Title: ABI5 Binding Proteins are substrates of key components in the ABA core signaling pathway

Short title: SnRK2s and PP2Cs regulate AFP2 phosphorylation

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One-Sentence Summary: An inhibitor of ABA-INSENSITIVE5 is proposed to act as a hub in ABA signaling, and its stability, localization and function are regulated by changes in phosphorylation state.

Author contributions: RRF conceived the project and supervised construction of genetic lines and physiological assays; all authors performed experiments; RRF wrote the article, with contributions from BJEM and TL, and serves as the author responsible for contact.

ABSTRACT

The central components of the ABA core signaling pathway are families of receptors, clade A type 2C protein phosphatases (PP2Cs), SNF1-Related Protein Kinases (SnRK2s), and diverse sets of proteins regulated by phosphorylation via these kinases, including bZIP transcription factors such as ABA-INSENSITIVE(ABI)5. The larger network of ABA signaling factors includes additional kinases and E3 ligases that modify these components to affect their activity and stability. The ABI5-Binding Proteins (AFPs) are negative regulators of ABA response. This study shows that the AFPs interact with specific family members of all components of this pathway and are substrates for SnRK2s and PP2Cs. AFPs also interact with subsets of MAP kinases (MPKs) and 14-3-3 proteins previously found to regulate activity of the ABI5-related clade of transcription factors. Residues predicted to be phosphorylated are conserved between AFPs, but are located within regions predicted to be unstructured. ABA promotes phosphorylation of AFP2, but conditions that prevent phosphorylation of AFP2 result in decreased stability, a shift in localization toward dispersed foci, and reduced effectiveness for inhibiting ABA response at germination. Thus, AFP2 appears to be an important hub in the ABA core signaling pathway.

45 **INTRODUCTION**

46

47 The phytohormone ABA regulates diverse responses in plants, in part mediated by large changes in gene
48 expression. Although transcriptome experiments in diverse tissues at various stages of growth have
49 identified many thousands of ABA-regulated genes, very few of these are consistently ABA responsive
50 across all of these conditions (reviewed in Finkelstein, 2013). However, some loci encode core
51 components regulating these diverse responses. The central components of the ABA core signaling
52 pathway are families of receptors, protein phosphatases, protein kinases, and diverse sets of proteins
53 regulated by phosphorylation via these kinases. In the absence of ABA, PP2C clade A protein
54 phosphatases bind to SNF1-Related Protein Kinase2s (SnRK2s), sterically preventing them from becoming
55 phosphorylated and active. When ABA is present, PYRABACTIN RESISTANT (PYR/PYL)/REGULATORY
56 COMPONENT OF ABA RECEPTOR (RCAR) receptors bind and inactivate these PP2C phosphatases, thereby
57 permitting phosphorylation and activation of the SnRK2 class kinases (Cutler et al., 2010). These kinases
58 can then phosphorylate regulatory proteins such as the ABA-INSENSITIVE(ABI)5/ABA RESPONSE ELEMENT
59 BINDING FACTOR (AREB/ABF) clade of bZIP transcription factors, leading to ABA-induced changes in gene
60 expression. Several of these bZIP proteins are bound in a phosphorylation-dependent manner by 14-3-3
61 proteins, and this interaction can be required for gene activation by the bZIP factor (Schoonheim et al.,
62 2007; Camoni et al., 2018).

63

64 The clade A PP2Cs are monomeric Ser/Thr phosphatases that were initially identified genetically by
65 dominant mutations in ABA-INSENSITIVE(ABI)1 and ABI2 (Koornneef et al., 1984), resulting in ABA
66 resistance due to their inability to be inactivated (reviewed in Finkelstein, 2013). Additional members of
67 the clade, e.g. ABA-HYPERSENSITIVE GERMINATION(AHG)1 and AHG3, were identified based on loss of
68 function mutations conferring hypersensitivity to ABA, reflecting their role as negative regulators of ABA
69 response (Yoshida et al., 2006; Nishimura et al., 2007). These PP2Cs have overlapping expression patterns
70 and functions, such that double mutants are more hypersensitive than the single mutants. Interactome
71 and other studies have identified numerous substrates for these PP2Cs, including members of multiple
72 families of kinases and transcription factors involved in response to ABA and abiotic stresses (Lumba et
73 al., 2014; Yilmaz et al., 2022).

74

75 The SnRK2 Ser/Thr kinases are a plant-specific subfamily of the SnRKs, comprising 10 family members in
76 *Arabidopsis thaliana* (reviewed in Kulik et al., 2011). Although all SnRK2s function in stress responses,
77 this subfamily is further divided into three groups distinguished by their response to ABA. Group 1 does

78 not respond to ABA, group 2 shows minimal response to ABA, and group 3 is strongly activated by ABA.
79 In Arabidopsis, group 3 consists of SnRK2.2, SnRK2.3 and SnRK2.6. These kinases have partially
80 redundant functions, with SnRK2.2 and SnRK2.3 playing major roles in seed dormancy, control of
81 germination and seedling growth, while SnRK2.6 has greater effects on stomatal regulation. Triple
82 mutants defective in all three are highly resistant to ABA and exhibit extreme drought sensitivity and
83 severely stunted growth. In contrast, SnRK2.10 is a member of group 1 and responds to osmotic stress,
84 but is not part of the ABA core signaling pathway.

85 Mitogen activated protein kinases (MAPKs) are another conserved family of Ser/Thr kinases, some of
86 which regulate abiotic stress and ABA response. MAPKs are the final step in the MAPK cascade,
87 composed of MAP kinase kinase kinases (MAPKKKs/MAP3Ks/MEKKs) that phosphorylate and activate
88 MAP kinase kinases (MAPKKs/MAP2Ks/MEKs), which in turn phosphorylate/activate the MAPKs (also
89 known as MPKs) (Jagodzik et al., 2018). Over 100 genes encode members of these families in
90 *Arabidopsis thaliana,* regulating diverse processes with substantial overlaps in function. Although not
91 part of the ABA core signaling pathway, cross-talk between these pathways occurs at various levels:
92 SnRK2s phosphorylate some MAPKs (Umezawa et al., 2013), while MAP3Ks phosphorylate SnRK2s and
93 are required for their activation by ABA (Takahashi et al., 2020). MAPK cascades regulate ABA response
94 through effects on gene expression, including induction of transcription factors such as ABI3 and ABI5
95 (Lee et al., 2014), and activation or stabilization of other factors, e.g. ABI4, by phosphorylation (Guo et
96 al., 2016; Eisner et al., 2021; Maymon et al., 2022). MPK3 and MPK6 function in diverse pathways
97 including stomatal development, root growth, pollen tube growth, immune response, and response to
98 ABA.
99
100 Although not initially characterized as part of the core signaling pathway, a group of ABI5-binding
101 proteins (AFPs) identified by a yeast two-hybrid screen using ABI5 as bait were shown to be important
102 ABA-inducible negative regulators of ABA response (Garcia et al., 2008). One of these, AFP3, is among the
103 few genes that are ABA-induced in many different contexts, but especially during vegetative growth
104 (Finkelstein, 2013). In contrast, AFP1 and AFP2 are most active during germination and seedling
105 establishment. The AFPs have three highly conserved domains, including an ethylene-responsive element
106 binding factor-associated amphiphilic repression (EAR) domain in the "A domain" and a nuclear
107 localization signal in the "B domain," in addition to the C-terminal domain initially shown to interact with
108 the ABI5/ABF/AREB transcription factors (Garcia et al., 2008). Overexpression of the AFPs results in
109 extreme ABA resistance, including decreased expression of many seed maturation genes, failure to

110 acquire desiccation tolerance, and ability to germinate on 100-fold higher ABA concentrations than wild-
111 type seeds (Lynch et al., 2017).
112
113 Studies addressing the mechanism by which AFPs inhibit ABA response have demonstrated interactions
114 with co-repressors including TOPLESS/TOPLESS-RELATED (TPL/TPR) and Histone Deacetylase (HDAC)
115 subunits, suggesting effects on chromatin remodeling (Lynch et al., 2017). Although mechanistically
116 more obscure, the AFPs also interact directly with DELLA proteins, antagonizing their inhibitory effects
117 on germination (Finkelstein and Lynch, 2022). The AFPs have also been proposed to confer ABA
118 resistance by promoting ABI5 degradation, functioning as adaptors for E3 ligases that ubiquitinate ABI5
119 and possibly related bZIP proteins (Lopez-Molina et al., 2003). However, tests of the timecourse of
120 germination in the presence of low concentrations of ABA show that germination mostly precedes
121 degradation of ABI5 (Lynch et al., 2022). Furthermore, the interactions with several such E3 ligases
122 (DWA1, DWA2 and KEG1) are rather weak and loss of function mutations for these ligases do not block
123 the ABA resistance conferred by AFP2 overexpression.
124
125 Outside of the conserved domains, much of the AFP sequences are predicted to be intrinsically
126 disordered (Lynch et al., 2017; Varadi et al., 2023), raising the possibility that the AFPs can assume a
127 variety of structures due to post-translational modifications, consistent with the diversity of their
128 observed protein interactions. Furthermore, much recent work highlights the tendency of
129 intrinsically disordered regions to drive phase transitions forming bimolecular condensates (reviewed
130 in Liu et al., 2023). The current work reports interactions between AFPs and the PP2Cs, SnRK2s and
131 MPKs previously shown to regulate ABA response, and addresses the functional significance of these
132 interactions. Additional interactions with ABA receptors and 14-3-3 proteins may affect the activity
133 of signaling complexes containing some of these factors.
134
135 **RESULTS**
136
137 **Interactions between AFPs and components of the ABA core signaling pathway**
138
139 Our early studies of the AFPs revealed no obvious biochemical function, so we used AFP2 as bait in a
140 yeast two-hybrid screen to identify additional interacting partners that might provide clues regarding
141 function. This screen produced a diverse set of potential interactors including multiple ABI5/ABF/AREB
142 clade bZIP proteins, ABI clade protein phosphatases (PP2Cs), and a variety of kinases. Further specific
143 pairwise yeast two-hybrid assays, expanding this study to include all four members of the AFP family and

144 seven members of the ABI PP2C clade, showed strong interactions between all AFPs and AHG1 and
145 AHG3. When the AFPs were present as Binding Domain (BD)-fusions, additional interactions were limited
146 to either AFP1 or AFP2 for only one other PP2C tested, ABI1 (Fig.1). Interactions mapped to the C domain
147 of AFP1, but to several regions of AFP2.
148
149 Tests of interactions with kinases showed all AFPs except AFP4 interacted with SnRK2.3, SnRK2.6, and
150 MPK3, but not with SnRK2.10 (Fig.2). The BD-MPK6 fusion conferred growth without requiring an AD-
151 interaction partner, but reporter activation was again enhanced by interaction with AD-fusions to AFP1,2
152 and 3. Surprisingly, activity was impaired when BD-MPK6 was co-expressed with AFP4 or AFP2-C. Only
153 AFP2 interacted with SnRK2.2 in this assay. Interactions with subdomains were very weak, suggesting
154 that regions across the entire protein were required for these interactions.
155
156 In contrast to the strong interactions between AFPs and bZIPs, PP2Cs and kinases, relatively weak
157 interactions were observed between AFPs and a few members of the PYR/PYL/RCAR family of ABA
158 receptors: PYLs 2, 7, 9, 11 and 12 (Fig. 3A, Supp Fig. 1). All of these are expressed in maturing, dry
159 and/or imbibing seeds, coinciding with peak expression of AFP1, AFP2, and ABI5 (Supp. Fig. 2).
160 Furthermore, PYLs 11 and 12 promote ABI5-mediated ABA response during germination and their
161 expression is regulated by ABI5 (Zhao et al., 2020), and PYLs 7 and 9 are members of the small subset
162 of receptors that can interact with AHG1 (Tischer et al., 2017). This result suggests that several AFPs
163 can interact with all components of the ABA core signaling pathway.
164
165 Many phosphorylation-dependent protein activities, including hormone signaling, are regulated by 14-3-
166 3 proteins that bind to phosphorylated Ser or Thr residues within specific contexts (Camoni et al., 2018).
167 The Arabidopsis genome encodes thirteen expressed 14-3-3 proteins, designated GENERAL REGULATORY
168 FACTORS(GRFs), many of which are also expressed in maturing, dry and germinating seeds (Supp Fig 2).
169 Previous studies in multiple cereal grains and Arabidopsis have shown a role for 14-3-3 proteins in ABA-
170 regulated transcriptional complexes, including direct interactions with members of the ABI5/ABF clade of
171 bZIPs (Jaspert et al., 2011). We initially tested the Arabidopsis 14-3-3 proteins with the highest homology
172 to the barley and rice proteins shown to interact with HvABI5 and HvABF1 (Schoonheim et al., 2007), and
173 that are expressed in dry, imbibed or germinating seeds: GRF1 (14-3-3 chi), GRF2 (14-3-3 omega), GRF3
174 (14-3-3 psi), GRF4 (14-3-3 phi), and GRF7 (14-3-3 nu). AD fusions with GRF1,2,3,4, and 7 all interacted
175 weakly with a BD-AFP1 fusion, but only the AD-GRF3 and -GRF4 fusions interacted above background
176 with BD-AFP2 (Fig. 3BC). Interaction with ABI5 was substantially stronger (Supp Fig 3). Consistent with the

177 importance of phosphorylation for 14-3-3 binding, a BD-fusion to AFP2 with alanine substitution
178 mutations in 2 binding sites predicted by 14-3-3-Pred
179 (https://www.compbio.dundee.ac.uk/1433pred/)(Madeira et al., 2015) had greatly reduced interactions
180 with the GRFs (Supp Fig 3). In addition to their relatively strong interactions with AFP1 and AFP2,
181 transcripts for both GRF3 and GRF4 are present at similar levels to those of these AFPs in dry and imbibed
182 seeds (Supp. Fig. 2), so our further studies focused on interactions with these family members.
183
184 The interactions between AFP1, AFP2, PP2Cs, kinases, receptors and GRFs were further tested by BiFC
185 assays, using split YFP fusions in transiently transformed *N. benthamiana*. These studies showed
186 interactions between both AFPs and SnRK2.3, SnRK2.6, MPK3, MPK6, AHG1, AHG3, GRF3, GRF4, PYL2 and
187 PYL9, confirming the yeast two-hybrid results, but interactions with GRF7 and AHG3 were much weaker
188 in these assays (Fig.4). Although none of the 14-3-3 proteins showed any specific interaction with PP2Cs
189 or a SnRK that interacts with AFP2 in the yeast two-hybrid assay, GRF4 interacted strongly with MPK3 and
190 SnRK2.6, and very weakly with AHG1, in BiFC assays (Supp Fig 3). Further mapping of the BiFC
191 interactions between domains of the AFPs with AHG1 and SnRK2.3 showed that all AFP domains
192 interacted with AHG1 (Supp Fig 4) (Erickson McNally, 2016). In contrast, SnRK2.3 interacted with only the
193 B and C domains of AFP2, and the A and B domains of AFP1.
194
195 The BiFC assays also showed some differences in localization. Fluorescence was diffuse throughout the
196 nuclei when AFPs and kinases were co-expressed, but localized to nuclear punctae when AFPs and AHG1
197 were combined (Fig 4 and Supp Fig4). Although GRF4 was distributed throughout the cell when forming
198 homodimers, or heterodimers with GRF3 and GRF7, their interactions with AFPs were mostly limited to
199 nuclei (Fig.4), suggesting that the AFPs promote nuclear localization of these 14-3-3 proteins.
200
201 **AFPs are substrates for SnRK2s and PP2Cs**
202
203 We had previously observed that YFP-AFP2 fusions migrate as doublets and occasionally even triplets on
204 SDS-PAGE, suggesting the possibility of post-translational modifications (Erickson McNally, 2016). These
205 doublets are especially pronounced in any fusions containing the B domain of AFP2 (Fig. 5A). To
206 determine whether the observed interactions between AFP2 and these kinases and phosphatases
207 reflected a substrate role of AFP2, these proteins were transiently co-expressed in *N. benthamiana*. Co-
208 expression with SnRK2.3 shifted the YFP-AFP2-AB fusion to predominantly the slower mobility form,
209 whereas co-expression with AHG1 or AHG3 resulted in a shift toward the higher mobility form (Fig.5B).
210 YFP-AFP2-AB affinity purified with GFP-trap beads cross-reacted with an anti-phosphoserine antibody,

211 and this reaction was enhanced in the slower migrating form, confirming that the altered mobility was
212 due at least in part to phosphorylation (Fig.5C).
213
214 AFP2 contains 40 serines and 22 threonines, constituting nearly 18% of the total amino acids in AFP2,
215 many of which are conserved between AFP1 and AFP2 (Supp. Fig.5). Of these, 17 serines and 11
216 threonines were predicted to be phosphorylated by the PhosPhAt database (Zulawski et al., 2012), but
217 only 9 serines and 1 threonine are predicted at high stringency by http://musite.sourceforge.net/ (Table
218 1). PhosPhAt lists only three documented phosphorylated residues, following ionizing radiation stress:
219 T76, S232 and S234 (Roitinger et al., 2015). Of these, only T76 was predicted by both programs. Based on
220 the consensus target sites of R-X-X-S and S-D for ABA-induced phosphorylation by SnRK2s (Wang et al.,
221 2013), potential targets in AFP2 are S85, S112, S150, S187, and S259. Of these, only S85 and S112 are
222 conserved between AFP1 and AFP2. Similarly, two consensus sequences, P-X-S/T-P and S/T-P, are highly
223 abundant in protein substrates of MAPKs (Seger and Krebs, 1995) such that potential targets of MAPKs
224 are T76, S221 and T319 of AFP2, all of which are conserved between AFP1 and AFP2. Three consensus
225 motifs have been proposed for 14-3-3 target binding sites, two of which are present in AFP2: S112 fits
226 the mode I consensus and both S346 and T347 fit the C-terminal mode III consensus (reviewed in Camoni
227 et al., 2018). The 14-3-3 Pred program scores S112 and S85 as highly likely binding sites, with relatively
228 moderate scores for T16, S38, and S259.
229
230 We took three approaches to identifying actual phosphorylation sites: *in vivo* phosphorylation in *N.*
231 *benthamiana*, *in vitro* phosphorylation with purified recombinant proteins, and site-directed mutagenesis
232 of predicted phosphorylation sites. The *in vivo* approach transiently co-expressed YFP-AFP2 and Myc-
233 SnRK2.3, then used GFP-trap beads and size fractionation by SDS-PAGE to enrich the YFP-AFP2 fusion for
234 mass spectrometric analysis. Multiple runs generated a diverse set of possible phosphorylated residues,
235 possibly reflecting activity of endogenous kinases in addition to the transiently expressed SnRK2.3.
236 Consequently, we focused on specific target sites of SnRK2s by incubating recombinant His6- AFP2-AB
237 (aa1-149) fusions with GST-SnRK2.6 *in vitro*, resulting in a shift of the His-AFP2-AB fusion to a higher
238 apparent molecular weight (Fig. 5D). Mass spectrometric analysis of this slower mobility form confirmed
239 that S112 was phosphorylated (Supp Fig 6). *In vitro* kinase assays including gamma-labeled ATP,
240 comparing wild-type to either S112A or phosphomimetic S112E or S112D mutants of His6-AFP2-AB,
241 showed that phosphorylation by SnRK2.6 was still possible until the S85 was also converted to alanine
242 (Fig. 5E). The phosphomimetic mutants appeared to have an intermediate degree of phosphorylation

243 between the wild type and S112A forms, suggesting that true phosphorylation at S112 affected the
244 likelihood of additional phosphorylation. In contrast, a His6-AFP2-C fusion (aa-149-348) was not
245 phosphorylated by SnRK2.6 (Supp Fig 7A).
246
247 Immunoblot analyses of possible *in vivo* phosphorylation by Myc-MPK3 showed either no shift in mobility
248 for the YFP-AFP2-AB domain fusion or an apparent compression to the higher mobility form for the YFP-
249 AFP2-BC domain fusion (Fig.5F). Surprisingly, although neither the S85A, S112A double mutant nor the
250 T76A, S85A, S112A triple mutant of the His6-AFP2-AB domain fusion could be phosphorylated by SnRK2.6
251 *in vitro* (Fig 5G), only the double mutant ran as a single high mobility form when expressed as a YFP-
252 fusion *in vivo* (Fig.5H). The triple mutant ran at the same mobility as wild-type when expressed as a YFP
253 fusion *in vivo*. The T76A mutant of the His6-AFP2-AB domain fusion showed a slight shift toward the
254 lower mobility form, relative to the wild-type fusion (Fig. 5H). However, similar to the wild-type fusion, its
255 mobility was not altered by co-expression with Myc-MPK3 (Supp. Fig.7B).

256 *In vitro* kinase assays with His6-AFP2-BC domain fusions showed that an S112A substitution greatly
257 reduced phosphorylation of this domain by SnRK2.6 (Fig.5I). Similarly, S112A mutations blocked *in vivo*
258 phosphorylation of YFP-AFP2-BC fusions by SnRK2.3 when transiently co-expressed in *N. benthamiana*
259 (Supp Fig.8A). Phosphorylation of S112A in the *in vitro* reactions was not affected by phosphomimetic
260 mutations in S221 or T319 ("2xD"), but these mutations reduced mobility of the fusion (Fig. 5I).
261 Transiently expressed YFP-AFP2-BC mutant fusions converting S112, S259, S221 or T319 to alanine all still
262 appeared as doublets in immunoblot analysis, as did double mutants combining predicted SnRK2 targets
263 (S112A, S259A) or potential MPK targets (S221A, T319A) (Fig. 5J). However, both single and double
264 mutants affecting S221 and T319 reduced the shift toward lower mobility, and both double mutants were
265 under-accumulated relative to the wild type. The triple mutant (S112A,S221A,T319A) fusion was rarely
266 detectable. In contrast, YFP-AFP2-BC fusions with phosphomimetic mutations converting S221 alone or in
267 combination with S112 and T319 to aspartic acid all resulted in compression to a single intermediate
268 mobility form (Fig.5K). Co-expression with MPK3 also converts most of the wild type AFP2-BC fusion
269 toward higher mobility forms, but has little effect on the S221A,T319A mutant which already displays a
270 higher mobility than the wild type (Fig.5F,K). In contrast to the higher mobility on Laemmli SDS-PAGE of
271 AFP2-BC co-infiltrated with MPK3, analysis on Phos-tag gels showed a shift toward decreased mobility,
272 reflecting increased phosphorylation (Supp Fig 8B). The S221A mutation alone blocked the
273 phosphorylation seen in the wild-type BC domain, but a phosphorylated form reappeared in the
274 S221A,T319A mutant. Although the S221A,T319A mutant migrated similarly to the wild-type BC domain,

275 co-expression with MPK3 actually resulted in the appearance of a novel phosphorylated form, visible in
276 both gel systems, suggesting that loss of both “consensus” MPK targets led to phosphorylation of an as-
277 yet unknown alternative residue, possibly by an unidentified endogenous kinase.
278
279 Given that ABA activates the group 3 SnRK2s, we tested whether these mutations affected any post-
280 translational modifications induced by ABA treatment. Transiently expressed wild type YFP-AFP2-AB
281 domain fusions shifted toward slower mobility forms after 4 hrs exposure to 30 µM ABA, but the S112A
282 mutation alone, or in combination with T76A and S85A (“3xA”), blocked this shift (Fig. 6A). Similarly, the
283 highest mobility form of the YFP-AFP2-BC domain fusion shifted to an intermediate slower mobility
284 following ABA treatment and this was blocked in the S112A,S259A mutant. In contrast, the S221A,T319A
285 mutant continued to show a shift of the highest mobility form in response to ABA, but had greatly
286 reduced expression of the slowest mobility form under any condition. In stable transgenic Arabidopsis
287 lines carrying the wild-type YFP-AFP2-BC domain fusion, this shift in mobility was induced by exposure to
288 as little as 1 µM ABA for 6 hrs or by 1 hr of treatment with 20 µM ABA (Supp Fig 9AB). Similar to the
289 transient expression assays, only those lines with the S112A mutation blocked the shift to slower mobility
290 (Fig. 6B), suggesting that this residue is modified in response to ABA treatment. Phos-tag gel analysis
291 showed that the ABA-induced shift in mobility correlated with S112-dependent changes in
292 phosphorylation (Supp Fig 8C), but surprisingly, “phosphomimetic” Asp substitutions at S112, S221, and
293 T319 did not prevent phosphorylation of this fusion. A similar mobility shift of the YFP-AFP2-BC domain
294 fusion was seen in response to salinity stress imposed by at least 50 mM NaCl (Fig.6C).

295 Comparison of transcript levels for wild-type vs. mutant fusions in tissue harvested in parallel to that used
296 for protein extracts showed that levels were somewhat variable for all constructs, as expected for a
297 transient system, but the reductions in protein levels of the mutant forms were far greater than any
298 changes in transcript levels (Supp Fig 10). Although some transiently expressed transgene transcripts
299 were two- to three-fold lower for the mutant constructs, this was not sufficient to explain the at least
300 ten-fold decreases in YFP-AFP2-AB protein or the five- to ten-fold decreases in YFP-AFP2-BC protein
301 fusions. Stable transformants carrying the S221A,T319A mutant YFP-AFP2-BC fusion also showed at least
302 five-fold less protein despite a similar transcript level as the wild-type fusion. Similarly, the YFP-AFP2-
303 BC(S112A) mutant form showed a roughly four-fold reduction in protein/transcript in stable
304 transformants. All of these results are consistent with possible defects in stability or translation of the
305 fusions.
306

307 **Functional tests of physiological significance of phosphorylation**
308
309 To further address the functional relevance of these differences in phosphorylation, we tested stability
310 and potential proteasomal degradation by assaying accumulation following treatment with either the
311 translation inhibitor cycloheximide (CHX) or the proteasome inhibitor MG132. In transient expression
312 assays, the highest mobility form of the AB domain decreased in CHX, resulting in a shift toward a higher
313 fraction in the lower mobility form, but the high mobility form was stabilized by MG132 (Fig.6D),
314 consistent with proteasomal degradation of the high mobility/unphosphorylated form. This preferential
315 stability of the low mobility form was still seen in the T76A mutant, but was reduced in all the mutant
316 fusions affecting SnRK2 targets: S85A, S112A, and the T76A,S85A,S112A (3xA) triple mutant, consistent
317 with a role for SnRK2-mediated phosphorylation in regulating protein stability. The S85A mutant
318 appeared to have an even higher mobility than the wild-type fusion forms (Fig.5H,6D).
319
320 The YFP-AFP2-BC domain fusions were tested in both transient and stably transformed tissue. All stable
321 transformants showed reduced fusion accumulation following CHX treatment and increased
322 accumulation in MG132-treated seedlings (Fig. 6E). The fusion combining S221A and T319A mutations
323 had severely decreased accumulation in both stable and transiently transformed tissue (Fig. 6E and
324 Supp.Fig.10). ABA treatment also increased accumulation of the fusions and this was especially
325 pronounced in the S221A,T319A transgenic lines, indicating that this effect did not depend on
326 phosphorylation at either of these residues (Supp Fig 11). Although the double mutants were stabilized
327 by MG132 treatment, this treatment was not sufficient to increase accumulation of the S112A, S221A,
328 T319A (3xA) mutant in transiently transformed tissue (Supp.Fig.12). Furthermore, the limited YFP-AFP2-
329 BC(3xA) fusion visible was present in only a small fraction of cells, mostly in small punctae throughout
330 the cytoplasm, not concentrated in the nuclei as for all other YFP-AFP2 fusion constructs.
331
332 Having observed that co-expression of nYFP-AFP fusions with cYFP-AHG1 led to formation of nuclear
333 punctae in the BiFC assay, we tested whether mutations preventing phosphorylation would lead to
334 similar changes in localization. Whereas transiently overexpressed full-length YFP-AFP2 fusions appear
335 mostly diffuse through nuclei, but generally excluded from nucleoli, YFP-AFP2-AB domain fusions display
336 as dense bundles of foci (Fig.7). YFP-AFP2-AB(S112A) and (3xA) mutants appear to form fewer foci.
337 Although not uniform across the leaf, the two double mutants of the YFP-AFP2-BC domain fusions tend
338 toward more discrete areas of accumulation, appearing either more excluded from some regions
339 (S112A,S259A) or more focused in a few foci (S221A,T319A), consistent with effects of phosphorylation

340 state on changes in aggregation state.
341
342 Complexes of signaling proteins may be scaffolded by 14-3-3 proteins such as the GRFs, which recognize
343 phosphorylation in several consensus motifs. The S85 and S112 residues of AFP2 are both SnRK2 target
344 sites and predicted binding sites for 14-3-3 proteins. Similar to the yeast two-hybrid result, comparison of
345 BiFC assays with GRF4 and either wild type AFP2 or a S85A,S112A mutant show greatly reduced
346 interactions with the mutant (Fig.7). However, GRF4 still interacts with the S112A mutant of the AFP2-BC
347 domain, consistent with the potential for additional 14-3-3 binding sites in this domain.
348
349 We previously showed that overexpression of YFP fusions to either full-length AFPs or just the BC
350 domains were sufficient to confer resistance to ABA inhibition of germination in transgenic Arabidopsis,
351 but AB-domain fusions had almost no effect (Lynch et al., 2017). The full-length AFP2 fusions conferred up
352 to 100-fold reductions in ABA sensitivity and produced desiccation intolerant seeds, while the AFP2-BC
353 domain fusions conferred up to 30-fold reductions in ABA sensitivity and did not impair seed viability.
354 Arabidopsis carrying transgenes with mutations either blocking phosphorylation or creating
355 phosphomimetics were tested for effects on ABA sensitivity of germination and AFP protein stability.
356 Although overexpression of full-length AFP2 with a variety of mutations permitted germination on up to
357 50 µM ABA, lines carrying phosphomimetic (S85E,S112E) or non-phosphorylatable (S85A,S112A) mutant
358 YFP-AFP2 fusions differed from those with the wild-type fusion in that seedling greening was still greatly
359 impaired by ABA (Fig. 8 and Supp Fig 13). The poor viability of seeds and seedlings expressing the full-
360 length YFP-AFP2 resulted in partial silencing of these transgenes over generations, and led us to focus on
361 AFP2-BC domain fusions for additional mutant analyses. When overexpressed in a wild-type background,
362 all YFP-AFP2-BC domain fusions conferred similar resistance up to 10 µM ABA, but the mutants with
363 alterations in the residues predicted to be phosphorylated by MPKs were less effective than the wild-type
364 fusion in permitting germination on ABA higher than 30 µM (Fig.8). Comparison of protein levels showed
365 that only the S221A,T319A mutant had reduced fusion protein accumulation, but accumulation could be
366 increased by MG132 treatment (Fig.6E). The well-expressed transgenes from the wild-type background
367 were backcrossed into the *afp2* mutant to create isogenic lines with similar transgene expression levels
368 (Supp Fig. 9C), resulting in similar resistance to ABA as seen in the wild-type background (Fig. 8).
369
370 Germination *sensu stricto* is defined as complete when the radicle emerges from the seed coat, and is
371 usually followed by cotyledon greening during seedling establishment in the light. However, some ABA
372 resistant lines including *abi5* mutants and AFP over-expressors often reverse this order when

373 germinating in the presence of ABA, such that their cotyledons turn green prior to radicle emergence
374 (Supp. Fig. 13). Consequently, at the higher ABA concentrations, the fraction of seeds that have turned
375 green may be higher than the fraction that has completed germination (Fig.8). This was especially true
376 for seeds expressing the wild-type and S112A mutants of the YFP-AFP2-BC fusion in the *afp2* mutant
377 background. In contrast, the S221A,T319A mutant was less effective than the other constructs in
378 reducing ABA sensitivity in the *afp2* background and severely delayed greening when germinating on
379 ABA.
380
381 In addition to decreasing ABA sensitivity at germination, AFP2 overexpression inhibits various aspects of
382 seed maturation including accumulation of storage proteins (e.g. cruciferins) and some late
383 embryogenesis abundant (LEA) proteins (Lynch et al., 2022). To determine whether the phosphorylation
384 state of AFP2 was important for this regulation, we compared storage protein accumulation in wild-type
385 vs transgenic seeds with either wild-type or mutant AFP2 fusions. Surprisingly, even though most
386 mutants were less effective than wild-type AFP2 for conferring ABA resistance, most of the well-
387 expressed mutant forms resulted in a much greater decrease in storage protein accumulation than seen
388 when overexpressing the wild-type AFP2 fusion (Fig. 9). However, examination of multiple ages of seed
389 lots showed that the major differences in storage protein levels reflected loss during aging (Supp Fig 14).
390 Furthermore, this was not directly related to the level of ABI5 accumulation in that lines with very little
391 storage protein still had ample ABI5. ABI5 is activated by phosphorylation and already appears as a
392 triplet in dry seeds of all genotypes, but shifts to a more highly phosphorylated state when incubated on
393 ABA (Supp Fig 15). The two BC domain mutant fusions producing the greatest reduction of storage
394 protein accumulation ((S112A,S259A) and "3xD") had similar effects on ABA sensitivity of germination
395 and seedling establishment (Fig. 8 and Supp Fig 13). However, they differ in that the S112A,S259A
396 mutations also adversely affect seed longevity, resulting in decreased viability within 4 months and
397 complete lethality by 6 months (Fig.9, Supp. Fig 14). It is noteworthy that the S112A,S259A mutant form
398 of AFP2-BC accumulates to higher levels in seeds than the wild-type construct, suggesting that this form
399 might have greater effects on disrupting seed maturation, but this difference fades away during seedling
400 growth (Supp Fig 15).
401
402 **DISCUSSION**
403
404 **Post-translational modification of ABA core signaling components**
405

406 The core signalosomes of ABA signaling comprise the PYR/PYL/RCAR receptors, clade A PP2C protein
407 phosphatases, and SnRK2 kinases (reviewed in Lim et al., 2022; Née and Krüger, 2023). Each of these
408 components is encoded by a multigene family that is differentially expressed, with different interaction
409 characteristics and ABA sensitivities, creating the potential for a range of responses at different ABA
410 concentrations in different tissues (Tischer et al., 2017). In the canonical view, ABA bridges the receptors
411 with PP2Cs, effectively inactivating the PP2Cs and releasing the SnRK2s to become phosphorylated and
412 active. When active, the SnRK2 kinases regulate the activity of many effector proteins, including the
413 ABI5/ABF/AREB clade of bZIP transcription factors and ion channels. However, AHG1 differs from the
414 other clade A PP2Cs in that it does not bind to the active sites of group 3 SnRK2s, interacts with only a
415 subset of receptors and requires high ABA concentrations to do so (Tischer et al., 2017; Krüger et al.,
416 2024).
417
418 All signalosome components are also subject to post-translational modifications including
419 phosphorylation, which may be activating or inhibitory (reviewed in Lim et al., 2022). Upstream elements
420 of the pathway can be phosphorylated by a variety of kinases. TOR, *Arabidopsis* Early flowering 1 (EL1)-
421 like casein kinase (AEL), and C-terminally encoded peptide receptor 2 (CEPR2) modify distinct sites on
422 various ABA receptors, but all negatively regulate ABA response, whereas cytosolic ABA receptor kinase
423 1 (CARK1) activates several ABA receptors. At least two PP2Cs, ABI1 and ABI2, are phosphorylated by the
424 Pathogenesis Related 5 Receptor-like Kinase 2 (PR5K2), resulting in negative regulation of ABA response
425 (Baek et al., 2019). Although the SnRK2 kinases can auto-phosphorylate, they can also be activated by
426 glycogen synthase kinase (GSK)3-like kinases (Cai et al., 2014) and RAF/MAPKKKinases (M3Ks) as part of
427 ABA-induced responses (Nguyen et al., 2019; Takahashi et al., 2020).
428
429 Ubiquitination leading to proteasomal degradation has also been documented for several ABA receptors
430 (PYR1, PYL4, and PYL8) and clade A PP2Cs (ABI1 and PP2CA/AHG3) (reviewed in Lim et al., 2022). SnRK2
431 stability can also be modified by ubiquitination (Maszkowska et al., 2021) and SnRK2 activity may even be
432 modified by non-covalent interactions with ubiquitin (Shao et al., 2023). Ubiquitin-specific proteases
433 (UBPs) can also modify ABA response by removing ubiquitin from targets (Zhao et al., 2016; Liu et al.,
434 2022). Although not limited to effects on ABA response, UBP12 and UBP13 fine-tune ABA signaling by
435 modifying stability of factors that in turn regulate stability of an ABA receptor.
436
437 Activity and stability of the ABI5/ABF/ABRE clade of bZIP transcription factors is also regulated by a
438 variety of post-translational modifications including phosphorylation, ubiquitination, sumoylation, and S-

439 nitrosylation (reviewed in Yu et al., 2015). Multiple kinases, including SnRK2s, CBL-interacting protein
440 kinase (CIPK)26, SOS2-like protein kinase 5 (PKS5), BRASSINOSTEROID INSENSITIVE(BIN)2, and MPK3,
441 phosphorylate overlapping and discrete subsets of amino acid residues within ABI5 (Wei et al., 2022;
442 Lyzenga et al., 2013; Zhou et al., 2015; Bhagat et al., 2021). Our immunoblots detected multiple forms of
443 ABI5 in dry seeds and seeds germinating in the presence of ABA, but it is currently not clear which
444 modifications are present on these ABI5 proteins.

445 The AFPs interact with multiple members of this clade of bZIPs (Garcia et al., 2008) and AFP1 was initially
446 proposed to assist degradation of these bZIPs by promoting interactions with E3 ligases that ubiquitinate
447 ABI5 (Lopez-Molina et al., 2003). Although these proposed E3 ligase interactions appear weak at most
448 (Lynch et al., 2022), subsequent studies including the present one have expanded the list of AFP
449 interactors to include proteins representing all elements of the ABA signalosome, some MPKs, several
450 14-3-3 proteins, the DELLA repressors of GA signaling (Finkelstein and Lynch, 2022), the flowering
451 regulator CONSTANS (Chang et al., 2019), WRKY36 and AHG1 in dormancy regulation (Deng et al., 2023;
452 Krüger et al., 2024), several chromatin modifying factors (Pauwels et al., 2010; Lynch et al., 2017), and
453 the protein kinase SALT OVERLY SENSITIVE(SOS)2 in regulating germination under salt stress (Wang et al.,
454 2024). This extensive list of interactions is consistent with a role for AFPs as signaling hubs in a variety of
455 processes, but raises the question of how specificity is achieved. Some of the likely options are regulated
456 expression affecting availability of these proteins in different organs or stages of growth, and changes in
457 phosphorylation state or other post-translational modifications leading to changes in structure of the
458 disordered regions.
459
460 **Kinases regulating ABA response target AFPs**
461
462 The kinase families most highly associated with ABA or osmotic stress signaling are the SnRK2 kinases
463 acting in the ABA core signalosome, SnRK3/CBL interacting kinases (CIPKs), MAP kinases and calcium-
464 dependent protein kinases (CDPKs). Many proteins can be phosphorylated at multiple sites, by multiple
465 kinases, and the different sites may be phosphorylated sequentially with initial modifications altering the
466 efficiency of subsequent events (reviewed in Miller and Turk, 2018). For example, mammalian GSK3
467 kinases often require “priming” by phosphorylation at a residue 4 or 5 amino acids C-terminal to the
468 target (Sutherland, 2011). Furthermore, modifications at different sites may have opposing effects on
469 activity of the substrate.
470
471 We focused on specific SnRK2s and MAP kinases that interacted with AFP1 and/or AFP2 in yeast two-

472 hybrid and/or BiFC assays, using *in vitro* kinase assays and co-expression to test for altered mobility on
473 SDS-PAGE and Phos-tag gels. Mass spectrometric analysis identified Ser-112 of AFP2 as the sole residue
474 phosphorylated by SnRK2.6, consistent with results reported for a phosphorylated site conserved in all
475 four AFPs (Krüger et al., 2024). Mutagenesis converting this residue to either alanine or a
476 phosphomimetic amino acid reduced, but did not eliminate, phosphorylation of the AFP2-AB domain.
477 This indicated that SnRK2.6 could phosphorylate additional residues, albeit not efficiently enough to be
478 detected by mass spectrometry. Mutagenesis converting an additional predicted SnRK2 target, Ser-85, to
479 alanine eliminated phosphorylation of the AFP2-AB domain. In contrast, combining the S112A mutation
480 with alanine substitution of Ser-259, the residue with the next highest match to the SnRK2 consensus
481 target in the AFP2-BC domain, was not sufficient to eliminate phosphorylation of this domain *in vitro*, but
482 did block ABA-induced changes in mobility of the YFP-AFP2-BC fusion in stable transgenic plants.
483 Mutations affecting predicted MAP kinase target residues did not block ABA-induced changes in mobility
484 of protein fusions, but severely reduced stability and consequently accumulation of these proteins.
485 Based on the structure predicted by AlphaFold, nearly all the predicted SnRK2 and MAPK
486 phosphorylation sites are in regions predicted to be disordered (Supp. Fig.14), consistent with the
487 observation that substrates are often bound to the kinase active site in an extended conformation
488 (reviewed in Miller and Turk, 2018).
489
490 **AFPs form biomolecular condensates**
491
492 Predictions of AFP structures show a series of likely protein binding regions separated by intrinsically
493 disordered regions (Lynch et al., 2017) and the AFPs have been reported to interact with diverse proteins.
494 Intrinsically disordered regions are recognized as drivers of biomolecular condensate formation, often
495 through multivalent interactions, i.e. with multiple other molecules (Emenecker et al., 2021). Post-
496 translational modifications such as phosphorylation alter the charge distribution, and consequently the
497 potential for interactions leading to condensate formation. Initial descriptions of the AFPs showed a
498 variety of localization patterns, depending on the experimental conditions. Transient over-expression of
499 AFP(1)::CFP in either onion epidermal cells or Arabidopsis seedlings treated with ABA produced diffuse
500 nuclear localization, but co-bombardment with 35S::ABI5 resulted in co-localization in nuclear bodies
501 (Lopez-Molina et al., 2003). Although many of these nuclear bodies also contained COP1, no direct
502 interactions with COP1 were shown. Stable expression of *AFP2pro:AFP2:GFP* in an *afp2* mutant
503 background was also primarily nuclear, but excluded from nucleoli, in ABA- or NaCl-treated seedlings
504 (Garcia et al., 2008). These conditions enhanced accumulation of both AFP2 and ABI5, but did not result

505 in restriction to nuclear bodies, consistent with diffuse nuclear localization of AFPs likely to be in a
506 phosphorylated state due to ABA or stress exposure. However, within 7 hrs of removal from these stress
507 treatments AFP2:GFP was no longer visible in the nuclei, but still detected by immunoblot. In addition,
508 some punctate fluorescence was visible in the cytoplasms of a few cells near the root meristem.
509 Subsequent studies of interactions between AFPs and ABI5/ABF proteins by split YFP BiFC assays using
510 transient over-expression in *N. benthamiana* also showed nuclear localization, but no foci (Lynch et al.,
511 2017).
512
513 In the current BiFC assays, we found that co-expression of AFPs with kinases revealed interactions in
514 nuclei, but excluded from nucleoli, whereas co-expression with PP2Cs resulted in formation of nuclear
515 bodies over a broad range of sizes (Fig.4), suggesting that dephosphorylated AFPs were more prone to
516 form condensates. However, YFP-AFP2 co-expressed with cYFP-GRF4 also formed numerous small
517 nuclear condensates, consistent with interactions dependent on phosphorylation. In an effort to
518 determine which residues were most important for regulating condensate formation, we tested
519 localization of YFP-AFP fusions with alanine substitutions at known or predicted phosphorylation sites.
520 Surprisingly, the wild-type YFP-AFP2-AB domain fusion formed clusters of nuclear bodies, similar to the
521 "grapebunch-like" condensates observed for ARF proteins (Powers et al., 2019), but the alanine-
522 substituted mutant AFP2-AB domain fusions formed fewer clusters. This might reflect formation of
523 complexes by phosphorylation- dependent interactions with 14-3-3 proteins that are reduced in the
524 mutants. In contrast, the wild-type YFP-AFP2-BC fusion was mostly diffuse, but YFP-AFP2-BC fusions with
525 mutations preventing phosphorylation of the predicted kinase target residues were more likely to form
526 condensates, sometimes with small internal areas of exclusion (donut-shaped). When co-expressed with
527 the tagged PP2C phosphatase myc-AHG1, full-length AFP2 formed punctae whether or not the SnRK2 or
528 GRF target residues could be phosphorylated. The AFP2-AB domain also continued to form punctae
529 when co- expressed with AHG1, but fewer punctae formed when AHG1 and the AFP2-BC domain were
530 co- expressed. In combination with the previously described diffuse nuclear localization in ABA-treated
531 roots, this suggests that condensate formation is negatively correlated with ABA-induced
532 phosphorylation.
533
534 **Physiological significance of AFP phosphorylation state**
535
536 The AFPs have recently been reported to function as a downstream element of DOG1 control of
537 dormancy. Proteomic and genetic studies showed that DOG1 maintains dormancy by repressing AHG1

538 and AHG3 activity (Krüger et al., 2024). In non-dormant or *dog1* mutant seeds AHG1 dephosphorylates
539 AFP2 at S112 and AFP1 at the corresponding conserved serine, allowing the AFPs to function as
540 repressors of ABA response, resulting in germination. These results are consistent with our observation
541 that ABA promotes a shift to the phosphorylated state, whereas co-expression with AHG1 has the
542 opposite effect. Our studies used a gain of function approach, such that overexpression of the AFP
543 domains and mutant forms leads to ABA resistance if the AFPs are physiologically active.
544
545 Functional tests of the physiological significance of these phosphorylation options focused on effects on
546 ABA sensitivity of germination, cotyledon greening, storage protein accumulation, and seed longevity. We
547 previously reported that overexpression of YFP-AFP2 could reduce ABA sensitivity nearly 100-fold and
548 was so extreme in a wild-type background that homozygous transgenic lines failed to complete seed
549 maturation and become desiccation tolerant (Lynch et al., 2017). Homozygous YFP-AFP2 transgenes are
550 tolerated in an *afp2* mutant background, where the total AFP2 concentration is lower, and YFP-AFP2-BC
551 domain fusions are sufficient to reduce sensitivity only 30- to 50-fold, also resulting in viable seeds. The
552 reduced impact of the truncated protein might reflect the loss of the EAR domain, and therefore
553 interactions with TPL/TPR co-repressors. However, these fusions could still inhibit ABI5 and related
554 AREB/ABF clade transcription factors via interactions with their C domains (Garcia et al., 2008).

555 Comparison of endogenous AFP and ABI5 levels over a range of ABA concentrations showed that ABI5 is
556 usually in excess post-stratification, ranging from 8-10 fold higher than AFP1/2 when exposed to 1 µM
557 ABA, but increasing to as much as 30 fold higher after 5d post-stratification on media containing 10 µM
558 ABA (Garcia et al., 2008). Extended incubation on the higher ABA concentrations led to accumulation of
559 more AFP protein, effectively reducing the ABI5/AFP ratio, which was accompanied by increased
560 germination. If this ratio is a sensor of physiological state that regulates germination, the severe
561 disruption of the ratio by the AFP over-expression approach might be acting by titrating their interacting
562 factors.
563
564 Mutations of the BC domain fusions converting only the SnRK2 targets, S112 or both S112 and S259, to
565 alanine had minimal effect on ABA sensitivity of germination. However, the double mutant substantially
566 reduced seed longevity. In contrast, mutants preventing phosphorylation of both predicted MPK target
567 residues, S221 and T319, were less effective than the wild-type BC domain in conferring ABA resistance
568 to concentrations higher than 10 µM ABA. This might reflect the reduced accumulation of this mutant
569 form due at least in part to its reduced stability. Overexpression of phosphomimetic mutants did not

570 confer greater ABA resistance than provided by the wild-type YFP-AFP2-BC. Although the mutants with
571 the strongest effects on ABA sensitivity (S112A,S259A and 3xD) had cruciferin levels similar to wild-type
572 seeds when fresh, they showed accelerated loss of these storage proteins during storage. Cruciferins
573 have been found to serve as protectants from oxidative stress leading to seed deterioration during
574 ageing (Nguyen et al., 2015), but the 3xD mutants remained viable despite a massive loss of cruciferins
575 within a year, suggesting that they rely on alternative protectants. A more detailed comparison of the
576 proteomes or metabolomes of these mutants might identify components critical for seed longevity.
577
578 Although phosphomimetic substitutions are often used to simulate phosphorylated forms of proteins,
579 they frequently do not confer the expected activities, especially if the phosphorylation is a binding site
580 for an adaptor protein, e.g. 14-3-3 proteins (Kozeleková et al., 2022; Dephoure et al., 2013).
581 Consequently, it is not surprising that the alanine and aspartic acid substitutions have some similar
582 effects since both types of mutations prevent true phosphorylation. Given that AFP2 phosphorylation is
583 required to interact with the 14-3-3 proteins, this might represent a sequestered state induced by ABA,
584 maintaining ABI5 and other downstream signaling factors active. As ABA levels decrease post-
585 stratification, increased activity of the AHG PP2Cs could release the AFPs from complexes with the 14-
586 3-3 proteins, permitting more inhibitory interactions with ABI5/ABFs/AREBs.
587
588 Finally, what is the physiological significance of these condensates? In other well-characterized plant
589 systems, condensates include miRNA processing bodies (Xie et al., 2021), light response complexes
590 regulating translation (Jang et al., 2019), high temperature-induced de-repression of gene expression
591 (Bohn et al., 2024), sensors of hydration regulating germination (Dorone et al., 2021), and cytoplasmic
592 sequestering of inactive transcription factors (Powers et al., 2019). Thus, they may represent complexes
593 required for activity or inactivity of the relevant proteins. In the current work, all AFPs or AFP domains
594 tested were present as overexpressed fusions to all or part of YFP. A trivial explanation is that at least
595 some of these simply reflect high protein concentrations produced by over-expression in transient
596 systems. However, different mutations or co-expression conditions led to different localizations, and the
597 BiFC studies permit visualization of only the fusion pairs being tested. As discussed above, the AFPs
598 interact with many different proteins and are likely to be modified at additional sites by kinases not yet
599 tested. Consequently, it is possible that these AFPs, and their variants created by distinct post-
600 translational modifications, are present in diverse complexes with different functions, i.e. acting as
601 signaling hubs.
602

603 **Materials and Methods**

604

605 **Plant Materials and Transgenes**

606

607 Arabidopsis plants were grown in pots in growth chambers under continuous light at 22°C. The
608 *35S::YFP:AFP2* fusion and split YFP fusions for AFP1 and AFP2 were described in (Lynch et al., 2017). The
609 split YFP fusions for SnRK2s and AHGs were described in (Lynch et al., 2012). The *35S::YFP:AFP2* fusion
610 lines in the wild-type and *afp2* mutant backgrounds were lines #4A2 and H1, respectively, reported in
611 (Lynch et al., 2017). Mutations affecting potential phosphorylation sites were created by PCR using
612 primers shown in Supp. Table 1 with plasmids encoding cDNAs for full-length AFP2 or specific
613 subdomains as templates. *Agrobacterium tumefaciens*-mediated direct transformation of constructs
614 with mutations in potentially phosphorylated residues was performed by the floral dip method (Clough
615 and Bent, 1998), followed by selection of BASTA-resistant seedlings. Homozygous lines were identified
616 by production of 100% BASTA-resistant progeny. Following crosses between *35S:YFP:AFP2-BC* fusion
617 lines and *afp2* mutants, YFP-AFP2-BC overexpressing progeny were selected by BASTA resistance and
618 homozygous *afp2* segregants were identified by PCR-based genotyping.

619

620 Split YFP fusions for MPKs, GRFs and PYLs were constructed using the Gateway compatible pSITE-nEYFP-
621 C1 (GenBank Acc# GU734651) and pSITE-cEYFP-C1 (Acc# GU734652) vectors and PCR products with attL
622 ends added as described in (Fu et al., 2008), following manufacturer's instructions for LR Clonase
623 reactions (Invitrogen). BiFC assays were conducted as described in (Lynch et al., 2017).

624

625 **Yeast Two-Hybrid Constructs and Assays**

626

627 Fusions between the GAL4 activation domain (AD) and full-length PP2C cDNAs were described in (Lynch
628 et al., 2012). Fusions between the AD domain and full-length and partial AFP cDNAs were described in
629 (Lynch et al., 2017). Fusions between the AD domain and 14-3-3 cDNAs were constructed by CRE-lox
630 recombination between pUNI clones and the pACT2-lox vector (Liu et al., 1998). Fusions between the
631 GAL4 DNA binding domain (BD) and either AFP or PYL cDNAs were constructed using the pGBKT7-DEST
632 vector (Lu et al., 2010) and PCR products with attL ends. BD fusions with SnRK2.2, 2.3 and MPK6 were
633 constructed by CRE-lox recombination using the pAS2lox vector and by LR Clonase reactions with the
634 pGBKT7-DEST vector and pDONR clones for SnRK2.6, SnRK2.10, and MPK3.

635

636 BD fusions were transformed into yeast line PJ69-4A selecting for growth on yeast synthetic medium

637 (YSM) without trp. AD fusions were transformed into Y187, selecting for growth on YSM without leu.
638 Interactions were tested by matings between pairs of lines carrying BD- and AD-fusions; following
639 overnight incubation on YPD plates, matings were replica plated to YSM lacking leu and trp to select for
640 diploids or YSM also missing histidine and, in some cases, adenine to screen for diploids that had
641 activated the HIS3 and ADE2 reporter genes. Diploids carrying BD-PYLs and AD vector or AD-AFPs were
642 grown in YSM lacking leu and trp, then diluted to an OD600 of 0.9, before making serial 10-fold dilutions
643 that were spotted in replicates on YSM lacking lacking leu and trp, or also lacking his. Diploids combining
644 BD-AFPs with AD vector or AD-GRFs were also analyzed by growth assays with serially diluted cultures.
645
646 **Plant growth conditions**
647
648 Germination assays testing ABA sensitivity of age-matched seeds were performed on minimal nutrient
649 media supplemented with ABA at concentrations over the range from 0-50 µM, as described in (Lynch et
650 al., 2017). Accumulation of fusion proteins was assayed by immunoblots of seeds or seedlings harvested
651 after 5d incubation on Germination Medium (GM: 0.5x MS salts and vitamins, 1% sucrose) or minimal
652 media solidified with 0.7% agar, then subjected to ABA, cycloheximide or MG132 for the indicated times.
653
654 For transient expression *Agrobacterium* lines expressing fusion proteins were combined with GV3101
655 expressing the P19 protein of tomato bushy stunt virus to enhance transient expression, as described in
656 (Voinnet et al., 2003). Cultures of all bacteria to be used in infiltration were grown overnight in YEP
657 media with appropriate selective antibiotics, diluted to an OD_{600} of 1.0 in 10 mM $MgCl_2$, 10 mM MES pH
658 5.6, 0.2 mM acetosyringone and rocked at room temperature for 2-3 hrs prior to mixing and infiltration
659 of leaves on 2-3 week old *Nicotiana benthamiana* plants. Fluorescence was scored 2-4 days later, using
660 an Olympus AX70 microscope or a Leica SP8 Resonant Scanning Confocal microscope. Confocal images
661 were processed with Fiji (Image J) to produce maximum intensity Z-stack composites.
662
663 **Immunoblots**
664 Arabidopsis seedlings or *N. benthamiana* leaf tissue were ground directly in 2x Laemmli loading buffer
665 (1.5-2 µL/mg FW), microfuged 10 min at 4°C to pellet debris, then the supernatants were boiled 5 min
666 prior to fractionation by SDS-PAGE or SuperSep™ Phos-tag™ PAGE (Fujifilm Wako Chemicals). Proteins
667 were transferred to nitrocellulose filters and stained with Ponceau S, as described in (Lynch et al., 2017).
668 Filters were blocked with Casein blocking buffer (LI-COR Biosciences, Lincoln, NE), then co-incubated with
669 anti-GFP mAb (1:10000, UBPBio, Aurora, CO) and/or anti-actin mAb (A0480, Sigma) primary antibodies,
670 followed by anti-mouse secondary IRDye 800 conjugated IgGs, and visualized using the 800 channel of

671 the Licor Odyssey Infrared Imaging System. Mature dry Arabidopsis seeds were ground in 20 µL/mg FW
672 1x Laemmli loading buffer, then processed as above. ABI5 was detected with anti-ABI5pAb (1:10000,
673 Ab98831, AbCam) primary antibodies, followed by anti-rabbit secondary IRDye 700 conjugated IgGs, and
674 visualized using the 700 channel of the Licor Odyssey Infrared Imaging System.
675
676 **Recombinant protein purification**
677
678 Fusions between His6-tags and full-length, partial and mutant AFP coding sequences were constructed by
679 CRE-lox recombination between pUNI cDNAs and the pHB3-His6 vector. Primers used for subcloning and
680 mutagenesis are shown in Supplementary Table 1. Plasmids carrying fusion genes were transformed into
681 *E.coli* BL21C+ and expression was induced by growth overnight at 19-20°C in Overnight ExpressTM Instant
682 TB medium (Novagen). Cells were harvested, resuspended in Lysis Buffer (8.3 M urea, 0.1 M
683 NaPhosphate pH 8). His6-fusions were affinity purified in batch mode with HIS-select Nickel Affinity beads
684 (Millipore P6611) according to the manufacturer's protocol for denaturing conditions, eluting with 8 M
685 urea, 0.1 M NaPhosphate pH 5.5.
686
687 GST fusions to full length SnRK2.6 also made use of the pUNI vector system, and induction overnight in
688 TB Express, but proteins were extracted under native conditions with CelLytic B in 1x PBS, 10 mM DTT, 83
689 units/mL Benzonase nuclease, 1 mM PMSF, 5 mg/mL lysozyme , followed by purification on glutathione-
690 agarose beads (UBP-Bio, P3060-10) and elution with 30 mM Tris pH9, 300 mM NaCl, 5 mM DTT, 0.5%
691 Triton-X100, 1 mM PMSF, 40 mM glutathione.
692
693 **In vitro kinase assays**
694
695 Purified GST-SnRK2.6 was activated by 1 hr pre-incubation in kinase buffer (25mM Tris pH 7.5, 12 mM
696 MgCl2, 2 mM DTT) with 1 mM ATP, then combined with His-AFP fusions maintaining the buffer and ATP
697 concentrations for a further hr of incubation. Phosphorylation was monitored in wild-type and mutant
698 constructs by incorporation of ^{32}P from gamma-labeled ATP (0.1-0.2 µCi/rxn) during the latter incubation.
699 SDS sample buffer was added to 1x final, samples were boiled 5 min., then fractionated on 13% SDS-
700 PAGE, dried and autoradiographed. Following assays with only cold ATP, gel slices containing lower
701 mobility products were excised and sent to UC Davis for mass spec analysis. Additional SnRK assays with
702 mutant AFP fusion proteins were analyzed by ProQ Diamond Phosphoprotein staining according to
703 manufacturer's instructions (Invitrogen).
704

705 **qRT-PCR analysis**

706

707 RNA was isolated from *N. benthamiana* leaf tissue or Arabidopsis seedlings as described in (Lynch et al.,
708 2017). DNA-free total RNA was prepared by incubation with RQ1 DNase (Promega) and RNAsin for 15
709 min. at room temperature, followed by inactivation of the DNase by addition of EGTA, then clean-up
710 over Qiagen RNeasy or Zymo-Clean columns according to manufacturers' instructions. Approximately 0.5
711 µg of RNA was used as template for cDNA synthesis by GoScript reverse transcriptase (Promega), using a
712 10:1 mix of random hexamers and oligo dT as primers. cDNA concentrations were checked by qRT-PCR,
713 using Forget-Me-Not master mix (Biotium) in an iQ5 cycler (Bio-Rad), according to manufacturer's
714 instructions. Primers used for normalizing were selected for uniform expression in Arabidopsis seeds or
715 young seedlings subjected to a variety of treatments: AT5G46630 and At1g13320 (Czechowski et al.,
716 2005) or a validated reference gene for *N. benthamiana* (Pombo et al., 2019). Equal amounts of cDNA
717 were used as templates for reactions with all other primer sets (listed in Supp. Table 1), quantified
718 relative to a standard curve spanning the range of concentrations present in all genotypes of transgene
719 expression.

720

721

722

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Table 1. AFP2 predicted phosphorylation sites.

Residue	NetPhos 2.0 score	PhosPhAt score	MUsite score	SnRK2 target	MPK target	in vitro phosphorylated
Y28	0.315	1.59				
S38	0.02	0.11	0.487			
T51	0.781	0.21	0.212			
T76	0.973	1.1	0.841		T-P	
S83	0.971		0.769			
S84	0.947		0.787			
S85	0.997	0.81	0.881	R-S-S-S		Yes
T89	0.014	0.84	0.292			
Y103	0.622	0.97				
T104	0.041	0.19	0.196			
T111	0.9		0.21			
S112	0.321	0.37	0.77	R-T-T-S		Yes!
S129	0.988		0.469			
S150	0.753	0.61	0.46	S-D		
T154	0.084	0.14	0.383			
S156	0.966	0.45	0.737			
T164	0.471	0.75	0.113			
S187	0.179	0.67	0.578	S-D		
T193	0.137	0.91	0.298			
S197	0.323	0.44	0.277	G-S?		
S198	0.725	0.52	0.227			
S199	0.063	0.21	0.154			
S200	0.992	0.65	0.539			
S202	0.996		0.274			
S213	0.572	0.03	0.249			
S215	0.916	0.8	0.402			
S221	0.772	1.13	0.654		S-P	
S232	0.844	0.46	0.47			
S234	0.745	0.56	0.536	G-S?		
T243	0.046	0.21	0.137			
S259	0.985	1.28	0.869	R-V-R-S		
S262	0.763	0.63	0.803			
S273	0.044	0.18	0.348			
S284	0.303	0.34	0.103			
Y299	0.137	0.46				
T319	0.94	0.27	0.247		T-P	
T343	0.689		0.173			

748 **Figure Legends**

749

750 Fig.1 Interactions between AFPs and PP2Cs detected by y2h assays. Fusions were combined by matings
751 between Y187 carrying AD fusions and PJ69-4a carrying BD fusions. After overnight incubation on YPD
752 media, yeast were replica-plated onto selective media lacking leu and trp (-LW) to maintain the AD- and
753 BD- fusion plasmids, and lacking histidine and adenine (-HALW) to score interactions between the fusion
754 proteins.

755

756 Fig.2 Interactions between AFPs and kinases detected by y2h assays. The indicated fusions were
757 combined as described in Fig. 1.

758

759 Fig.3 Interactions between AFPs, receptors and GRFs detected by y2h assays. Following matings, the
760 indicated diploid lines were grown overnight in media lacking leu and trp. After measuring the OD600,
761 cultures were diluted to the same concentration, then serially diluted 10-fold three times. The diluted
762 cultures were then replica-spotted onto selective media. The -LW plate serves as a control for accuracy
763 of the dilutions. Growth on the -HLW plate requires interaction between the AD- and BD- fusions to
764 complement the *his3-200* allele of these yeast.

765

766 Fig.4 Interactions between AFPs and SnRK2s , PP2Cs, GRFs and receptors detected by split YFP assays.
767 Low magnification images were obtained with an Infinity camera on an Olympus AX70 epifluorescence
768 microscope at 100x magnification. Insets are 400x magnification. High magnification images of individual
769 nuclei were obtained with a Leica SP8 Resonant Scanning Confocal microscope. Scale bars = 10 μm for
770 interactions with kinases, 5 μm for interactions with AHG1

771

772 Fig.5 AFP2 is a substrate for both specific kinases and PP2Cs. Immunoblot of extracts from transiently
773 expressed full-length or subdomains of AFP2 as YFP fusions (A). The YFP-AFP2-AB fusion was transiently
774 co-expressed with fusions to either SnRK2.3 or AHG1 (B) Transiently expressed YFP-AFP2-AB was
775 immunoprecipitated using GFP-Trap agarose beads; upper panels are Westerns with indicated
776 antibodies, lower panels are Ponceau stain (C) In vitro kinase reactions with GST-SnRK2.6 and wt or
777 phosphomutant His-fusions of AFP2-AB resulting in reduced mobility (D,G), incorporation of radioactivity
778 (E), or reactivity with Pro-Q Diamond stain (G). The 2xA mutant of the AB domain is (S85, S112)A; the
779 3xA mutant also includes T76A. Co-expression of the wild-type AB and BC domains of AFP2 with MyC-
780 MPK3 (F) and phosphomutant BC fusions (K). Mobility of transiently expressed wild-type and

781 phosphomutant AFP2-AB domain fusions (H) and AFP2-BC domain fusions (J,K). In vitro kinase reactions
782 with GST-SnRK2.6 and wt or mutant His fusions of AFP2-BC, viewed with Pro-Q Diamond stain (I). Lower
783 panels are Ponceau (A,C,F,H,J,K) or Coomassie (D,E,I) stain. Immunoblots were probed with anti-GFP. For
784 the BC domain, 2xA = (S112,S259)A; 3xA = (S112,S221,T319)A; 2xD = (S221,T319)D
785
786 Fig.6 *In vivo* expression assays with mutant AFPs testing relative significance of specific residues for
787 ABA/stress-induced modification and stability. Transient expression in *N.benthamiana*, followed by 4 hrs
788 incubation in GM +/- 30 µM ABA (A). Stable *A. thaliana* transgenic seedlings grown 5d on GM, then
789 incubated in GM +/- 20 µM ABA for 4.5 hrs (B) or min +/- NaCl (50 - 300 mM) for 2.75 hr (C). Transient
790 expression of YFP-AFP2-AB fusions in *N.benthamiana*, followed by 6 hrs incubation in GM +/- 20 µM CHX
791 or MG132 (D). Stable *A. thaliana* transgenic seedlings grown 5d on GM, then incubated in GM +/- CHX or
792 MG132 for 6 Hrs (E). All upper panels were probed with anti-GFP. Lower panels show either anti-actin
793 reactivity or Ponceau stain (pink).
794
795 Fig.7 Localization of YFP-fusions to wild-type and phosphomutant AFP2-AB and –BC domains, and split
796 YFP assays with GRF4, in transiently transformed *N. benthamiana*. Confocal images of nuclei: scale bars
797 = 5 um. nYFP-GRF4/cYFP-GRF4 image: scale bar = 20 um
798
799 Fig. 8 Physiological analysis comparing germination (A,C,D) or cotyledon greening (B,E,F) of transgenic
800 lines overexpressing wild-type (wt) vs phosphomutant or phosphomimetic AFP2 fusions in wt(Col)
801 background (A,B,C,E) or *afp2* background (A,B,D,F). Data displayed is the average of at least
802 triplicate assays for each genotype and treatment $\pm$ S.E. Bars with different letters represent
803 statistically different values using Tukey's HSD post-hoc test ($p<0.01$).
804
805 Fig.9 Effects of phosphomutant or phosphomimetic YFP-AFP2 fusions on storage protein accumulation
806 and seed longevity. Top: comparison of dry seed protein extracts from lines either segregating a full-
807 length wild type AFP2 fusion or homozygous for S85E,S112E (S>E) or S85A,S112A (S>A) mutant full-
808 length AFP2 fusions (left) or AFP2-BC fusions with the indicated mutations (right). Upper panels show
809 immunoblots of YFP-AFP2(-BC) fusions detected by α-GFP, ABI5, and Actin as a loading control. Lower
810 panels show Coomassie staining of a comparably loaded higher 15% SDS-PAGE to retain and resolve the
811 storage proteins. The "old" extracts were from early generations of these lines with high transgene
812 expression and poor seed viability, while "new" extracts were from later generations with partially

813 silenced transgenes permitting better viability but still conferring ABA-resistant germination. Bottom:
814 seedlings from seed stocks of the indicated ages and genotypes, and their % germination, after 5d
815 growth on minimal medium.
816
817 Figure 10. Changing relationships in the ABA core signaling pathway during the transition from seed
818 maturation to germination. At high ABA concentrations, PP2Cs (e.g. AHG1 & AHG3) are inactive, kinases
819 (e.g. SnRK2s & MPKs) are active, bZIPs (e.g. ABI5 & ABFs) and AFPs are phosphorylated and are bound by
820 GRFs (14-3-3 proteins). When the bZIP:AFP ratio is high, ABA-regulated gene expression proceeds and
821 germination is blocked (Garcia et al, 2008). As ABA levels decrease, ABA induces repressors including AFPs
822 and PP2Cs in a feedback mechanism, the bZIP:AFP ratio decreases and seed-expressed gene expression
823 declines. Interaction between AFPs and co-repressors (e.g. TPL/TPR and HDAC components) leads to
824 inactivation of seed-expressed genes (Lynch et al. 2016). At low ABA levels, the PP2Cs are active and
825 inactivate the SnRK2s, bZIPs (Lynch et al. 2012) and AFPs, and the latter two classes are proteasomally
826 degraded. Many additional interactors are not included due to space constraints. The figure was created
827 with BioRender.com.
828
829 **Supplementary Figures**
830
831 Supp. Fig. 1. Yeast two-hybrid test of interactions between ABA receptors and AFPs, presented as GAL4BD-
832 PYR/PYL and GAL4AD-AFP fusions. The AD fusion to the PP2C HAB1 is included as a positive control, and
833 pACT is a negative control. Fusions were combined by matings between Y187 carrying AD fusions and PJ69-
834 4a carrying BD fusions. After overnight incubation on YPD media, yeast were replica plated onto selective
835 media lacking leu and trp (-LW) to maintain the AD- and BD-fusion plasmids, then replica plated again to
836 media lacking histidine (-HLW) and supplemented with ABA to score interactions between the fusion
837 proteins. Lower panel: test of potential positives by dilution assay, as in Fig.3.
838
839 Supp. Fig. 2 Expression of PYLs, GRFs and AFPs in maturing embryos, dry seeds and either 24 h post-2d-
840 stratification or after 24h imbibition in water following 2-4 months after-ripening. Data from (Narsai et
841 al., 2011; Nakabayashi et al., 2005; Hofmann et al., 2019) displayed on http://bar.utoronto.ca/efp//cgi-
842 bin/efpWeb.cgi (Winter et al., 2007)
843
844 Supp. Fig. 3 Interactions between 14-3-3 proteins (GRFs) and ABA core signaling elements. Top: Yeast

845 two-hybrid assays combining AD-fusions to 14-3-3 proteins with BD-fusions to wild-type and mutant
846 AFP2, PP2Cs, SnRK2.3 and ABI5 by matings, as described for Supp.Fig.1.
847 Bottom: BiFC assays testing GRF interactions with kinases and phosphatases.
848
849 Supp. Fig. 4 Biomolecular fluorescence complementation of full length AFP2 (middle) and AFP1 (bottom)
850 and subdomains versus ABA core signaling components in *N. benthamiana* leaves (100X magnification).
851 Top panel shows localization of YFP-fused phosphatase and kinase for reference. Color differences are
852 due to different versions of image capture software. Inset images show examples of nuclear speckles
853 observed at 200X or 400X magnification
854
855 Supp. Fig. 5 Predicted phosphorylated residues in AFP1 and AFP2. Top: PhosPhAt shows possible
856 phosphorylation sites of AFP1 highlighted in green, with documented sites identified by black letters.
857 Middle: Alignment of AFP1 and AFP2. A, B and C domains are underlined in blue, green and red,
858 respectively. Residues fitting consensus for MAPK phosphorylation are in blue, those fitting SnRK2
859 consensus are in red. Bottom: Disordered regions (score <8) in AFP2 predicted by Attention based
860 DisOrder PredicTor (ADOPT) (Redl et al., 2023).
861
862 Supp. Fig. 6 In vitro kinase reaction of His-AFP2-AB incubated with GST-SnRK2.6 used for mass
863 spectrometric analysis of phosphorylation site(s). Top: Coomassie-stained SDS-PAGE; the
864 phosphorylated substrate bands were excised from the gel, and sent to the UC Davis Mass
865 Spectrometry Facility for analysis following tryptic digestion. Bottom: Summary of Mass Spec analysis
866 by Scaffold software.
867
868 Supp. Fig. 7 Tests of potential phosphorylation sites by kinase reactions with mutant AFP substrates. In
869 vitro kinase assay comparing phosphorylation of wild-type AFP2-AB domain with phosphomutants or
870 AFP2-C domain (A). Top two panels are autoradiographs of the Coomassie-stained gel shown in the
871 bottom panel. Affinity-purified GST-SnRK2.6 was pre-activated, then reactions set up as pool before
872 aliquoting for combination with His-AFP2 substrates. Coomassie shows loading of AB fusions and SnRK.
873 “4xA” = (S85,T111,S112,S129)A; as shown in Fig. 5E, (S85,S112)A gives the same result as the 4xA
874 mutant.
875 Comparison of wild type and phosphomutant YFP-AFP2-AB domain mobility when transiently expressed
876 in *N. benthamiana*, either alone or in combination with either Myc-SnRK2.3 or Myc-MPK3 (B). “3xA” =

877 (T76,S85,S112)A

878

879 Supp. Fig. 8 Comparison of phosphorylation status of AFP2-BC domain by Phos-tag gels. YFP-AFP2-BC
880 fusions were expressed transiently in *N.benthamiana* (A,B,C) or stably in *A.thaliana* (C). Fusions were
881 expressed alone or in combination with Myc-SnRK2.3 (A), Myc-MPK3 (B) or Myc-AHG1 (C). ABA or GM
882 incubations were as described in Fig. 6AB. The "3xD" mutant is altered at S112, S221, and T319. The
883 unphosphorylated AFP2-BC domain is identified by the arrows.

884

885 Supp. Fig. 9 Effects of ABA, mutations and genetic background on expression and mobility of YFP-AFP2-
886 BC transgenes in stably transformed *A. thaliana*. Time course of changes in mobility of wild-type AFP2-
887 BC in response to 20 µM ABA (A). Dose response of ABA-induced changes in mobility of wild-type AFP2-
888 BC domain in stably transformed *A. thaliana* incubated for 6 hrs in GM with or without ABA ranging from
889 1-20 µM (B). Expression of wild-type (wt) YFP-AFP2-BC and transgenes with the indicated mutations in wt
890 and *afp2* seedlings following germination and 7d growth on minimal media supplemented with 3 µM
891 ABA (C). Upper panels are immunoblots probed with anti-GFP. Lower panels in B and C are the RbcL
892 bands seen on Ponceau stained filters

893

894 Supp. Fig. 10 Comparison of protein and transcript levels for AFP2-AB or AFP2-BC domain fusions.
895 Transient expression in *N. benthamiana* (A-C) or stable transformants in *A. thaliana* (D,E). The standard
896 series of wt AFP2-AB shows the volume of extract loaded for the standard curve. Amounts of YFP fusion
897 protein were normalized relative to actin. YFP-fusion transcripts were normalized relative to these stably
898 expressed endogenous genes: PP2A (Niben101Scf09716g01002.1) of *N.benthamiana* and the geometric
899 mean of PP2AA3 (At1g13320) and AP2M (At5g46630) of Arabidopsis.

900

901 Supp. Fig. 11. Confocal images of root tips of transgenic Arabidopsis expressing the indicated YFP-AFP2-
902 BC fusions. Seedlings were grown 4d post-stratification on minimal medium, then transferred to GM
903 with or without 20 µM ABA for 1 day. Scale bar = 20 µm

904

905 Supp. Fig. 12. Comparison of stability and accumulation of transiently expressed wild type, double
906 (S112A,S259A or S221A,T319A) or triple (3xA=S112A,S221A,T319A) mutant AFP2-BC domain fusions to
907 YFP. Following infiltration, *N. benthamiana* leaf discs were infiltrated with GM (0.5x MS medium) with or
908 without 20 µM cycloheximide (CHX) or MG132(MG), and incubated 6 hrs prior to harvest for extraction.

909 Immunoblots were probed with anti-GFP (top panels) after staining with Ponceau (lower panels) (A,B).
910 Micrographs of *N. benthamiana* leaves used for assay in B, taken with 10x objective on an Olympus AX70
911 epifluorescence microscope (C). Gain was set at 11.9 for 3xA mutant vs. 2.0 for wild-type. Inset is wild-
912 type nucleus taken with 40x objective.
913
914 Supp. Fig. 13. Germination phenotypes of wild type (Col) and transgenic seeds expressing the
915 indicated YFP-AFP2 fusions after 7d incubation on either 10 or 50 µM ABA.
916
917 Supp. Fig. 14. Effects of phosphomutant or phosphomimetic YFP-AFP2 fusions on storage protein
918 accumulation and seed longevity. Dry seed protein extracts of the indicated genotypes and ages were
919 separated on 10% SDS-PAGE for immunoblot or 15% SDS-PAGE for resolution of storage proteins.
920 Germination was scored at 4d post-stratification incubation on minimal nutrient media.
921
922 Supp. Fig. 15. Effect of ABA on accumulation and phosphorylation of ABI5 in seeds with wild type or
923 mutant YFP-AFP2-BC fusions in an *afp2* mutant background. Protein extracts were separated on 10%
924 SDS-PAGE (upper panels) or a 7.5% Phos-tag gel, then transferred to filters for immunoblots.
925 Germination on minimal media supplemented with 3 mM ABA (A3) was scored just prior to harvest.
926
927 Supp. Fig. 16. Alphafold prediction of AFP2 structure (Model AF-Q9LMX5-F1) with locations of predicted
928 SnRK2 or MPK phosphorylation sites labeled.
929
930
931
932

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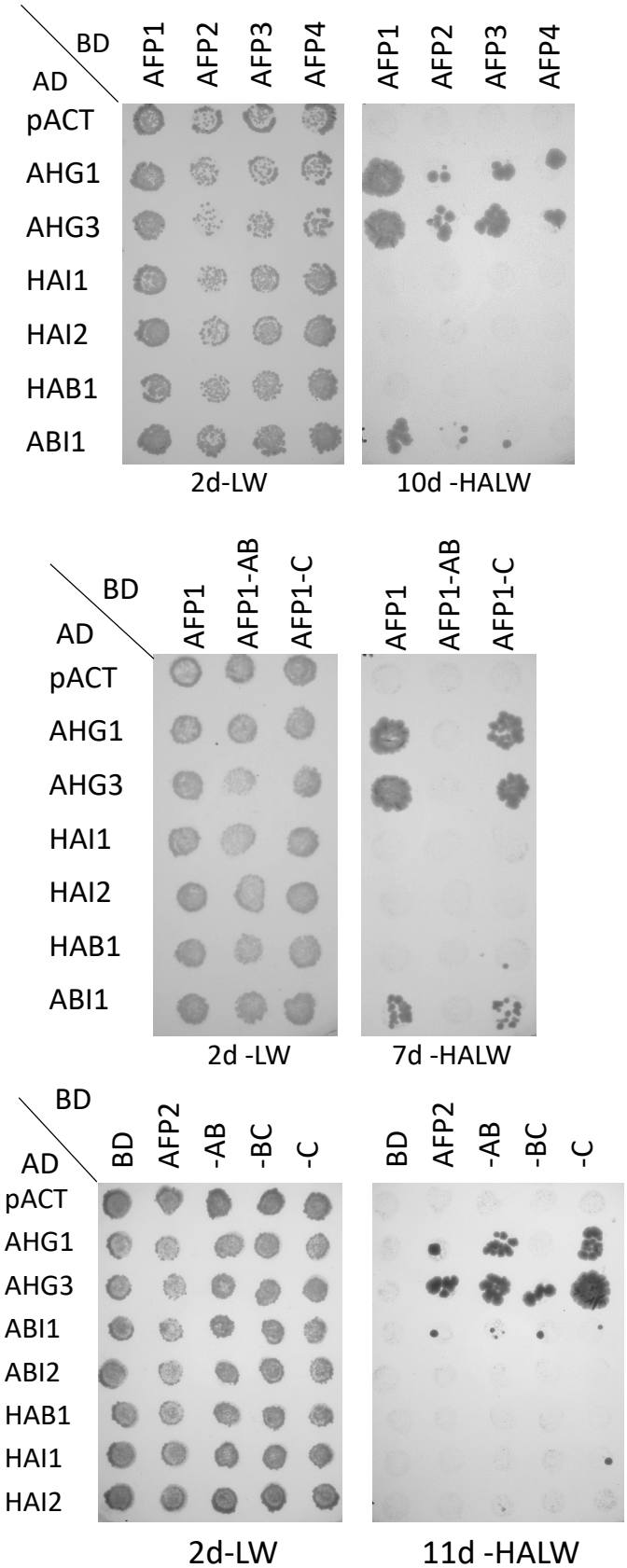
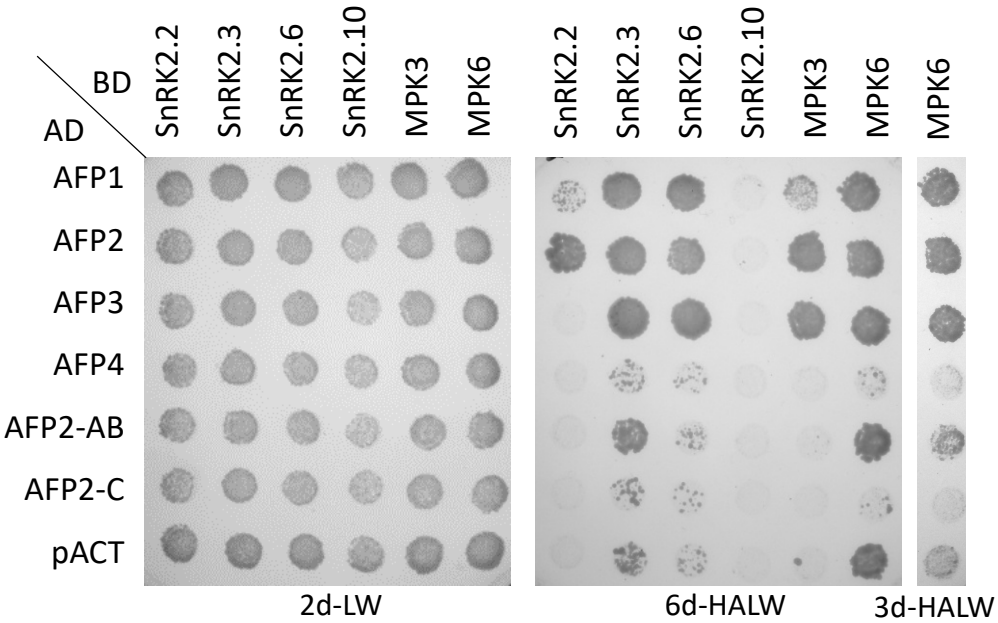


Figure 1 interactions between AFPs and PP2Cs detected by y2h assays. Fusions were combined by matings between Y187 carrying AD fusions and PJ69-4a carrying BD fusions. After overnight incubation on YPD media, yeast were replica plated onto selective media lacking leu and trp (-LW) to maintain the AD- and BD-fusion plasmids, and lacking histidine and adenine (-HALW) to score interactions between the fusion proteins.



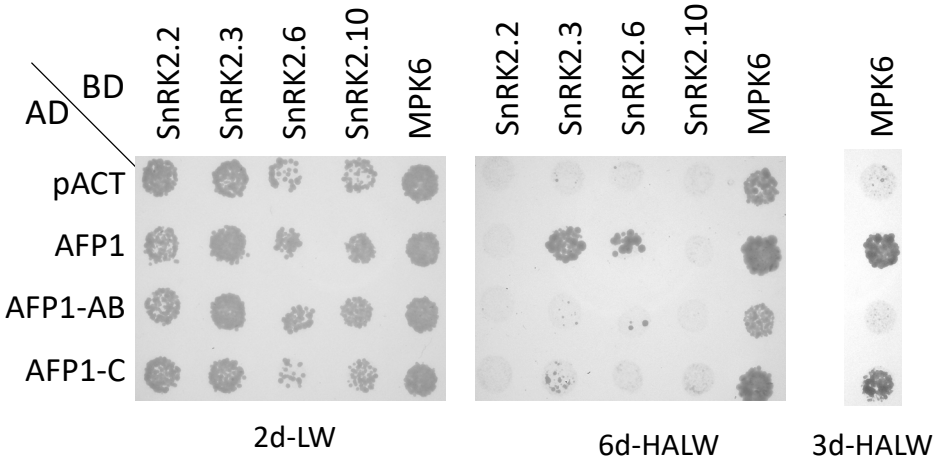


Figure 2 Interactions between AFPs and kinases detected by y2h assays. The indicated fusions were combined as described in Fig. 1.

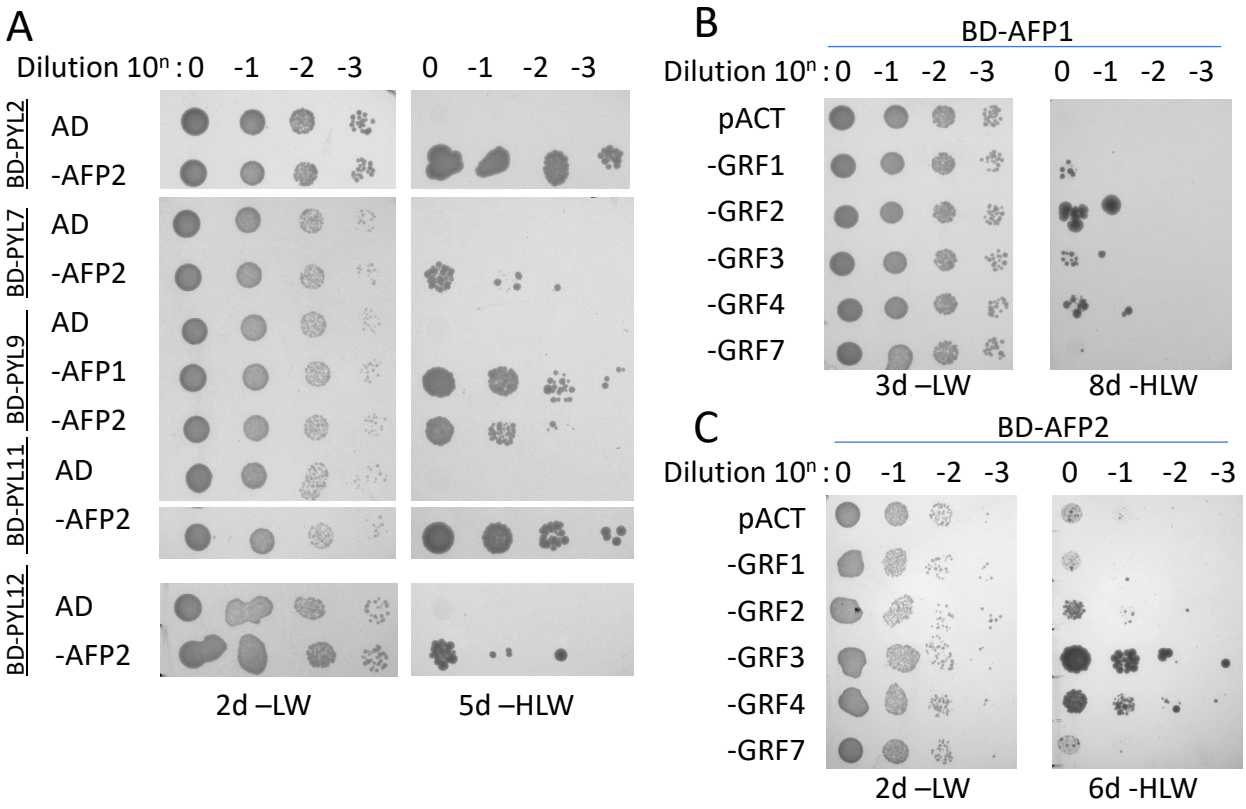


Figure 3. Interactions between AFPs, receptors and GRFs detected by y2h assays. Following matings, the indicated diploid lines were grown overnight in media lacking leu and trp. After measuring the OD600, cultures were diluted to the same concentration, then serially diluted 10-fold three times. The diluted cultures were then replica-spotted onto selective media. The -LW plate serves as a control for accuracy of the dilutions. Growth on the -HLW plate requires interaction between the AD- and BD- fusions to complement the *his3*- of these yeast.

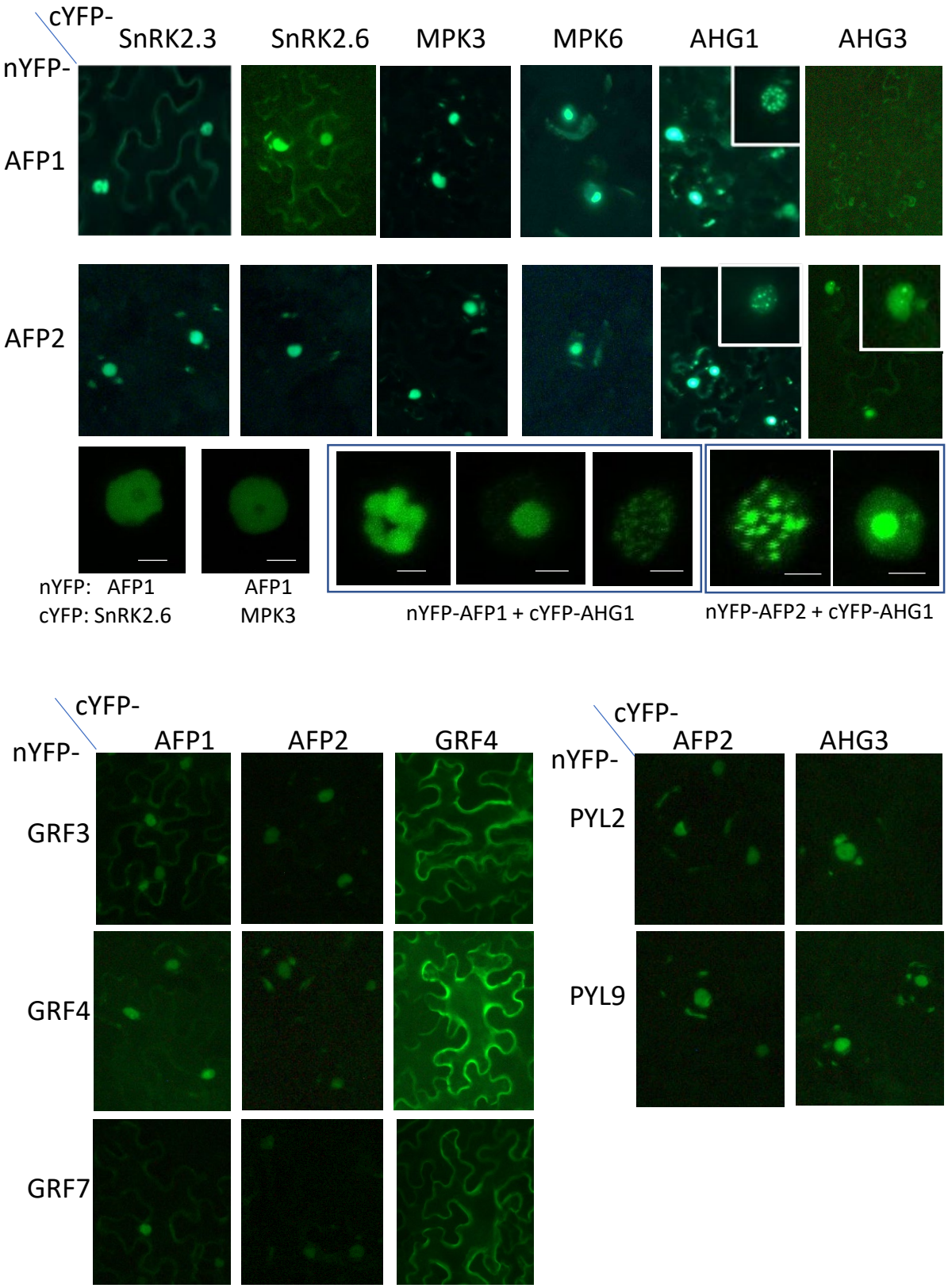


Figure 4. Interactions between AFPs and SnRK2s , PP2Cs, GRFs and receptors detected by split YFP assays. Low magnification images were obtained with an Infinity camera on an Olympus AX70 epifluorescence microscope at 100x magnification. Insets are 400x magnification. High magnification images of individual nuclei were obtained with a Leica SP8 Resonant Scanning Confocal microscope. Scale bars = 10 μm for interactions with kinases, 5 μm for interactions with AHG1

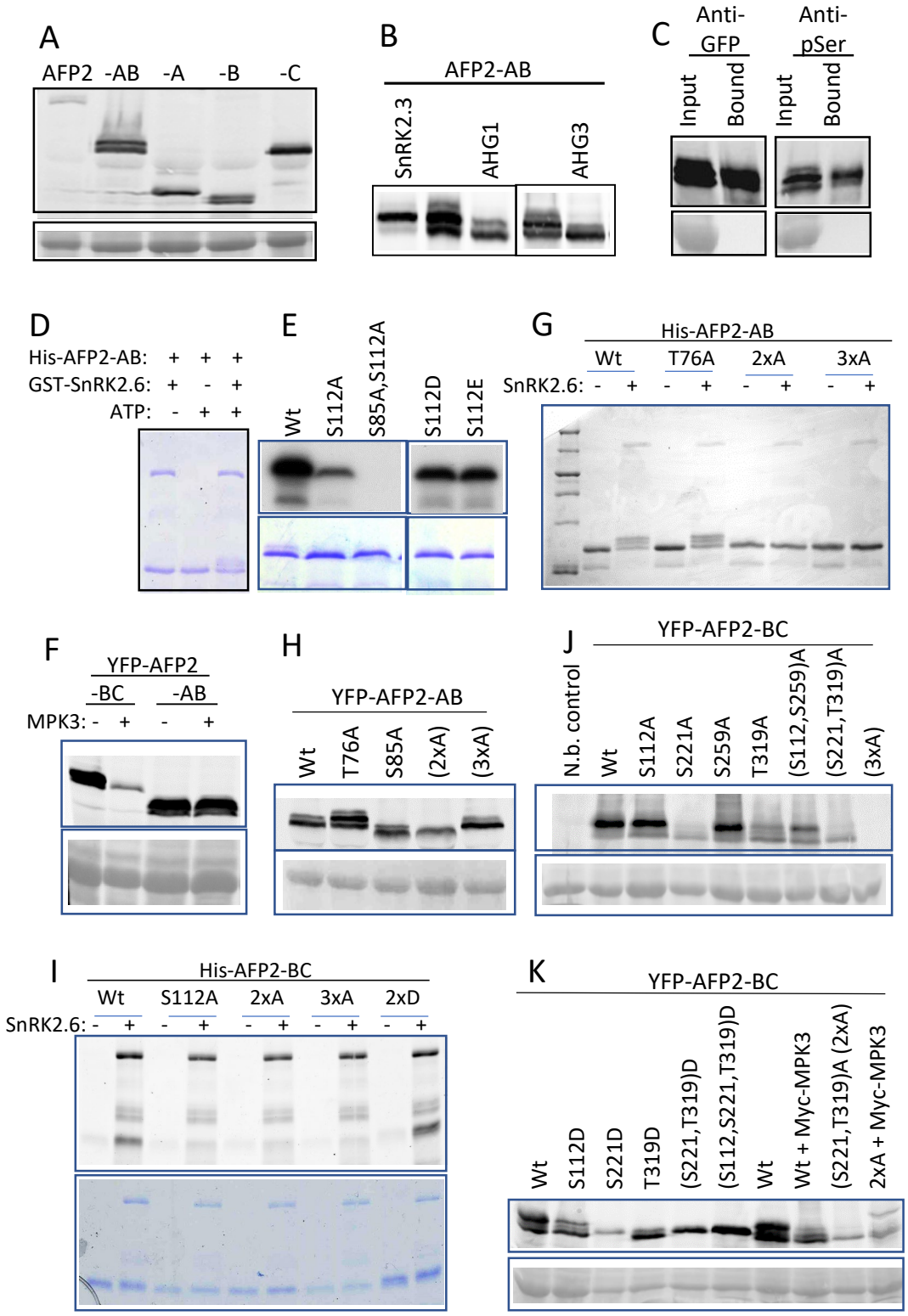


Fig.5 AFP2 is a substrate for both specific kinases and PP2Cs. Immunoblot of extracts from transiently expressed full-length or subdomains of AFP2 as YFP fusions (A). The YFP-AFP2-AB fusion was transiently co-expressed with fusions to either SnRK2.3, AHG1 or AHG3 (B). Transiently expressed YFP-AFP2-AB was immunoprecipitated using GFP-Trap agarose beads; upper panels are Westerns with indicated antibodies (C). In vitro kinase reactions with GST-SnRK2.6 and wt or phosphomutant His-fusions of AFP2-AB resulting in reduced mobility (D,G), incorporation of radioactivity (E), or reactivity with Pro-Q Diamond stain (G). The 2xA mutant of the AB domain is (S85, S112)A; the 3xA mutant also includes T76A. Co-expression of the wild-type AB and BC domains of AFP2 with MyC-MPK3 (F) and phosphomutant BC fusions (K). Mobility of transiently expressed wild-type and phosphomutant AFP2-AB domain fusions (H) and AFP2-BC domain fusions (J,K). In vitro kinase reactions with GST-SnRK2.6 and wt or mutant His fusions of AFP2-BC, viewed with Pro-Q Diamond stain (I). Lower panels are Ponceau (A,C,F,H,J,K) or Coomassie (D,E,I) stain. Immunoblots were probed with anti-GFP. For the BC domain, 2xA = (S112,S259)A; 3xA = (S112,S221,T319)A; 2xD = (S221,T319)D

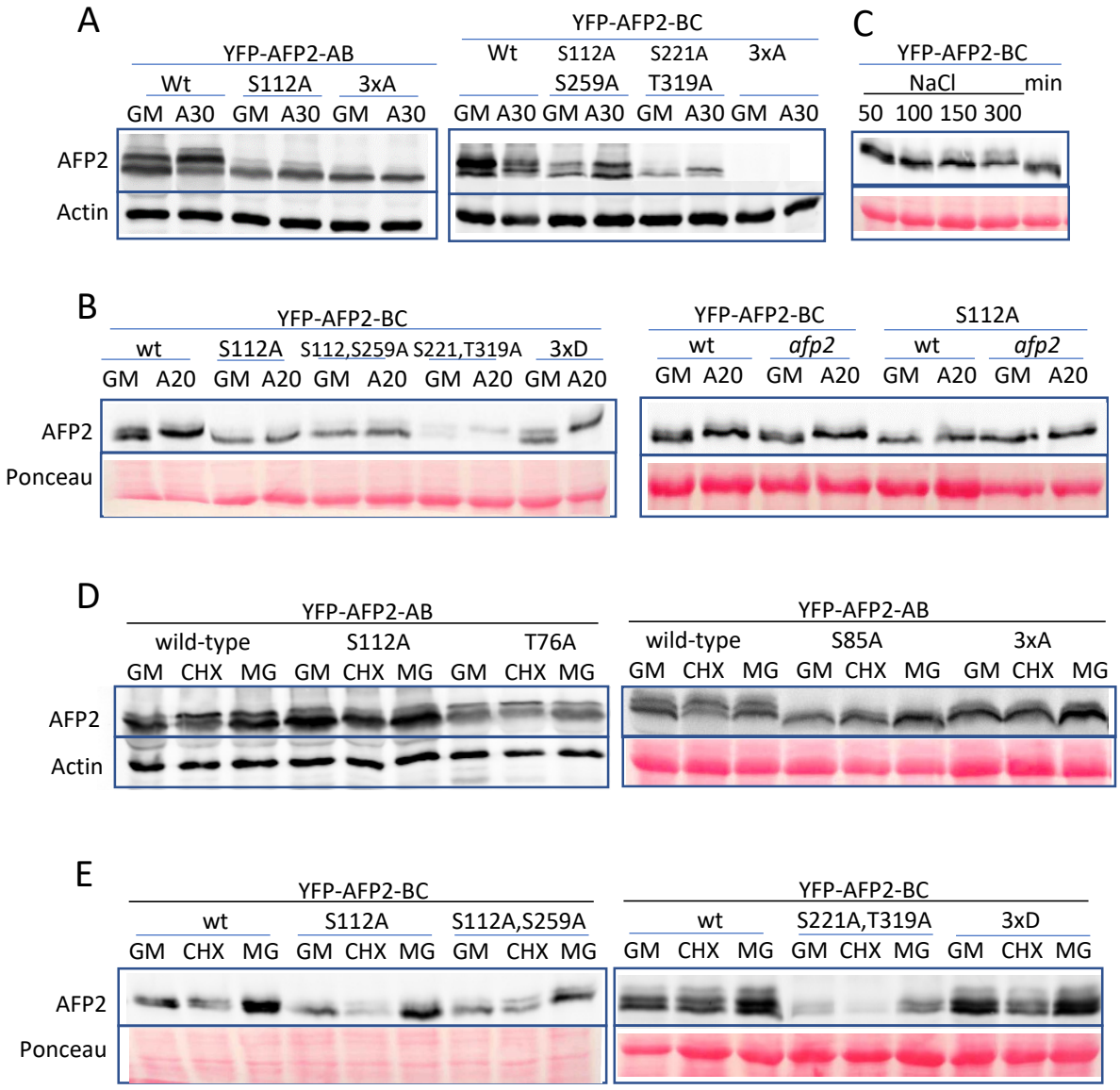


Figure 6. *In vivo* expression assays with mutant AFPs testing relative significance of specific residues for ABA/stress-induced modification and stability. Transient expression in *N.benthamiana*, followed by 4 hrs incubation in GM +/- 30 µM ABA (A). Stable *A. thaliana* transgenic seedlings grown 5d on GM, then incubated in GM +/- 20 µM ABA for 4.5 hrs (B) or min +/- NaCl (50 - 300 mM) for 2.75 hr (C). Transient expression of YFP-AFP2-AB fusions in *N.benthamiana*, followed by 6 hrs incubation in GM +/- 20 µM CHX or MG132 (D). Stable *A. thaliana* transgenic seedlings grown 5d on GM, then incubated in GM +/- CHX or MG132 for 6 Hrs (E). All upper panels were probed with anti-GFP. Lower panels show either anti-actin reactivity or Ponceau stain (pink).

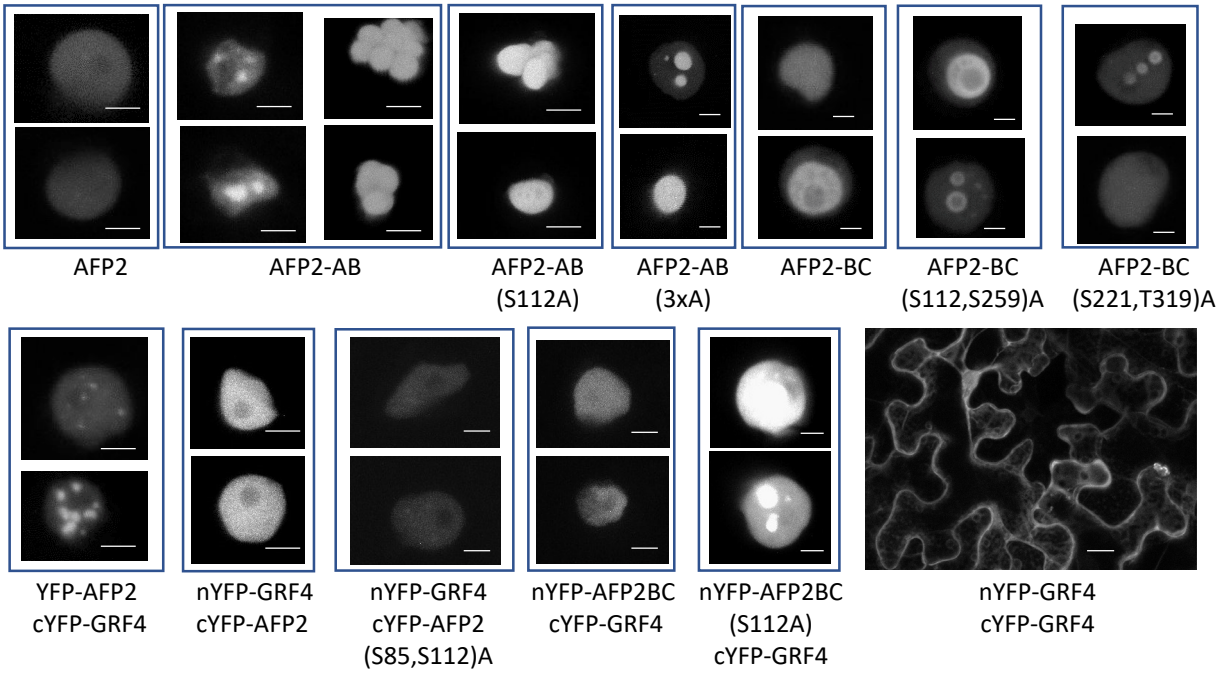


Figure 7. Localization of YFP-fusions to wild-type and phosphomutant AFP2-AB and –BC domains, and split YFP assays with GRF4, in transiently transformed *N. benthamiana*. Confocal images of nuclei: scale bars = 5 um. nYFP-GRF4/cYFP-GRF4 image: scale bar = 20 um

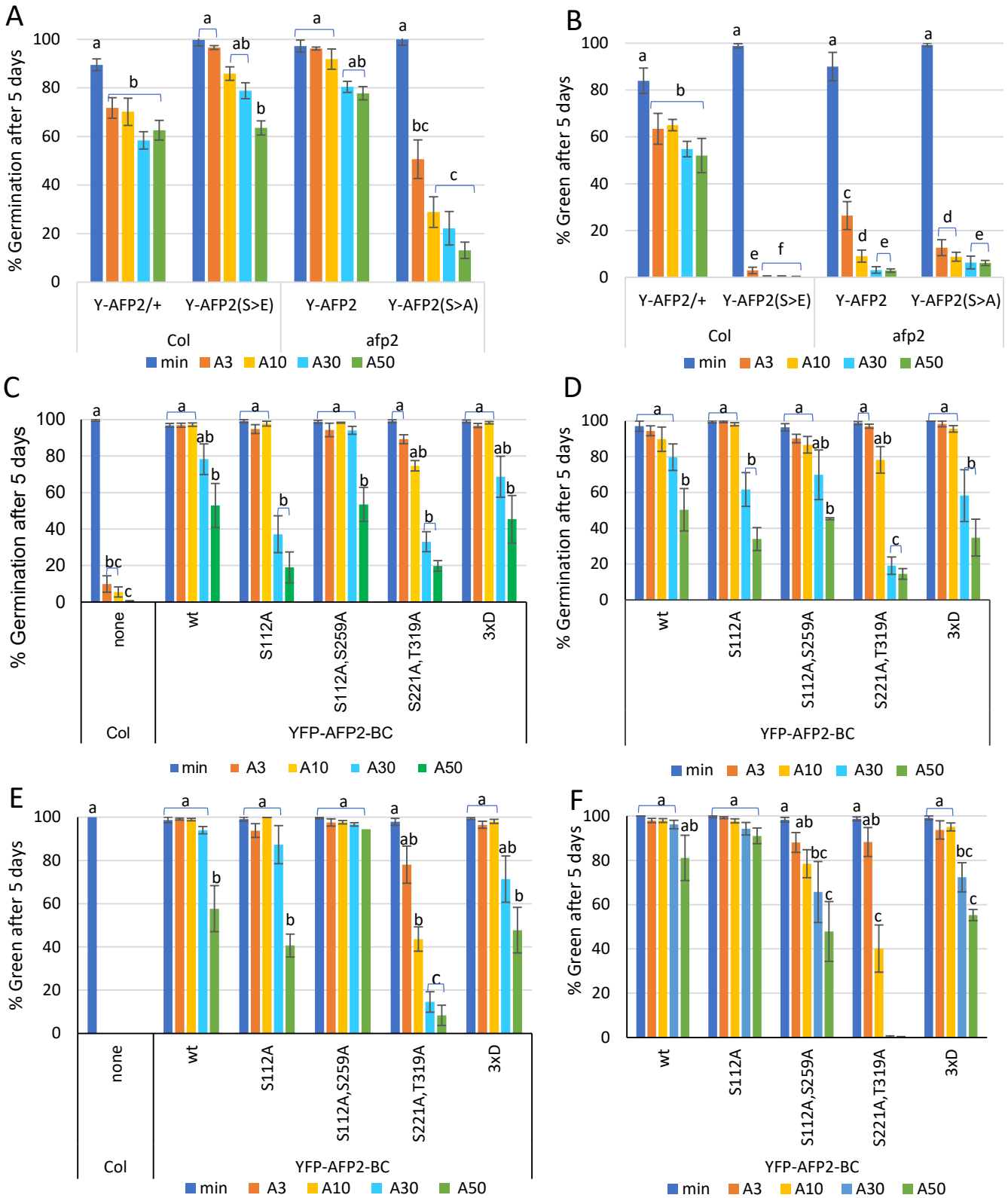
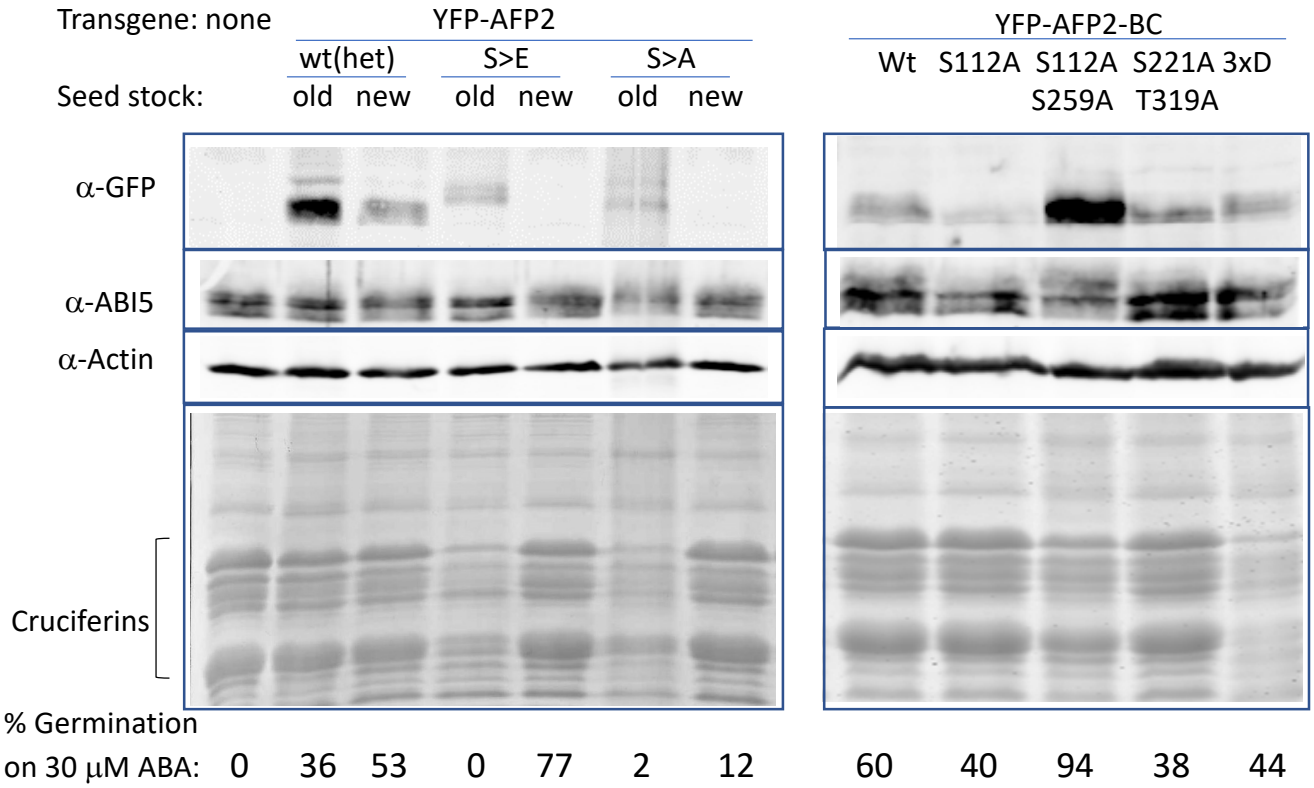


Figure 8. Physiological analysis comparing germination (A,C,D) or cotyledon greening (B,E,F) of transgenic lines overexpressing wild-type (wt) vs phosphomutant or phosphomimic AFP2 fusions in wt(Col) background (A,B,C,E) or *afp2* background (A,B,D,F). Data displayed is the average of at least triplicate assays for each genotype and treatment ± S.E. Bars with different letters represent statistically different values using Tukey's HSD post-hoc test ($p<0.01$).



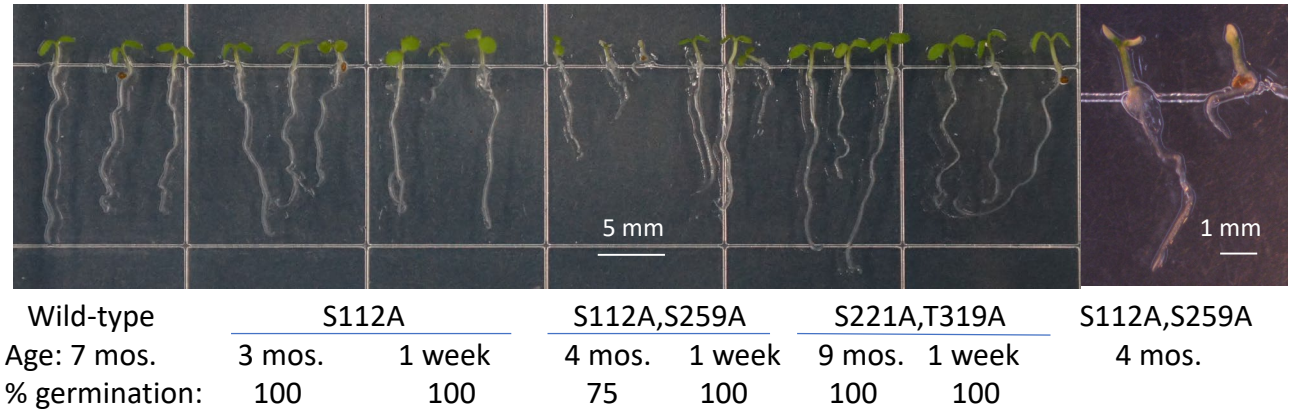


Figure 9. Effects of phosphomutant or phosphomimetic YFP-AFP2 fusions on storage protein accumulation and seed longevity. Top: comparison of dry seed protein extracts from lines either segregating a full-length wild type AFP2 fusion or homozygous for S85E,S112E (S>E) or S85A,S112A (S>A) mutant full-length AFP2 fusions (left) or AFP2-BC fusions with the indicated mutations (right). Upper panels show immunoblots of YFP-AFP2(-BC) fusions detected by α-GFP, ABI5, and Actin as a loading control. Lower panels show Coomassie staining of a comparably loaded higher (15%) SDS-PAGE to retain and resolve the storage proteins. The "old" extracts were from early generations of these lines with high transgene expression and poor seed viability, while "new" extracts were from later generations with partially silenced transgenes permitting better viability but still conferring ABA-resistant germination. Bottom: YFP-AFP2-BC seedlings from seed stocks of the indicated ages and genotypes, and their % germination, after 5d growth on minimal medium.

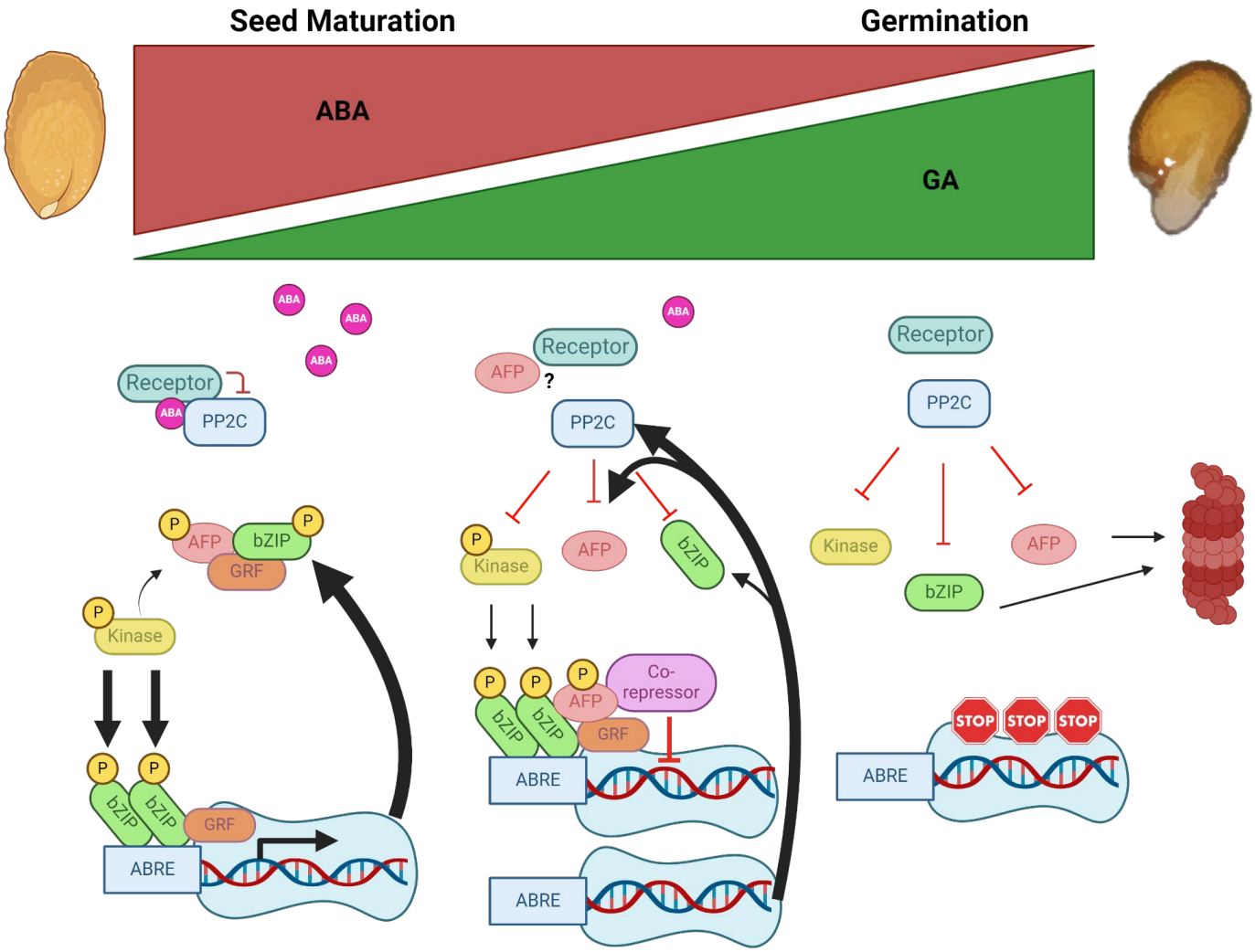


Figure 10. Changing relationships in the ABA core signaling pathway during the transition from seed maturation to germination. At high ABA concentrations, PP2Cs (e.g. AHG1 & AHG3) are inactive, kinases (e.g. SnRK2s & MPKs) are active, bZIPs (e.g. ABI5 & ABFs) and AFPs are phosphorylated and are bound by GRFs (14-3-3 proteins). When the bZIP:AFP ratio is high, ABA-regulated gene expression proceeds and germination is blocked (Garcia et al, 2008). As ABA levels decrease, ABA induces repressors including AFPs and PP2Cs in a feedback mechanism, the bZIP:AFP ratio decreases and seed-expressed gene expression declines. Interaction between AFPs and co-repressors (e.g. TPL/TPR and HDAC components) leads to inactivation of seed-expressed genes (Lynch et al. 2016). At low ABA levels, the PP2Cs are active and inactivate the SnRK2s, bZIPs (Lynch et al. 2012) and AFPs, and the latter two classes are proteasomally degraded. Many additional interactors are not included due to space constraints. The figure was created with BioRender.com.

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