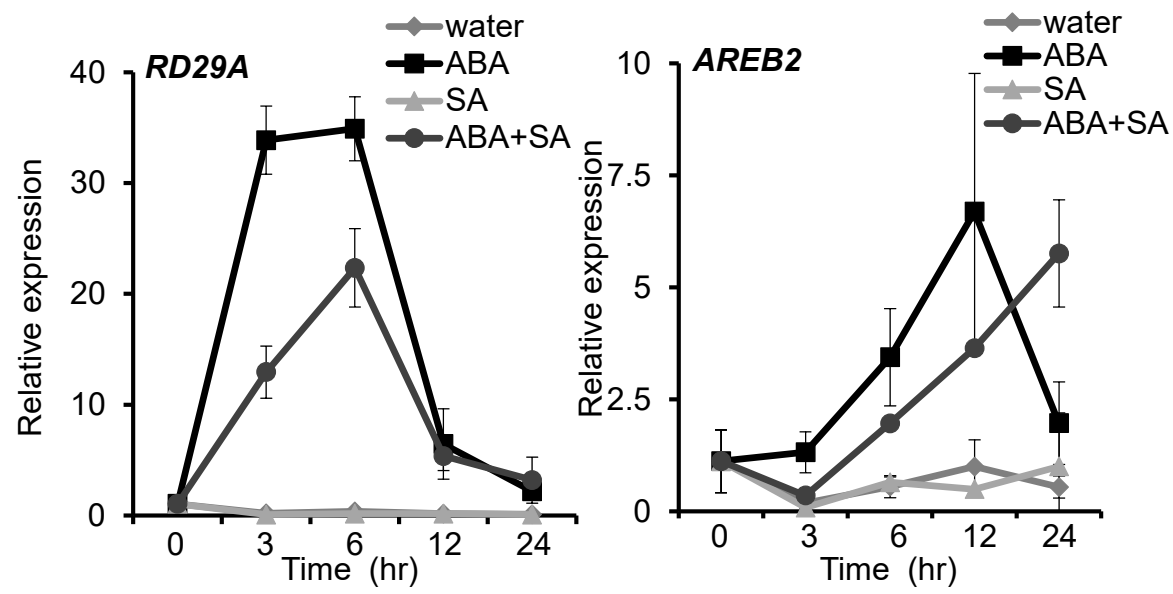
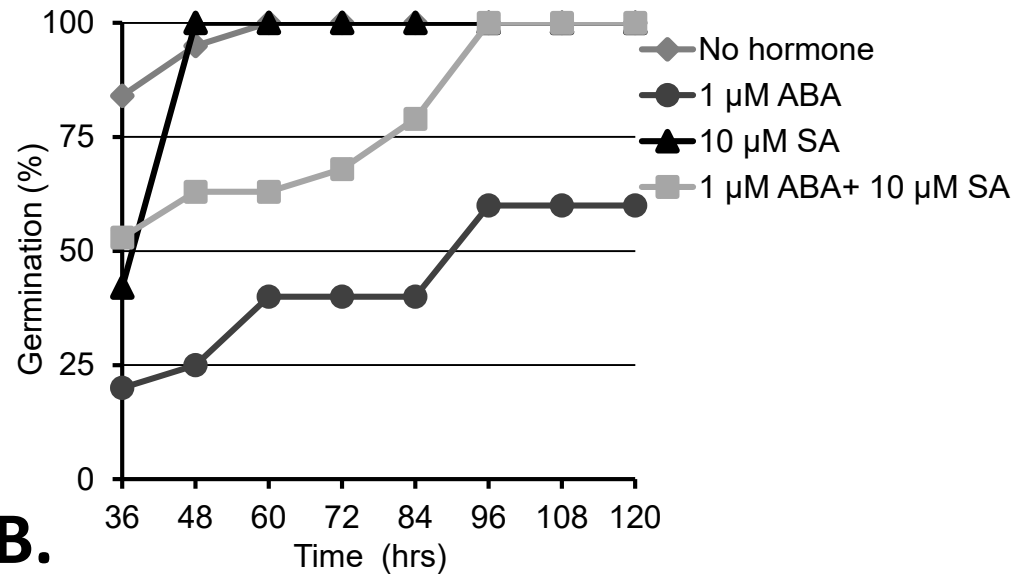


Figure 6

A.



B.

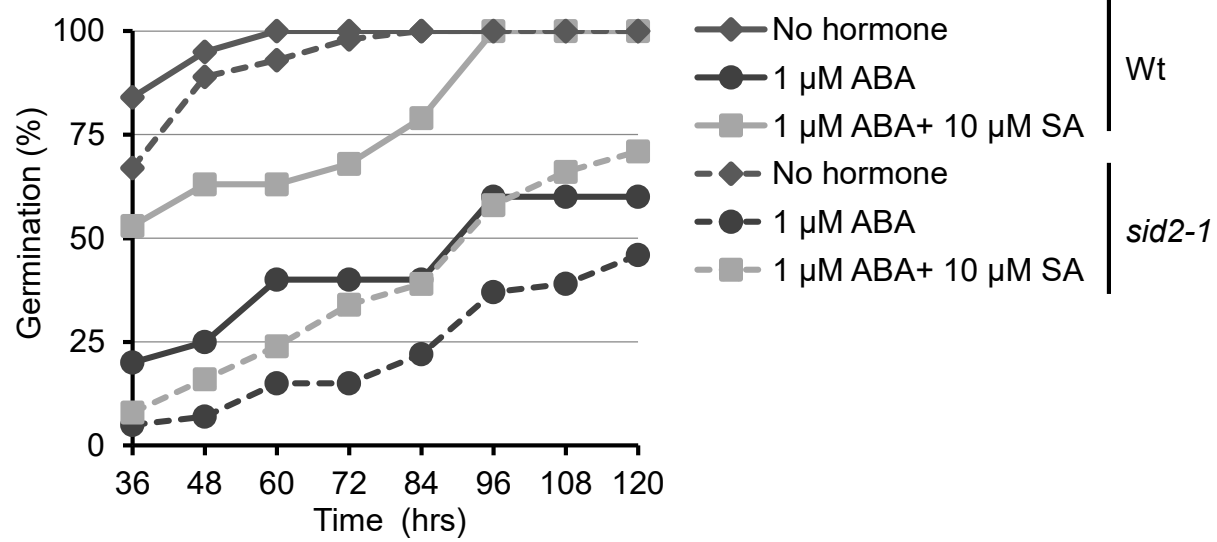
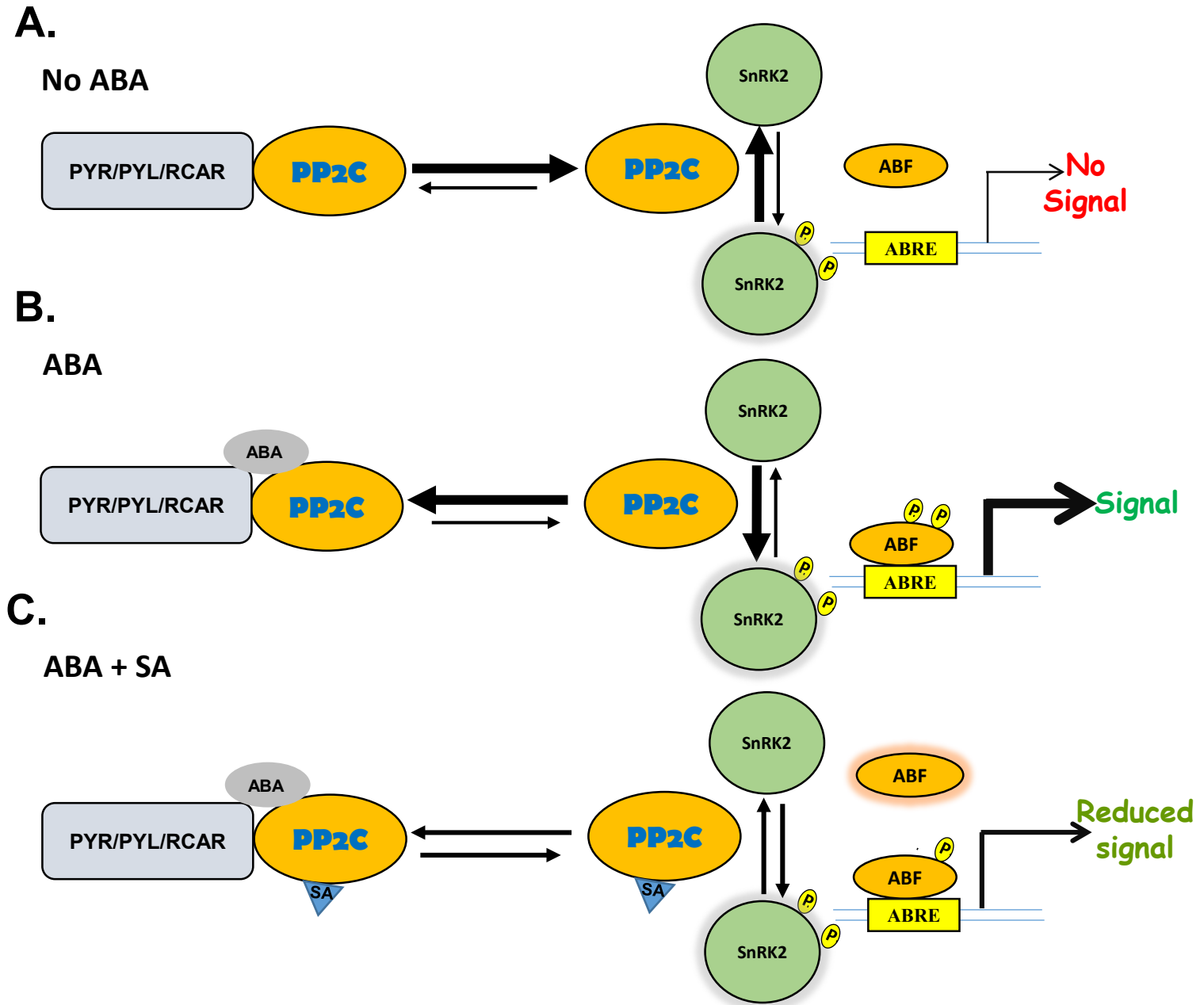
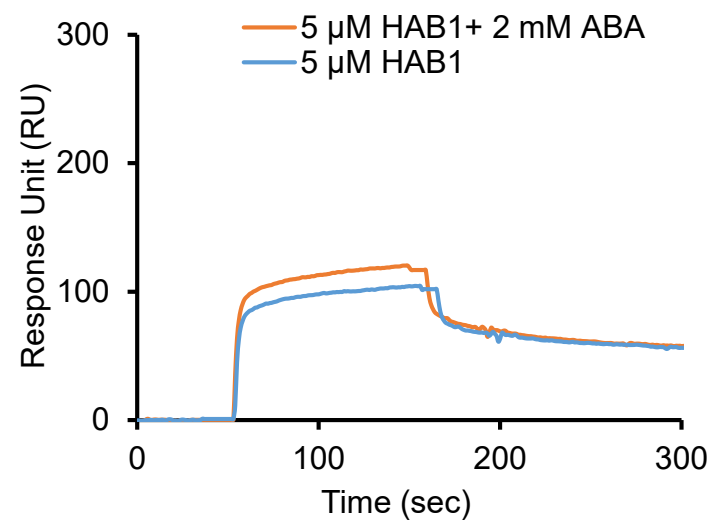


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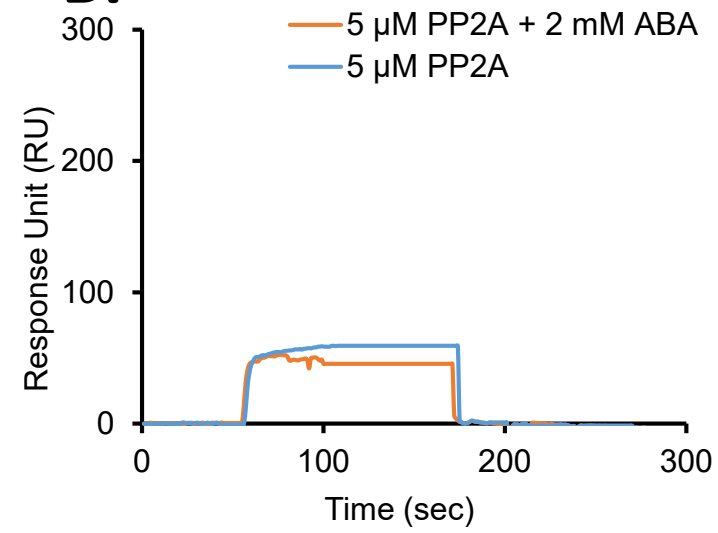


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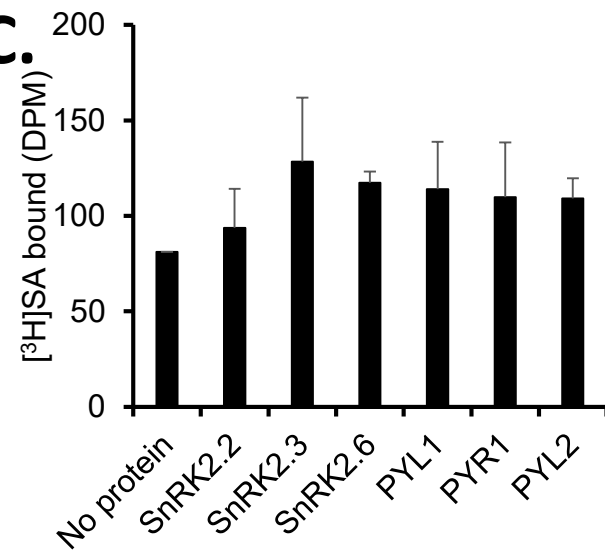
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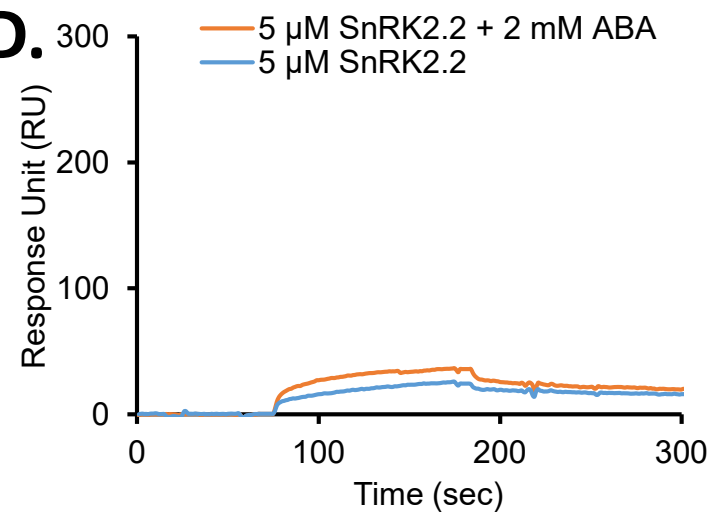
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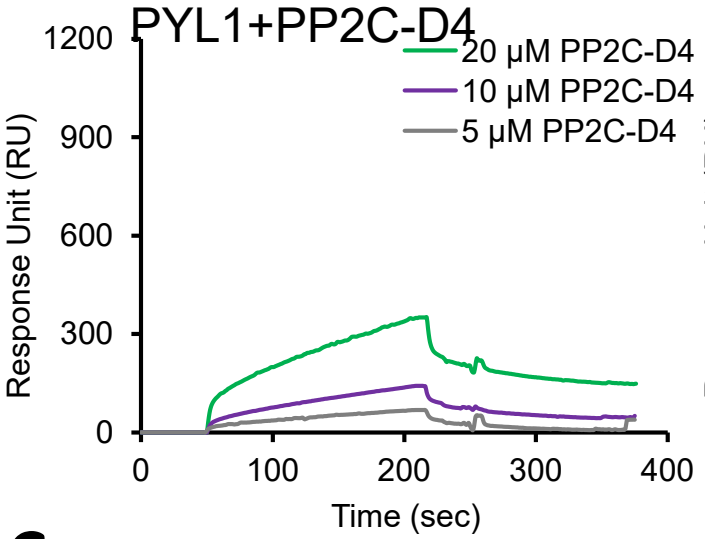


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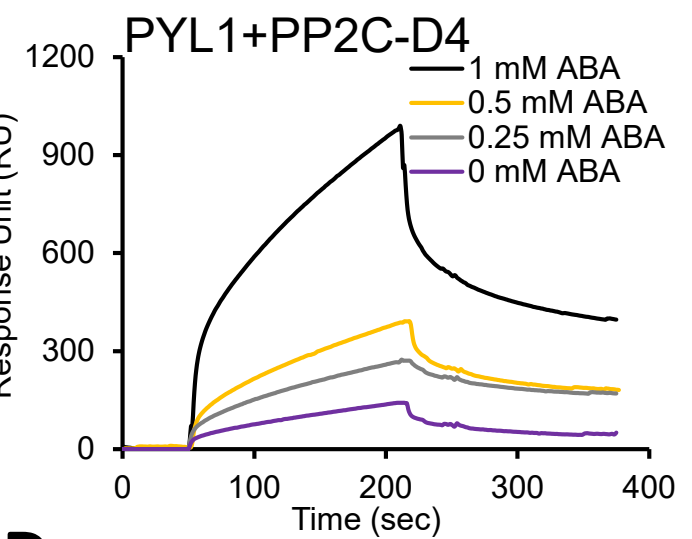


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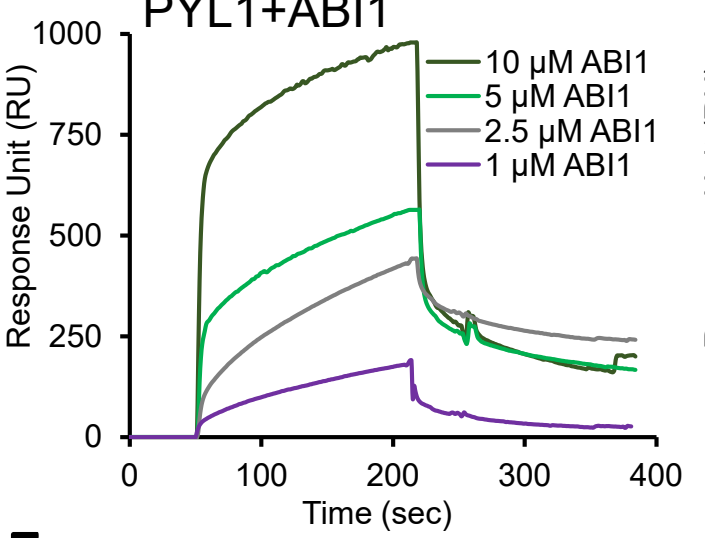
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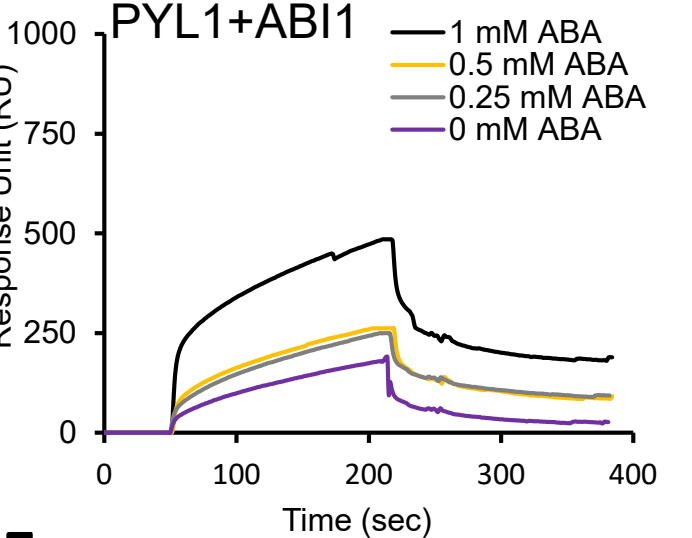
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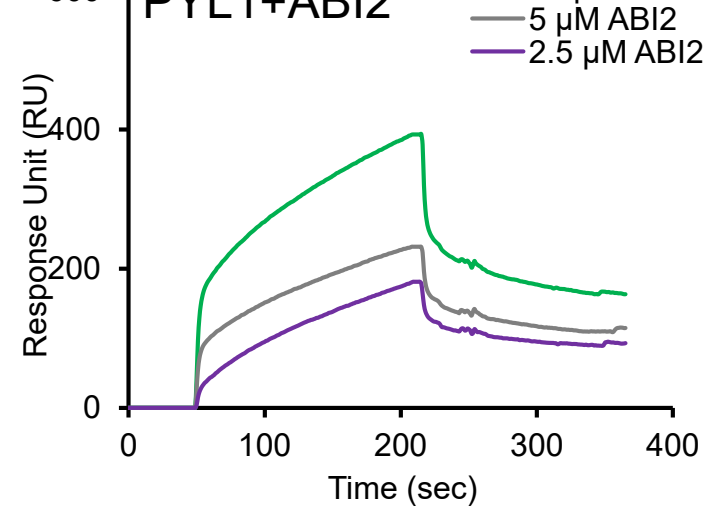
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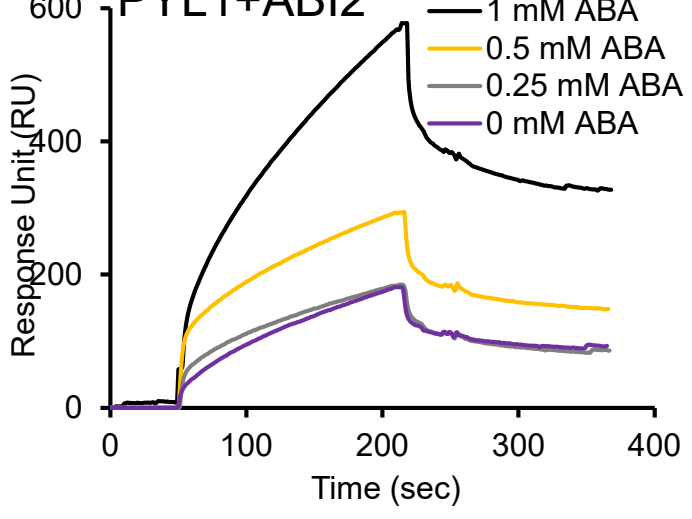
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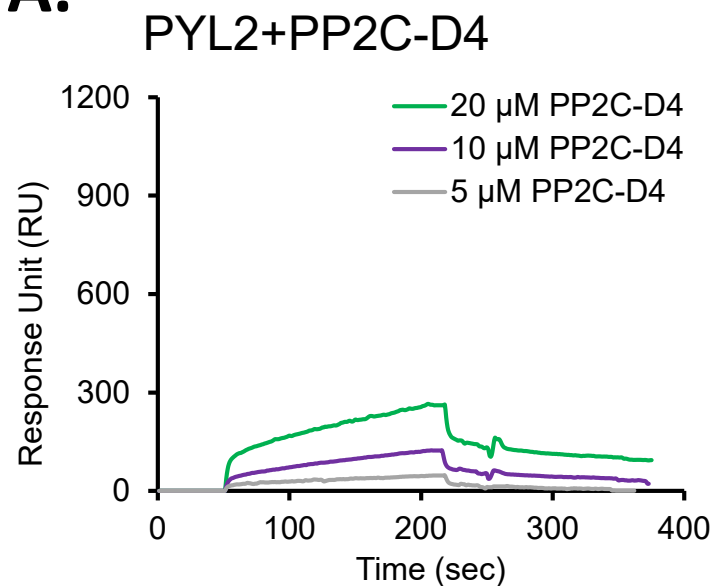
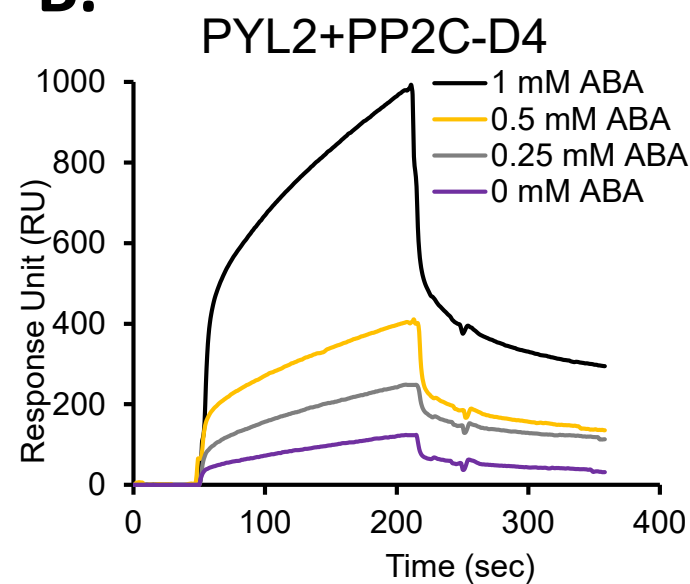
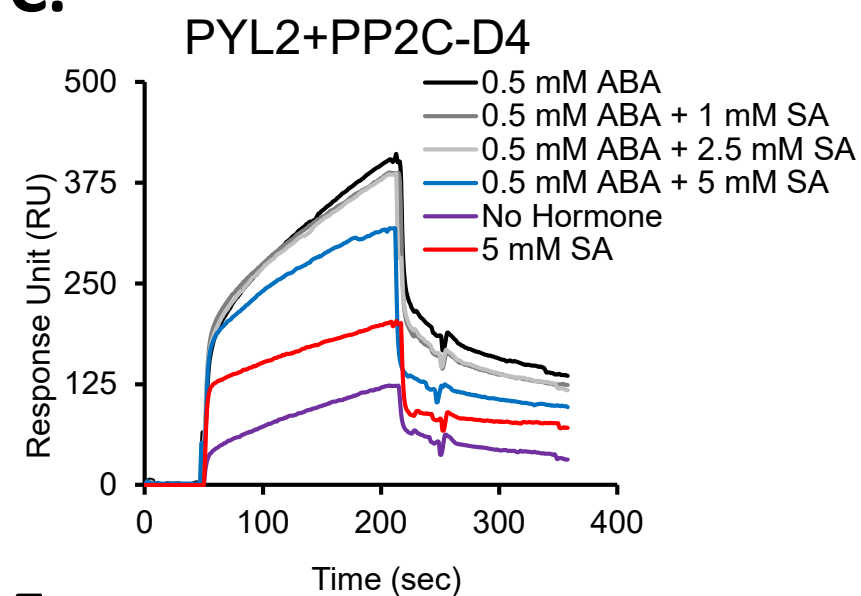
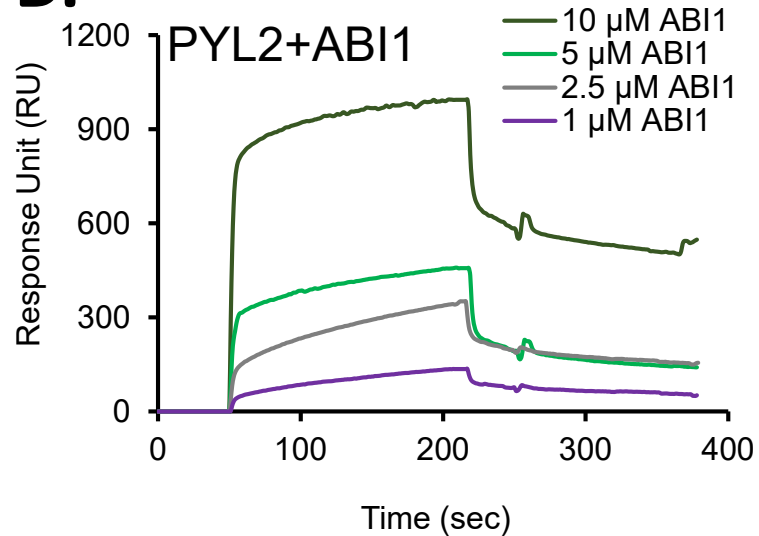
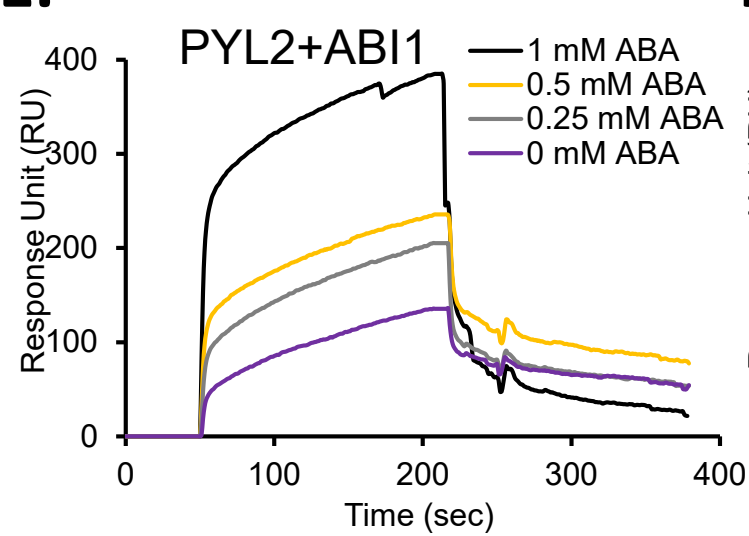
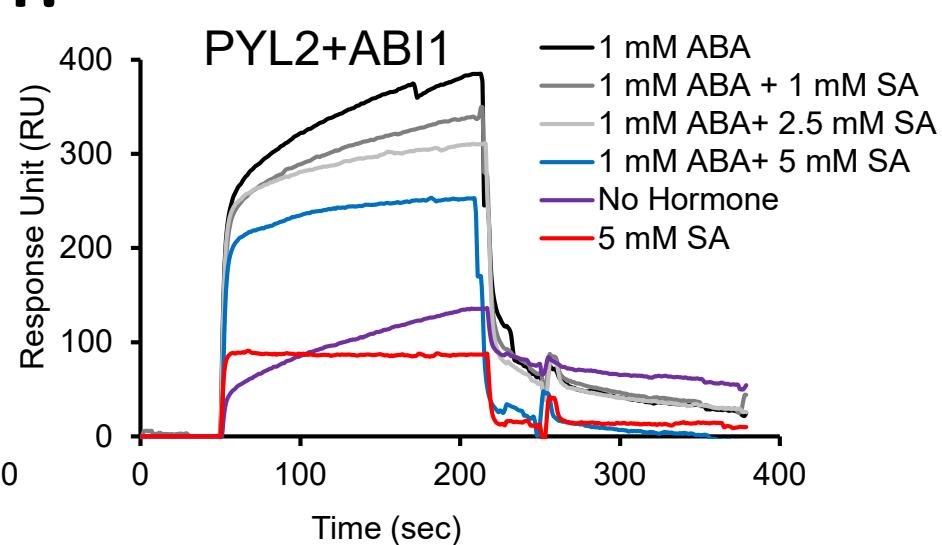
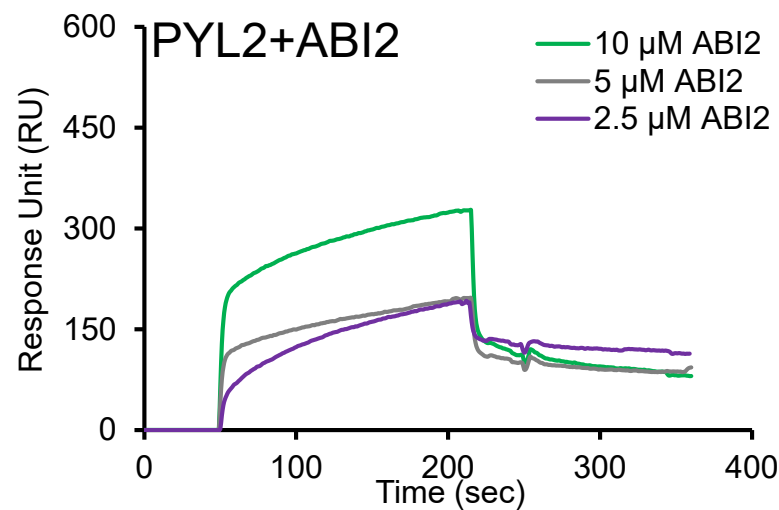
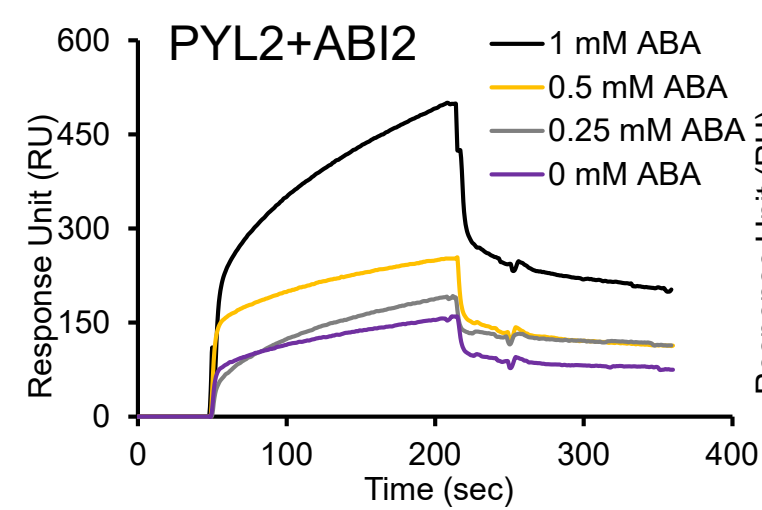
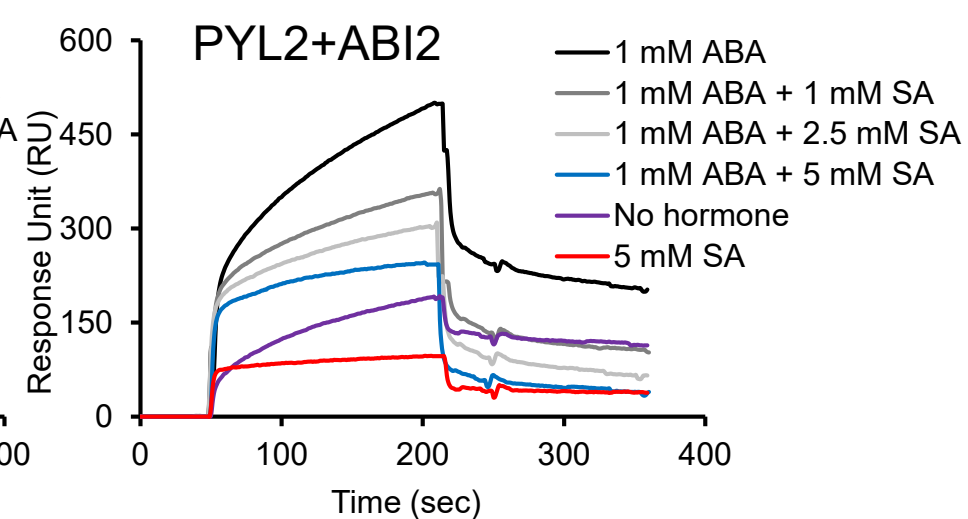
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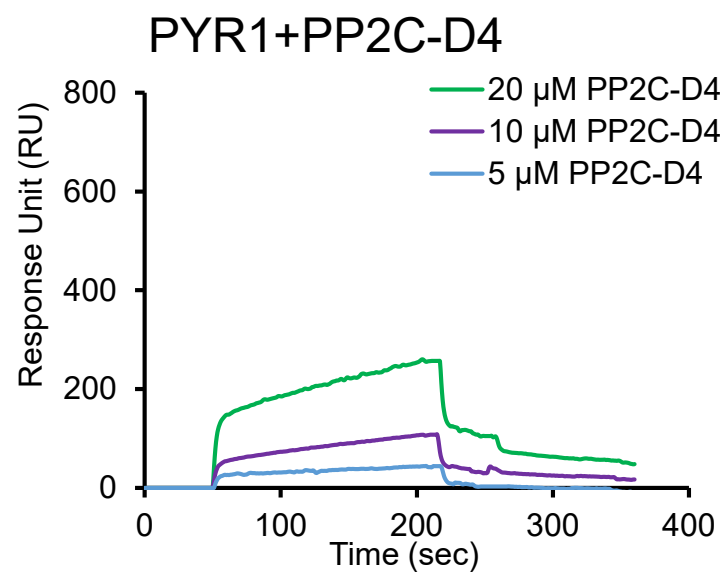


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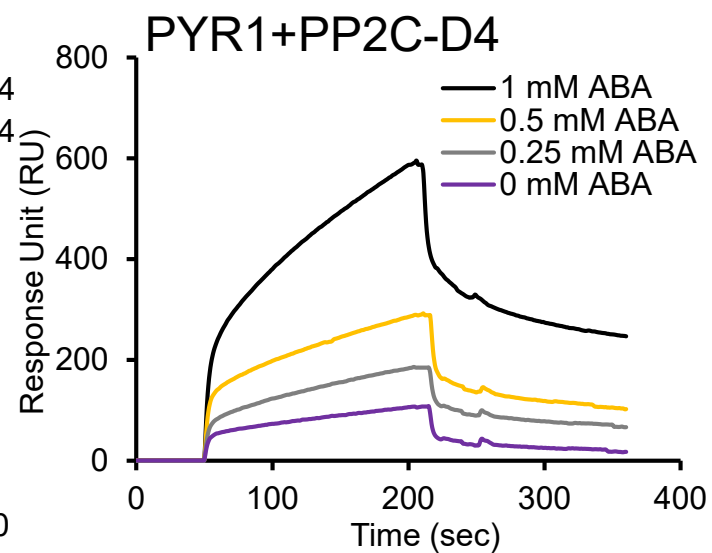
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Supporting Figure 4

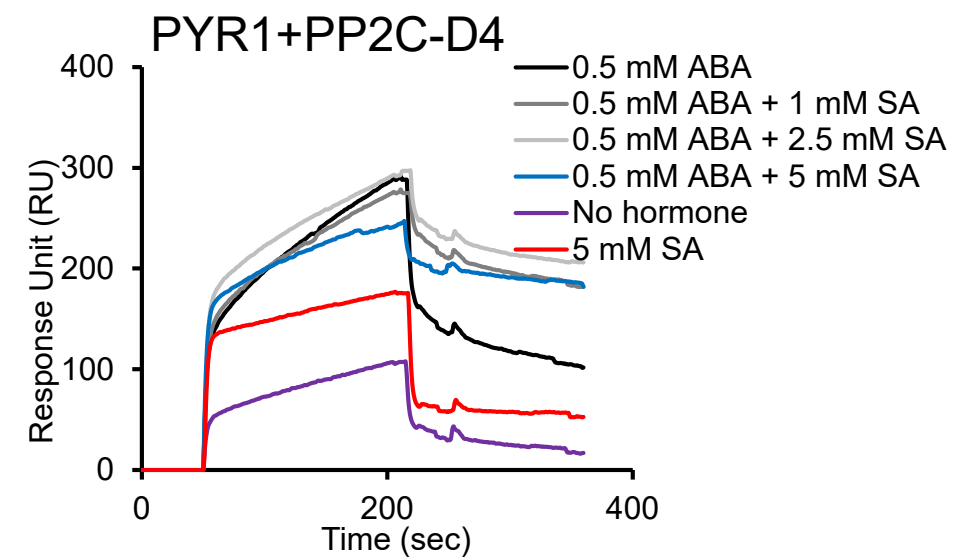
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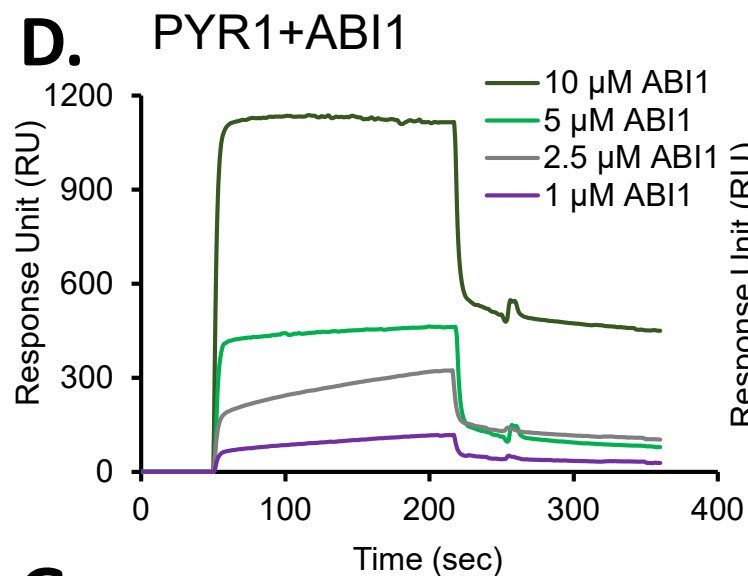
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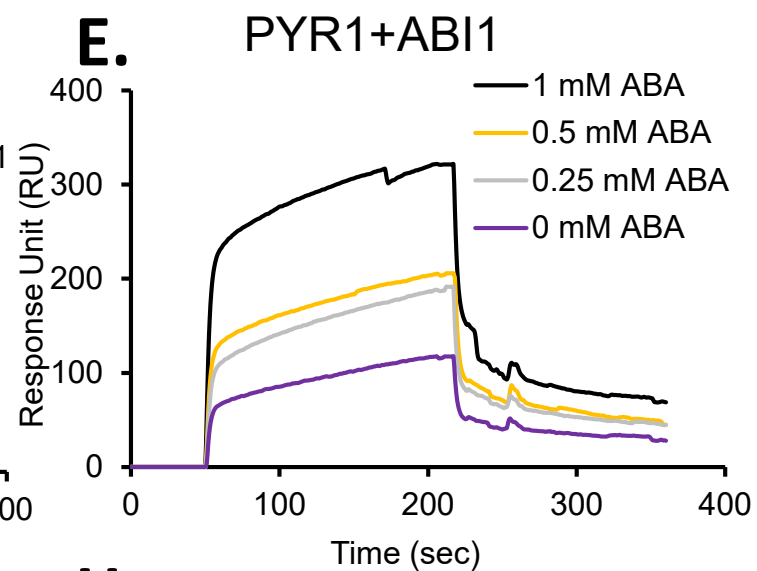
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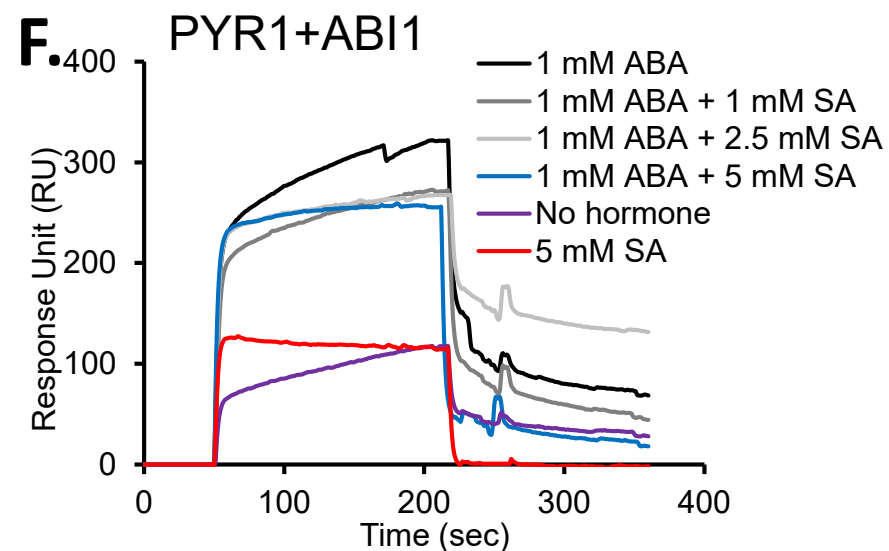
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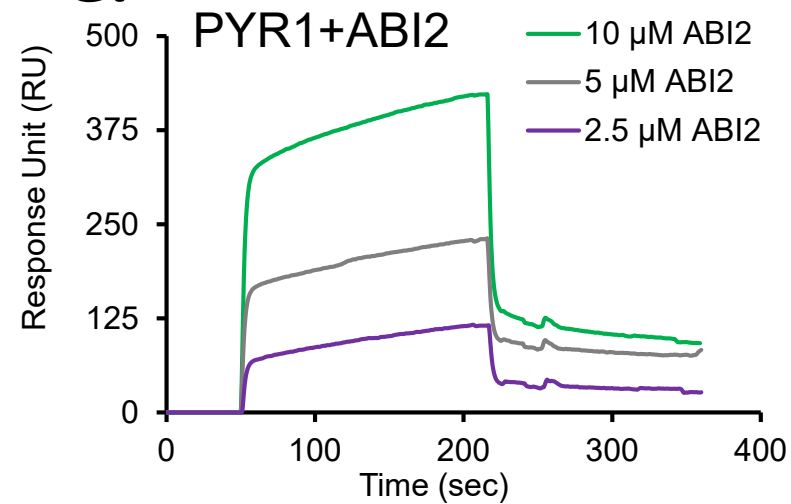
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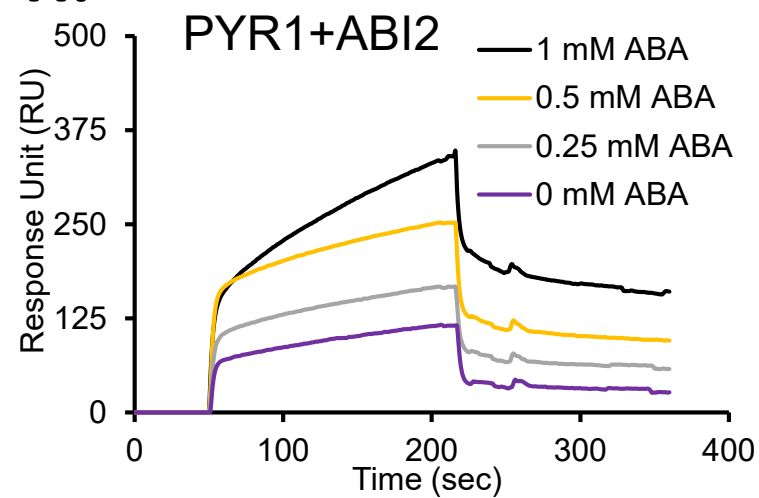
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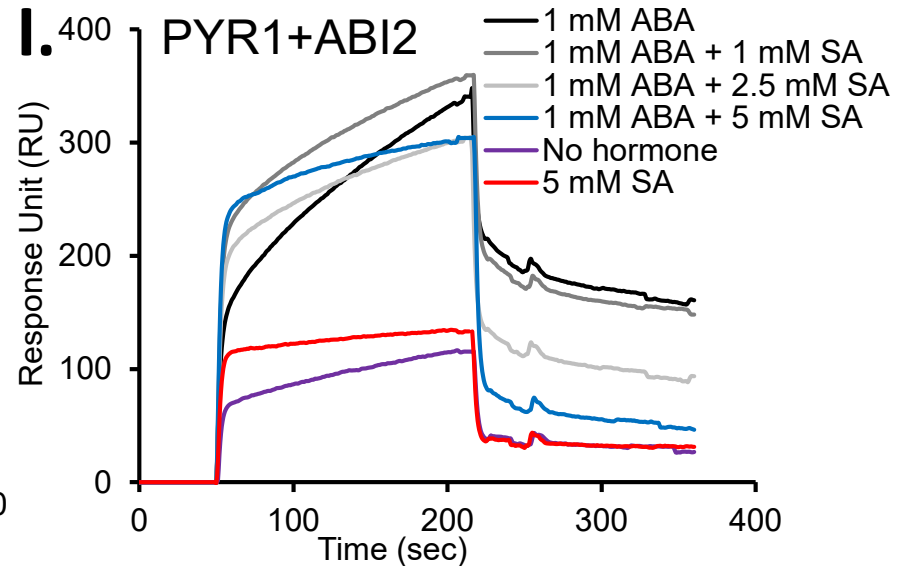
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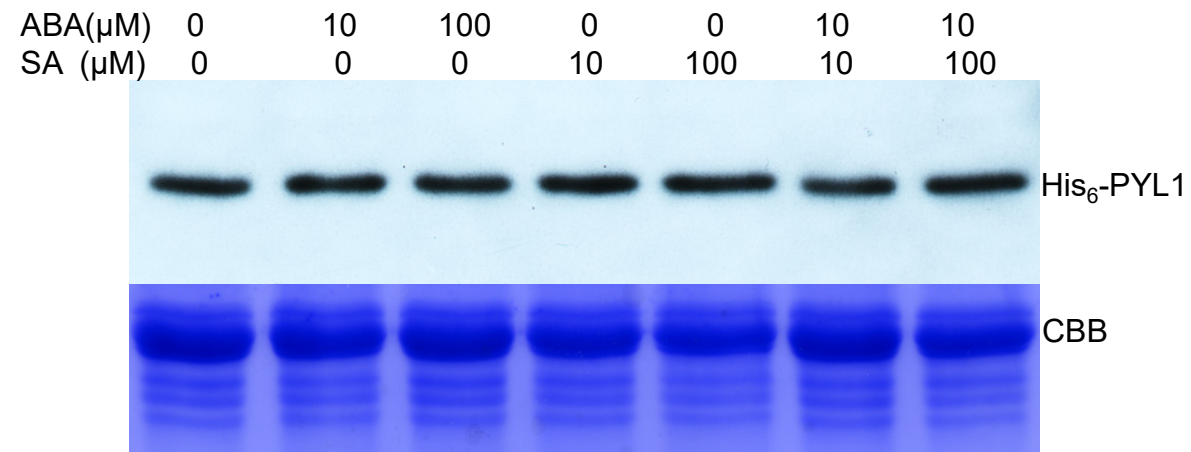
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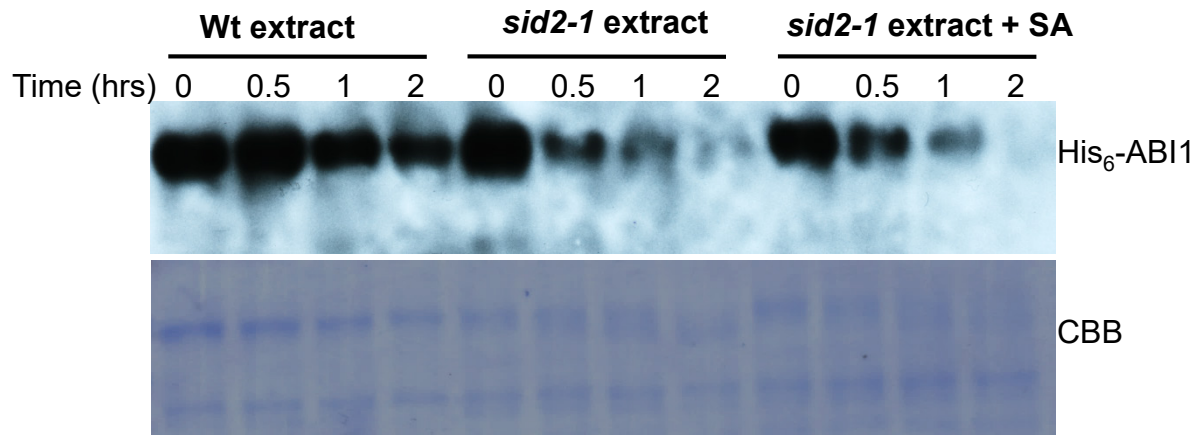


Supporting Figure 5

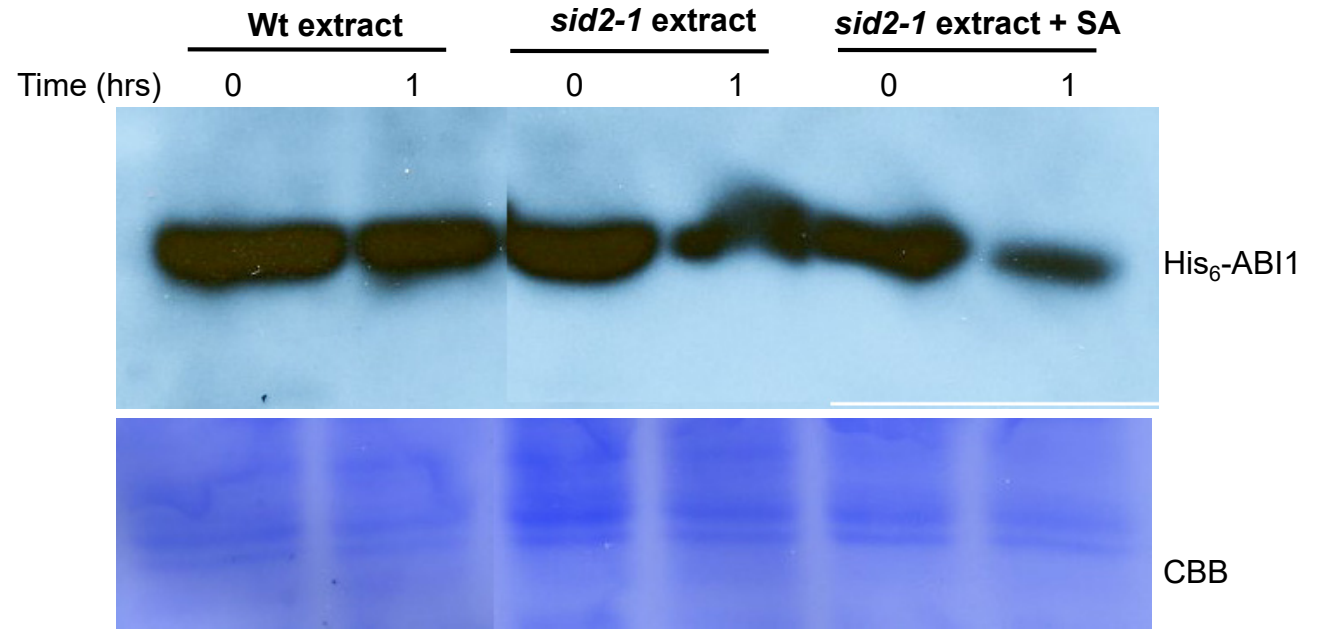


Supporting Figure 6

A.



C.



B.

