

1 **Arabidopsis ACINUS is O-glycosylated and regulates transcription and alternative**  
2 **splicing of regulators of reproductive transitions**

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25 **Abstract**

26 O-GlcNAc modification plays important roles in metabolic regulation of cellular status.  
27 Two homologs of O-GlcNAc transferase, SECRET AGENT (SEC) and SPINDLY (SPY),  
28 which have O-GlcNAc and O-fucosyl transferase activities, respectively, are essential in  
29 *Arabidopsis* but have largely unknown cellular targets. Here we show that AtACINUS is  
30 O-GlcNAcylated and O-fucosylated and mediates regulation of transcription, alternative  
31 splicing (AS), and developmental transitions. Knocking-out both AtACINUS and its  
32 distant paralog AtPININ causes severe growth defects including dwarfism, delayed seed  
33 germination and flowering, and abscisic acid (ABA) hypersensitivity. Transcriptomic and  
34 protein-DNA/RNA interaction analyses demonstrate that AtACINUS represses  
35 transcription of the flowering repressor *FLC* and mediates AS of *ABH1* and *HAB1*, two  
36 negative regulators of ABA signaling. Proteomic analyses show AtACINUS's O-  
37 GlcNAcylation, O-fucosylation, and association with splicing factors, chromatin  
38 remodelers, and transcriptional regulators. Some AtACINUS/AtPININ-dependent AS  
39 events are altered in the *sec* and *spy* mutants, demonstrating a function of O-  
40 glycosylation in regulating alternative RNA splicing.

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## 47 Introduction

48 Posttranslational modification of intracellular proteins by O-linked *N*-  
49 acetylglucosamine (O-GlcNAc) is an important regulatory post-translational modification  
50 (PTM) that modulates protein activities and thereby control cellular functions according  
51 to nutrient and energy status<sup>1,2</sup>. Extensive studies in animals have shown that  
52 thousands of proteins involved in diverse biological processes are modified on serine  
53 and threonine residues by O-GlcNAcylation, which is catalyzed by O-GlcNAc  
54 transferase (OGT) using UDP-GlcNAc as donor substrate<sup>1,3</sup>. As a sensor of primary  
55 metabolic status, O-GlcNAcylation plays key roles in cellular homeostasis and  
56 responses to nutritional and stress factors<sup>1,2,4,5</sup>, whereas dysregulation of O-  
57 GlcNAcylation has been implicated in many diseases including cancer, diabetes,  
58 cardiovascular and neurodegenerative diseases<sup>5,6</sup>. The *Arabidopsis* genome encodes  
59 two OGT homologs: SPINDLY (SPY) and SECRET AGENT (SEC). The *spy* mutant was  
60 identified as a gibberellin (GA) response mutant with phenotypes of enhanced seed  
61 germination, early flowering, increased stem elongation, and hyposensitivity to the  
62 stress hormone abscisic acid (ABA)<sup>7,8</sup>. The *sec* mutants show no dramatic phenotype,  
63 but the double loss-of-function *spy sec* mutants are embryo lethal<sup>9</sup>. SEC and SPY were  
64 recently reported to have O-GlcNAc and O-fucosyl transferase activities, respectively,  
65 and they antagonistically regulate DELLAs, the repressors of GA signaling<sup>10</sup>. The lethal  
66 phenotype of *spy sec* double mutants suggests that SPY and SEC have broader  
67 functions, which remain to be investigated at the molecular level<sup>10-12</sup>. Our recent study  
68 identified the first large set of 971 O-GlcNAcylated peptides in 262 *Arabidopsis*  
69 proteins<sup>13</sup>. The functions of these O-GlcNAcylation events remain to be characterized.

70 One of the O-GlcNAcylated proteins is AtACINUS, an *Arabidopsis* homolog of  
71 the mammalian apoptotic chromatin condensation inducer in the nucleus (Acinus)<sup>14</sup>. In  
72 animals, Acinus forms the apoptosis and splicing-associated protein (ASAP) complex by  
73 recruiting RNA-binding protein S1 (RNPS1), a peripheral splicing factor, and Sin3-  
74 associated protein of 18 kDa (SAP18), a chromatin remodeler, through its conserved  
75 RNPS1-SAP18 binding (RSB) domain<sup>14</sup>. Another RSB-containing protein, Pinin, forms a  
76 similar protein complex named PSAP, which has distinct biological functions<sup>14,15</sup>. The

77 ASAP and PSAP complexes are believed to function at the interface between histone  
78 modification, transcription, and alternative splicing (AS) in metazoans<sup>14,16,17</sup>. In  
79 *Arabidopsis*, AtRNPS1, also known as ARGinine/Serine-Rich 45 (SR45), has been  
80 implicated in splicing, transcription and RNA-dependent DNA methylation, with effects  
81 on multiple aspects of plant development as well as stress and immune responses<sup>18-23</sup>.  
82 AtSAP18 has been shown to associate with transcription factors involved in stress  
83 responses and embryo development<sup>24,25</sup>. AtACINUS, AtSAP18 and SR45 have been  
84 shown to associate with a transcription factor involved in flowering<sup>26</sup>. While sequence  
85 analysis predicted similar ASAP complex in plants<sup>23</sup>, interactions among SR45,  
86 AtSAP18, and AtACINUS remain to be tested experimentally and the functions of  
87 AtACINUS and AtPININ remain to be characterized genetically.

88 Our finding of O-GlcNAcylation of AtACINUS suggests that the functions of  
89 AtACINUS are regulated by O-linked glycosylation<sup>13</sup>. We therefore performed genetic,  
90 genomic, and proteomic experiments to understand the functions of AtACINUS and its  
91 regulation by O-linked sugar modifications. Our results demonstrate key functions of  
92 AtACINUS and its distant homology AtPININ in regulating seed germination, ABA  
93 sensitivity, and flowering, through direct involvement in AS of two key components of  
94 the abscisic acid (ABA) signaling pathway and in the transcriptional regulation of the  
95 floral repressor *FLC*. Our results further show that AtACINUS is modified by both O-  
96 GlcNAc and O-fucose, is part of the ASAP complex, and associates with splicing and  
97 transcription factors. A subset of AtACINUS-dependent AS events is altered in the *spy*  
98 and *sec* mutants, providing genetic evidence for regulation of AS by the O-linked  
99 glycosylations.

## 100 **Result**

### 101 ***AtACINUS* and *AtPININ* play genetically redundant roles**

102 The *Arabidopsis* AtACINUS (AT4G39680) protein is 633 amino-acid long, and it  
103 shares sequence similarity to all the known motifs of the human Acinus including the N-  
104 terminal SAF-A/B, Acinus and PIAS (SAP) motif, the RNA-recognition motif (RRM) and  
105 the C-terminal RSB motif (Fig. 1a, Supplementary Fig. 1a)<sup>14,16,27</sup>. AtACINUS is a unique



106 gene in *Arabidopsis* with no homolog detectable using standard BLAST (Basic Local  
107 Alignment Search Tool) search of the *Arabidopsis* protein database. However, another  
108 *Arabidopsis* gene (AT1G15200, AtPININ) contains the RSB domain and is considered a  
109 homolog of mammalian Pinin<sup>14</sup>. AtACINUS and AtPININ share 12 amino acids within  
110 the 15-amino acid region of RSB motif (Fig. 1b), but no sequence similarity outside this  
111 motif.

112 To study the biological function of AtACINUS, we obtained two mutant lines that  
113 contain T-DNA insertions in the exons of *AtACINUS*, *Salk\_078854* and  
114 *WiscDsLoxHs108\_01G*, which are designated *acinus-1* and *acinus-2*, respectively (Fig.  
115 1c). These mutants showed no obvious morphological phenotypes except slightly  
116 delayed flowering (Fig. 1d,e). The weak phenotype of *acinus* is surprising considering  
117 the important function of its mammalian counterpart and the absence of any close  
118 homolog in *Arabidopsis*.

119 We did not expect AtACINUS and AtPININ to have redundant functions,  
120 considering their very limited sequence similarity and the fact that mammalian Acinus  
121 and Pinin have distinct functions<sup>14</sup>. AtPININ shares extensive sequence similarity with  
122 human Pinin surrounding the RSB domain<sup>14</sup> (Supplementary Fig. 1b). Phylogenetic  
123 analysis indicated that AtPININ and human Pinin belong to one phylogenetic branch  
124 that is distinct from that of AtACINUS and human Acinus (Supplementary Fig. 1c),  
125 suggesting independent evolution of ACINUS and PININ before the separation of the  
126 metazoan and plant kingdoms. However, Pinin can, through its RSB domain, interact  
127 with RNPS1 and SAP18 to form a complex (PSAP) similar to the ASAP complex.  
128 Therefore, we tested the possibility that the weak phenotype of *Arabidopsis acinus*  
129 mutants is due to functional redundancy with AtPININ.

130 We obtained a T-DNA insertion mutant of AtPININ (*pinin-1*, T-DNA line  
131 *GABI\_029C11*). The *pinin-1* mutant also showed no obvious morphological phenotype  
132 (Fig. 1d). We then crossed *pinin-1* with *acinus-1* and *acinus-2* to obtain double mutants.  
133 Both *acinus-1 pinin-1* and *acinus-2 pinin-1* double mutants displayed pleiotropic  
134 phenotypes including severe dwarfism, short root, pale leaves, narrow and twisted  
135 rosette leaves with serrated margin, severely delayed flowering, altered phyllotaxis,

136 increased numbers of cotyledons and petals, and reduced fertility (Fig. 1d,e, and  
137 Supplementary Fig. 2). The *acinus-2 pinin-1* double mutants transformed with  
138 *35S::AtACINUS-GFP* or *35S::YFP-AtPININ* displayed near wild-type morphology (Fig.  
139 1f), confirming that the phenotypes of the double mutants are due to loss of both  
140 *AtACINUS* and *AtPININ*, and the two genes play genetically redundant roles. The  
141 *AtACINUS-GFP* and *YFP-AtPININ* proteins are localized in the nucleus outside the  
142 nucleolus (Supplementary Fig. 3).

143 We also noticed that the seed germination was delayed in the *acinus pinin*  
144 mutant (Fig. 2a). This, together with the pale leaf and dwarfism phenotypes, suggests  
145 an alteration in ABA response. Indeed, on 0.25  $\mu\text{mol/L}$  ABA, germination of the *acinus-2*  
146 *pinin-1* double mutant seeds was further delayed compared to the wild type and the  
147 single mutants (Fig. 2b). Dose response experiment indicate that seed germination of  
148 the *acinus-1 pinin-1* and *acinus-2 pinin-1* double mutants is about three fold more  
149 sensitive to ABA than wild type and the *acinus* and *pinin* single mutants (Fig. 2c).  
150 Similarly, post-germination seedling growth of *acinus-2 pinin-1* was more inhibited by  
151 ABA (Supplementary Fig. 4a). These ABA-hypersensitive phenotypes were rescued by  
152 expression of either *AtACINUS-GFP* or *YFP-AtPININ* in the *acinus-2 pinin-1*  
153 background (Fig. 2d and Supplementary Fig. 4b). These results indicate that the *acinus-*  
154 *2 pinin-1* double mutant is hypersensitive to ABA, and that *AtACINUS* and *AtPININ* are  
155 redundant negative regulators of ABA responses.

### 156 **AtACINUS and AtPININ are involved in AS of specific introns**

157 We conducted RNA-seq analysis of the transcriptome of the *acinus-2 pinin-1*  
158 double mutant. Wild-type and *acinus-2 pinin-1* seedlings were grown under constant  
159 light for 14 days, and RNA-seq was performed with three biological replicates, each  
160 yielding a minimum of 22.4 million uniquely mapped reads. The RNA-seq data  
161 confirmed the truncation of the *AtACINUS* and *AtPININ* transcripts in the double mutant  
162 (Supplementary Fig. 5). Compared to wild type, the *acinus-2 pinin-1* double mutant  
163 showed significantly decreased expression levels for 786 genes and increased levels of  
164 767 genes (fold change>2, multiple-testing corrected p-value<0.05), which include the  
165 flowering repressor *FLC*<sup>28</sup> (Supplementary Data 1).

166 A significantly higher proportion of reads was mapped to the intron regions in the  
167 *acinus-2 pinin-1* double mutant than in the wild type (Supplementary Fig. 6a). Further  
168 analyses using the RACKJ software package revealed increase of retention of 258  
169 introns in 225 genes and decreased retention of 31 introns in 31 genes in the *acinus-2*  
170 *pinin-1* double mutant compared to wild type (Fig. 3a, Supplementary Data 2). Intron  
171 retention was the dominant form of splicing defect in the *acinus-2 pinin-1* double mutant  
172 (Fig. 3a, Supplementary Fig. 6b). About 99% of these genes contain multiple introns,  
173 and the defects tend to be retention of a specific single intron among many introns of  
174 each gene, indicating defects in alternative splicing rather than general splicing. Among  
175 the RNAs showing increased intron retention, 26 RNAs also showed decreased levels  
176 of RNA abundance, and their retained introns introduce in-frame stop codons  
177 (Supplementary Fig. 7), consistent with non-sense-mediated decay<sup>29</sup>. The results show  
178 that AtACINUS and AtPININ function in AS, primarily by enhancing splicing of a specific  
179 intron among many introns of each transcript.

180 We found a significant overlap between ABA-induced genes and the genes  
181 overexpressed in *acinus-2 pinin-1* (p-value by random chance <2.42E-13) (Fig. 3b).  
182 Only four of these RNAs were mis-spliced in *acinus-2 pinin-1*. One possibility is that  
183 intron retention in RNAs encoding components of ABA synthesis or signaling pathway  
184 leads to expression of ABA-responsive genes. Indeed, we found retention of the 10<sup>th</sup>  
185 intron of *ABA HYPERSENSITIVE 1 (ABH1)* in the *acinus-2 pinin-1* double mutant (Fig.  
186 4a).

187 *ABH1* encodes the large subunit of the dimeric *Arabidopsis* mRNA cap-binding  
188 complex (NUCLEAR CAP-BINDING PROTEIN SUBUNIT 1, CBP80) and functions as a  
189 negative regulator of ABA responses including inhibition of seed germination<sup>30,31</sup>. The  
190 retention of the 10<sup>th</sup> intron of *ABH1* introduces a pre-mature stop codon that truncates  
191 the C-terminal 522 amino acids of *ABH1* (Fig. 4a). Quantification using qRT-PCR  
192 analysis in 12-day-old seedlings showed that the intron-containing *ABH1.2* transcript  
193 was about 8-10% of the total *ABH1* transcripts in the wild type, about 11% in *pinin-1*,  
194 about 15% in *acinus-2*, but more than 50% in *acinus-2 pinin-1* (Fig. 4b,c). Expression of  
195 either YFP-AtPININ or AtACINUS-GFP in the *acinus-2 pinin-1* background rescued the

196 *ABH1* intron retention phenotype (Fig. 4b,c). Consistent with compromised *ABH1*  
197 activity, the gene expression changes in *acinus-2 pinin-1* show a strong correlation to  
198 those in *abh1*, with Spearman's correlation=0.74 as calculated by AtCAST3.1  
199 (Supplementary Fig. 8)<sup>32,33</sup>.

200 Intron retention in *HAB1* has been reported to cause ABA hypersensitive  
201 phenotypes<sup>34,35</sup>. *HAB1* did not display any apparent splicing defects in our RNA-seq and  
202 RT-PCR analysis of 12-day old seedling. However, after ABA treatment, *HAB1* intron  
203 retention is significantly increased in *acinus pinin* compared to the wild type. While the  
204 expression level of *HAB1* transcripts was increased similarly in wild type and *acinus*  
205 *pinin*, the wild-type seedlings maintained relatively similar ratios between different splice  
206 forms of *HAB1* before and after ABA treatment, whereas the *acinus pinin* mutant  
207 accumulated a much increased level of the intron-containing *HAB1.2* and a reduced  
208 level of fully spliced *HAB1.3* after ABA treatment (Fig. 4d,e). *HAB1.2* encodes a  
209 dominant negative form of HAB1 protein that activates ABA signaling<sup>34,35</sup>. Therefore, the  
210 accumulation of HAB1.2 should contribute to the ABA hypersensitivity of the *acinus*  
211 *pinin* mutant.

212 To test whether AtACINUS is directly involved in AS of *ABH1* and *HAB1*, we  
213 carried out an RNA immunoprecipitation (RIP) experiment using an *AtACINUS-*  
214 *GFP/acinus-2* transgenic line, with *35S::GFP* transgenic plants as the negative control.  
215 Immunoprecipitation using an anti-GFP antibody pulled down significantly more *ABH1*  
216 and *HAB1* RNAs in *AtACINUS-GFP/acinus-2* than in the *35S::GFP* control (Fig. 4f,g),  
217 indicating that AtACINUS interacts with *ABH1* and *HAB1* RNAs *in vivo* and is involved in  
218 their splicing.

### 219 **AtACINUS regulates flowering through repression of *FLC***

220 Consistent with the late flowering phenotype of *acinus pinin* (Fig. 1e, 5a), our  
221 RNA-seq data showed an increased expression level of the floral repressor *FLC*,  
222 without obvious alteration of the splicing pattern (Supplementary Fig. 9a). RT-qPCR  
223 analysis confirmed the increased levels of *FLC* RNA that correspond to the severity of  
224 the late-flowering phenotypes in the single and double mutants (Fig. 5b). As *FLC*  
225 expression is also controlled by its anti-sense RNA, which undergoes AS<sup>36,37</sup>, we

226 analyzed the anti-sense *FLC* RNAs using RT-qPCR. The results showed a dramatic  
227 increase of the class I anti-sense RNA and a slight increase of the class II anti-sense  
228 RNA of *FLC*, but no obvious change of the splicing efficiency of the *FLC* anti-sense  
229 RNAs (Supplementary Fig. 9b-d). AtACINUS was recently reported to associate with  
230 VAL1 and VAL2, which bind to the *FLC* promoter to repress transcription<sup>26</sup>. We thus  
231 performed chromatin immunoprecipitation (ChIP) assays to test whether AtACINUS is  
232 associated with the *FLC* locus, and our results show that AtACINUS interacts with the  
233 DNA of the promoter and first intron regions but not the 3' region of *FLC in vivo* (Fig. 5c).  
234 Together our results provide evidence for a role of AtACINUS in regulating the  
235 transcription of *FLC*.

### 236 **AtACINUS-dependent AS events are altered in *spy* and *sec***

237 To study how O-linked sugar modification affects the function of AtACINUS, we  
238 tested if the AtACINUS-dependent AS events are altered in the *spy* and *sec* mutants. Of  
239 the ten AtACINUS-dependent intron splicing events we have tested, four showed  
240 alterations in the *spy* mutant and one showed alteration in the *sec* mutant (Fig. 6).

241 In the 7-day-old light-grown plants, splicing of the 12<sup>th</sup> intron and the 15<sup>th</sup> intron of  
242 *TRNA METHYLTRANSFERASE 4D* (*TRM4D*, *At4g26600*) was enhanced in the *acinus-2*  
243 *pinin* double mutant compared to that in the WT. In the loss-of-function mutants *spy-4*  
244 and *spy-t1* (SALK\_090580), the splicing efficiency of these two introns were also  
245 enhanced. In contrast, the loss-of-function mutants *sec-2* and *sec-5* showed an  
246 increased retention of the 12<sup>th</sup> intron (Fig. 6). These results suggest that SPY and SEC  
247 have opposite effects on AtACINUS function in *TRM4D* splicing. The *spy-t1* and *spy-4*  
248 mutants accumulated more *HAB1.3* and less *HAB1.2* than wild type, while *acinus-2*  
249 *pinin* accumulated more *HAB1.2* than the wild type (Fig. 6), consistent with their  
250 opposite seed germination phenotypes. In addition, the splicing efficiency of the 14<sup>th</sup>  
251 intron of *EMBRYO DEFECTIVE 2247* (*Emb2247*, *AT5G16715*) was reduced in the  
252 *acinus-2 pinin* double mutant, but was increased in the *spy-t1* and *spy-4* mutants  
253 compared to wild type (Fig. 6). These results support that the O-linked sugar  
254 modifications of AtACINUS modulate its functions in alternative splicing of specific  
255 RNAs.

## 256 **AtACINUS associates with transcriptional and splicing factors**

257 To understand the molecular mechanisms of AtACINUS function, we conducted  
258 two immunoprecipitations followed by mass spectrometry (IP-MS) experiments. In the  
259 first experiment, immunoprecipitation was performed in three biological replicates using  
260 the AtACINUS-GFP/*acinus-2* plants and the anti-GFP nanobody. Transgenic plants  
261 expressing a Tandem-Affinity-Purification-GFP (TAP-GFP) protein was used as  
262 control<sup>38</sup>. The proteins co-immunoprecipitated with AtACINUS-GFP was identified  
263 based on enrichment (FDR=0.01, S0=2) relative to the TAP-GFP control, quantified by  
264 label-free mass spectrometry analysis. In the second experiment, AtACINUS-associated  
265 proteins were identified by <sup>15</sup>N stable-isotope-labeling in *Arabidopsis* (SILIA) quantitative  
266 MS. Wild-type and *acinus-2* mutant seedlings were metabolically labelled with <sup>14</sup>N and  
267 <sup>15</sup>N, and immunoprecipitation was performed using the anti-AtACINUS antibody,  
268 followed by mass spectrometry analysis. The isotope labels were switched in the two  
269 biological replicates. AtACINUS-associated proteins were identified based on  
270 enrichment in the wild type compared to the *acinus* mutant control. These IP-MS  
271 experiments consistently identified 46 AtACINUS-associated proteins (Fig. 7a,  
272 Supplementary Fig. 10a and Supplementary Data 3). These included SR45 and  
273 AtSAP18, supporting the existence of an evolutionarily conserved ASAP complex in  
274 *Arabidopsis*. The AtACINUS interactome also included a large number of proteins  
275 homologous to known components of the spliceosome, including five Sm proteins, one  
276 protein of the U2 complex, four proteins in the U5 complex, seventeen proteins of the  
277 nineteen complex (NTC) and NTC-related complex (NTR)<sup>39-41</sup>. In addition, AtACINUS  
278 associated with six proteins of the exon junction complex (EJC) core and the EJC-  
279 associated TRanscription-EXport (TREX) complex, three proteins of the small nucleolar  
280 ribonucleoprotein (snoRNP) complexes, and four other splicing-related proteins (Fig. 7a,  
281 Supplementary Data 3)<sup>41-45</sup>. AtACINUS interactome also included a component of the  
282 RNA Polymerase II Associated Factor 1 Complex (PAF1C) (Fig. 7a, Supplementary  
283 Data 3). The interactome data suggests that, similar to mammalian Acinus, AtACINUS  
284 plays dual roles in AS and transcriptional regulation.



285 The AtACINUS interactome includes five proteins that are genetically involved in  
286 regulating *FLC* and flowering (Fig. 7a, Supplementary Data 3). These are BRR2 and  
287 PRP8 of the U5 complex, ELF8 of the PAF1C, and SR45 and AtSAP18 of the ASAP  
288 complex<sup>19,37,46,47</sup>. These results suggest that AtACINUS may regulate *FLC* expression  
289 through a complex protein network involving multiple regulatory pathways.

290 We have previously identified O-GlcNAcylation modification on Thr79 on  
291 AtACINUS<sup>13</sup> (Fig. 7b) after LWAC enrichment. Mass spectrometry analysis following  
292 affinity purification of AtACINUS identified additional O-GlcNAc modification on the  
293 peptide containing amino acids 407-423 (Fig. 7c, Supplementary Fig. 10b), as well as  
294 O-fucosylation on the peptide containing amino acids 169-197 (Fig. 7d). These results  
295 confirm that AtACINUS is a target of both O-GlcNAc and O-fucose modifications.

296 Using targeted mass spectrometry analysis, we confirmed that the *acinus-2 pinin*  
297 double mutant expressed only the AtACINUS's N-terminal peptides (at about 20% wild-  
298 type level), but no detectable peptides of the C-terminal region (after T-DNA insertion)  
299 (Supplementary Fig 11, Supplementary Table 5). Both N- and C-terminal peptides of  
300 AtPININ were undetectable in the *acinus-2 pinin* mutant (Supplementary Fig 12,  
301 Supplementary Table 5). Meanwhile, SR45 and AtSAP18 protein levels were  
302 dramatically reduced to 3.9% and 2.7% of wild-type levels, respectively (Supplementary  
303 Fig. 13 and 14, Supplementary Table 5). Together, these results indicate that the  
304 stability of the other members of the ASAP and PSAP complexes is dependent on  
305 AtACINUS and AtPININ.

## 306 Discussion

307 Our recent identification of O-GlcNAcylated proteins in *Arabidopsis* enabled  
308 functional study of this important signaling mechanism in plants<sup>13</sup>. Here our systematic  
309 analysis of one of these O-GlcNAcylated proteins, AtACINUS, demonstrates its  
310 functions as a target of O-GlcNAc and O-fucose signaling and a component of the  
311 evolutionarily conserved ASAP complex that regulates transcription and RNA alternative  
312 splicing thereby modulating stress responses and developmental transitions. Our  
313 comprehensive genetic, transcriptomic, and proteomic analyses provide a large body of  
314 strong evidence illustrating a molecular pathway in which nutrient sensing O-

315 GlcNAcylation and O-fucosylation modulate specific functions of the evolutionarily  
316 conserved RSB-domain protein AtACINUS to modulate stress hormone sensitivity, seed  
317 germination, and flowering in plants (Fig. 7e).

318 Studies in animals have identified Acinus and Pinin as essential cellular  
319 components that bridge chromatin remodeling, transcription and splicing through the  
320 formation of analogous ASAP and PSAP complexes<sup>14,16,17,48-51</sup>. Sequence alignment  
321 and phylogenetic analysis show that the *Arabidopsis* orthologs, AtACINUS and AtPININ,  
322 share higher levels of sequence similarity to their animal counterparts than to each  
323 other and appear to have evolved independently since the separation of the plant and  
324 metazoan kingdoms<sup>14</sup>. Considering their evolutionary distance and limited sequence  
325 similarity (12 amino acid residues in the RSB motif), it was surprising that the functions  
326 of AtACINUS and AtPININ are genetically redundant. This represents likely the least  
327 sequence similarity between two redundant genes and raises cautions for prediction of  
328 genetic redundancy based on the level of sequence similarity.

329 The developmental functions in seed germination and flowering seem to involve  
330 AtACINUS's distinct activities in splicing and transcription of key components of the  
331 regulatory pathways. Specifically, AS events in *ABH1* and *HAB1* are likely the major  
332 mechanisms by which AtACINUS modulates ABA signaling dynamics to control seed  
333 germination and stress responses. *ABH1* is an mRNA cap-binding protein that  
334 modulates early ABA signaling<sup>30,31</sup>. The loss-of-function *abh1* mutant with a T-DNA  
335 insertion in the 8<sup>th</sup> intron is ABA hypersensitive with enhanced early ABA signaling<sup>30</sup>.  
336 Similarly, the retention of the 10<sup>th</sup> intron of *ABH1* in *acinus pinin* mutant is expected to  
337 truncate its C-terminal half and cause loss of *ABH1* function and thus increase of ABA  
338 sensitivity. Supporting the functional role of the ASAP/PSAP-*ABH1* pathway, we  
339 observed a significant correlation between the transcriptomic changes in *abh1* and the  
340 *acinus pinin* double mutant (Supplementary Fig. 8)<sup>32,33</sup>. A recent proteomic study  
341 showed that the *ABH1* protein level was decreased in the *sr45* mutant<sup>23</sup>, whereas a  
342 reduction of *ABH1* RNA level to ~30% caused obvious phenotypes in potato<sup>52</sup>.

343 AtACINUS-mediated AS of *HAB1* switches a positive feedback loop to a negative  
344 feedback loop in the ABA signaling pathway. *HAB1* encodes a phosphatase that



345 dephosphorylates the SNF1-related protein kinases (SnRK2s) to inhibit ABA responses,  
346 and the ligand-bound ABA receptor inhibits HAB1 to activate ABA responses<sup>53,54</sup>. The  
347 intron-containing *HAB1.2* encodes a dominant negative form of HAB1 protein that lacks  
348 the phosphatase activity but still competitively interacts with SnRK2, thus activating,  
349 instead of inhibiting, ABA signaling<sup>34,35</sup>. As ABA signaling feedback increases the *HAB1*  
350 transcript level, the AtACINUS-mediated AS switches a positive feedback loop that  
351 reinforces ABA signaling to a negative feedback loop that dampens ABA signaling.  
352 Such a switch is presumably important the different ABA signaling dynamics required  
353 for the onset of and recovery from stress responses or dormancy.

354 The relative contributions of intron retention of *ABH1* and *HAB1* to ABA  
355 sensitivity will need to be quantified by genetic manipulation of each splicing event.  
356 Additional mechanisms may contribute to the ABA-hypersensitivity phenotypes of  
357 *acinus pinin*. For example, the level of SR45 is significantly decreased in *acinus pinin*,  
358 while loss of SR45 has been reported to cause accumulation of SnRK1 which is a  
359 positive regulator of stress and ABA responses<sup>55</sup>.

360 The late-flowering phenotype of the *acinus pinin* mutant correlated with increased  
361 *FLC* expression. A role of AtACINUS in repressing *FLC* has been suggested based on  
362 its association with the VAL1 transcription factor, which binds to the *FLC* promoter<sup>26</sup>.  
363 Our results provide genetic evidence for the function of AtACINUS in repressing *FLC*  
364 expression. Further, our ChIP-PCR analysis shows that AtACINUS associates with  
365 genomic DNA of the promoter region and the first intron of *FLC*, confirming a direct role  
366 in transcriptional regulation of *FLC*. These results provide critical evidence for the  
367 hypothesis that the AtACINUS represses *FLC* by AtSAP18-mediated recruitment of the  
368 Sin3 histone deacetylase complex (HDAC)<sup>26</sup>. It's worth noting that overexpression of  
369 AtSAP18 in the *sr45* mutant increased *FLC* expression and further delayed flowering<sup>23</sup>.  
370 It's possible that the transcriptional repression function of AtSAP18 requires the  
371 ASAP/PSAP complex. It's also worth noting that the AtACINUS interactome includes  
372 several proteins known to be involved in regulating *FLC* expression and flowering.  
373 Among these, BRR2 and PRP8 are components of the U5 complex and mediate  
374 splicing of the sense and anti-sense transcripts of *FLC* to inhibit and promote flowering,

375 respectively<sup>37,46</sup>. ELF8 is a component of the PAF1 complex and promotes histone  
376 methylation of *FLC* chromatin<sup>47</sup>. The identification of additional *FLC*-regulators as  
377 AtACINUS-associated proteins suggests that AtACINUS may regulate *FLC* expression  
378 through complex protein networks. Genetic evidence supports that ELF8/PAF1C and  
379 SR45 also have dual functions in regulating *FLC* expression and ABA  
380 responses<sup>18,19,22,56</sup>, suggesting that the functions of AtACINUS in seed germination and  
381 flowering may involve overlapping protein networks.

382 Structural studies in metazoan systems showed that the RSB domains of Acinus  
383 and Pinin directly interact with RNPS1 and SAP18, forming a ternary ASAP and PSAP  
384 complexes that have both RNA- and protein-binding properties as well as abilities to  
385 interact with both RNA splicing machinery and histone modifiers<sup>14</sup>. ASAP and PSAP  
386 function as EJC peripheral protein complexes to modulate RNA processing<sup>15,57</sup>. Our  
387 quantitative proteomic analysis of the AtACINUS interactome indicates strong  
388 interaction with SR45 (ortholog of RNPS1) and AtSAP18, as well as components of EJC.  
389 Further, levels of SR45 and AtSAP18 proteins are greatly reduced in *acinus pinin*. In  
390 contrast, the *sr45* mutation leads to a similar near absence of AtSAP18 but only a mild  
391 decrease of the AtACINUS protein level<sup>23</sup>. Together these observations support the  
392 notion that AtACINUS and AtPININ mediate formation of similar ASAP and PSAP  
393 complexes and stabilize SR45 and AtSAP18 in plants.

394 Studies in human cells have shown that Acinus and Pinin mediate splicing of  
395 distinct RNAs and that Acinus cannot rescue the splicing defects caused by knockdown  
396 of Pinin<sup>15</sup>. In contrast, AtACINUS and AtPININ appear to have largely redundant and  
397 interchangeable functions. It's possible that both AtACINUS and AtPININ, through their  
398 RSB domain, recruit SR45 and AtSAP18, which determine target specificities. However,  
399 AtACINUS and AtPININ may have subtle differences in their functions. Like human  
400 Acinus, AtACINUS contains two additional conserved domains that are absent in  
401 AtPININ. Further, the regions of AtACINUS and AtPININ, as well as human Acinus and  
402 Pinin, outside the RSB domain contain mostly divergent intrinsically disordered  
403 sequences<sup>58</sup> (Supplementary Fig. 15). These distinct sequences may provide specificity  
404 in interactions with target transcripts and partner proteins or in regulation by PTMs<sup>58</sup>.

405 Indeed, O-GlcNAcylated residues (Thr79 and amino acids 407-423) and the O-  
406 fucosylated site (amino acids 169-197) were in the intrinsically disordered regions of  
407 AtACINUS, whereas no O-GlcNAc or O-fucose modification was detected in AtPININ,  
408 though this could be due to partial sequence coverage of our mass spectrometry  
409 analysis. Deep RNA-seq analysis with higher sequence coverage of the single and  
410 double mutants of *acinus* and *pinin* will be required to fully understand their functional  
411 overlap and specificities.

412 How SEC/O-GlcNAc and SPY/O-fucose modulate development and physiology  
413 of plants is not fully understood at the molecular level. The mechanism of regulating GA  
414 signaling involves antagonistic effects of O-fucosylation and O-GlcNAcylation of the  
415 DELLA proteins<sup>10</sup>. Similarly, we observed opposite effect of *spy* and *sec* on the splicing  
416 of the 12th intron of *TRM4D*, suggesting distinct effects of O-GlcNAcylation and O-  
417 fucosylation on AtACINUS functions. Consistent with their different phenotype severities,  
418 more AS events were affected in *spy* than *sec*. The *spy* mutant showed increased  
419 splicing for four of the ten introns analyzed; two of these introns (in *TRM4D*) were more  
420 spliced and the other two (*HAB1* and *EMB2247*) were less spliced in the *acinus pinin*  
421 mutant than in wild type, suggesting that the SPY-mediated O-fucosylation may have  
422 different effects on AtACINUS activities on different transcripts. The two O-GlcNAc-  
423 modified residues (Thr79 and amino acids 407-423) and the O-fucose modified residue  
424 (amino acids 169-197) are in different regions of the intrinsically disordered sequence<sup>58</sup>  
425 (Supplementary Fig. 15), suggesting that PTMs in the disordered sequences play roles  
426 in substrate-specific splicing activities.

427 The high percentage of AtACINUS-dependent AS events affected in *spy* and *sec*  
428 supports an important function of AtACINUS in mediating the regulation of AS by O-  
429 glycosylation. On the other hand, AtACINUS-independent mechanisms may also  
430 contribute to the regulation, as the O-GlcNAcylated *Arabidopsis* proteins include  
431 additional RNA-binding and splicing factors<sup>13</sup>, such as SUS2 which is in the AtACINUS  
432 interactome. Deep transcriptomic analysis of *spy*, *sec*, and conditional double *spy sec*  
433 mutants will be required to better understand how O-GlcNAc and O-fucose modulate  
434 RNA processing and AtACINUS function. Genetic analyses have suggested that SPY

435 acts upstream of the ABA insensitive 5 (ABI5) transcription factor in regulating seed  
436 germination<sup>8</sup>. The molecular link between SPY/O-fucose and ABA signaling has  
437 remained unknown. Our results support a hypothesis that O-fucose modification  
438 modulates AtACINUS activity in splicing a subset of transcripts including *HAB1* to  
439 modulate ABA sensitivity. The biological function of this SPY-AtACINUS pathway  
440 remains to be further evaluated by genetic analyses including mutagenesis of the O-  
441 fucosylation sites of AtACINUS. It is likely that parallel pathways also contribute to the  
442 regulation of ABA sensitivity and seed germination by O-fucosylation and O-  
443 GlcNAcylation. For example, increased GA signaling was thought to contribute to ABA  
444 hyposensitivity in the *spy* mutant<sup>59</sup>. Further, the ABA response element binding factor 3  
445 (ABF3) is also modified by O-GlcNAc<sup>13</sup>. The function of O-glycosylation in stress  
446 responses seems to be conserved, as large numbers of molecular connections between  
447 O-GlcNAc and stress response pathways have been reported in metazoans<sup>5</sup>.

448 How O-linked glycosylation of AtACINUS affect its transcriptional activity at the  
449 *FLC* locus remains to be investigated. Both *spy* and *sec* mutants flower early, opposite  
450 to *acinus pinin*. While *spy* shows strong early flowering phenotype, the *FLC* expression  
451 level was unaffected in *spy* under our experimental conditions (Supplementary Fig. 16),  
452 suggesting that SPY regulates flowering independent of *FLC*. The *FLC* level was  
453 decreased in *sec*<sup>60</sup>, supporting the possibility that O-GlcNAcylation affects AtACINUS  
454 transcription activity. However, the effect of *sec* on *FLC* expression could also be  
455 mediated by other O-GlcNAc-modified flowering regulators<sup>13,60</sup>.

456 Our study reveals important functions of AtACINUS in developmental transitions  
457 and a previously unknown function of O-linked glycosylation in regulating RNA  
458 alternative splicing. While we were getting our revised manuscript ready for submission,  
459 evidence was reported for similar function of O-GlcNAc in intron splicing in metazoan  
460 and for broad presence of stress-dependent intron retention in plants. Interestingly,  
461 inhibition of OGT was found to increase splicing of detained introns in human cells<sup>61</sup>.  
462 Detained introns are a novel class of post-transcriptionally spliced (pts) introns, which  
463 are one or few introns retained in transcripts where other introns are fully spliced<sup>62</sup>.  
464 Transcripts containing pts introns are retained on chromatin and are considered a

465 reservoir of nuclear RNA poised to be spliced and released when rapid increase of  
466 protein level is needed, such as in neuronal activities<sup>62,63</sup>. A recent study uncovered a  
467 large number of pts introns in *Arabidopsis*. A significant portion of these pts introns  
468 show enhanced intron retention under stress conditions. Several splicing factors  
469 involved in pts intron splicing, MAC3A, MAC3B and SKIP<sup>64</sup>, are parts of the AtACINUS  
470 interactome. Among the introns retained in the *acinus pinin* mutant, 114 are pts introns,  
471 which is about 1.7-fold the random probability ( $p$  value < 3.0e-9). These pts introns  
472 include the intron retained in *ABH1* but not that in *HAB1*, consistent with translation of  
473 the dominant negative form of HAB1.2<sup>34,35</sup>. Together with these recent developments,  
474 our study raises the possibility that AtACINUS plays important roles in the splicing of pts  
475 introns, acting downstream of the metabolic signals transduced by SPY/O-fucose and  
476 SEC/O-GlcNAc. Our study supports an evolutionarily conserved function of O-  
477 glycosylation in regulating RNA splicing, thereby linking metabolic signaling with  
478 switches of cellular status between normal and stress conditions as well as during  
479 developmental transitions.

## 480 **Material and Methods**

### 481 **Plant materials**

482 All the *Arabidopsis thaliana* plants used in this study were in the Col-0 ecotype  
483 background. The plants were grown in greenhouses with a 16-h light/8-h dark cycle at 22-24°C  
484 for general growth and seed harvesting. For seedlings grown on the medium in Petri dishes, the  
485 sterilized seeds were grown on ½ Murashige and Skoog (MS) medium and supplemented with  
486 0.7% (w/v) phytoagar. Plates were placed in a growth chamber under the constant light  
487 condition at 21-22 °C. T-DNA insertional mutants for AtACINUS(AT4G39680), *atacinus-1*  
488 (Salk\_078854, insertion position +674 relative to the genomic translational start site), *atacinus-2*  
489 (WiscDsLoxHs108\_01G, insertion position +1744), and for AtPININ (AT1G15200) *atpinin-1*  
490 (GABI\_029C11, insertion position +1817), *spy-t1* (Salk\_090580), and *sec-5* (Salk\_034290)  
491 were obtained from *Arabidopsis* Biological Resource Center. The *spy-4* and *sec-2* seeds that  
492 have been backcrossed to Columbia for six generations were provided by Neil Olszewski lab.

### 493 **Germination assay**

494 Seeds were surface sterilized with 70% (v/v) ethanol and 0.1% (v/v) Triton X-100  
495 sterilization solution for 5 mins. The sterilization solution was then removed and seeds were re-

496 suspended in 100% ethanol and dried on a filter paper. The sterilized seeds were then plated on  
497 ½ MS medium supplemented with mock or ABA. The seeds were placed in 4°C cold room for 3  
498 days for stratification before moving into a growth chamber to germinate. Germination was  
499 defined as obvious radicle emergence from the seed coat.

## 500 **Gene cloning and plant transformation**

501 The *AtACINUS* cDNA was initially cloned into the vector pENTR-D/TOPO and  
502 subsequently into the binary vector pGWB5 to generate the *35S::AtACINUS-GFP* plasmid. The  
503 *35S::AtACINUS-GFP* binary plasmid was transformed into *acinus-2* plants by floral dipping with  
504 *A. tumefaciens* strain GV3101. A homozygous *35S::AtACINUS-GFP/acinus-2* plant was  
505 selected for similar protein expression level to the endogenous *AtACINUS* protein of wild-type  
506 plants using a native α-*AtACINUS* antibody, and crossed with *acinus-2 pinin-1* to obtain  
507 *35S::AtACINUS-GFP/acinus-2 pinin-1* transgenic lines. Similarly, *35S::AtACINUS-YFP-TurboID*  
508 plasmid was generated by LR reaction of gateway-compatible *35S::YFP-TbID*<sup>65</sup> with *pENTR-*  
509 *AtACINUS* and transformed to *acinus-2 pinin-1* to obtain transgenic lines.

510 The *AtPININ* cDNA was acquired from *Arabidopsis* stock center and subsequently  
511 cloned into the binary vector pEarleyGate104 to generate the *35S::YFP-AtPININ* vector. The  
512 *35S::YFP-AtPININ* binary plasmid was transformed into *acinus-2 pinin-1+* plants by floral  
513 dipping with *A. tumefaciens* strain GV3101. Transgenic plants were genotyped for *pinin-1* allele  
514 to obtain *35S::YFP-AtPININ/acinus-2 pinin-1* transgenic lines.

## 515 **Bioinformatics analysis**

516 Dendrogram of *AtACINUS* and *AtPININ* homologs in different species was constructed  
517 using the “simple phylogeny” web tool of EMBL-EBI website with UPMGA method using default  
518 settings ([https://www.ebi.ac.uk/Tools/phylogeny/simple\\_phylogeny/](https://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny/)). The protein alignment was  
519 generated using MUSCLE from EMBL-EBI website with default setting  
520 (<https://www.ebi.ac.uk/Tools/msa/muscle/>)<sup>66,67</sup>. Pairwise protein sequence alignment was  
521 performed with Blastp from the NCBI blastp suite with E-value set to 0.01.  
522 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>).

523 Protein disorderness was predicted based on amino acid sequences using PrDOS  
524 (<http://prdos.hgc.jp/cgi-bin/top.cgi>) with the default setting<sup>68</sup>.

525 Gene expression correlation was analyzed with AtCAST3.1 using default settings  
526 (<http://atpbsmd.yokohama-cu.ac.jp/cgi/atcast/home.cgi>)<sup>33</sup>.



## 527 RNA sequencing and data analysis

528 RNA was extracted from 14-day-old wild-type and *acinus-2 pinin-1* seedlings using  
529 RNeasy mini kit (Qiagen) and treated with TURBO DNA-free Kit (Ambion) to remove any  
530 genomic DNA contamination. The mRNA libraries were constructed using NEBNext RNA  
531 Library Prep Kit for Illumina following the standard Illumina protocol. Illumina sequencing was  
532 performed in the Sequencing Center for Personalized Medicine, Department of Genetics in  
533 Stanford University using an Illumina HiSeq 2000 System. The RNA-seq data have been  
534 deposited at the NCBI Gene Expression Omnibus (GEO) database under the accession number  
535 GSE110923.

536 Differential gene expression was analyzed using STAR and Deseq2. Trimmed and  
537 quality control-filtered sequence reads were mapped to the *Arabidopsis* reference genome  
538 (TAIR10) using STAR (v.2.54) in two pass mode (parameters: `-outFilterScoreMinOverLread 0.3`,  
539 `-outFilterMatchNminOverLread 0.3`, `-outSAMstrandField intronMotif`, `-outFilterType BySJout`, `-`  
540 `outFilterIntronMotifs RemoveNoncanonical`, `-quantMode TranscriptomeSAM GeneCounts`)<sup>69</sup>. To  
541 obtain uniquely mapping reads, these were filtered by mapping quality (q20), and PCR  
542 duplicates were removed using Samtools rmdup (v.1.3.1). Gene expression was analyzed in R  
543 (v.3.4.1) using DEseq2 (v.1.16.1)<sup>70</sup>. Significant differentially expressed genes are selected  
544 based on  $\text{adjP-value} < 0.02$  and fold change  $> 2$ .

545 Alternative splicing analysis was performed with RACKJ using default setting (online  
546 manual available at <http://rackj.sourceforge.net/>)<sup>71</sup>. Raw intron retention data was analyzed and  
547 filtered to reduce false positives with 2 criteria: 1) fold change of intron retention  $> 2$ , p-  
548 value  $< 0.05$  in a two-tail T-test and 2) Intron RPKM  $> 1$  and estimated percentage of IR  $> 5\%$  in  
549 the sample that shows increased IR in the intron. Raw exon skipping (ES) data was analyzed  
550 and filtered with 2 criteria: 1) fold change of ES rate  $> 2$ , p-value  $< 0.05$  in a two-tail T-test, and 2)  
551 Increased ES event is supported by reads with RPKM  $> 1$  and ES rate  $> 5\%$ . For alternative  
552 donor/acceptor usage discovery, only events that appear significantly different in each pair-wise  
553 comparison between wild-type and *acinus-2 pinin-1* (fisher's exact test p-value  $< 0.05$ ) were  
554 considered significant and were further filtered with 2 criteria: 1) fold change  $> 2$ , and 2)  
555 Increased alternative donor/acceptor usage is supported by reads with RPKM  $> 1$  and rate  $> 5\%$ .

## 556 RNA extraction, reverse transcription PCR

557 RNA was extracted from seedlings using Spectrum™ Plant Total RNA Kit (Sigma) and  
558 treated with TURBO DNA-free Kit (Ambion) to remove any genomic DNA contaminants. Purified

559 RNA (500ng) is subjected to cDNA synthesis using RevertAid Reverse Transcriptase (Thermo)  
560 with Oligo(dT)<sub>18</sub> primer. The synthesized cDNA was used for PCR and qPCR analyses. PCR  
561 products were analyzed by gel electrophoresis and the PCR band intensities were quantified  
562 using ImageJ. The qPCR analyses were performed with the SensiMix™ SYBR® & Fluorescein  
563 Kit (Bioline) on a LightCycler 480 (Roche). For each sample, 2 technical replicates were  
564 performed. The comparative cycle threshold method was used for calculating transcript level.  
565 Primers used for *FLC* antisense analysis are the same as in previous publication<sup>37</sup>. Sequences  
566 of oligo primers are listed in Supplementary data 4.

### 567 **RNA immunoprecipitation**

568 RNA immunoprecipitation (RNA-IP) was performed using a protocol modified based on  
569 published procedures<sup>22</sup>. Briefly, 3 grams of tissues of 7-day-old *35S::AtACINUS-GFP/acinus-2*  
570 and *35S::GFP* seedlings were cross-linked with 1% (v/v) formaldehyde for 15 mins. Cross-  
571 linked RNA-protein complexes were extracted in NLB buffer (20 mmol/L Tris-HCl, pH 8.0, 150  
572 mmol/L NaCl, 2 mmol/L EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 1 mmol/L PMSF and 2X  
573 Protease Inhibitor (Roche)) and sheared by sonication (25% amplitude, 0.5"on/0.5"off for  
574 2minX3 cycles on a Branson Digital Sonifier). Immunoprecipitation was carried out with Protein  
575 A magnetic beads (Thermo Fisher) that were pre-incubated overnight with homemade anti-GFP  
576 antibody (5 µg for each sample) for 1 hr on a rotator. Beads were washed 5 times with 1ml NLB  
577 buffer (no SDS, 0.5% (v/v) Triton X-100) with 80 U/ml RNase inhibitor. To elute the immuno-  
578 complex, 100ul Elution Buffer (20 mmol/L Tris-HCl, pH 8.0, 10 mmol/L EDTA, 1% (w/v) SDS,  
579 800U/ml RNase inhibitor) was added to the beads and incubated at 65 °C for 15 mins. The elute  
580 was incubated with 1ul 20 mg/ml Protease K at 65 °C for 1hr for protein digestion and reverse-  
581 crosslinking. RNA was purified and concentrated using the RNA Clean & Concentrator™ kit  
582 (Zymo). On-column DNase digestion was performed to remove DNA contaminations. Samples  
583 were kept on ice whenever possible during the experiment. Three biological replicates were  
584 performed and the co-immunoprecipitated *ABH1* transcripts were quantified with RT-qPCR, and  
585 the results were normalized to 25S rRNA<sup>72</sup>.

### 586 **ChIP-PCR**

587 Chromatin immunoprecipitation (ChIP) analysis was performed using a similar protocol  
588 to previous publications<sup>73</sup>. Briefly, tissue crosslinking, protein extraction, and  
589 immunoprecipitation were carried out as described above for RNA-IP. The beads were washed  
590 with low-salt buffer (50 mmol/L Tris-HCl at pH 8.0, 2 mmol/L EDTA, 150 mmol/L NaCl and 0.5%



591 (v/v) Triton X-100), high-salt buffer (50 mmol/L Tris-HCl at pH 8.0, 2 mmol/L EDTA, 500 mmol/L  
592 NaCl and 0.5% (v/v) Triton X-100), LiCl buffer (10 mmol/L Tris-HCl at pH 8.0, 1 mmol/L EDTA,  
593 0.25 mol/L LiCl, 0.5% (w/v) NP-40 and 0.5% (w/v) sodium deoxycholate) and TE buffer (10  
594 mmol/L Tris-HCl at pH 8.0 and 1 mmol/L EDTA), and eluted with elution buffer (1% (w/v) SDS  
595 and 0.1 mmol/L NaHCO<sub>3</sub>). After reverse cross-linking and proteinase K digestion, the DNA was  
596 purified with a PCR purification kit (Thermo Fisher) and analyzed by PCR. Three biological  
597 replicates were performed. *FLC* primers were based on previous publications<sup>47</sup>.

## 598 **SILIA-MS quantitative analysis of the AtACINUS interactome**

599 Stable-isotope-labeling in *Arabidopsis* mass spectrometry (SILIA-MS) was used for  
600 quantitative analysis of the AtACINUS interactome. The WT and *acinus-2* plants were grown for  
601 two weeks at 21°C under constant light on vertical plates of <sup>14</sup>N or <sup>15</sup>N medium (Hogland's No. 2  
602 salt mixture without nitrogen 1.34g/L, 6g/L phytoblend, 2 μmol/L propiconazole, and 1g/L KNO<sub>3</sub>  
603 or 1g/L K<sup>15</sup>NO<sub>3</sub> (Cambridge Isotope Laboratories), pH5.8). About 5 g of tissue was harvested for  
604 each sample, ground in liquid nitrogen and stored in -80°C. Immunoprecipitation was performed  
605 as described previously with slight modifications<sup>74</sup>. Briefly, proteins were extracted in 10 mL  
606 MOPS buffer (100 mmol/L MOPS, pH 7.6, 150 mmol/L NaCl, 1% (v/v) TritonX-100, 1 mmol/L  
607 phenylmethylsulfonyl fluoride (PMSF), 2X Complete protease inhibitor cocktail, and PhosStop  
608 cocktail (Roche)), centrifuged, and filtered through two layers of Miracloth. The flow through was  
609 incubated with 20 μg anti-AtACINUS antibody for one hour at 4 °C, then 50 μL protein A  
610 agarose beads were added and incubated for another hour, followed by four 2-min washes with  
611 immunoprecipitation buffer. At the last wash, <sup>14</sup>N-labeled Wild-type and <sup>15</sup>N-labeled *acinus-2* IP  
612 samples or reciprocal <sup>15</sup>N-labeled Wild-type and <sup>14</sup>N-labeled *acinus-2* IP samples were mixed,  
613 and eluted with 2x SDS buffer. The eluted proteins were separated by SDS-PAGE. After  
614 Coomassie Brilliant blue staining, the whole lane of protein samples was excised in ten  
615 segments and subjected to in-gel digestion with trypsin.

616 The peptide mixtures were desalted using C18 ZipTips (Millipore) and analyzed on a  
617 LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher), equipped with a NanoAcquity liquid  
618 chromatography system (Waters). Peptides were loaded onto a trapping column (NanoAcquity  
619 UPLC 180 μm X 20 mm; Waters) and then washed with 0.1% (v/v) formic acid. The analytical  
620 column was a BEH130 C18 100 μm X 100 mm (Waters). The flow rate was 600 nL/min.  
621 Peptides were eluted by a gradient from 2-30% solvent B (100% (v/v) acetonitrile/ 0.1% (v/v)  
622 formic acid) over 34 min, followed by a short wash at 50% solvent B. After a precursor scan was  
623 measured in the Orbitrap by scanning from mass-to-charge ratio 350 to 1500, the six most

624 intense multiply charged precursors were selected for collision-induced dissociation in the linear  
625 ion trap.

626 Tandem mass spectrometry peak lists were extracted using an in-house script PAVA,  
627 and data were searched using Protein Prospector against the *Arabidopsis* Information Resource  
628 (TAIR10) database, to which reverse sequence versions were concatenated (a total of 35,386  
629 entries) to allow estimation of a false discovery rate (FDR). Carbamidomethyl cysteine was  
630 searched as a fixed modification and oxidation of methionine and N-terminal acetylation as  
631 variable modifications. Data were searched with a 10 ppm tolerance for precursor ion and 0.6  
632 Da for fragment ions. Peptide and protein FDRs were set as 0.01 and 0.05. <sup>15</sup>N labeled amino  
633 acids were also searched as a fixed modification for <sup>15</sup>N data. <sup>15</sup>N labeling efficiency was  
634 calculated as about 96%, by manually comparing experimental peak envelop data of the <sup>15</sup>N  
635 labeled peptide from top 10 proteins in the raw data to theoretical isotope distributions using  
636 Software Protein-prospector (MS-Isotope app). Quantification was done using Protein  
637 Prospector which automatically adjusts the L/H ratio with labeling efficiency. The SILIA ratio  
638 (WT/*acinus-2*) was normalized using the average ratios of non-specific interactor ribosomal  
639 proteins (with more than five peptides). <sup>15</sup>N labeling samples in general have less identification  
640 rates of proteins because of 96% labeling efficiency. The data has been deposited to PRIDE  
641 with project accession: PXD020700.

#### 642 **Label-free mass spectrometric analysis of AtACINUS and its interactome**

643 The *AtACINUS-GFP/acinus-2* and TAP-GFP seedlings<sup>38</sup> were grown for 7 days at 21°C  
644 under constant light on ½ MS medium. Tissues were harvested, ground in liquid nitrogen and  
645 stored in -80°C.

646 Immunoprecipitation was performed as described previously with slight modifications<sup>74</sup>.  
647 Briefly, proteins were extracted in MOPS buffer (100 mmol/L MOPS, pH 7.6, 150 mmol/L NaCl,  
648 1% (v/v) TritonX-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2X Complete protease  
649 inhibitor cocktail, and PhosStop cocktail (Roche) and 20 µmol/L PUGNAc inhibitor (Sigma),  
650 centrifuged, and filtered through two layers of Miracloth, then incubated with a modified version  
651 of LaG16-LaG2 anti-GFP nanobody<sup>75</sup> conjugated to dynabeads (Invitrogen), for 3 hr at 4°C,  
652 followed by four 2-min washes with immunoprecipitation buffer and eluted with 2% (w/v) SDS  
653 buffer containing 10 mmol/L tris(2-carboxyethyl) phosphine (TCEP) and 40 mmol/L  
654 chloroacetamide at 95°C for 5 mins. The eluted proteins were separated by SDS-PAGE. After

655 Colloidal blue staining, the whole lane of protein samples was excised in two segments and  
656 subjected to in-gel digestion with trypsin. Three biological experiments were performed.

657 The peptide mixtures were desalted using C18 ZipTips (Millipore) and analyzed on a Q-  
658 Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher) equipped with an  
659 Easy LC 1200 UPLC liquid chromatography system (Thermo Fisher). Peptides were separated  
660 using analytical column ES803 (Thermo Fisher). The flow rate was 300nL/min and a 120-min  
661 gradient was used. Peptides were eluted by a gradient from 3 to 28% solvent B (80% (v/v)  
662 acetonitrile/0.1% (v/v) formic acid) over 100 mins and from 28 to 44% solvent B over 20 mins,  
663 followed by short wash at 90% solvent B. Precursor scan was from mass-to-charge ratio ( $m/z$ )  
664 375 to 1600 and top 20 most intense multiply charged precursor were selection for fragmentation.  
665 Peptides were fragmented with higher-energy collision dissociation (HCD) with normalized  
666 collision energy (NCE) 27.

667 The raw data were processed by MaxQuant using most of preconfigured settings<sup>76</sup>. The  
668 search was against the same TAIR database as mentioned above. Carbamidomethylcysteine  
669 was searched as a fixed modification and oxidation of methionine and N-terminal acetylation as  
670 variable modifications. Data were searched with a 4.5ppm tolerance for precursor ion and 20  
671 ppm for fragment ions. The second peptide feature was enabled. A maximum of two missed  
672 cleavages was allowed. Peptide and protein FDRs were set as 0.01. Minimum required peptide  
673 length was seven amino acids. Multiplicity was set to 1. Label-free quantification (LFQ) was  
674 enabled. The match between runs option was enabled with a match time window of 0.7 min  
675 and alignment time window as 20 mins. Quantification was done on unique and razor peptides  
676 and a minimum ratio count was set to 2.

677 The proteinGroups.txt generated by MaxQuant were loaded to Perseus<sup>77</sup>. The results  
678 were filtered by removing identified proteins by only modified sites, or hits to reverse database  
679 and contaminants. LFQ intensity values were logarithmized. The pull-downs were divided to  
680 AtACINUS-GFP and TAP-GFP control. Samples were grouped in triplicates and identifications  
681 were filtered for proteins having at least three values in at least one replicate group. Signals that  
682 were originally zero were imputed with random numbers from a normal distribution (width 0.3,  
683 shift =1.8). Volcano plot was performed with x axis representing the logarithmic ratios of protein  
684 intensities between AtACINUS-GFP and TAP-GFP. The hyperbolic curve that separates  
685 AtACINUS specific interactor from background was drawn using threshold value FDR 0.01 and  
686 curve bend S0 value 2.

687 LFQ data and SILIA data were combined and filtered to get a high-confidence list of  
688 interactors: 1) Significant enrichment in LFQ three biological replicates (FDR=0.01, S0=2); 2)  
689 Enrichment of over 2 folds in both SILIA biological experiment; or over 2 folds in one SILIA  
690 experiment, but not identified in second SILIA experiment. If the proteins are only identified and  
691 quantified by LFQ three biological replicates, then a higher stringency cut off (enrichment > 16  
692 fold, t test >4) is used. The data was deposited to PRIDE with project accession: PXD020748.

693 For affinity purification of AtACINUS using *in vivo* biotinylation, the *acinus pinin* mutant  
694 was transformed with a T-DNA construct that expresses AtACINUS as a fusion with TurboID  
695 from the 35S promoter<sup>65</sup>. The *AtACINUS-YFP-TurboID/acinus-2 pinin-1* seedlings were treated  
696 with 0 or 50  $\mu$ mol/L biotin for 3 hours. The AtACINUS-YFP-Turbo protein was affinity purified  
697 using streptavidin beads as previously described<sup>65</sup> using a modified extraction buffer containing  
698 20  $\mu$ mol/L PUGNAC and 1 x PhosphoStop. After on-bead tryptic digestion, the samples were  
699 analyzed as described above in the label-free IP-MS section on a Q-Exactive HF instrument.  
700 Data were searched as described above but allowing additional modifications: O-GlcNAcylation  
701 modification on S/T and neutral loss, O-fucosylation on S/T and neutral loss, phosphorylation on  
702 S/T and biotinylation on lysine. The data was deposited to PRIDE with accession number:  
703 PXD020749.

#### 704 **Targeted quantification comparing wild-type and the *acinus-2 pinin-1* double mutant**

705 The wild-type and *acinus-2 pinin-1* plants were grown Hoagland medium containing <sup>14</sup>N or <sup>15</sup>N  
706 (1.34g/L Hogland's No2 salt mixture without nitrogen, 6g/L phytoblend, and 1g/L KNO<sub>3</sub> or 1g/L  
707 K<sup>15</sup>NO<sub>3</sub> (Cambridge Isotope Laboratories), pH5.8). Proteins were extracted from 6 samples (one  
708 <sup>14</sup>N-labelled Col, two of <sup>15</sup>N-labelled Col, two of <sup>14</sup>N-labelled *acinus-2 pinin-1* and one <sup>15</sup>N-  
709 labelled *acinus-2 pinin-1*) individually using SDS sample buffer and mixed as the followings: one  
710 forward sample F1 (<sup>14</sup>N Col/ <sup>15</sup>N *acinus-2 pinin-1*) and two reverse samples R2 and R3 (<sup>14</sup>N  
711 *acinus-2 pinin-1*/<sup>15</sup>N Col) and separated by the SDS-PAGE gel with a very short run. Two  
712 segments (upper part (U) ranging from the loading well to ~ 50KD; lower part (L) ranging from ~  
713 50KD to the dye front) were excised, trypsin digested and analyzed by liquid chromatography  
714 mass spectrometry (LC-MS) as described above in the label-free IP-MS section on a Q-  
715 Exactive HF instrument using an ES803A analytical column. Data-dependent acquisition was  
716 used first to get the peptide information from multiple proteins with peptide mass/charge (*m/z*),  
717 retention time and MS2 fragments. PININ peptide information was from an IP-MS experiment.  
718 For targeted analysis, parallel reaction monitoring (PRM) acquisition<sup>78</sup> using a 20 min window  
719 was scheduled with an orbitrap resolution at 60,000, AGC value 2e5 and maximum fill time of

720 200 ms. The isolation window for each precursor was set at 1.4  $m/z$  unit. Data processing was  
721 similar to the previous report<sup>79</sup> with a 5 ppm window using skyline from <sup>14</sup>N- and <sup>15</sup>N -labeled  
722 samples. Peak areas of fragments were calculated from each sample, the sum of peak areas  
723 from upper segment and lower segment was used to calculate *acinus-2 pinin-1/Col* ratios for  
724 each peptide, normalized to TUBULIN2 to get the normalized ratios. Median number of multiple  
725 ratio measurements is used for each protein.

## 726 **Data Availability**

727 Proteomic Data that support the findings of this study have been deposited in Proteomics  
728 Identification Database (PRIDE) with the accession codes: PXD020700, PXD020748,  
729 PXD020749. The RNA-seq data that support the findings of this study have been deposited in  
730 the National Center for Biotechnology Information Gene Expression Omnibus and are  
731 accessible through the GEO series accession number GSE110923. All other related data are  
732 available from the corresponding authors upon request.

## 733 **Supplementary information**

734 Supplementary Figures 1-16

735 Supplementary data 1: Summary of reads mapping quality and differentially expressed genes in  
736 *acinus-2 pinin-1* identified by RNA-seq analysis.

737 Supplementary data 2: Alternative splicing events in *acinus-2 pinin-1* identified by RNA-seq  
738 analysis.

739 Supplementary data 3: AtACINUS interactome.

740 Supplementary data 4: List of primers used in the study.

741 Supplementary data 5: Targeted quantification on AtACINUS, AtPININ, SR45 and AtSAP18  
742 between Col and *acinus-2 pinin-1* mutant.

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#### 751 **Author Contributions:**

752 Z.D., K.L, J.O, and A.L.B. identified AtACINUS; Z.D., S.L.X., and S.P. analyzed the *acinus*  
753 mutant; Y.B., S.L.X. and D.S. characterized the *acinus pinin* double mutants; Y.B performed  
754 RNA-seq and T.H helped with data analysis; W.N. performed proteomic analysis of AtACINUS  
755 interactome under supervision by A.L.B., P.H.Q and S.L.X. R.S performed targeted  
756 quantification, S.H. performed affinity purification of biotinylated protein and R.S prepared  
757 spectra. Z-Y.W and S.L.X conceived the projects; Y.B., S.L.X. and Z-Y.W. wrote the manuscript.

758 **Competing financial interests:** The authors declare no competing financial interests.

759

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958 transcription factor PIF3. *Nat Commun* **8**, 15236, doi:10.1038/ncomms15236 (2017).

## 959 **Figure legends**

960 **Figure 1 | AtACINUS and AtPININ are genetically redundant.** (a) Diagrams of the  
961 domain structures of AtACINUS and AtPININ. SAP: SAF-A/B, Acinus and PIAS motif.  
962 RRM: RNA-recognition motif. RSB: RNPS1-SAP18 binding domain. G and F indicates  
963 the position of O-GlcNAcylation and O-fucosylation modifications respectively. (b) The  
964 sequence alignment of the RSB domains of AtACINUS and AtPININ. Conserved amino  
965 acids are highlighted in green. (c) Diagrams of the AtACINUS and AtPININ (translation  
966 start at position 1) with T-DNA insertion sites in *acinus-1*, *acinus-2* and *pinin-1* mutants.  
967 (d) Plant morphologies of wild type (WT), *acinus-1*, *acinus-2*, *pinin-1*, *acinus-1 pinin-1*  
968 and *acinus-2 pinin-1* grown on soil for 20 days. (e) Five-week old WT, *acinus-1*, *acinus-*  
969 *2*, *pinin-1*, *acinus-1 pinin-1* and *acinus-2 pinin-1* plants grown under long day condition.  
970 Inset shows enlarged view of the *acinus-1 pinin-1* and *acinus-2 pinin-1* mutants. (f)

971 Expression of either AtACINUS-GFP or YFP-AtPININ suppresses the growth defects in  
972 *acinus-2 pinin-1* double mutant (*ap*).

973 **Figure 2 | The *acinus pinin* double mutants showed ABA hypersensitive**  
974 **phenotypes. (a,b)** Germination rates of wild-type, *acinus-2*, *pinin-1* and *acinus-2 pinin-*  
975 *1* after different days on ½ MS medium without ABA (a) or with 0.25 µmol/L ABA (b). The  
976 data points of wild-type, *acinus-2* and *pinin-1* overlap. **(c)** Seed germination rates of the  
977 indicated genotypes on ½ MS medium supplemented with increasing concentrations of  
978 ABA after five days. Note that the data points of *acinus-1 pinin-1* and *acinus-2 pinin-1*  
979 overlap and those of wild-type, *acinus-1*, *acinus-2* and *pinin-1* overlap. **(d)** Seed  
980 germination and development of the indicated genotypes on ½ MS medium with or  
981 without 0.5 µmol/L ABA. The pictures were taken 6 days after germination. Error bars  
982 indicate SD calculated from 3 biological replicates (n=3). Asterisks indicate significant  
983 differences to wild type (two-sided Student's t-test, \*P<0.05, \*\* P<0.01, \*\*\* P<0.001 ).

984 **Figure 3 | RNA-sequencing analysis of *acinus-2 pinin-1* showed differential intron**  
985 **retention and expression level of many genes. (a)** Number of introns that showed  
986 increased or decreased intron retention in *acinus-2 pinin-1* and the number of genes  
987 that contain these introns. **(b)** Comparison between genes differentially expressed in  
988 *acinus-2 pinin-1* and ABA-responsive genes. RNA-seq was conducted using 14-day-old  
989 light-grown seedlings for both genotypes.

990 **Figure 4 | *ABH1* and *HAB1* showed increased intron retention in *acinus-2 pinin-1***  
991 **and *ABH1* and *HAB1* mRNAs are associated with AtACINUS. (a)** Integrative  
992 genomic viewer (IGV) display of increased intron retention of the *ABH1* 10<sup>th</sup> intron in  
993 *acinus-2 pinin-1* compared to WT. **(b)** RT-PCR of *ABH1* in 12-day-old seedlings of the  
994 indicated genotypes using primers at positions indicated by arrowheads in panel (a). **(c)**  
995 Intron retention ratio of *ABH1* 10<sup>th</sup> intron as determined by RT-qPCR in 12-day-old  
996 seedlings of the indicated genotypes. The intron-containing form *ABH1.2* was highly  
997 accumulated while the spliced form *ABH1.1* was reduced in *acinus-2 pinin-1* compared  
998 to WT, the single mutants, or the double mutant complemented by *YFP-AtPININ* or  
999 *AtACINUS-GFP*. **(d)** RT-PCR of *HAB1* in 12-day-old WT and *acinus-2 pinin-1* seedlings  
1000 treated with ABA (100 µmol/L for 3 hrs). **(e)** RT-qPCR quantification of the fold changes

1001 of expression levels of each splice forms of *HAB1* after ABA treatment of 12-day-old WT  
1002 and *acinus-2 pinin-1* seedlings. (f,g) Quantification of *ABH1* and *HAB1* mRNAs by qPCR  
1003 after RNA-IP using  $\alpha$ -GFP antibody in 7-day-old *AtACINUS-GFP/acinus-2* seedlings,  
1004 compared to *35S::GFP* as a negative control. Error bars in this figure indicate SD  
1005 calculated from 3 biological replicates (n=3). Asterisks indicate significant differences to  
1006 wild type or between indicated samples (two-sided Student's t-test, \*P<0.05, \*\* P<0.01,  
1007 \*\*\* P<0.001).

1008 **Figure 5 | The *acinus-2 pinin-1* double mutant is late flowering with increased *FLC***  
1009 **expression.** (a) Rosette leaf number of WT, *acinus-2*, *pinin-1* and *acinus-2 pinin-1* at  
1010 bolting stage grown in long day condition. Error bars indicate SD calculated from n>12.  
1011 (b) *FLC* expression level relative to *PP2a* in WT, *acinus-2*, *pinin-1* and *acinus-2 pinin-1*,  
1012 determined by RT-qPCR in 12-day-old seedlings. Error bars indicate SD calculated from  
1013 3 biological replicates (n=3). (c) Analysis of *AtACINUS-GFP* association with the *FLC*  
1014 locus by CHIP-PCR in 12-day-old *AtACINUS-GFP/acinus-2* seedlings. Wild type (WT)  
1015 serves as the negative control. Bars below the gene structure diagram show regions  
1016 analyze by PCR (blue bars indicate positive binding detected). GFP IP shows PCR  
1017 products using immunoprecipitated DNA. *CO-FACTOR FOR NITRATE, REDUCTASE*  
1018 *AND XANTHINE DEHYDROGENASE 5 (CNX5)* serves as an internal control to show  
1019 non-specific background DNA after immunoprecipitation. Asterisks indicate significant  
1020 differences to wild type (two-sided Student's t-test, \*P<0.05, \*\* P<0.01, \*\*\* P<0.001).

1021 **Figure 6 | A subset of *AtACINUS*-dependent intron splicing events are affected in**  
1022 **the *spy* and *sec* mutants.** RT-PCR of *HAB1*, *EMB2247* and *TRM4D* in 7-day-old WT,  
1023 *acinus-2 pinin*, *spy-t1*, *spy-4*, *sec-2* and *sec-5* seedlings with exon-spanning primers  
1024 flanking the targeted introns.

1025 **Figure 7 | *AtACINUS* is O-GlcNAc and O-Fucose modified and associates with**  
1026 **spliceosomal complexes, transcriptional regulators and chromatin remodeling**  
1027 **proteins.** (a) Diagram shows functional groups of *AtACINUS*-associated proteins.  
1028 Proteins are grouped in boxes based on their association with known complexes or  
1029 functions. Positive regulators of *FLC* are highlighted in red and negative regulators in  
1030 blue. Seven-day-old seedlings were used for the label-free IP-MS experiments and 14-

1031 day-old seedlings were used for the <sup>15</sup>N Stable-isotope-labeling in *Arabidopsis* (SILIA)  
1032 quantitative MS experiments. **(b,c)** Higher energy collisional dissociation (HCD) mass  
1033 spectra shows O-GlcNAcylation on Thr79 and a sequence spanning amino acid 400-  
1034 423 of AtACINUS. The sequence ion series that retains this modification (shifted by  
1035 203Da) are labeled in blue (b). The sequence ion series that have lost the modification  
1036 are labeled in red. HexNAc oxonium ion (*m/z* 204) and its fragments masses are  
1037 labeled in red. **(d)** HCD spectrum shows O-fucosylation on a sequence spanning amino  
1038 acid 169-197 of AtACINUS with neutral loss. **(e)** Proposed model of a molecular  
1039 pathway in which nutrient sensing O-GlcNAcylation and O-fucosylation modulate the  
1040 evolutionarily conserved RSB-domain protein AtACINUS, which controls transcription  
1041 and alternative RNA splicing of specific target genes to modulate stress hormone  
1042 sensitivity and developmental transitions such as seed germination and flowering in  
1043 plants.

1044

1045



Fig.1

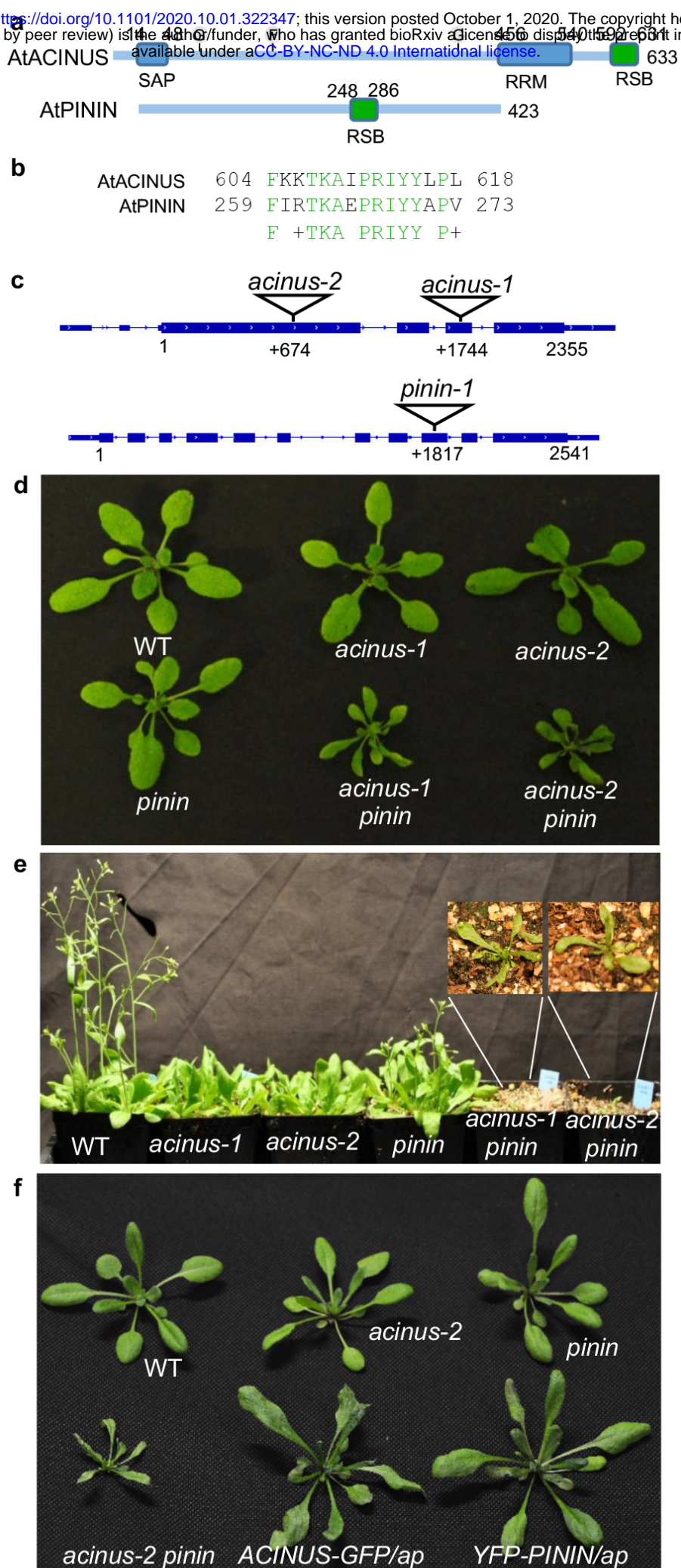


Fig.2

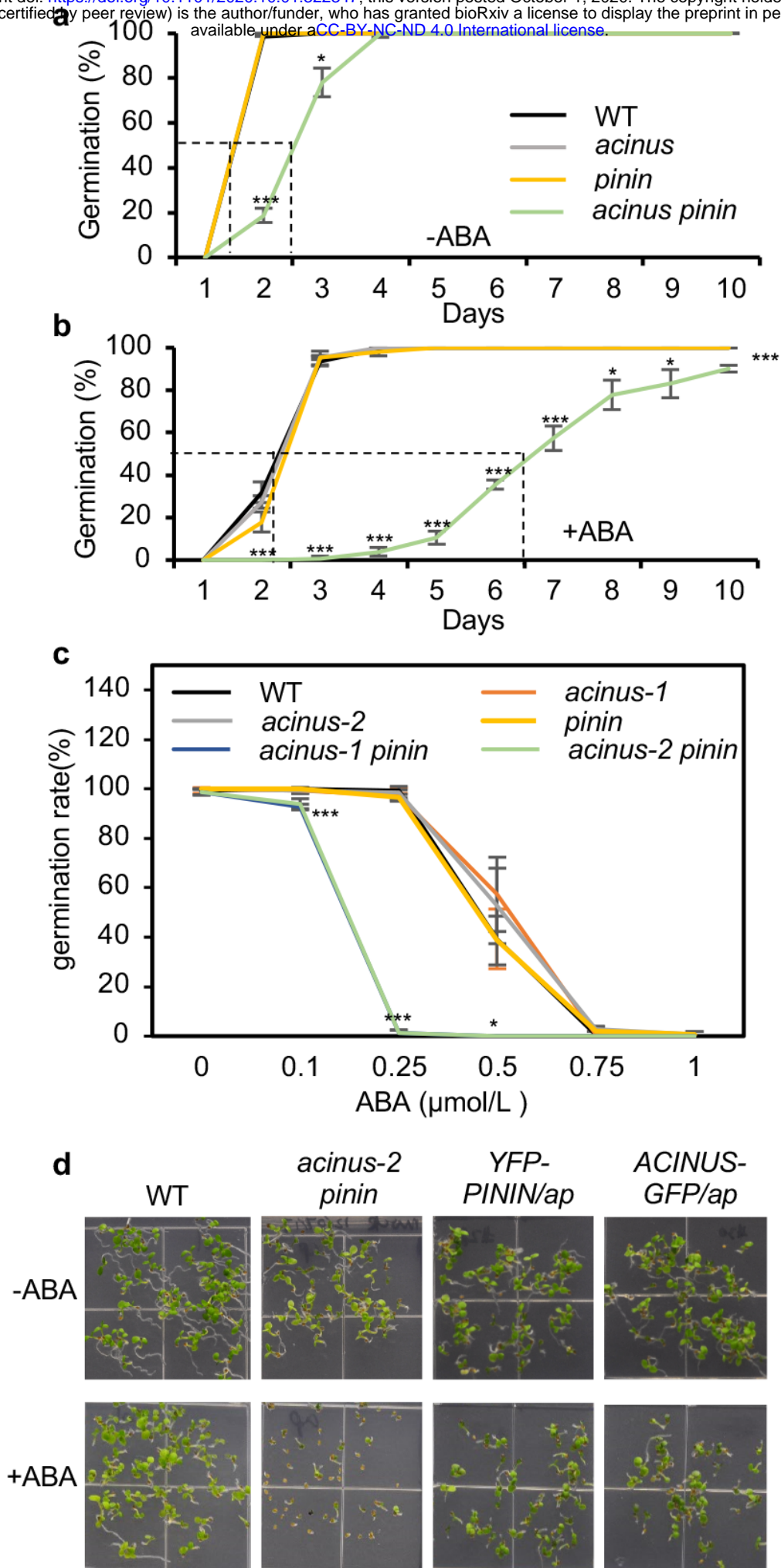


Fig.3

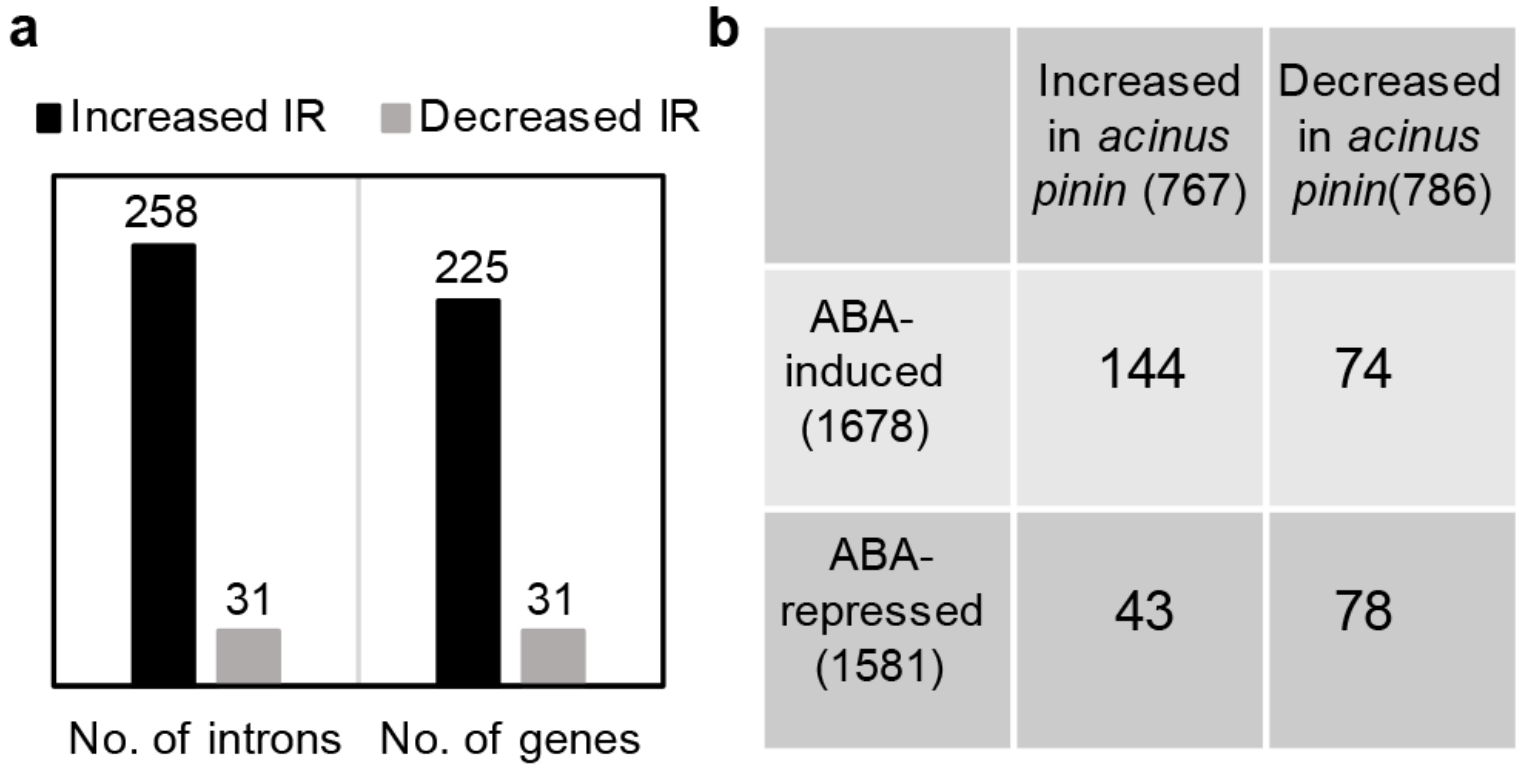




Fig.4

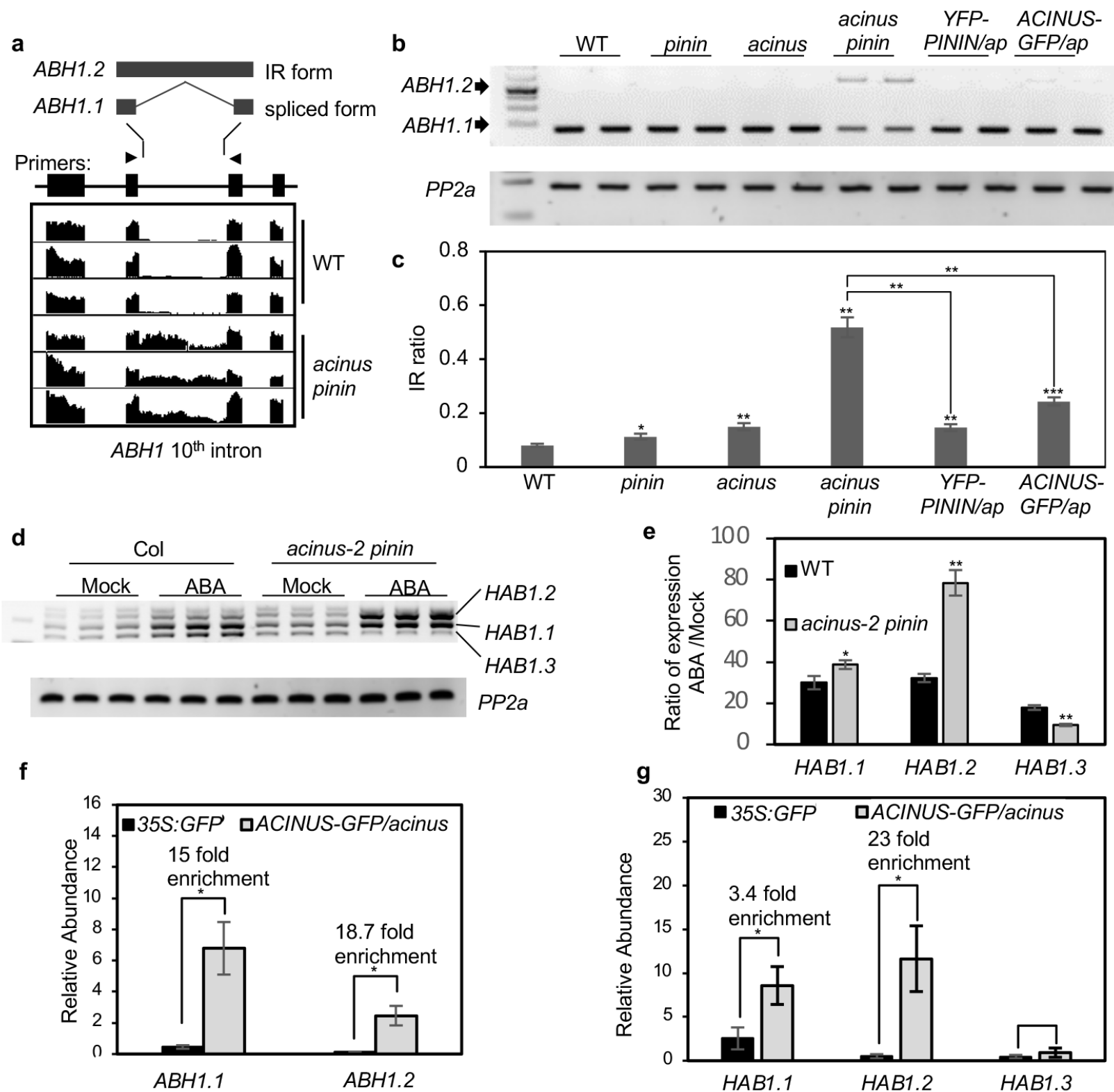


Fig.5

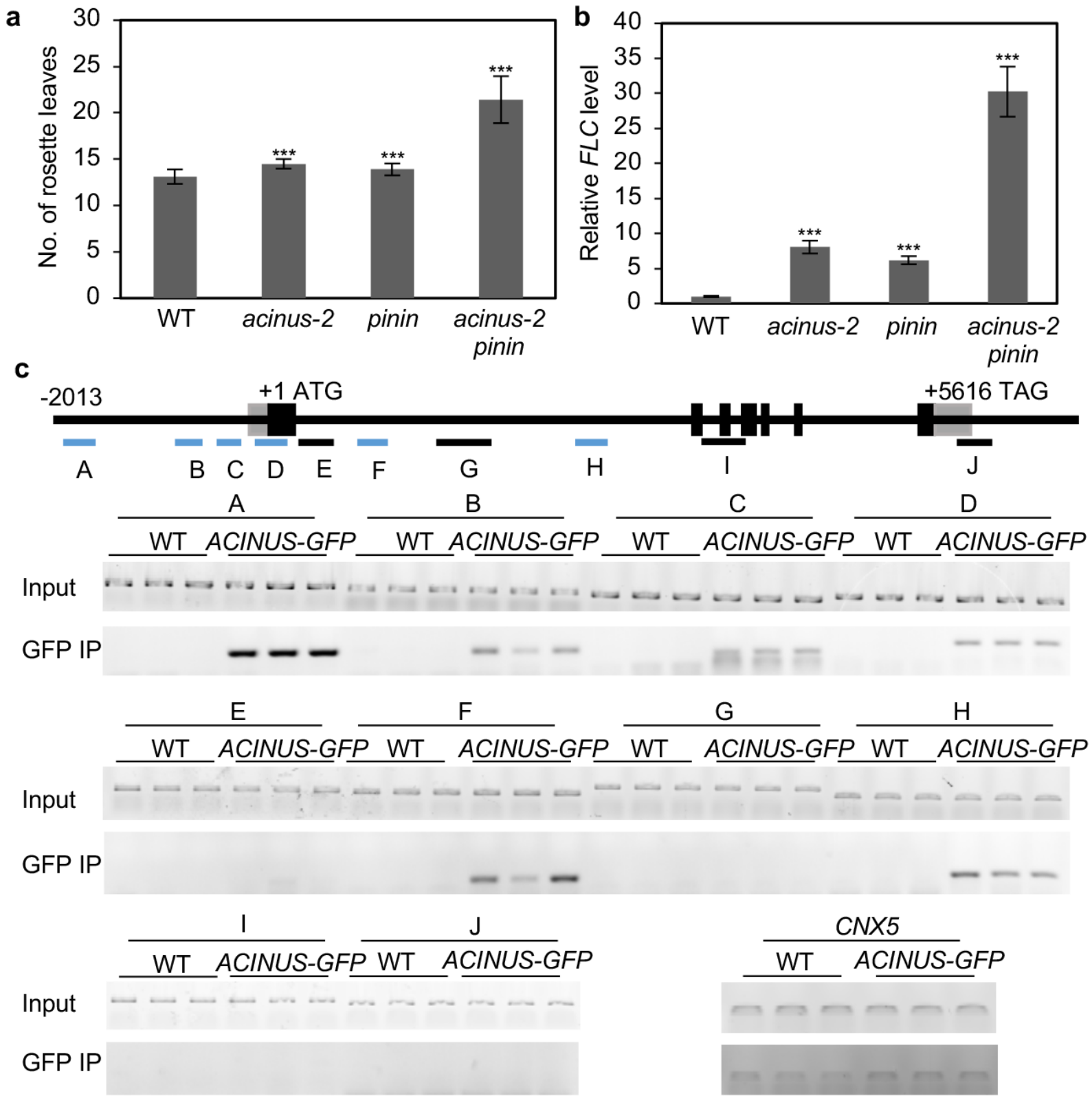


Fig.6

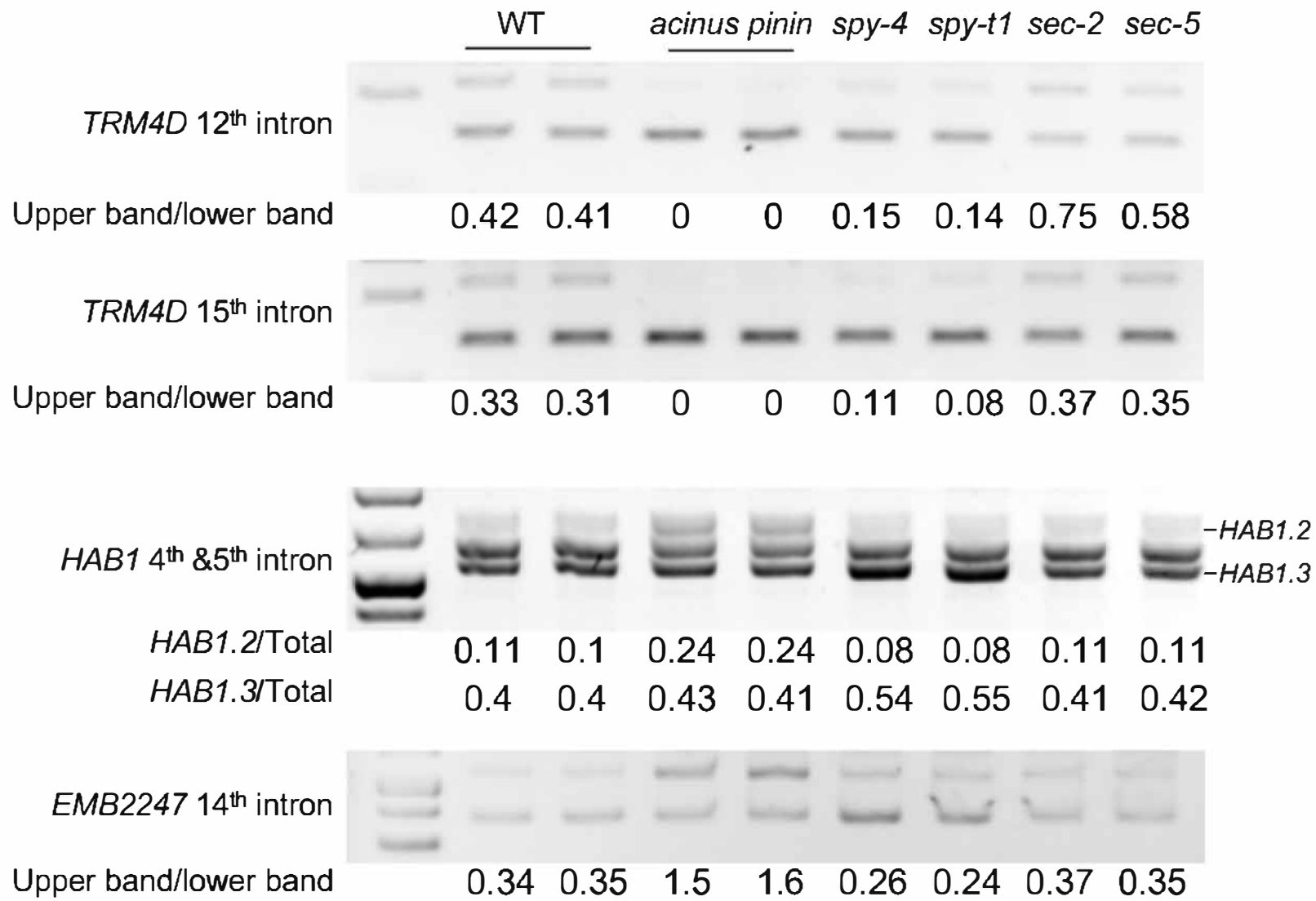
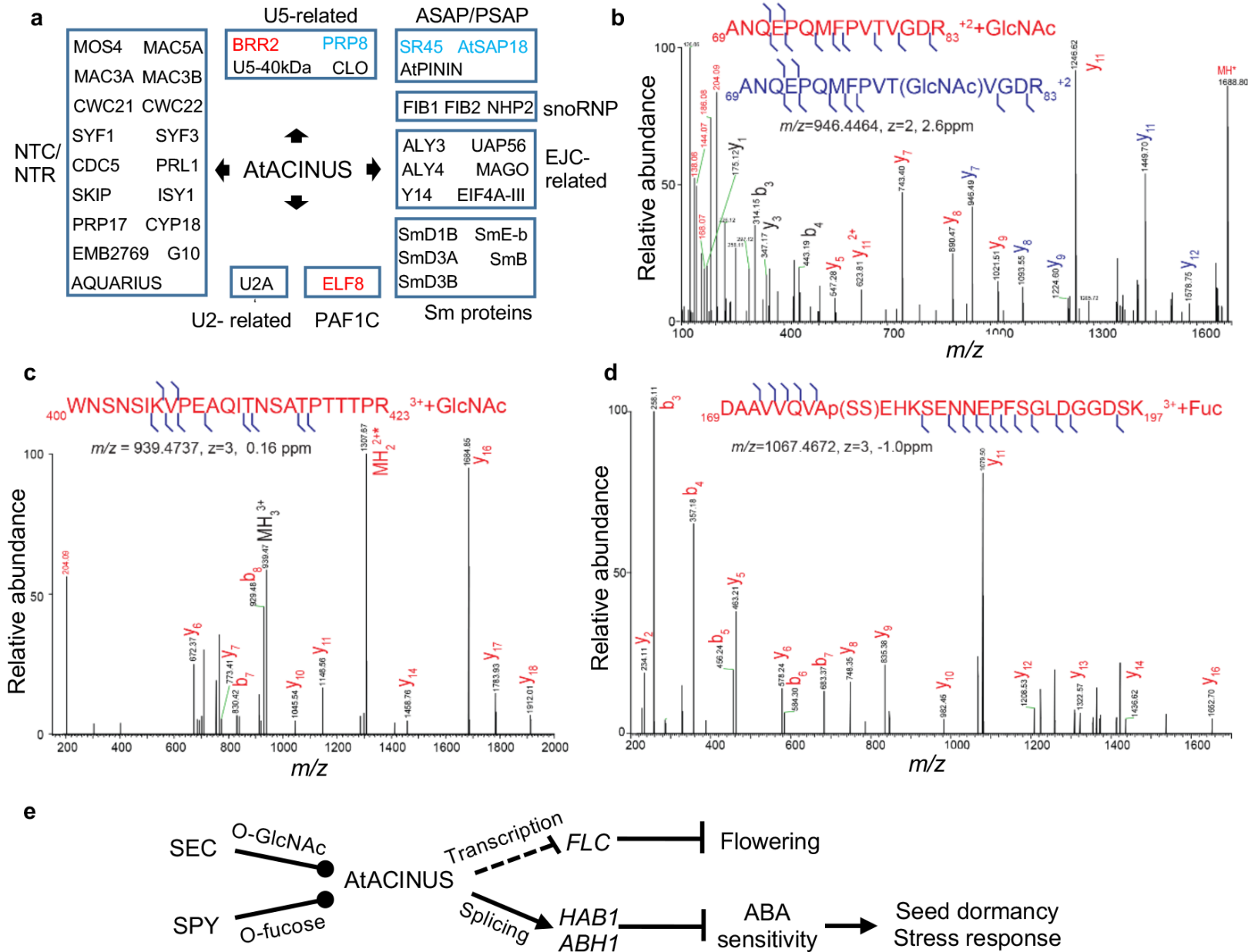


Fig.7



**a** Alignment of Acinus and AtACINUS, around SAP domain

Score	Expect	Method	Identities	Positives	Gaps
34.3 bits(77)	3e-05	Compositional matrix adjust.	18/41(44%)	25/41(60%)	0/41(0%)

Acinus: 66 TLDGKPLQALRVTDLKAALBQRGLAKSQKLSALVKRLKCAL 106  
 LD +P+ +VT+LK L+R L G K LV+RL AL  
 AtACINUS:8 VLDNRPIDKWKVTELKEELKRRRLTRGLKEELVRLDEAL 48

Alignment of Acinus and AtACINUS, around RRM domain

Score	Expect	Method	Identities	Positives	Gaps
90.1 bits(222)	2e-22	Compositional matrix adjust.	35/82(43%)	58/82(70%)	2/82(2%)

Acinus: 1011 SMIVHISNLRVPTLQQLKELLGRCTLVVEAFWIDKIKSHCFVITYSTVEEAVATRTALH 1070  
 +N + I +RPFTL ++ELLC+TC + +FW+D IK+HC+V+Y +VEEA ATR A++  
 AtACINUS:456 TNSLRIDRFLEPFTLKA+VQELLC+KCNVT—SFWMDHIKTHCYVSYFVVEEAATREAVY 513

Acinus: 1071 GWKFPQSNFKFLCADYAEQDEL 1092  
 ++WP + + L A++ +E+  
 AtACINUS:514 NLQWPPNGGRHLIAEFVRAEEV 535

Alignment of Acinus and AtACINUS, around RSB domain

Score	Expect	Method	Identities	Positives	Gaps
44.3 bits(103)	3e-08	Compositional matrix adjust.	18/29(62%)	23/29(79%)	0/29(0%)

Acinus: 1211 LDDLFRKTKAAPCIYWLPLTDSQIVQKEA 1239  
 LDDLFRKTKA P IY+LPL++ Q+ K A  
 AtACINUS:600 LDDLFRKTKAIPRIYYLPLSEBQVAAKLA 628

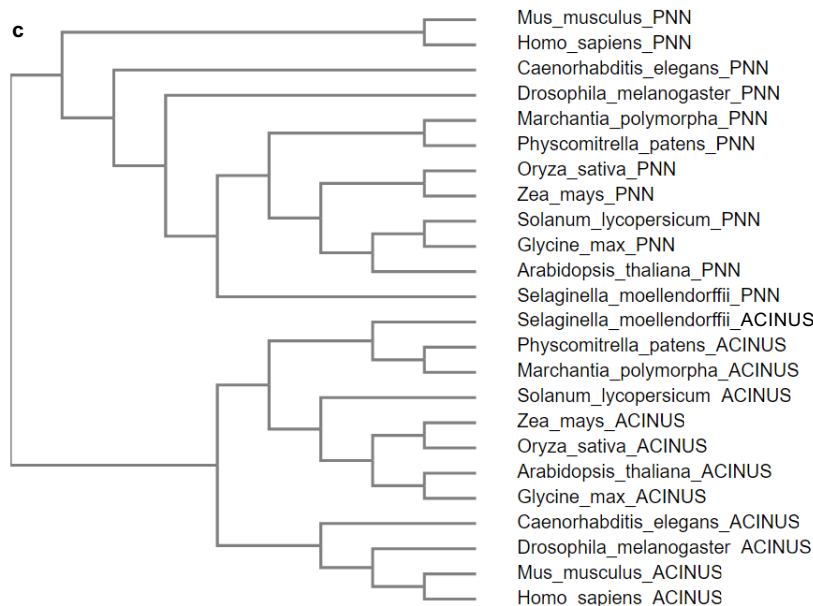
**b** Alignment of Pinin and AtPININ, around RSB domain

Score	Expect	Method	Identities	Positives	Gaps
67.4 bits(163)	3e-16	Compositional matrix adjust.	45/122(37%)	69/122(56%)	10/122(8%)

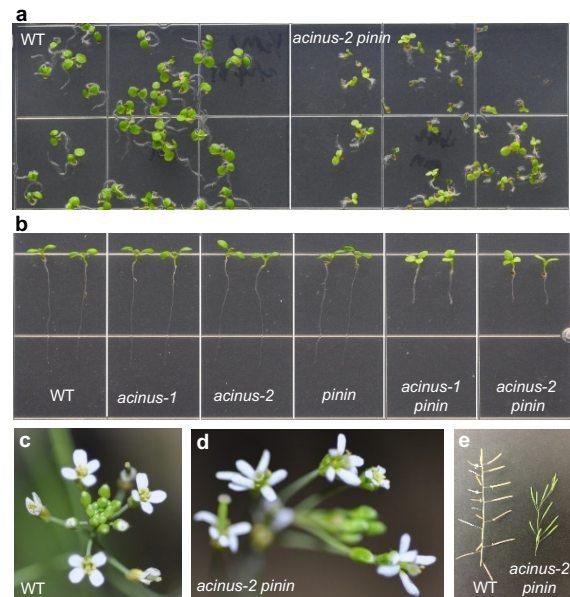
Pinin: 132 QNMDEKQKQRNRIFCLLMCTLQKFKQEST—VATERQKRQEIIBQKLEVQAEERKQWE 189  
 +N D K RNR+ C L+C+L+K+P+E T+ RR Q+ E +A EE +++  
 AtPININ: 153 KNEDPKLVNRNRMLCNLLC+LEKFRKEDKQRSGTDAYARRTAALQRAEAKAREESERLR 212

Pinin: 190 NERRLEFERRAKQTELRL—EQKVELAQLQEWNEHNAKIKYIRTKTPHLY 243  
 + RE E+R + LR ++K+EL LQ W+EH K+ +IRTK +P +Y  
 AtPININ: 213 LQERENLTEKRRRDLTLRARVAAKAQQKLELLFLQ—WSEHQKLSNFRTKAEPRIYY 270

Pinin: 244 IP 245  
 P  
 AtPININ: 271 AP 272

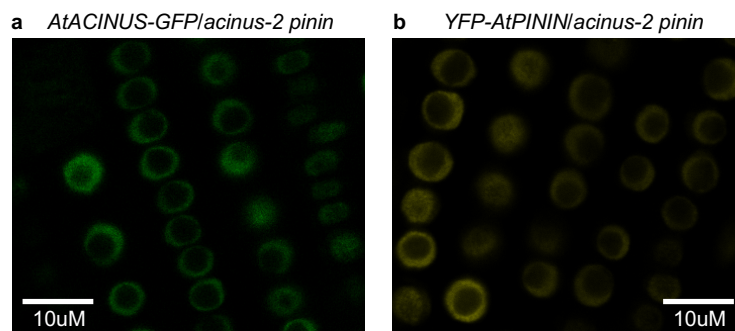


**Supplementary Fig. 1 | Protein sequence analysis of AtACINUS and AtPININ.** (a,b) Pairwise sequence alignment between human Acinus and AtACINUS and between human Pinin and AtPININ using Blastp from NCBI blastp suite. Hits with E value<0.01 are shown. (c) Dendrogram of AtACINUS and AtPININ homologs from various species. PNN=PININ.

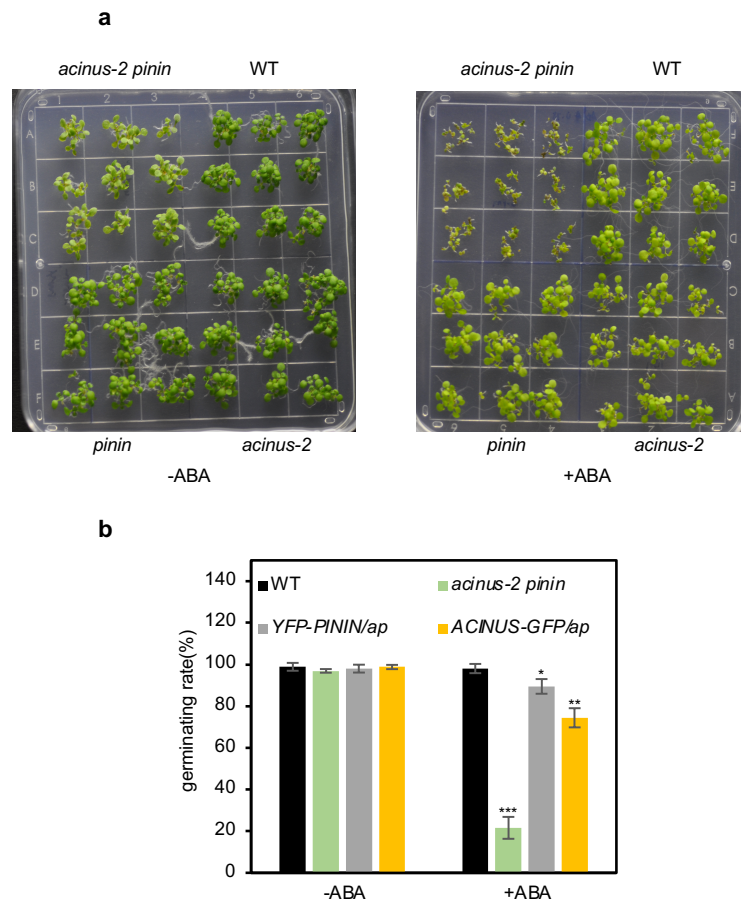


**Supplementary Fig. 2 | Pleiotropic developmental defects in the *acinus-2 pinin-1* mutant.** (a) Germination of *acinus-2 pinin-1* seeds was slightly delayed compared to WT. (b) The *acinus-2 pinin-1* mutants showed short root and tri-cotyledon phenotypes. (c,d) The *acinus-2 pinin-1* double mutant (d) showed increased number of petals compared to WT (c). (e) The *acinus-2 pinin-1* double mutant (right) showed phyllotaxis defects compared to WT (left).

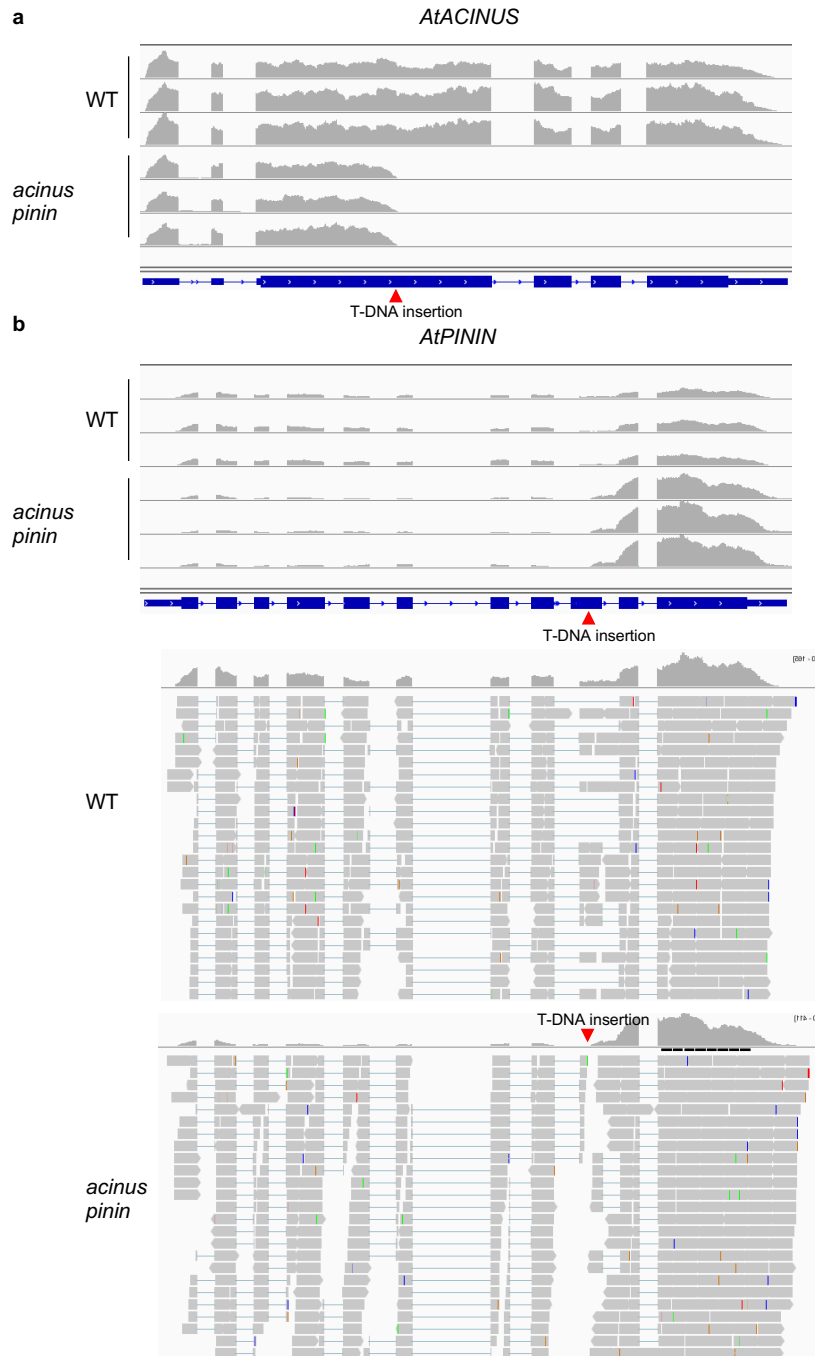




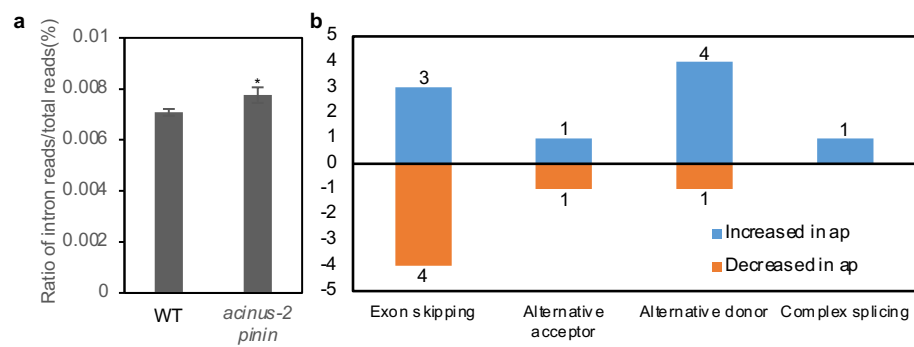
**Supplementary Fig. 3** | Confocal image of AtACINUS-GFP localization in the root of *AtACINUS-GFP/acinus-2 pinin-1* seedlings (a) and YFP-PININ localization in the root of *YFP-PININ/acinus-2 pinin-1* seedlings (b).



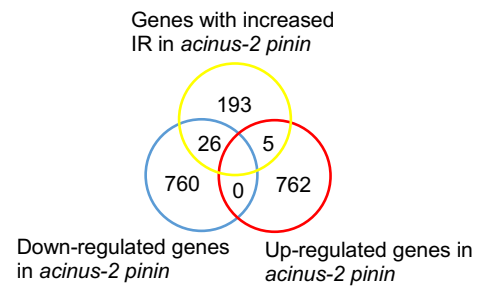
**Supplementary Fig. 4 | AtACINUS and AtPININ redundantly inhibit ABA responses.** (a) Post-germination seedling growth is inhibited by ABA in *acinus-2 pinin-1*. Seeds of WT, *acinus-2*, *pinin-1* and *acinus-2 pinin-1* were germinated on filtered paper, transferred to medium containing no ABA or 1  $\mu\text{mol/L}$  ABA for 5 days. (b) Germination rate of the indicated genotypes after six days on  $\frac{1}{2}$  MS medium containing 0 or 0.5  $\mu\text{mol/L}$  ABA. Error bars indicate SD calculated from 3 biological replicates (n=3). The data points of wild-type, *acinus-2* and *pinin-1* overlap. Asterisks indicate significant differences to wild type (two-sided Student's t-test, \*P<0.05, \*\* P<0.01, \*\*\* P<0.001 ).



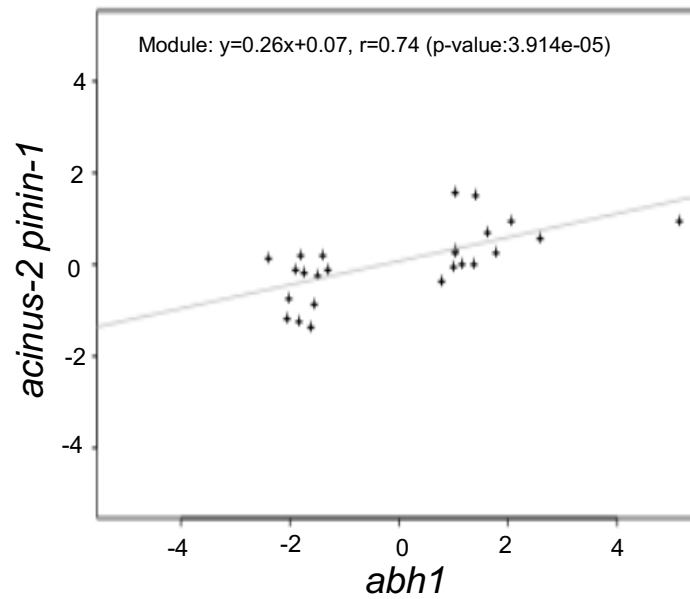
**Supplementary Fig. 5 | Full length *AtACINUS* and *AtPININ* were not transcribed in *acinus-2 pinin-1*.** (a) A partial *AtACINUS* transcript from the 5' transcription start site until T-DNA insertion site was detected in *acinus-2 pinin-1*. (b) A partial *AtPININ* transcript from the 5' transcription start site until T-DNA insertion site was detected at a reduced level in *acinus-2 pinin-1*. Transcription was initiated from the T-DNA insertion to transcribe the 3' end of *AtPININ* after the T-DNA insertion site at an increased level. However, there was no full length *AtPININ* produced because transcripts were discontinuous and showed a gap in the 9<sup>th</sup> exon at the position marked by the red triangle. No reads spanning (gray bar or blue line) this region was detected in *acinus-2 pinin-1* while a large number of reads spanning this region were detected in wild-type.



**Supplementary Fig. 6** (a) The percentage of intron reads in WT and the *acinus-2 pinin-1* double mutant. Error bars indicate SD calculated from 3 biological replicates (n=3). (b) A summary of other types of splicing defects in *acinus-2 pinin-1* compared to WT. Asterisks indicate significant differences to wild type (two-sided Student's t-test, \*P<0.05, \*\* P<0.01, \*\*\* P<0.001 ).

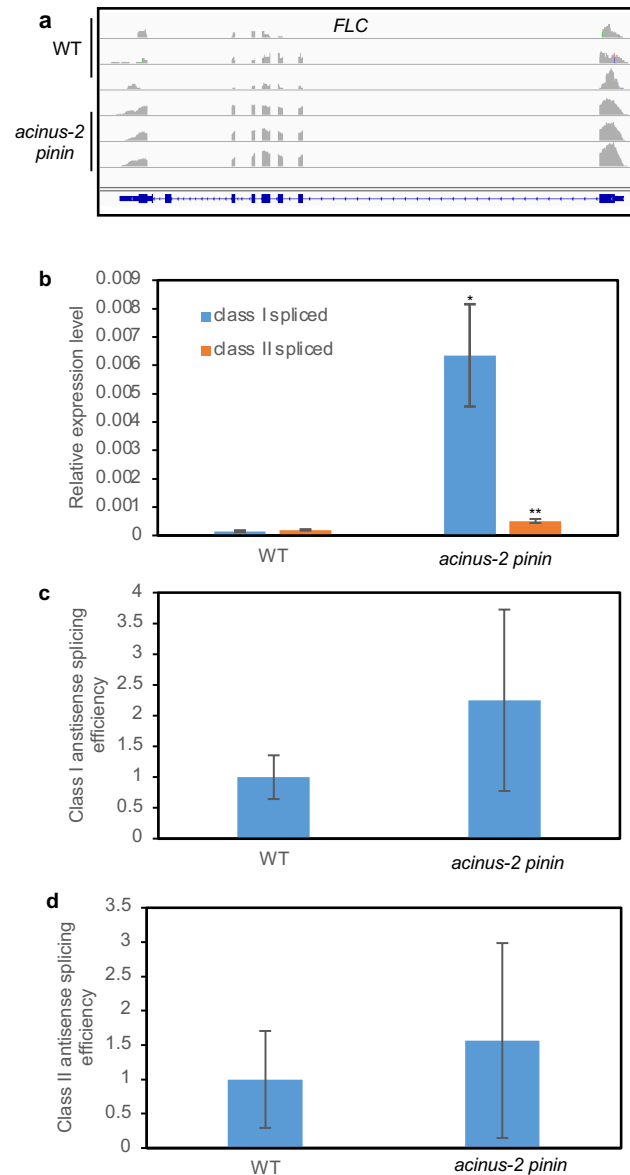


**Supplementary Fig. 7** | Overlap between differentially expressed genes in *acinus-2 pinin-1* and genes with increased intron retention in *acinus-2 pinin-1*.

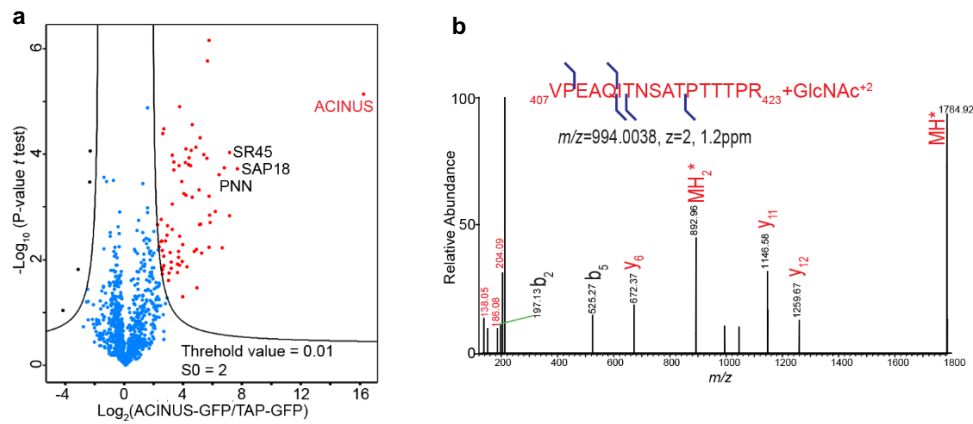


**Supplementary Fig. 8** | The genes mis-expressed in *abh1* (data from Kuhn *et al.*, 2008 [32]) show a strong correlation to genes mis-regulated in *acinus-2 pinin-1*, with Spearman's correlation=0.74.



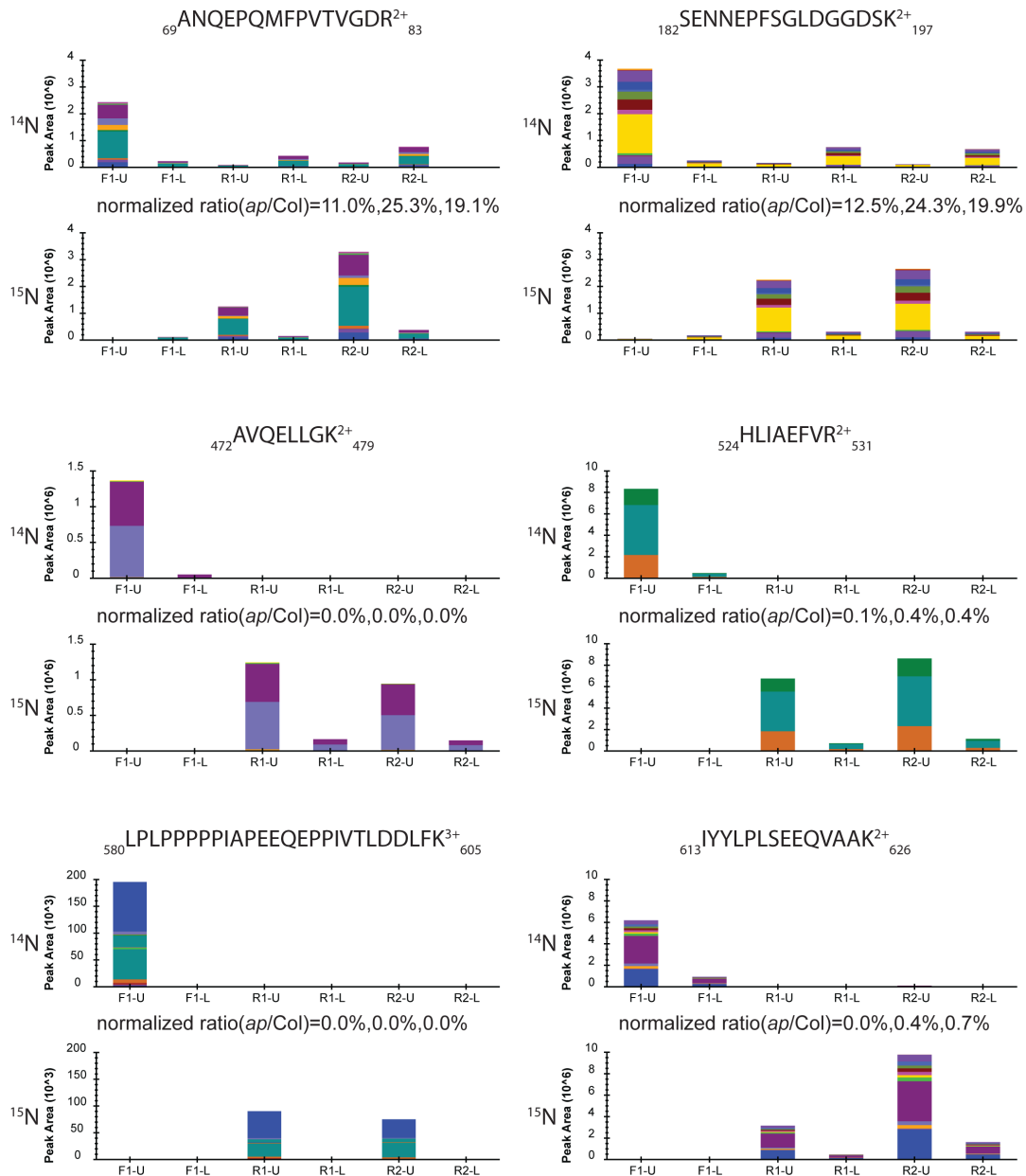


**Supplementary Fig. 9 | *FLC* antisense I is increased relative to antisense II in *acinus-2 pinin-1* while the splicing efficiency is not significantly changed. (a)** Reads coverage of *FLC* locus in WT and *acinus-2 pinin-1*. Track height is set to 15 in WT and 200 in *acinus-2 pinin-1*. **(b)** Expression levels of *FLC* spliced class I antisense and spliced class II antisense relative to *PP2A* in wild-type and *acinus-2 pinin-1*. **(c)** Class I antisense splicing efficiency calculated from class I spliced/class I unspliced. WT is set to 1. **(d)** Class II antisense splicing efficiency calculated from class II spliced/class II unspliced. WT is set to 1. In our experimental conditions, only class II-II is detected and used for calculation for class II antisense. RNA was extracted from 12-day-old seedlings. Error bars indicate SD calculated from 3 biological replicates (n=3). Asterisks indicate significant differences to wild type (two-sided Student's t-test, \*P<0.05, \*\* P<0.01, \*\*\* P<0.001 ).



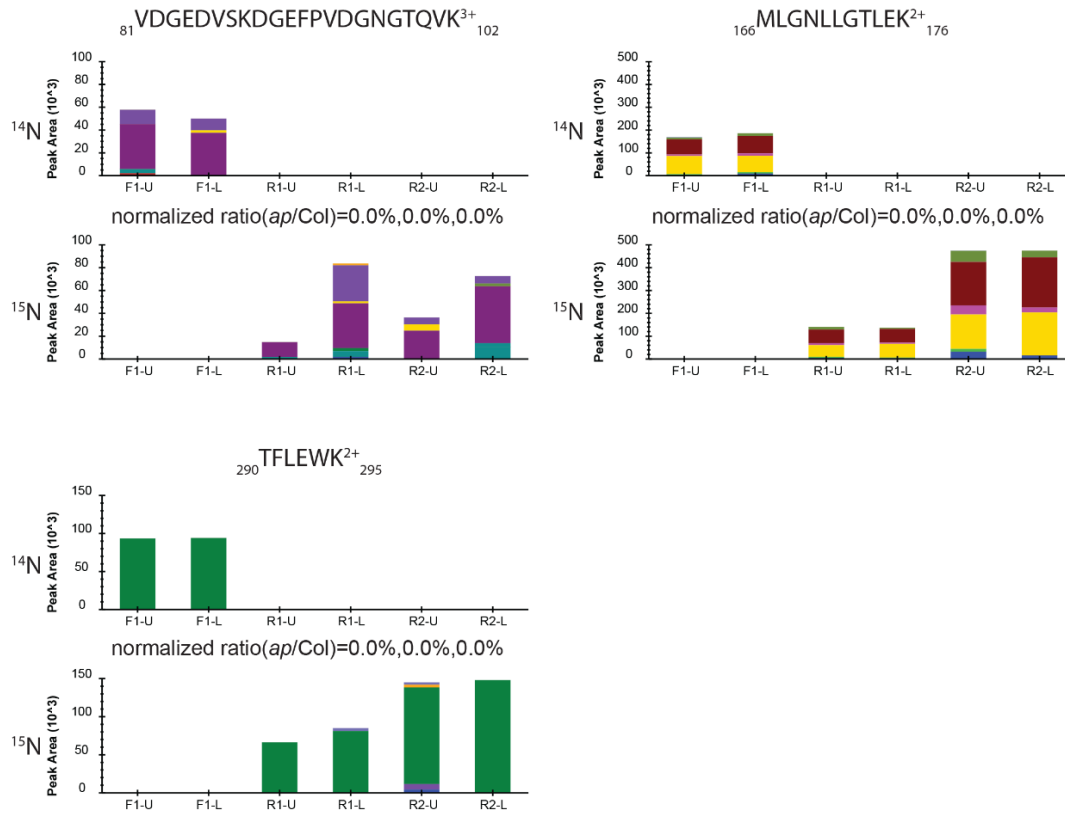
**Supplementary Fig. 10 | (a)** Volcano plot of the IP-MS analysis of the AtACINUS interactome. The logarithmic ratios of protein signal intensities between AtACINUS-GFP and TAP-GFP (negative control) are plotted against negative logarithmic p-values of the *t*-test of triplicate IP-MS. The hyperbolic curves are based on an FDR estimation 0.01 and  $S_0=2$ . The curves separate bait AtACINUS and its specific interactors (red dots) from background proteins (blue dots) and possible false positive (black dots) that are enriched in the TAP-GFP control. Additional information is in Supplemental Data 1. **(b)** HCD spectra detected O-GlcNAcylation on a sequence spanning amino acid 407 to 423 of AtACINUS with neutral loss.

## AtACINUS

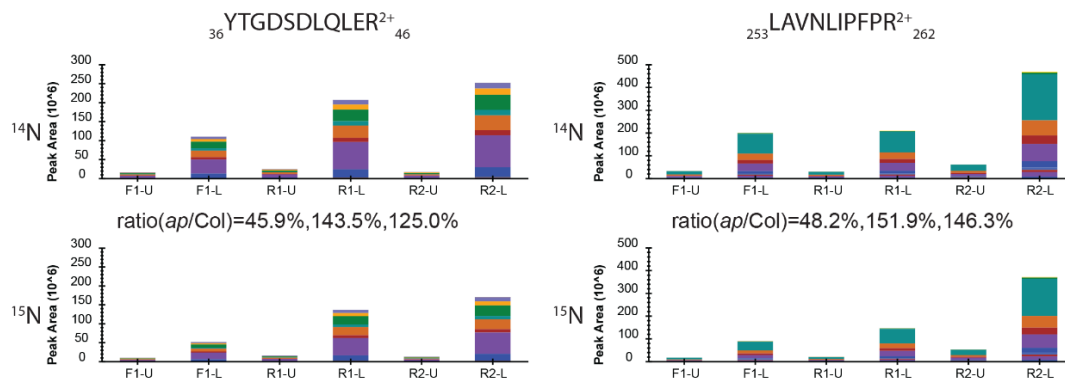


**Supplementary Fig.11** Targeted quantifications using Parallel Reaction Monitoring (PRM) show AtACINUS N-terminal has reduced expression and C-terminal is undetectable in *acinus-2 pinin-1* mutant. Two gel segments (upper part (U) and lower part(L)) were excised from each mixed samples and subjected to trypsin digestion. Proteins were quantified from both segments of each mixed sample, including F1 (<sup>14</sup>N Col/ <sup>15</sup>N *acinus-2 pinin-1*) and R1, R2 samples (<sup>14</sup>N *acinus-2 pinin-1*/ <sup>15</sup>N Col). Peak areas of fragments were extracted for the <sup>14</sup>N and <sup>15</sup>N labeled peptides of targeted proteins using 5 ppm mass window and integrated across the elute profile using Skyline platform. The sum of peak areas from two segments were calculated from Col and *acinus-2 pinin-1* peptides and ratios were calculated and normalized to TUBULIN2.

## AtPININ

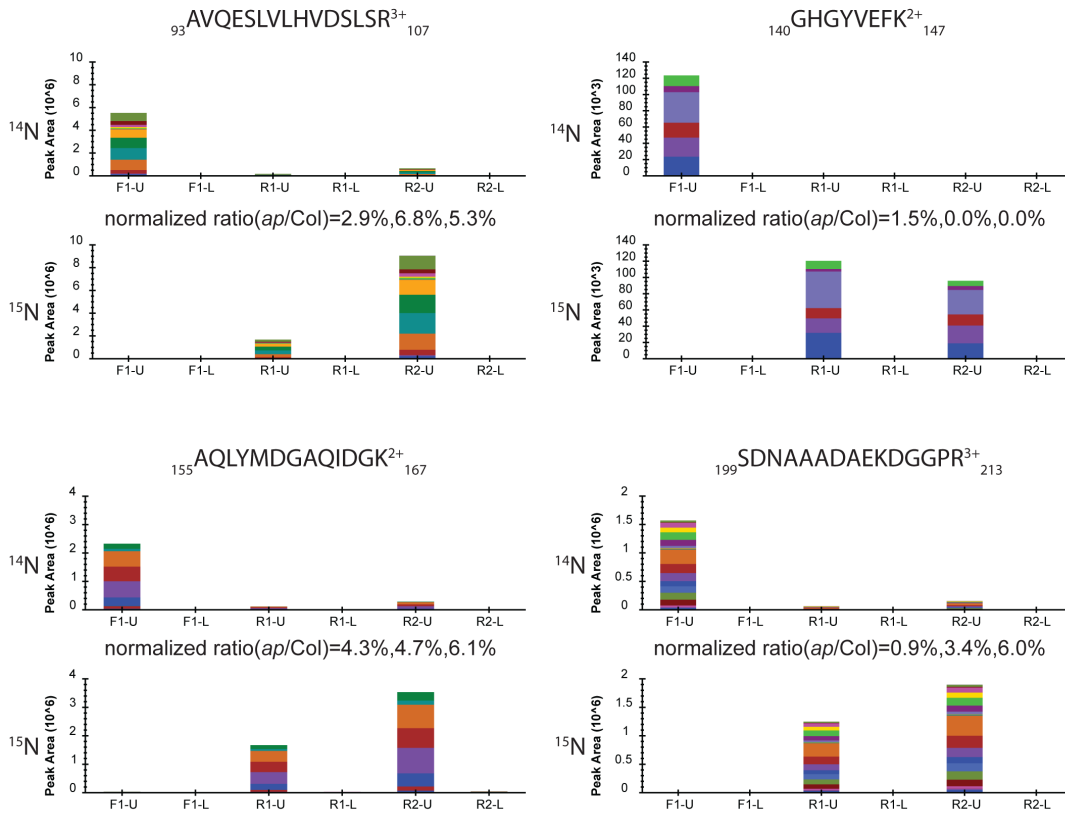


## Control TUBULIN2



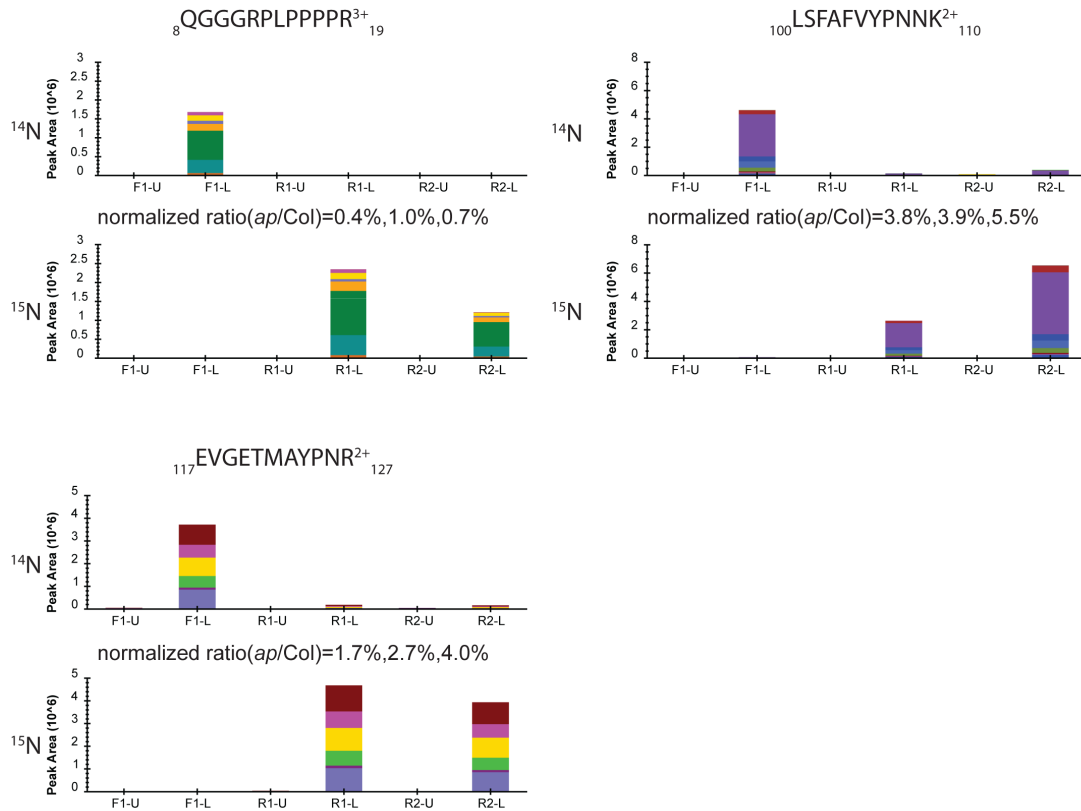
**Supplementary Fig.12** Targeted quantifications using Parallel Reaction Monitoring (PRM) show AtPININ protein level is non-detectable in *acinus-2 pinin-1* mutant. Two gel segments (upper part (U) and lower part(L)) were excised from each mixed samples and subjected to trypsin digestion. Proteins were quantified from both segments of each mixed sample, including F1 (<sup>14</sup>N Col/ <sup>15</sup>N *acinus-2 pinin-1*) and R1, R2 samples (<sup>14</sup>N *acinus-2 pinin-1* <sup>15</sup>N Col). Peak areas of fragments were extracted for the <sup>14</sup>N and <sup>15</sup>N labeled peptides of targeted proteins using 5 ppm mass window and integrated across the elute profile using Skyline platform. The sum of peak areas from two segments were calculated from Col and *acinus-2 pinin-1* peptides and ratios were calculated and normalized to TUBULIN2.

SR45



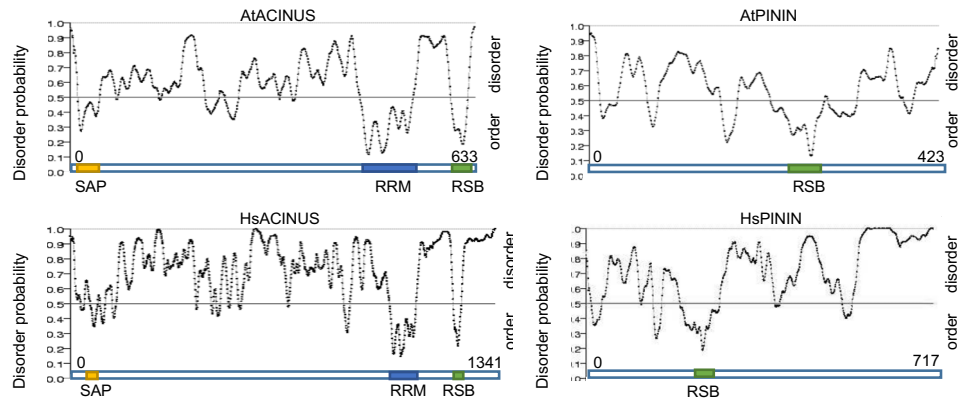
**Supplementary Fig.13** Targeted quantifications using Parallel Reaction Monitoring (PRM) show much reduced SR45 protein levels in *acinus-2 pinin-1* mutant. Two gel segments (upper part (U) and lower part(L)) were excised from each mixed samples and subjected to trypsin digestion. Proteins were quantified from both segments of each mixed sample, including F1 ( $^{14}\text{N}$  Col/  $^{15}\text{N}$  *acinus-2 pinin-1*) and R1, R2 samples ( $^{14}\text{N}$  *acinus-2 pinin-1*/  $^{15}\text{N}$  Col). Peak areas of fragments were extracted for the  $^{14}\text{N}$  and  $^{15}\text{N}$  labeled peptides of targeted proteins using 5 ppm mass window and integrated across the elute profile using Skyline platform. The sum of peak areas from two segments were calculated from Col and *acinus-2 pinin-1* peptides and ratios were calculated and normalized to TUBULIN2.

## SAP18

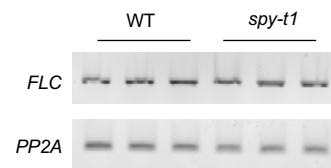


**Supplementary Fig.14** Targeted quantifications using Parallel Reaction Monitoring (PRM) show much reduced SAP18 protein levels in *acinus-2 pinin-1* mutant. Two gel segments (upper part (U) and lower part(L)) were excised from each mixed samples and subjected to trypsin digestion. Proteins were quantified from both segments of each mixed sample, including F1 ( $^{14}\text{N}$  Col/  $^{15}\text{N}$  *acinus-2 pinin-1*) and R1, R2 samples ( $^{14}\text{N}$  *acinus-2 pinin-1*/  $^{15}\text{N}$  Col). Peak areas of fragments were extracted for the  $^{14}\text{N}$  and  $^{15}\text{N}$  labeled peptides of targeted proteins using 5 ppm mass window and integrated across the elute profile using Skyline platform. The sum of peak areas from two segments were calculated from Col and *acinus-2 pinin-1* peptides and ratios were calculated and normalized to TUBULIN2.





**Supplementary Fig. 15** | ACINUS and PININ are predicted to be highly disordered proteins with small ordered regions that overlap with functional domains.



**Supplementary Fig.16** Semi-quantitative RT-PCR of *FLC* in WT and *spy-t1*. *PP2A* serves as an internal control.