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

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O-GlcNAc modification plays important roles in metabolic regulation of cellular status. Two homologs of O-GlcNAc transferase, SECRET AGENT (SEC) and SPINDLY (SPY), which have O-GlcNAc and O-fucosyl transferase activities, respectively, are essential in Arabidopsis but have largely unknown cellular targets. Here we show that AtACINUS is O-GlcNAcylated and O-fucosylated and mediates regulation of transcription, alternative splicing (AS), and developmental transitions. Knocking-out both AtACINUS and its distant paralog AtPININ causes severe growth defects including dwarfism, delayed seed germination and flowering, and abscisic acid (ABA) hypersensitivity. Transcriptomic and protein-DNA/RNA interaction analyses demonstrate that AtACINUS represses transcription of the flowering repressor FLC and mediates AS of ABH1 and HAB1, two negative regulators of ABA signaling. Proteomic analyses show AtACINUS’s O-GlcNAcylation, O-fucosylation, and association with splicing factors, chromatin remodelers, and transcriptional regulators. Some AtACINUS/AtPININ-dependent AS events are altered in the sec and spy mutants, demonstrating a function of O-glycosylation in regulating alternative RNA splicing.
Posttranslational modification (PTM) of intracellular proteins by O-linked N-acetylglucosamine (O-GlcNAc) is an important regulatory PTM that modulates protein activities and thereby controls cellular functions according to nutrient and energy status1,2. Extensive studies in animals have shown that thousands of proteins involved in diverse biological processes are modified on serine and threonine residues by O-GlcNAcylation, which is catalyzed by O-GlcNAc transferase (OGT) using UDP-GlcNAc as donor substrate1,3. As a sensor of primary metabolic status, O-GlcNAcylation plays key roles in cellular homeostasis and responses to nutritional and stress factors1,2,4,5, whereas dysregulation of O-GlcNAcylation has been implicated in many diseases including cancer, diabetes, cardiovascular and neurodegenerative diseases4,6. The Arabidopsis genome encodes two OGT homologs: SPINDLY (SPY) and SECRET AGENT (SEC). The ASAP and PSAP component of the ASAP18 kDa (SAP18), a chromatin remodeler, through its conserved RSB motif (Fig.1b), but no sequence similarity outside this motif. To study the biological function of AtACINUS, we obtained two mutant lines that contain T-DNA insertions in the exons of AtACINUS, Salk_078554 and WiscDsLoxHs108_01G, which are designated acinus-1 and acinus-2, respectively (Fig. 1c). These mutants showed no obvious morphological phenotypes except slightly delayed flowering (Fig. 1d, e). The weak phenotype of acinus is surprising considering the important function of its mammalian counterpart and the absence of any close homolog in Arabidopsis.

We did not expect AtACINUS and AtPININ to have redundant functions, considering their very limited sequence similarity and the fact that mammalian Acinus and Pinin have distinct functions14. AtPININ shares extensive sequence similarity with human Pinin surrounding the RSB domain14 (Supplementary Fig. 1b). Phylogenetic analysis indicated that AtPININ and human Pinin belong to one phylogenetic branch that is distinct from that of AtACINUS and human Acinus (Supplementary Fig. 1c), suggesting independent evolution of ACINUS and PININ before the separation of the metazoan and plant kingdoms. However, Pinin can, through its RSB domain, interact with RNPS1 and SAP18 to form a complex (PSAP) similar to the ASAP complex. Therefore, we tested the possibility that the weak phenotype of Arabidopsis acinus mutants is due to functional redundancy with AtPININ.

We obtained a T-DNA insertion mutant of AtPININ (pinin-1, T-DNA line GABI_029C11). The pinin-1 mutant also showed no obvious morphological phenotype (Fig. 1d). We then crossed pinin-1 with acinus-1 and acinus-2 to obtain double mutants. Both acinus-1 pinin-1 and acinus-2 pinin-1 double mutants displayed pleiotropic phenotypes including severe dwarfism, short root, pale leaves, narrow and twisted rosette leaves with a serrated margin, severely delayed flowering, altered phyllotaxis, altered phyllotaxis, short root, pale leaves, narrow and twisted rosette leaves with a serrated margin, severely delayed flowering, altered phyllotaxis, increased numbers of cotyledons and petals, and reduced fertility (Fig. 1d, e and Supplementary Fig. 2). The acinus-2 pinin-2 double mutants transformed with 35S::AtACINUS-GFP or 35S::YFP-AtPININ displayed near wild-type (WT) morphology (Fig. 1f), confirming that the phenotypes of the double mutants are due to loss of both AtACINUS and AtPININ, and the two genes play genetically redundant roles. The AtACINUS-GFP and YFP-AtPININ proteins are localized in the nucleus outside the nucleolus (Supplementary Fig. 3).

We also noticed that the seed germination was delayed in the acinus pinin mutant (Fig. 2a). This, together with the pale leaf and dwarfism phenotypes, suggests an alteration in ABA response. Indeed, on 0.25 μmol/L ABA, germination of the acinus-2 pinin-1 double mutant seeds was further delayed compared to the WT and the single mutants (Fig. 2b).
Fig. 1 AtACINUS and AtPININ are genetically redundant. a Diagrams of the domain structures of AtACINUS and AtPININ. SAP: SAF-A/B, Acinus, and PIAS motifs. RRM: RNA-recognition motif. RSB: RNPS1–SAP18 binding domain. G and F indicates the position of O-GlcNAcylation and O-fucosylation modifications, respectively. b The sequence alignment of the RSB domains of AtACINUS and AtPININ. Conserved amino acids are highlighted in green. c Diagrams of the AtACINUS and AtPININ (translation start at position 1) with T-DNA insertion sites in acinus-1, acinus-2, and pinin-1 mutants. d Plant morphologies of wild-type (WT), acinus-1, acinus-2, pinin-1, acinus-1 pinin-1, and acinus-2 pinin-1 grown on soil for 20 days. e Five-week-old WT, acinus-1, acinus-2, pinin-1, acinus-1 pinin-1, and acinus-2 pinin-1 plants grown under long day condition. Inset shows enlarged view of the acinus-1 pinin-1 and acinus-2 pinin-1 mutants. f Expression of either AtACINUS-GFP or YFP-AtPININ suppresses the growth defects in acinus-2 pinin-1 double mutant (ap).

Fig. 2 The acinus pinin double mutants showed ABA hypersensitive phenotypes. a, b Germination rates of WT, acinus-2, pinin-1, and acinus-2 pinin-1 after different days on ½ MS medium without ABA (a) or with 0.25 μmol/L ABA (b). The data points of WT, acinus-2, and pinin-1 overlap. Statistically significant differences between WT and acinus-2 pinin-1 were determined by two-tailed t test. The P values for a and b in a are 1.24E−5 and 2.96E−2. The P values for a, b, c, d, e, f, g, h, and i in b are 7.69E−3, 5.60E−6, 2.88E−6, 3.92E−4, 3.17E−4, 6.00E−3, 3.10E−2, 4.77E−2, and 8.57E−3. c Seed germination rates of the indicated genotypes on ½ MS medium supplemented with increasing concentrations of ABA after 5 days. Note that the data points of acinus-1 pinin-1 and acinus-2 pinin-1 overlap and those of WT, acinus-1, acinus-2, and pinin-1 overlap. Statistically significant differences between WT and acinus-2 pinin-1 were determined by two-tailed t test. The P values for a, b, and c are 3.51E−2, 5.14E−8, and 2.99E−2. d Seed germination and development of the indicated genotypes on ½ MS medium with or without 0.5 μmol/L ABA. The pictures were taken 6 days after germination. Values represent mean ± SD calculated from three biological replicates (n = 3) for a–c.

indicates that seed germination of the acinus-1 pinin-1 and acinus-2 pinin-1 double mutants is about threefold more sensitive to ABA than WT and the acinus and pinin single mutants (Fig. 2c). Similarly, post-germination seedling growth of acinus-2 pinin-1 was more inhibited by ABA (Supplementary Fig. 4a). These ABA-hypersensitive phenotypes were rescued by expression of either AtACINUS-GFP or YFP-AtPININ in the acinus-2
**Fig. 3 RNA-sequencing analysis of acinus-2 pinin-1 showed differential intron retention and expression level of many genes.** a Number of introns that showed increased or decreased intron retention in acinus-2 pinin-1 and the number of genes that contain these introns. b Comparison between genes differentially expressed in acinus-2 pinin-1 and ABA-responsive genes. RNA-seq was conducted using 14-day-old light-grown seedlings for both genotypes.

**AtACINUS and AtPININ are involved in AS of specific introns.** We conducted an RNA-seq analysis of the transcriptome of the acinus-2 pinin-1 double mutant. WT and acinus-2 pinin-1 seedlings were grown under constant light for 14 days, and RNA-seq was performed with three biological replicates, each yielding a minimum of 22.4 million uniquely mapped reads. The RNA-seq data confirmed the truncation of the AtACINUS and AtPININ transcripts in the double mutant (Supplementary Fig. 5). Compared to WT, the acinus-2 pinin-1 double mutant showed significantly decreased expression levels for 786 genes and increased levels of 767 genes (fold change >2, multiple-testing corrected p-value <0.05), which include the flowering repressor FLC28 (Supplementary Data 1).

A significantly higher proportion of reads was mapped to the intron regions in the acinus-2 pinin-1 double mutant than in the WT (Supplementary Fig. 6a). Further analyses using the RACK1 software package revealed an increase of retention of 258 introns in 225 genes and decreased retention of 31 introns in 31 genes in the acinus-2 pinin-1 double mutant compared to WT (Fig. 3a and Supplementary Data 2). Intron retention was the dominant form of splicing defect in the acinus-2 pinin-1 double mutant (Fig. 3a and Supplementary Fig. 6b). About 99% of these genes contain multiple introns, and the defects tend to be retention of a specific single intron among many introns of each gene, indicating defects in AS rather than general splicing. Among the RNAs showing increased intron retention, 26 RNAs also showed decreased levels of RNA abundance, and their retained introns introduce in-frame stop codons (Supplementary Fig. 7), consistent with non-sense-mediated decay29. The results show that AtACINUS and AtPININ function in AS, primarily by enhancing splicing of a specific intron among many introns of each transcript.

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

**Abbreviations.** ABH1 encodes the large subunit of the dimeric Arabidopsis mRNA cap-binding complex (NUCLEAR CAP-BINDING PROTEIN SUBUNIT 1, CBP80) and functions as a negative regulator of ABA responses including inhibition of seed germination30,31. The retention of the 10th intron of ABH1 introduces a premature stop codon that truncates the C-terminal 522 amino acids of ABH1 (Fig. 4a). Quantification using qRT-PCR analysis in 12-day-old seedlings showed that the intron-containing ABH1.2 transcript was about 8–10% of the total ABH1 transcripts in the WT, about 11% in pinin-1, about 15% in acinus-2, but more than 50% in acinus-2 pinin-1 (Fig. 4b, c). Expression of either YFP-AtPININ or AtACINUS-GFP in the acinus-2 pinin-1 background rescued the ABH1 intron retention phenotype (Fig. 4b, c). Consistent with compromised ABH1 activity, the gene expression changes in acinus-2 pinin-1 show a strong correlation to those in abh1, with Spearman’s correlation = 0.74 as calculated by ATCAST3.1 (Supplementary Fig. 8).

Intron retention in ABH1 has been reported to cause ABA hypersensitive phenotypes34,35. ABH1 did not display any apparent splicing defects in our RNA-seq and RT-PCR analysis of the 12-day-old seedling. However, after ABA treatment, HAB1 intron retention is significantly increased in acinus pinin compared to the WT. While the expression level of HAB1 transcripts was increased similarly in WT and acinus pinin, the WT seedlings maintained relatively similar ratios between different splice forms of HAB1 before and after ABA treatment, whereas the acinus pinin mutant accumulated a much increased level of the intron-containing HAB1.2 and a reduced level of fully spliced HAB1.3 after ABA treatment (Fig. 4d, e). HAB1.2 encodes a dominant negative form of HAB1 protein that activates ABA signaling8,34,35. Therefore, the accumulation of HAB1.2 should contribute to the ABA hypersensitivity of the acinus pinin mutant.

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

**AtACINUS regulates flowering through repression of FLC.** Consistent with the late-flowering phenotype of acinus pinin (Figs. 1e, 5a), our RNA-seq data showed an increased expression level of the floral repressor FLC, without obvious alteration of the splicing pattern (Supplementary Fig. 9a). The RT-qPCR analysis confirmed the increased levels of FLC mRNA that correspond to the severity of the late-flowering phenotypes in the single and double mutants (Fig. 5b). As FLC expression is also controlled by its anti-sense RNA, which undergoes AS, we analyzed the anti-sense FLC RNAs using RT-qPCR. The results showed a dramatic increase of the class I anti-sense RNA and a slight increase of the class II anti-sense RNA of FLC, but no obvious change of the splicing efficiency of the FLC anti-sense RNAs (Supplementary Fig. 9b–d). AtACINUS was recently reported to associate with VAL1 and VAL2, which bind to the FLC promoter to repress transcription26. We thus performed chromatin immunoprecipitation (ChIP) assays to test whether AtACINUS is associated with the FLC locus, and our results show that AtACINUS interacts with the DNA of the promoter and first intron regions but not the 3’ region of FLC in vivo (Fig. 5c). Together our results provide evidence for a role of AtACINUS in regulating the transcription of FLC.
AtACINUS-dependent AS events are altered in spy and sec. To study how O-linked sugar modification affects the function of AtACINUS, we tested if the AtACINUS-dependent AS events are altered in the spy and sec mutants. Of the ten AtACINUS-dependent intron splicing events we have tested, four showed alterations in the spy mutant and one showed alteration in the sec mutant (Fig. 6).

In the 7-day-old light-grown plants, splicing of the 12th intron and the 15th intron of TRNA METHYLTRANSFERASE 4D (TRM4D, At4g26600) was enhanced in the acinus-2 pinin double mutant compared to that in the WT. In the loss-of-function mutants spy-4 and spy-t1 (SALK_090580), the splicing efficiency of these two introns were also enhanced. In contrast, the loss-of-function mutants sec-2 and sec-5 showed an increased retention of the 12th intron (Fig. 6). These results suggest that SPY and SEC have opposite effects on AtACINUS function in TRM4D splicing. The spy-t1 and spy-4 mutants accumulated more HAB1.3 and less HAB1.2 than WT, while acinus-2 pinin accumulated more HAB1.2 than the WT (Fig. 6), consistent with their opposite seed germination phenotypes. In addition, the splicing efficiency of the 14th intron of EMBRYO DEFECTIVE 2247 (Emb2247, AT5G16715) was reduced in the acinus-2 pinin double mutant, but was increased in the spy-t1 and spy-4 mutants compared to WT (Fig. 6). These results support that the O-linked sugar modifications of AtACINUS modulate its functions in AS of specific RNAs.

AtACINUS associates with transcriptional and splicing factors. To understand the molecular mechanisms of AtACINUS function, we conducted two immunoprecipitations followed by mass spectrometry (IP-MS) experiments. In the first experiment, immunoprecipitation was performed in three biological replicates using the AtACINUS-GFP/acus-2 plants and the anti-GFP nanobody. Transgenic plants expressing a Tandem-Affinity-Purification-GFP (TAP-GFP) protein were used as control.38,
The proteins co-immunoprecipitated with AtACINUS-GFP were identified based on enrichment (FDR = 0.01, S0 = 2) relative to the TAP-GFP control, quantified by label-free mass spectrometry analysis. In the second experiment, AtACINUS-associated proteins were identified by $^{15}$N stable-isotope-labeling in Arabidopsis (SILIA) quantitative mass spectrometry. WT and acinus-2 mutant seedlings were metabolically labeled with $^{14}$N and $^{15}$N, and immunoprecipitation was performed using the anti-AtACINUS antibody, followed by mass spectrometry analysis. The isotope labels were switched in the two biological replicates. AtACINUS-associated proteins were identified based on enrichment in the WT compared to the acinus mutant control. These IP-MS experiments consistently identified 46 AtACINUS-associated proteins (Fig. 7a, Supplementary Fig. 10a, and Supplementary Data 3). These included SR45 and AtSAP18, supporting the existence of an evolutionarily conserved ASAP complex in Arabidopsis. The AtACINUS interactome also included a large number of proteins homologous to known components of the spliceosome, including five Sm proteins, one protein of the U2 complex, four proteins in the U5 complex, 17 proteins of the nineteen complex (NTC) and NTC-related complex (NTR)39–41.

In addition, AtACINUS associated with six proteins of the exon junction complex (EJC) core and the EJC-associated TRanscription-Export (TREX) complex, three proteins of the small nucleolar ribonucleoprotein (snoRNP) complexes, and four other splicing-related proteins (Fig. 7a and Supplementary Data 3)41–45. AtACINUS interactome also included a component of the RNA Polymerase II Associated Factor 1 complex (PAF1C) (Fig. 7a and Supplementary Data 3). The interactome data suggest that, similar to mammalian Acinus, AtACINUS plays dual roles in AS and transcriptional regulation.

The AtACINUS interactome includes five proteins that are genetically involved in regulating FLC and flowering (Fig. 7a and Supplementary Data 3). These are BRR2 and PRP8 of the U5 complex, ELF8 of the PAF1C, and SR45 and AtSAP18 of the ASAP complex. These results suggest that AtACINUS may regulate FLC expression through a complex protein network involving multiple regulatory pathways.

We have previously identified O-GlcNAc modification on Thr79 on AtACINUS13 (Fig. 7b) after LWAC enrichment. Mass spectrometry analysis following affinity purification of AtACINUS identified additional O-GlcNAc modification on the peptide.
containing amino acids 407–423 (Fig. 7c and Supplementary Fig. 10b), as well as O-fucosylation on the peptide containing amino acids 169–197 (Fig. 7d). These results confirm that AtACINUS is a target of both O-GlcNAc and O-fucose modifications.

Using targeted mass spectrometry analysis, we confirmed that the acinus-2 pinin double mutant expressed only the AtACINUS’s N-terminal peptides (at about 20% WT level), but no detectable peptides of the C-terminal region (after T-DNA insertion) (Supplementary Fig 11 and Supplementary Data 5). Both N- and C-terminal peptides of AtPININ were undetectable in the acinus-2 pinin mutant (Supplementary Fig 12 and Supplementary Data 5). Meanwhile, SR45 and AtSAP18 protein levels were decreased in the double mutant (Supplementary Fig. 8)32,33. A recent proteomic study showed that the ABH1 protein level was decreased in the sr45 mutant23, whereas a reduction of ABH1 RNA level to ~30% caused obvious phenotypes in potato52. The intron-containing 10th intron of ABH1 in acinus pinin mutant is expected to truncate its C-terminal half and cause loss of ABH1 function and thus increase of ABA sensitivity. Supporting the functional role of ABH1, a recent study showed that the ABH1 protein level was decreased in the sr45 mutant23, whereas a reduction of ABH1 RNA level to ~30% caused obvious phenotypes in potato52.

AtACINUS-mediated AS of HAB1 switches a positive feedback loop to a negative feedback loop in the ABA signaling pathway. HAB1 encodes a phosphatase that dephosphorylates the SNF1-related protein kinases (SnRK2s) to inhibit ABA responses, and the ligand-bound ABA receptor inhibits HAB1 to activate ABA responses33,34. The intron-containing HAB1.2 encodes a dominant negative form of HAB1 protein that lacks the phosphatase activity but still competely interacts with SnRK2, thus activating, instead of inhibiting, ABA signaling34,35. As ABA signaling feedback increases the HAB1 transcript level, the AtACINUS-mediated AS switches a positive feedback loop that...
accumulation of SnRK1 which is a positive regulator of stress and AtACINUS. The sequence ion series that retain this modification contribute to the ABA-hypersensitivity phenotypes of moter26. Our results provide genetic evidence for the function of the VAL1 transcription factor, which binds to the FLC repressing related with increased GlcNAcylation and O-fucosylation modulate the evolutionarily conserved RSB-domain protein AtACINUS, which controls transcription and alternative RNA sequence spanning amino acid 169.

**Fig. 7 AtACINUS is O-GlcNAc and O-fucose modified and associates with spliceosomal complexes, transcriptional regulators, and chromatin remodeling proteins.** a) Diagram shows functional groups of AtACINUS-associated proteins. Proteins are grouped in boxes based on their association with known complexes or functions. Positive regulators of FLC are highlighted in red and negative regulators in blue. Seven-day-old seedlings were used for the label-free IP-MS experiments and 14-day-old seedlings were used for the 15N stable-isotope-labeling in known complexes or functions. Positive regulators of ABA signaling are labeled in red. HexNAc oxonium ion (m/z 1144) and its fragments masses are labeled in red.  b) Higher energy collisional dissociation (HCD) mass spectra show O-GlcNAcylation on Thr79 and a sequence spanning amino acid 400 – 1684. The sequence ion series that retain this modification (shifted by 203 Da) are labeled in blue (b). The sequence ion series that have lost the modification are labeled in red. HexNAc oxonium ion (m/z 204) and its fragments masses are labeled in red. d) HCD spectrum shows O-fucosylation on a sequence spanning amino acid 169–197 of AtACINUS with neutral loss. e) Proposed model of a molecular pathway in which nutrient sensing O-GlcNAcylation and O-fucosylation modulate the evolutionarily conserved RSB-domain protein AtACINUS, which controls transcription and alternative RNA splicing of specific target genes to modulate stress hormone sensitivity and developmental transitions such as seed germination and flowering in plants.

reinforces ABA signaling to a negative feedback loop that dampens ABA signaling. Such a switch is presumably important for the different ABA signaling dynamics required for the onset of and recovery from stress responses or dormancy.

The relative contributions of intron retention of ABH1 and HAB1 to ABA sensitivity will need to be quantified by genetic manipulation of each splicing event. Additional mechanisms may contribute to the ABA-hypersensitivity phenotypes of acinus pinin. For example, the level of SR45 is significantly decreased in acinus pinin, while loss of SR45 has been reported to cause accumulation of SnRK1 which is a positive regulator of stress and ABA responses23.

The late-flowering phenotype of the acinus pinin mutant correlated with increased FLC expression. A role of AtACINUS in repressing FLC expression. Further, our ChIP-PCR analysis shows that AtACINUS associates with genomic DNA of the promoter region and the first intron of FLC, confirming a direct role in transcriptional regulation of FLC. These results provide critical evidence for the hypothesis that the AtACINUS represses FLC by AtSAP18-mediated recruitment of the Sin3 histone deacetylase complex (HDAC)20. It is worth noting that overexpression of AtSAP18 in the sr45 mutant increased FLC expression and further delayed flowering23. It is possible that the transcriptional repression function of AtSAP18 requires the ASAP/PSAP complex. It is also worth noting that the AtACINUS interactome includes several proteins known to be involved in regulating FLC expression and flowering. Among these, BRR2 and PRP8 are components of the U5 complex and mediate splicing of the sense and anti-sense transcripts of FLC to inhibit and promote flowering, respectively27,46. ELF8 is a component of the PAF1 complex and promotes histone methylation of FLC chromatin47.
The identification of additional FLC-regulators as AtACINUS-associated proteins suggests that AtACINUS may regulate FLC expression through complex protein networks. Genetic evidence supports that ELF8/PAF1C and SR45 also have dual functions in regulating FLC expression and ABA responses, suggesting that the functions of AtACINUS in seed germination and flowering may involve overlapping protein networks.

Structural studies in metazoan systems showed that the RSB domains of Acinus and Pinin directly interact with RNPS1 and SAP18, forming a ternary ASAP and PSAP complexes that have both RNA- and protein-binding properties as well as abilities to interact with both RNA splicing machinery and histone modifiers. ASAP and PSAP function as EJC peripheral protein complexes to modulate RNA processing. We quantitatively proteomic analyses of the AtACINUS interactome provide strong evidence for interaction with SR45 (ortholog of RNPS1) and AtSAP18, as well as components of EJC and additional splicing factors. However, some proteins, such as SPY and SEC, may interact transiently and were not detected by IP-MS. While our proteomic data do not distinguish the proteins that directly interact with AtACINUS from those that associate indirectly as a subunit of the interacting protein complexes, the greatly reduced levels of SR45 and AtSAP18 proteins in acinus pinin are consistent with the direct interactions predicted based on the conserved RSB domain. Similarly, the sr45 mutation leads to a near absence of AtSAP18 and a mild decrease of the AtACINUS protein level in the inflorescence tissues. Together, these observations support the notion that AtACINUS and AtPININ mediate formation of similar ASAP and PSAP complexes and stabilize SR45 and AtSAP18 in plants.

Studies in human cells have shown that Acinus and Pinin mediate splicing of distinct RNAs and that Acinus cannot rescue the splicing defects caused by knockdown of Pinin. In contrast, AtACINUS and AtPININ appear to have largely redundant and interchangeable functions. It is possible that both AtACINUS and AtPININ, through their RSB domain, recruit SR45 and AtSAP18, which determine target specificities. However, AtACINUS and AtPININ may have subtle differences in their functions. Like human Acinus, AtACINUS contains two additional conserved domains that are absent in AtPININ. Further, the regions of AtACINUS and AtPININ, as well as human Acinus and Pinin, outside the RSB domain contain mostly divergent intrinsically disordered sequences (Supplementary Fig. 15). These distinct sequences may provide specificity in interactions with target transcripts and partner proteins or in regulation by PTMs. Indeed, O-GlcNAcylated residues (Thr79 and amino acids 407–423) and the O-fucosylated site (amino acids 169–197) were in the intrinsically disordered regions of AtACINUS, whereas no O-GlcNAc or O-fucose modification was detected in AtPININ, though this could be due to partial sequence coverage of our mass spectrometry analysis. Deep RNA-seq analysis with higher coverage of the single and double mutants of AtACINUS in seed germination and flowering may involve overlapping protein networks.

Our study reveals important functions of AtACINUS in developmental transitions and a previously unknown function of O-linked glycosylation in regulating FLC transcription. However, the effect of sec on FLC expression could also be mediated by other O-GlcNAc-modified flowering regulators. sec supports an important function of AtACINUS in mediating the regulation of AS by O-glycosylation. On the other hand, AtACINUS-independent mechanisms may also contribute to the regulation, as the O-GlcNAcylated Arabidopsis proteins include additional RNA-binding and splicing factors, such as SUS2 which is in the AtACINUS interactome. Deep transcriptomic analysis of sec, and conditional double spy sec mutants will be required to better understand how O-GlcNAc and O-fucose modulate RNA processing and AtACINUS function. Genetic analyses have suggested that the ABA insensitive 5 (ABI5) transcription factor in regulating seed germination is also modified by O-GlcNAc. The function of O-glycosylation in stress responses seems to be conserved, as large numbers of molecular connections between O-GlcNAc and stress response pathways have been reported in metazoans. How O-linked glycosylation of AtACINUS affects its transcriptional activity at the FLC locus remains to be investigated. Both spy and sec mutants flower early, opposite to acinus pinin. While spy shows a strong early flowering phenotype, the FLC expression level was unaffected in spy under our experimental conditions (Supplementary Fig. 16), suggesting that spy regulates flowering independent of FLC. The FLC level was decreased in sec, supporting the possibility that O-GlcNAcylation affects AtACINUS transcription activity. However, the effect of sec on FLC expression could also be mediated by other O-GlcNAc-modified flowering regulators.
introns retained in the actin1 pin mutant, 114 are pts introns, which is about 1.7-fold the random probability (p value <3.0E−9). These pts introns include the intron retained in ABH1 but not that in HAB1, consistent with translation of the dominant negative form of HAB1.2 (refs. 43, 44). Together with these recent developments, our study raises the possibility that ATACINUS plays important roles in the splicing of pts introns, acting downstream of the metabolic signals transduced by SPY/O-fucose and SEC/O-GlcNAc. Our study supports an evolutionarily conserved function of the metabolic signals transduced by SPY/O-fucose and SEC/O-GlcNAc.

Methods

Plant materials. All the Arabidopsis thaliana plants used in this study were in the Col-0 ecotype background. The plants were grown in greenhouses with a 16-h light/8-h dark cycle at 22–24 °C for general growth and seed harvesting. For seedlings grown on the medium in Petri dishes, the sterilized seeds were grown on ½ Murashige and Skoog (MS) medium and supplemented with 0.7% (w/v) phytagar. Plates were placed in a growth chamber under the constant light condition at 21–22 °C. T-DNA insertion mutants atacinus-1 (Salk_087554, insertion position +1744 relative to the genomic translational start site of At4G39690), atacinus-2 (WiscDLoxHs108_01G, insertion position +674), atipin1 (GABI_029C11/CS402723, insertion position +1817 of At1G15200), spy-1 (Salk_090580), and sec-5 (Salk_034290) were obtained from Arabidopsis Biological Resource Center. The spy-4 and sec-2 seeds that have been backcrossed to Columbia for six generations were provided by Neil Oszerski lab.

Germination assay. Seeds were surface sterilized with 70% (v/v) ethanol and 0.1% H2O2 for 5 min. The sterilized seeds were then plated on 1/2 MS medium supplemented with mock or ABA. The seeds were placed in 4 °C cold room for 3 days for stratification before moving into a growth chamber to germinate. Germination was defined as obvious radicle emergence from the seed coat.

Gene cloning and plant transformation. The ATACINUS cDNA was initially cloned into the vector PENTR-D/TOPO and subsequently into the binary vector pGWB5 to generate the 35S::AtACINUS-GFP plasmid. The 35S::AtACINUS-GFP binary plasmid was transformed into actin1-2 plants by floral dipping with Agrobacterium tumefaciens strain GV3101. A homogenous 35S::AtACINUS-GFP/actin1-2 plant was selected for similar protein expression level to the endogenous AtACINUS protein of WT plants using a native GFP

RNA sequencing and data analysis. RNA was extracted from 14-day-old WT and actin1-2 pin1-1 seedlings using RNEasy mini kit (Qiagen) and treated with TURBO DNA-free kit (Ambion) to remove any genomic DNA contamination. The mRNA libraries were constructed using NEBNext RNA Library Prep Kit for Illumina following the standard Illumina protocol. Illumina sequencing was performed in the Sequencing Center for Personalized Medicine, Department of Genetics at Stanford University, using an Illumina HiSeq 2000 System. The RNA-seq data have been deposited at the NCBI Gene Expression Omnibus (GEO) database under the accession number GSE19023.
AtACINUS antibody for 1 h at 4 °C, then 50 µL of protein A agarose beads were deposited to PRIDE with project accession: PXD020700. Label-free mass spectrometric analysis of AtACINUS and its interactome was performed as described previously with slight modifications. Briefly, proteins were extracted in 10 mL of MOPS buffer (100 mM/L MOPS, pH 7.6, 150 mM/L NaCl, 1% (v/v) Triton X-100, 1 mM/L phenylmethylsulfonyl fluoride (PMSF), 2× Complete-protease inhibitor cocktail, and PhosStop cocktail (Roche)), centrifuged, and filtered through two layers of Miracloth. The flow through was incubated with 20 µg of anti-AtACINUS antibody for 1 h at 4 °C, then 50 µL of protein A agarose beads were added and incubated for another hour, followed by four 2-min washes with immunopurification buffer. At the last wash WT and 15N labeled acinus-2 IP samples or reciprocal 15N-labeled WT and 14N-labeled acinus-2 IP samples were mixed, and eluted with 2× SDS buffer. The eluted proteins were separated by SDS-PAGE. After Coomassie Brilliant blue staining, the whole lane of protein samples was excised in ten segments and subjected to in-gel digestion with trypsin.

The peptide mixtures were desalted using C18 ZipTips (Millipore) and analyzed on an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher), equipped with a NanoAcuity liquid chromatography system (Waters). Peptides were loaded onto a trapping column (NanoAcuity UPLC 180 µm × 20 mm; Waters) and then washed with 0.1% (v/v) formic acid (FA) for 1 min. The analyte injection column was a BEH130 C18 100 µm × 100 mm (Waters). The flow rate was 600 nL/min. Peptides were eluted by a gradient from 2–30% solvent B (100% (v/v) acetonitrile/0.1% (v/v) formic acid) over 34 min, followed by a short wash at 50% solvent B. After a precursor scan was measured in the Orbitrap by scanning from mass-to-charge ratio 350 to 1500, the six most intense multiply charged precursors were selected for collision-induced dissociation in the linear trap.

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

Label-free mass spectrometric analysis of ATACINUS and its interactome. The ATACINUS-GFP/acinus-2 and TAP-GFP seedlings were grown for 7 days at 21 °C under constant light on ½ MS medium. Tissues were harvested, ground in liquid nitrogen, and stored at −80 °C. Immunoprecipitation was performed as described previously with slight modifications. Briefly, proteins were extracted in 100 mM/L MOPS, pH 7.6, 150 mM/L NaCl, 1% (v/v) Triton X-100, 1 mM/L phenylmethylsulfonyl fluoride (PMSF), 2× Complete-protease inhibitor cocktail, and PhosStop cocktail (Roche), centrifuged, and filtered through two layers of Miracloth. The flow through was incubated with 20 µg of anti-AtACINUS antibody for 1 h at 4 °C, then 50 µL of protein A agarose beads were added and incubated for another hour, followed by four 2-min washes with immunopurification buffer. At the last wash WT and 15N labeled acinus-2 IP samples or reciprocal 15N-labeled WT and 14N-labeled acinus-2 IP samples were mixed, and eluted with 2× SDS buffer. The eluted proteins were separated by SDS-PAGE. After Coomassie Brilliant blue staining, the whole lane of protein samples was excised in ten segments and subjected to in-gel digestion with trypsin. The peptide mixtures were desalted using C18 ZipTips (Millipore) and analyzed on an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher), equipped with a NanoAcuity liquid chromatography system (Waters). Peptides were loaded onto a trapping column (NanoAcuity UPLC 180 µm × 20 mm; Waters) and then washed with 0.1% (v/v) formic acid (FA) for 1 min. The analyte injection column was a BEH130 C18 100 µm × 100 mm (Waters). The flow rate was 600 nL/min. Peptides were eluted by a gradient from 2–30% solvent B (100% (v/v) acetonitrile/0.1% (v/v) formic acid) over 34 min, followed by a short wash at 50% solvent B. After a precursor scan was measured in the Orbitrap by scanning from mass-to-charge ratio 350 to 1500, the six most intense multiply charged precursors were selected for collision-induced dissociation in the linear trap.

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

Targeted quantification comparing WT and the acinus-2 pin1-1 double mutant. The WT and acinus-2 pin1-1 plants were grown on Hoagland medium containing 14N or 15N (1.34 g/L Hogland’s No. 2 salt mixture without nitrogen, 6 g/L Phytobolin, and 1 g/L KNO3 or 1 g/L K15NO3 (Cambridge Isotope Laboratories), pH 5.8). Proteins were extracted from multiple proteins with peptide mass/charge (m/z), retention time, and MS2 fragments. PININ peptide information was from an IP-MS experiment. For targeted analysis, parallel reaction monitoring (PRM) acquisition was used with a 20-min window using skyline from 14N- and 15N-labeled samples. Peak areas of fragments were calculated from each sample, the sum of peak areas from the upper gel segment and the lower gel segment was used to calculate the acinus-2 pin1-1/Col ratios for each peptide, normalized to TUBULIN2 to get the normalized ratios. The median number of multiple ratio measurements is used for each protein.

Statistics and reproducibility. Figure 4b, d and Supplementary Fig. 16 show representative results from two independent experiments, each with three biological repeats. Figure 6 and Supplementary Fig. 3a, b show representative results from two independent experiments. Figure 5c shows results from one experiment with three biological repeats.

Data availability. Source data are provided as a supplementary file. ProteinData. Data that support the findings of this study have been deposited in Proteomes Data Identification Database (PRIDE) with the accession codes: PXD020700, PXD020748, PXD020749. The RNA-seq data that support the findings of this study have been deposited in the National Center for Biotechnology Information (NCBI) under accession number GSE178702.
for Biotechnology Information Gene Expression Omnibus and are accessible through the GEO series accession number GSE110923. All other related data are available from the corresponding authors upon request.

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References

1. Hanover, J. A., Krause, M. W. & Love, D. C. The hexosamine signaling pathway, O-GlcNAc cycling in feast or famine. Biochem. Biophys. Acta 1800, 801–820 (2010).
2. Hart, G. W., Slawson, C., Ramirez-Correa, G. & Lagerlof, O. Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. Annu. Rev. Biochem. 80, 825–858 (2011).
3. Ma, J. & Hart, G. W. O-GlcNAc profiling: from proteins to proteomes. Clin. Proteomics 11, 43–54 (2014).
4. Yang, X. & Qian, K. Protein O-GlcNAcylation: emerging mechanisms and functions. Nat. Rev. Mol. Cell Biol. 18, 452–465 (2017).
5. Chen, P. H., Chi, J. T. & Boyce, M. Functional crosstalk among oxidative stress and O-GlcNAc signaling pathways. Glycobiology 28, 556–564 (2018).
6. Banerjee, P. S., Lagerlof, O. & Hart, G. W. Roles of O-GlcNAc in chronic diseases of aging. Mol. Asp. Med. 51, 1–15 (2016).
7. Jacobsen, S. E. & Olszewski, N. E. Mutations at the SPINDLY locus of Arabidopsis alter gibberellin signal transduction. Plant Cell 5, 887–896 (1993).
8. Liang, L. et al. SPINDLY is involved in ABA signaling bypassing the PYR/PYL/RCA1-mediated pathway and partly through functional ABAR. Environ. Exp. Bot. 151, 43–54 (2018).
9. Hartweck, L. M., Scott, C. L. & Olszewski, N. E. Two O-linked N-acetylgalactosamine transferase genes of Arabidopsis thaliana L. Heynh. have overlapping functions necessary for gamete and seed development. Genetics 161, 1279–1291 (2002).
10. Zentella, R. et al. Arabidopsis O-fucosyltransferase SPINDLY activates nuclear growth regulator DELLA. Nat. Chem. Biol. 13, 479–485 (2017).
11. Zentella, R. et al. O-GlcNAcylation of master growth repressor DELLA by SECRET AGENT modulates multiple signaling pathways in Arabidopsis. Genes Dev. 30, 164–176 (2016).
12. Olszewski, N. E., West, C. M., Sassi, S. O. & Hartweck, L. M. O-GlcNAc protein-modifying plant evolution and function. Biochem. Biophys. Acta 1800, 49–56 (2010).
13. Xu, S. L. et al. Proteomic analysis reveals O-GlcNAc modification on proteins with key regulatory functions in Arabidopsis. Proc. Natl Acad. Sci. USA 114, E1536–E1543 (2017).
14. Mezzalama, A. G., Ebert, J., Basquin, C., Le Hir, H. & Conti, E. The structure of the ASAP core complex reveals the existence of a Pinin-containing PSAP complex. Nat. Struct. Mol. Biol. 19, 378–386 (2012).
15. Wang, Z., Ballut, L., Barbosa, I. & Le Hir, H. Exon Junction Complexes can have distinct functional flavours to regulate specific splicing events. Sci. Rep. 8, 9509 (2018).
16. Schworer, C. et al. ASAP, a novel protein complex involved in RNA processing and apoptosis. Mol. Cell. Biol. 23, 2981–2990 (2003).
17. Deka, B. & Singh, K. K. Multifaceted regulation of gene expression by the apoptosis- and splicing-associated protein complex and its components. Int. J. Biol. Sci. 13, 545–560 (2017).
18. Carvalho, R. F., Carvalho, S. D. & Duque, P. The plant-specific SR45 protein negatively regulates glucose and ABA signaling during early seedling development in Arabidopsis. Plant Physiol. 154, 772–783 (2010).
19. Ali, G. S. et al. Regulation of plant developmental processes by a novel splicing factor. PLoS ONE 2, e471 (2007).
20. Avin, I., Greenberg, M. V., Li, C. F. & Jacobsen, S. E. The splicing factor SR45 affects the RNA-directed DNA methylation pathway in Arabidopsis. Epigenetics 7, 29–33 (2012).
21. Zhang, X. N. et al. Transcriptome analyses reveal SR45 to be a neutral splicing event. PLoS Pathog. 5, e1000526 (2009).
22. Koncz, C., Dejong, F., Villacorta, N., Szakonyi, D. & Koncz, Z. The spliceosome-activating complex: molecular mechanisms underlying the function of a pleiotropic regulator. Front. Plant Sci. 3, 9 (2012).
23. Reischow, S. L., Hama, T., Ferre-D’Amare, A. R. & Varani, G. The structure and function of small nuclear ribonucleoproteins. Nucleic Acids Res. 35, 1452–1464 (2007).
24. Boehm, V. & Gehring, N. H. Exon junction complexes: supervising the gene expression assembly line. Trends Genet. 32, 724–735 (2016).
25. Le Hir, H., Sauri, B. & Wang, Z. The exon junction complex as a node of post-transcriptional networks. Nat. Rev. Mol. Cell Biol. 17, 41–54 (2016).
26. Woodward, L. A., Mabin, J. W., Gangpras, P. & Singh, G. The exon junction complex play redundant roles in plant innate immunity. PLoS Pathog. 5, e1000526 (2009).
27. Mahrez, W. et al. BRR2a affects flowering time via FLC splicing. PLoS Genet. 12, e1005924 (2016).
28. He, Y., Doyle, M. R. & Amasino, R. M. PAF1-complex-mediated histone methylation of FLOWERING LOCUS C chromatin is required for the vernalization-responsive, winter-annual habit in Arabidopsis. Genes Dev. 18, 2774–2784 (2004).
29. Rodor, J., Pan, Q., Blencowe, B. J., Eyhas, E. & Caceres, J. F. The RNA-binding profile of Acinus, a peripheral component of the exon junction complex, reveals its role in splicing regulation. RNA 22, 1411–1426 (2016).
30. Vucetic, Z. et al. Acinus-S represses ribonucleic acid receptor (RAR)-regulated gene expression through interaction with the B domains of RARS. Mol. Cell. Biol. 25, 2549–2558 (2008).
31. Feng, W., Soprano, K. J. & Soprano, D. R. Role of Acinus in regulating ribonucleic acid-responsive gene pre-mRNA splicing. J. Cell Physiol. 230, 791–801 (2015).
32. Akin, D., Newman, J. R., McIntyre, L. M. & Sugre, S. P. RNA-seq analysis of impact of PNN on gene expression and alternative splicing in corneal epithelial cells. Mol. Vis. 22, 40–60 (2016).
33. Pieczynski, M. et al. Down-regulation of CBP80 gene expression as a strategy to engineer a drought-tolerant potato. Plant Biotechnol. J. 11, 459–469 (2013).
34. Stewart, J. et al. Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2CHAB1 reveal its role as a negative regulator of abscisic acid signalling. Plant J. 37, 354–369 (2004).

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54. Vlad, F. et al. Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscissic acid in Arabidopsis. *Plant Cell* **21**, 3170–3184 (2009).

55. Carvalho, R. F. et al. The Arabidopsis SR45 splicing factor, a negative regulator of sugar signaling, modulates Snf1-related protein kinase 1 stability. *Plant Cell* **28**, 1910–1925 (2016).

56. Liu, Y. et al. Identification of the Arabidopsis REDUCED DORMANCY 2 gene uncovers a role for the polymerase associated factor 1 complex in seed dormancy. *Plant Cell* **6**, 622241 (2011).

57. Tange, T. O., Shibuya, T., Jurica, M. S. & Moore, M. J. Biochemical analysis of the EJC reveals two new factors and a stable tetrameric protein core. *RNA* **11**, 1869–1883 (2005).

58. Oldfield, C. J. & Dunker, A. K. Intrinsically disordered proteins and intrinsically disordered protein regions. *Annu. Rev. Biochem.* **83**, 533–584 (2014).

59. Swain, S. M., Tseng, T. S. & Olszewski, N. E. Altered expression of SPINDLY affects gibberellin response and plant development. *Plant Physiol.* **126**, 1174–1185 (2001).

60. Xing, L. et al. Arabidopsis O-GlcNAc transferase SEC activates histone methyltransferase ATX1 to regulate flowering. *EMBO J.* **37**, e98115 (2018).

61. Tan, Z. W. et al. O-GlcNAc regulates gene expression by controlling detained intron splicing. *Nucleic Acids Res.* **48**, 5656–5669 (2020).

62. Couto, P. L., Bhutkar, A. & Sharp, P. A. Detained introns are a novel, widespread class of post-transcriptionally spliced introns. *Genes Dev.* **29**, 63–80 (2015).

63. Mauger, O., Lemoine, F. & Scheiffele, P. Targeted intron retention and excision for rapid gene regulation in response to neuronal activity. *Neuron* **92**, 1266–1278 (2016).

64. Jia, J. et al. Post-transcriptional splicing of nascent RNA contributes to widespread intron retention in plants. *Nat. Plants* **6**, 780–788 (2020).

65. Kim, T. W. et al. Application of TurboID-mediated proximity labeling for mapping the ribosomal signaling network in Arabidopsis. *Preprint* at bioRxiv https://doi.org/10.1101/636324 (2019).

66. Li, W. et al. The EMBL-EBI bioinformatics web and programmatic tools framework. *Nucleic Acids Res.* **43**, W580–W584 (2015).

67. Edgar, R. C. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **15**, 383 (2014).

68. Ishida, T. & Kinoshita, K. PrDOS: prediction of disordered protein regions from amino acid sequence. *Nucleic Acids Res.* **35**, W460–W464 (2007).

69. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).

70. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).

71. Li, W., Lin, W. D., Ray, P., Lan, P. & Schmidt, W. Genome-wide detection of condition-sensitive alternative splicing in Arabidopsis roots. *Plant Physiol.* **162**, 1750–1763 (2013).

72. Kojima, H. et al. Sugar-inducible expression of the nucleolin-1 gene of Arabidopsis thaliana and its role in ribosome synthesis, growth and development. *Plant J.* **49**, 1053–1063 (2007).

73. Oh, E., Zhu, J. Y. & Wang, Z. Y. Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. *Nat. Cell Biol.* **14**, 802–U864 (2012).

74. Ni, W. et al. Multisite light-induced phosphorylation of the transcription factor PIF3 is necessary for both its rapid degradation and concomitant negative feedback modulation of photoreceptor phyB levels in Arabidopsis. *Plant Cell* **25**, 2679–2698 (2013).

75. Fridy, P. C. et al. A robust pipeline for rapid production of versatile nanobody repertoires. *Nat. Methods* **11**, 1253–1260 (2014).

76. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **26**, 1367–1372 (2008).

77. Tyanova, S. et al. The Perseus computational platform for comprehensive analysis of (pro)teomics data. *Nat. Methods* **13**, 731–740 (2016).

78. Peterson, A. C., Russell, J. D., Bailey, D. J., Westphall, M. S. & Coon, J. J. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol. Cell. Proteom.* **11**, 1475–1488 (2012).

79. Ni, W. et al. PPKs mediate direct signal transfer from phytochrome photoreceptors to transcription factor PIF3. *Nat. Commun.* **8**, 15236 (2017).

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**Author contributions**

Z.D., K.L., J.O., and A.L.B. identified AtAICINUS, Z.D., S.L.X., and S.P. analyzed the acinus mutant; Y.B., S.L.X., and D.S. characterized the acinus pnn double mutants; Z.Z. identified the spy-t1 mutant. Y.B. performed the RNA-seq and RT-PCR analyses. T.H. helped with RNA-seq data analysis; W.N. performed the proteomic analysis of AtAICINUS interactome under supervision by A.L.B., P.H.Q, and S.L.X. R.S. performed the targeted mass spectrometry quantification, S.H. performed the affinity purification of biotinylated protein and R.S. prepared the spectra. Z.-Y.W. and S.L.X. conceived the projects; Y.B., S.L.X., and Z.-Y.W. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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