Direct ETTIN-auxin interaction controls chromatin state in gynoecium development

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Impact statement: Auxin binds to the ETTIN transcription factor to disrupt the interaction between ETT and a TPL/TPR co-repressor and subsequently affecting chromatin dynamics to ensure proper gynoecium development.

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Abstract

Hormonal signalling in animals often involves direct transcription factor-hormone interactions that modulate gene expression[1,2]. In contrast, plant hormone signalling is most commonly based on de-repression via the degradation of transcriptional repressors[3]. Recently, we uncovered a non-canonical signalling mechanism for the plant hormone auxin in organ development with strong similarity to animal hormonal pathways. In this mechanism, auxin directly affects the activity of the auxin response factor ETTIN (ETT) towards regulation of target genes without the requirement for protein degradation[4,5]. Here we show that auxin binds ETT to modulate gene expression and that this ETT-auxin interaction leads to the dissociation of ETT from co-repressor proteins of the TOPLESS/TOPLESS-RELATED family followed by histone acetylation and the induction of target gene expression. Whilst canonical ARFs are classified as activators or repressors[6], ETT is able to switch chromatin locally between repressive and de-repressive states in an instantly-reversible auxin-dependent manner.

43 Developmental programmes within multicellular organisms originate from a single cell (*i.e.* a
44 fertilised oocyte) that proliferates into numerous cells ultimately differentiating to make up
45 specialised tissues and organs. Tight temporal and spatial regulation of the genes involved
46 in these processes is essential for proper development of the organism. Changes in gene
47 expression are often controlled by mobile signals that translate positional information into
48 cell-type specific transcriptional outputs[7]. In plants, this coordination can be facilitated by
49 phytohormones such as auxin, which controls processes throughout plant development[8]. In
50 canonical auxin signalling, auxin-responsive genes are repressed when auxin levels are low
51 by Aux/IAA transcriptional repressors that interact with DNA-bound Auxin Response Factors
52 (ARFs). As auxin levels increase, the auxin molecule binds to members of the TIR1/AFB
53 family of auxin co-receptors[9,10]. This facilitates interaction with Aux/IAA repressors, Aux/IAA
54 ubiquitinylation and subsequent degradation by the 26S proteasome, while relieving the
55 repression of ARF-targeted loci[11,12].

56 We recently identified an alternative auxin-signalling mechanism whereby auxin directly
57 affects the activity of a transcription factor (TF) complex towards its downstream targets[4,5].
58 This mechanism mediates precise polarity switches during organ initiation and patterning
59 and includes the ARF, ETTIN (ETT/ARF3) as a pivotal component. However, ETT is an
60 unusual ARF lacking the Aux/IAA-interacting Phox/Bem1 (PB1) domain[4,13] and it is therefore
61 likely that ETT would mediate auxin signalling via an alternative pathway.

62 ETT can interact with a diverse set of TFs and these interactions are sensitive to the
63 naturally occurring auxin, indole 3-acetic acid (IAA). The region responsible for IAA-
64 sensitivity is situated within the C-terminal part of ETT, known as the ETT-Specific (ES)
65 domain[6]. A protein fragment containing 207 amino acids of the ES domain, $ES^{388-594}$,
66 sufficient for mediating IAA-sensitivity in ETT-protein interactions, was produced
67 recombinantly and shown to be intrinsically disordered[14]. The sensitivity of ETT-TF
68 interactions to IAA suggests a direct effect of the IAA molecule on the ETT protein.
69 Therefore, to test whether ETT binds IAA, we carried out heteronuclear single quantum

70 coherence (HSQC) nuclear magnetic resonance (NMR) experiments using ^{15}N-labelled
71 ES$^{388-594}$ protein. The HSQC spectrum, recorded at 5˚C, shows a prominent signal-dense
72 region consistent with the ES domain being largely intrinsically disordered. Interestingly, the
73 spectrum also shows dispersed peaks flanking the signal-dense region indicating that there
74 is nevertheless some propensity to form secondary structure, particularly with a helical
75 character (Fig. 1a). In addition to this overview of ETT structure, the HSQC NMR probes
76 chemical shifts of protein amide-NH bonds in response to the presence of ligand[15]. We
77 found that a number of residues shifted their position in the spectrum in response to the
78 addition of IAA, whereas addition of the related Benzoic Acid (BA) had no effect (Fig. 1a-c).
79 These shifts show that certain residues are experiencing a changed chemical environment
80 as a consequence of IAA-binding and this may include the conformational change of a
81 structural motif within the ETT protein. The HSQC experiment therefore demonstrates that
82 ETT binds IAA directly. This experiment has not allowed us to assign signals to specific
83 amino acids and hence there is some uncertainty associated with tracking the chemical
84 shifts of some residues. However, a particularly large change is observed when IAA is
85 added to the ETT fragment for the tryptophan NH cross peak (~10ppm, rectangle I in Fig.
86 1a,c). Since there is only one tryptophan in the ETT fragment used here (W505), this shift
87 can be assigned to this residue.

88 We also used the recombinant ETT fragment in an Isothermal Titration Calorimetry (ITC)
89 assay, which characterises binding of ligands to proteins by determining thermodynamic
90 parameters of the interaction as heat exchange. This experiment revealed interaction
91 between ETT and IAA, while control experiments titrating IAA into buffer without protein and
92 titrating buffer without IAA into the ETT fragment showed no heat exchange (Fig. 1d-f).
93 Together, these two independent biochemical methods demonstrate that ETT binds IAA
94 directly thus revealing a key molecular aspect of the non-canonical auxin-signalling pathway.

95 Previously, *PINOID* (*PID*)[16] and *HECATE1* (*HEC1*)[17] were identified as ETT target genes[4,5]
96 and both genes are upregulated in gynoecium tissue from the *ett-3* mutant compared to wild

97 type (Figure 2-figure supplement 1). We also observed that expression of both genes is
98 induced by IAA, but did not observe any additional induction beyond the constitutive
99 upregulation in the *ett-3* mutant background (Figure 2-figure supplement 1). This ETT-
100 dependent regulation does not require a functional TIR1/AFB machinery, since IAA-induction
101 of *PID* and *HEC1* is still observed in TIR1/AFB mutant combinations, whereas the known
102 TIR1/AFB-mediated auxin induction of the *IAA19* gene is completely abolished in these
103 mutants (Fig. 2a-c).

104 To further assess the TIR1/AFB independence of the ETT-mediated auxin signalling
105 pathway, we exploited a recently-developed synthetic auxin-TIR1 pair[18]. In this system, the
106 auxin-binding pocket of TIR1 has been engineered (ccvTIR1) to accommodate an IAA
107 derivative bearing a bulky side chain (cvxIAA). By expressing the ccvTIR1 in a *tir1 afb2*
108 mutant background, the canonical pathway will only respond to the addition of cvxIAA and
109 not IAA[18]. We performed an expression analysis on *ccvTIR1* gynoecia treated ±cvxIAA and
110 ±IAA as well as control plants with the same treatments. In this experiment, *IAA19* served as
111 a control gene whose expression is known to be regulated in a TIR1/AFB-dependent
112 manner. Indeed, *IAA19* was strongly upregulated by cvxIAA in the ccvTIR1 line, but not by
113 IAA (Fig. 2d). In contrast, *PID* and *HEC1* expression was not significantly affected by
114 cvxIAA, whilst still responding to IAA in the ccvTIR1 background (Fig. 2d). These data
115 demonstrate that ETT-mediated auxin signalling can occur independently of the canonical
116 TIR1/AFB signalling pathway.

117 In a phylogenetic analysis of ETT protein sequences across the angiosperm phylum, we
118 identified a number of regions that are highly conserved (Figure 3-figure supplement 1).
119 Unsurprisingly, the DNA-binding domain characteristic to B3-type TFs such as ARF proteins
120 was conserved across all ETT proteins. Towards the C terminus of the ES domain we
121 identified an EAR-like motif with a particularly high level of conservation (Fig. 3a, Figure 3-
122 figure supplement 1). Ethylene-responsive element binding factor-associated Amphiphilic
123 Repression (EAR) motifs are also found in Aux/IAA proteins. Interactions between Aux/IAA

124 and members of the TOPLESS and TOPLESS-RELATED (TPL/TPR) family of co-
125 repressors occur via this motif[19]. TPL/TPRs mediate their repressive effect by attracting
126 histone deacetylases (HDACs) to promote chromatin condensation[20]. Since ETT functions
127 independently of the canonical auxin pathway, it is possible that its role in chromatin
128 remodelling occurs via direct interaction with TPL/TPRs through the EAR-like motif. To test
129 this, we carried out Yeast 2-Hybrid (Y2H) assays in which ETT was found to interact with
130 TPL, TPR2 and TPR4 (Fig. 3b, Figure 3-figure supplement 1). Moreover, mutating residues
131 in the EAR-like motif abolished the interactions demonstrating its requirement for the ETT-
132 TPL/TPR interaction (Fig. 3b).

133 Given that several ETT-protein interactions are affected by IAA and that part of the ETT
134 transcriptome changes in response to IAA[4,5], we tested the IAA sensitivity of ETT-TPL/TPR
135 interactions. In both Y2H and in co-immunoprecipitation (Co-IP) experiments, we observed
136 that the interactions were reduced with increasing IAA concentrations (Fig. 3b,c and Figure
137 3-figure supplement 2). Moreover, as described previously for other ETT-protein
138 interactions, the sensitivity was specific to IAA as other auxinic compounds tested did not
139 show this effect (Figure 3-figure supplement 2). Henceforth, 'auxin' will refer to IAA unless
140 stated otherwise. These data suggest that in conditions with low auxin levels, ETT can
141 interact with TPL/TPR proteins to repress the expression of target genes. An increase in
142 cellular auxin causes ETT to bind auxin thereby undergoing a conformational change that
143 abolishes interaction with TPL/TPR co-repressors.

144 TPL was originally identified as a key factor involved in setting up the apical-basal growth
145 axis during embryo development[21,22]. Large-scale interaction studies suggest that the five
146 Arabidopsis TPL/TPRs have roles throughout plant development[20,23]. Whilst ETT has been
147 implicated in a wide array of developmental processes[24-27], the most dramatic phenotypes of
148 *ett* loss-of-function mutants are observed during gynoecium development[13,28,29]. In
149 accordance with this, *ETT* is highly expressed in the gynoecium (Fig. 4a)[6]. We produced
150 reporter lines of *TPL*, *TPR2* and *TPR4* promoters fused to the *GUS* gene to test if they

151 overlap with *ETT* expression in the gynoecium. Both *pTPL:GUS* and *pTPR2:GUS* exhibited
152 strong expression in the apical part of the gynoecium where *ETT* is also expressed, while no
153 *pTPR4:GUS* expression was observed (Fig. 4a-d). Single loss-of-function mutants in *TPL*
154 and *TPR2* do not show any abnormal phenotypes during gynoecium development. However,
155 the *tpl tpr2* double mutant has defects in the development of the apical gynoecium similar to
156 *ett* mutants (Fig. 4e-g) demonstrating that *TPL* and *TPR2* function redundantly in gynoecium
157 development. Together with the protein interaction data and the overlapping expression
158 patterns, these results suggest that ETT and TPL/TPR2 cooperate to regulate gynoecium
159 development.

160 TPL was shown previously to recruit histone deacetylase, HDA19, during early Arabidopsis
161 flower development to keep chromatin in a repressed state[20]. Moreover, HDA19 was also
162 recently shown to participate in repression of the meristem identity gene, *SHOOT*
163 *MERISTEMLESS* (*STM*)[30]. Here, our analysis of gynoecia from the *hda19-4* mutant
164 demonstrate that HDA19 is also required for gynoecium development as the *hda19-4* mutant
165 has strong style defects (Fig. 4h). In agreement with this, the *HDA19* gene was highly
166 expressed in gynoecium tissue, whereas another member of the *HDA* gene family, *HDA6*,
167 was not (Figure 4-figure supplement 1). Moreover, HDA19 recruitment likely involves *ETT*,
168 since expression of the ETT target genes, *PID* and *HEC1*, are increased in the *tpl tpr2* and
169 *hda19-4* mutants compared to wild type. Similar to the *ett* mutant, auxin treatments failed to
170 further induce expression in these mutants (Fig. 4i,j). These observations suggest that ETT,
171 TPL/TPR2 and HDA19 function in conjunction to control gene expression during gynoecium
172 development.

173 To test the direct interaction of ETT, TPL and HDA19 on chromatin, we performed
174 Chromatin-Immunoprecipitation (ChIP) using reporter lines expressing GFP fusion protein.
175 Although only ETT is expected to bind DNA, ChIP followed by qPCR revealed that all three
176 proteins associate with DNA elements in the same regions of the promoters of *PID* and
177 *HEC1* (Fig. 5a). This supports a model in which ETT recruits TPL/TPR2 and HDA19 to ETT

178 target loci to keep chromatin in a condensed state through histone deacetylation. When
179 auxin levels increase, the ETT-TPL/TPR2 interaction is broken, presumably preventing
180 HDA19 from deacetylating histones. To test this, we assayed for H3K27 acetylation, which is
181 a substrate for HDA19. H3K27 acetylation increased in the absence of ETT and upon
182 treatment with auxin. This occurred in the same regions of the *PID* and *HEC1* promoters
183 where the proteins were found to associate (Fig. 5b,c). In agreement with ETT mediating the
184 association of TPL/TPR and HDA19 with these regions, there was no further increase of
185 acetylation in the *ett-3* mutant upon treatment with auxin (Fig. 5b,c).

186 The data presented in this paper provide molecular insight as to how auxin levels are
187 translated into changes in gene expression of ETT target genes. Our data lead to a model in
188 which low levels of auxin maintain ETT associations with TPL/TPR2 to repress gene
189 expression via H3K27 deacetylation. As auxin levels increase, TPL/TPR2 (and hence
190 HDA19) disassociate from ETT, promoting H3K27 acetylation (Fig. 5d). This model
191 molecularly underpins the published association between auxin dynamics and *PID*
192 expression at the gynoecium apex where *PID* is repressed at early stages of development to
193 allow symmetry transition, but subsequently de-repressed as auxin levels rise to facilitate
194 polar auxin transport[4,31].

195 The direct binding of auxin allows ETT to switch the chromatin locally between repressive
196 and de-repressive states, whilst other ARFs have been categorised as either repressors or
197 activators[8]. The effect of auxin is therefore instantly reversible, making it possible to switch
198 between states immediately in response to changes in auxin levels. This feature, which is
199 reminiscent of animal hormonal signalling pathways such as the Thyroid Hormone and
200 Wnt/ß-catenin pathways[1,2], may be particularly important in controlling changes in tissue
201 polarity during plant organogenesis as observed in the Arabidopsis gynoecium[31].

202 The identification of a direct auxin-ETT interaction to control gene expression adds an
203 additional layer of complexity to auxin biology, which contributes towards explaining how
204 auxin imparts its effect on highly diverse processes throughout plant development. In a wider

205 context, this work also opens for the exciting possibility that direct transcription factor-ligand
206 interactions is a general feature in the control of gene expression in plants as found in
207 animals.
208
209 **MATERIAL AND METHODS**
210
211 **Plant materials and treatments**
212 Plants were grown in soil at 22 °C in long day conditions (16hrs day/8 hrs dark). All
213 mutations were in the *Col-0* background. Mutant alleles described before include e*tt-3*[4,13],
214 *hda19-4* (SALK_139443)[32], *pETT:ETT-GFP* in *ett-3*[4], *pTPL:TPL:GFP*[33], *p35S:HDA19:GFP*[33],
215 *pTIR1:ccvTIR1* in *tir1-1 afb2-3*[19] and *tir1-1 afb2-3 afb3-4*[34].The *tpl* mutant (SALK_034518C)
216 was obtained from the European Arabidopsis Stock Centre.
217 For both expression and ChIP analysis, auxin treatments were applied by spraying bolting
218 *Col-0* and *ett-3* inflorescences with a solution containing 100 µM IAA (Sigma) or cvxIAA and
219 0.015% Silwet L-77 (De Sangosse Ltd.). Treated samples were returned to the growth room
220 and incubated for two hours.
221
222 **Expression analysis**
223 Quantitative Real time PCR (qRT-PCR) was used for expression analysis. RNA was
224 extracted from floral buds using the RNeasy mini kit (Qiagen). Using the SuperScript™ IV
225 First-Strand Synthesis kit (ThermoFisher), cDNA was synthesised from 1 µg of total RNA.
226 Subsequently, qRT-PCR was carried out using SYBR Green JumpStart Taq ReadyMix
227 (Sigma) using the appropriate primers (Figure 2-source data 1). Relative expression values
228 were determined using the $2^{-\Delta\Delta Ct}$ method[35]. Data were normalised to *POLYUBIQUITIN 10*
229 (*UBQ10/*AT4G05320) expression.
230
231 **ETT protein analysis by alignment**

232 Published *ETT* sequences of 22 Angiosperm species were retrieved from Phytozome
233 version 12[36]. Nucleotide sequences were translated and aligned using MUSCLE in
234 Geneious version 6.1.8[37]. The EAR domain was extracted as a sequence logo (Fig. 3a;
235 Figure 3-figure supplement 1).
236
237 **Generation of the *tpl tpr2* CRISPR mutant**
238 The *tpl tpr2*^{ge} mutant was generated using CRISPR/Cas9 technology by a method previously
239 described[38]. Briefly, for the construction of the RNA-guided genome-editing plasmid, DNA
240 sequences encoding the gRNA adjacent to the PAM sequences were designed to target two
241 specific sites in *TPR2* (AT3G16830). DNA-oligonucleotides (Figure 2-source data 1)
242 containing the specific gRNA sequence were synthesised and used to amplify the full gRNA
243 from a template plasmid (AddGene #46966). Using Golden Gate cloning[39] each gRNA was
244 then recombined in a L1 vector downstream of U6 promoter[38]. Finally, the resulting gRNA
245 plasmids were then recombined with a L1 construct containing *pYAO:Cas9_3:E9t*[38] (kindly
246 provided by Jonathan Jones) and a L1 construct containing Fast-Red selection marker
247 (AddGene #117499) into a L2 binary vector (AddGene #112207).
248 The construct was transformed into *Agrobacterium tumefaciens* strain GV3101 by
249 electroporation, followed by plant transformation by floral dip into the *tpl* single mutant[40].
250 Transgenic T0 seeds appear red under UV light and were selected under a Leica M205FA
251 stereo microscope. T0 plants were genotyped using PCR and the *TPR2* locus sequenced
252 (Oligonucleotides in Figure 2-source data 1). Genome edited plants were selected and the
253 next generation grown (T1). Seeds of this generation were segregating in a 3:1 ratio for the
254 transgene. Transgene negative plants were selected and grown on soil. To find homozygous
255 mutations T1 plants were again genotyped. The T2 generation was again checked for the
256 absence of the transgene.
257
258 **Protein interaction**

259 For Yeast-two-Hybrid (Y2H) assays coding sequences were cloned into pDONR207 and
260 recombined into the pGDAT7 and pGBKT7 (Clontech). Using the co-transformation
261 techniques[41] these constructs were transformed into the AH109 strain (Clontech).
262 Transformations were selected on Yeast Selection Medium (YSD) lacking Tryptophan (W)
263 and Leucine (L) at 28°C for 3-4 days. Transformed yeast cells were serially diluted (10^0, 10^1,
264 10^2 and 10^4) and dotted on YSD medium lacking Tryptophan (W), Leucin (L), adenine (A)
265 and Histidine (H) to test for interaction. To examine interaction strength 3-amino-1,2,4-
266 triazole (3-AT) was supplemented to the YSD (-W-L-A-H) medium with different
267 concentrations (0, 5, 10 mM). To determine the effect of auxinic compounds on the protein-
268 protein interactions benzoic acid (BA), IAA, NAA and 2,4D (all Sigma) were dissolved in
269 ethanol and added directly to the medium at the desired concentrations. Pictures were taken
270 after 3 days of growth at 28°C.
271 For the β-Galactosidase assay transgenic yeast was grown in liquid YSD (-W-L) medium
272 supplemented with/-out 100 µM IAA or NAA, to an OD_{600} of 0.5. The cells were then
273 harvested and lysed using 150 µL Buffer Z with β-mercaptoethanol (100 mM Phosphate
274 buffer pH 7, 10 mM KCl, 1mM Mg2SO4, β-mercaptoethanol 50 mM), 50 µL chloroform and
275 20 µL of 0.1% SDS. After lysis, the sample was incubated with 700 µL pre-warmed ONPG
276 solution (1mg/mL ONPG (o-Nitrophenyl-β-D-Galactopyranoside, Sigma) prepared in Buffer Z
277 without β-mercaptoethanol at 28°C until a yellow colour developed in the samples without
278 auxin treatment. After stopping all reactions (using 500 µL Na_2CO_3) the supernatant was
279 collected and OD_{405} determined. The β-Galactosidase activity was calculated as follows:
280 (A405*1000)/(A600*min*mL)
281 For co-immunoprecipitation, ETT-FLAG was generated using Golden Gate cloning[39] by
282 recombining a previously described L0 clone for ETT with a 35S promoter (AddGene
283 #50266), a C-terminal 3xFLAG epitope (AddGene #50308) and a Nos-terminator (AddGene
284 #50266) into a L1 vector (AddGene #48000). The pGWB14 TPL-HA construct was provided
285 by Salomé Prat and has been used in previous studies[41]. The epitope-tagged proteins were
286 transiently expressed in four-week-old *N. benthamiana* leaves for two days. Co-

287 immunoprecipitation was performed as described previously[42]. After harvest, 1 g of fresh leaf
288 tissue was ground in liquid nitrogen. The powder was homogenised for 30 min in two
289 volumes of extraction buffer (10% glycerol, 25 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM
290 NaCl, 0.15% NP-40, 1mM PMSF, 10 mM DTT, 2% Polyvinylporrolidone, 1x cOmplete Mini
291 tablets EDTA-free Protease Inhibitor Cocktail (Roche). The homogenised samples were
292 cleared by centrifugation at 14,000 xg for 10 min and cleared lysates were incubated for 2h
293 with 20 µl anti-FLAG M2 magnetic beads (SIGMA-ALDRICH, M8823; lot: SLB2419). The
294 beads were washed five times with IP buffer (10% glycerol, 25 mM Tris-HCl pH 7.5, 1 mM
295 EDTA, 150 mM NaCl, 0.15% NP-40, 1 mM PMSF, 1 mM DTT, 1x cOmplete Mini tablets
296 EDTA-free Protease Inhibitor Cocktail (Roche)) and proteins were eluted by adding 80 µl 2x
297 SDS loading buffer followed by an incubation at 95 °C for 10 min. To examine auxin
298 sensitivity 4 g of fresh leaf tissue was collected, ground in liquid nitrogen and protein was
299 extracted. The lysate was then divided according to the number of treatments. The desired
300 concentration of IAA or NAA was added to each of the cleared lysates before the anti-FLAG
301 M2 magnetic beads were added. IAA or NAA at the desired concentration was also
302 supplemented to the IP buffer during the washes. The eluates were analysed by western
303 blot using an anti-FLAG antibody (M2, Abcam, ab49763, Lot: GR3207401-3) or an anti-HA
304 antibody (Abcam, ab173826, Lot: GR3255539-1). Both antibodies were used as 1:10000
305 dilutions. The antibodies were validated by the manufacturer.
306
307 **Scanning electron microscopy**
308 Whole inflorescences of *Col-0*, *ett-3*, *tpl tpr2*^{ge} and *hda19-4* were fixed overnight in FAA
309 (3.7% formaldehyde, 5% glacial acetic acid, 50% ethanol) and dehydrated through an
310 ethanol series (70% to 100%) as described previously[31]. The samples were then critical
311 point-dried, gynoecia dissected and mounted. After gold coating samples were examined
312 with a Zeiss Supra 55VP Field Emission Scanning electron microscope using an
313 acceleration voltage of 3 kV.
314

315 ***TPL*, *TPR2* and *TPR4* reporter lines**

316 For the construction of the promoter:GUS reporter plasmids of *TPL*, *TPR2* and *TPR4*, 2.5 kb
317 of promoter sequences were isolated from genomic DNA and inserted upstream of the ß-
318 glucoronidase gene of pCambia1301 vectors using the In-Fusion Cloning Recombinase kit
319 (Clontech). The constructs were transformed into *Agrobacterium tumefaciens* strain GV3101
320 by electroporation, followed by plant transformation by floral dip into *Col-0*[40].
321 The GUS histochemical assay was performed in at least three individual lines per construct.
322 Inflorescences of each GUS line were pre-treated with ice cold acetone for 1h at -20°C and
323 washed two times for 5 minutes with 100 mM sodium phosphate buffer followed by one
324 wash with sodium phosphate buffer containing 1 mM $K_3Fe(CN)_6$ and 1 mM $K_4Fe(CN)_6$ (both
325 Sigma) at room temperature. Subsequently, samples were vacuum infiltrated for 5 minutes
326 with X-Gluc solution (100 mM sodium phosphate buffer, 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 3
327 mM $K_4Fe(CN)_6$, 0.1% Triton X100) containing 1 mg/ml of ß-glucoronidase substrate X-gluc
328 (5-bromo-4-chloro-3-indolylglucuronide, Melford) and incubated at 37°C. *pTPL:GUS* were
329 incubated for 20 minutes and *pTPR2:GUS* lines for 45 minutes to prevent overstaining.
330 *pTPR4:GUS* lines were incubated for 16h. After staining, the samples were washed in 70%
331 ethanol until chlorophyll was completely removed. Gynoecia were dissected and mounted in
332 chloral hydrate (Sigma). Samples were analysed using a Leica DM6000 light microscope.
333

334 **Chromatin Immunoprecipitation**

335 Transcription factor ChIP was performed in triplicate using the *pETT:ETT:GFP*,
336 *pTPL:TPL:GFP* and *p35S:HDA19-GFP* lines and data analysed as described previously[43].
337 Additionally, a *WUS* promoter fragment was used as a negative control for ETT binding[44]. IP
338 was conducted using the anti-GFP antibody (Roche, 11814460001, Lot: 19958500) and
339 Pierce Protein G magnetic beads (ThermoFisher, 88847, Lot: SI253639) were used for IP.
340 Histone acetylation ChIP was carried out and data were analysed as described previously[45].
341 The experiment was carried out in triplicate using 3 g auxin-treated or untreated *Col-0* or *ett-*
342 *3* inflorescent tissue. The antibodies used for IP were anti-H3K27ac antibodies (Abcam,

343 ab4729, Lot: GR3231937-1) and anti-H3 (Abcam, ab1791, Lot: GR310541-1). All antibodies
344 were validated by the manufacturers.
345 In all ChIP experiments, DNA enrichment was quantified using quantitative PCR (qPCR)
346 with the appropriate primers (Supplementary Data). In case of H3K27ac, *ACTIN* was used
347 as an internal control and the data represented as ratio of (H3K27ac at *HEC1* or *PID* divided
348 by H3 at *HEC1or PID*) to H3K27ac at *ACT* divided by H3 at *ACT*).
349
350 **Statistical analyses and replication**
351 In all graphs error bars represent the standard deviation of the mean for all numerical
352 values. QRT-PCR and ChIP experiments have been carried out at least in triplicate. The
353 data presented here show an average of three replicates. For qRT-PCR data were analysed
354 using one-way ANOVA with post-hoc Tukey multiple comparison test. $ChIP_{qPCR}$ data were
355 analysed using two-way ANOVA with post hoc Bonferroni multiple comparison test. All
356 output of statistical tests can be found in the source data files. All statistical tests were
357 carried out using GraphPad Prism Version 5.04 (La Jolla California USA,
358 www.graphpad.com).
359
360 **Protein production**
361 The ES domain, $ES^{388-594}$, protein was isotopically labelled in preparation for NMR analysis.
362 The ES domain was expressed for as a fusion protein with a 6x Histidine tag in minimal
363 media with ^{15}N ammonium chloride. The ^{15}N isotope labelling of the expressed protein
364 involved a 125-fold dilution of cell culture in enriched growth media into minimal media with
365 ^{15}N ammonium chloride and grown for 16 hours (37 ˚C / 200 rpm); followed by a further 40-
366 fold dilution into minimal media for the final period of cell growth and protein expression
367 (induced with L-arabinose 0.2 % w/v / 18 ˚C / 200 rpm and grown for a further 12 hours).
368 The fusion protein was isolated from soluble cell lysate by Co-NTA affinity chromatography
369 with two His-Trap 1 mL TALON Crude columns (GE Healthcare Life Sciences, 28953766).

370 Chromatography buffers contained sodium phosphate 20 mM pH 8.0, NaCl 500 mM and
371 either no-imidazole or 500 mM imidazole for wash and elution buffers respectively. The
372 majority of the non-specifically bound protein was removed by passing 20 mL of the wash
373 buffer through the columns. The protein eluted on a gradient of increasing imidazole
374 concentration of up to 30% elution buffer over 20 mL.
375
376 **HSQC NMR**
377 The ES domain, $ES^{388-594}$, protein was analysed by NMR at 5°C under reducing conditions
378 (DTT 10 mM), buffered at pH 8.0 (Tris 20 mM). ^{1}H-^{15}N HSQC was performed at 950 MHz,
379 TCI probe, Bruker following the parameters described in Figure 1-figure supplement 1.
380
381 **Isothermal titration calorimetry (ITC)**
382 ITC was carried out on a MicroCal PEAQ-ITC (Malvern) at 25 °C in a Buffer A (sodium
383 phosphate 20 mM, pH 8.0; NaCl 500 mM). Ligand (2 mM IAA) was injected (19 × 4.0 µl) at
384 150-s intervals into the stirred (500 rpm) calorimeter cell (volume 270 µl) containing 50 µM
385 $ES^{388-594}$ protein. Titration of Buffer A into 50 µM $ES^{388-594}$ protein and IAA (2 mM) into Buffer
386 A served as negative controls. Measurements of the binding affinity of all the titration data
387 were analysed using the MicroCal Software (Malvern).
388
389 **Accessions**
390 ETT, AT2G33860; TPL, AT1G15750; TPR1, AT1G80490, TPR2, AT3G16830; TPR3,
391 AT5G27030; TPR4, AT3G15880; HDA6, AT5G63110; HDA19, AT4G38130; HEC1,
392 AT5G67060; PID, AT2G34650; WUS, AT2G17950.
393

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Author Contributions

A.K. and L.Ø. conceived the experiments. A.K., S.R.H. and H.M.M. performed the experiments. A.K., S.R.H., H.M.M., S.K. and L.Ø. analysed the data. A.K. and L.Ø. wrote the manuscript and S.R.H., H.M.M. and S.K. commented on it. All authors read and approved the manuscript.

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Competing Interests

No competing interests declared.

Ethics

Human subjects: No; Animal subjects: No

Dual-use research: No

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No

431 **FIGURE LEGENDS**

432

433 **Figure 1. ETT directly binds auxin (IAA).**

434 **a,** HSQC-NMR performed with $ES^{388-594}$ protein either alone (black), with indole-3-acetic acid
435 (IAA, orange) or benzoic acid (BA, blue). **b,** zoom-in of the indicated rectangular region in a.
436 **c,** zoom-in of the specific shifts (labelled I-V) in the indicated dotted rectangles in a and b.
437 Changes in chemical shifts are indicated by arrows from control to IAA treatment. **d-f,** ITC
438 spectre showing heat exchange between $ES^{388-594}$ protein and IAA (**d**), but not in controls
439 (**e,f**). See Figure 1-figure supplement 1 for parameters used in the HSQC-NMR experiment.

440

441 **Figure 2. ETT regulates target gene expression independently of TIR1/AFB auxin**
442 **receptors.**

443 Expression of the canonical auxin responsive *IAA19* gene (**a**) and the ETT-target genes
444 *HEC1* (**b**) and *PID* (**c**) in control-treated or 100 μM IAA-treated gynoecia assayed using qRT-
445 PCR. **a,** *IAA19* expression is up-regulated in response to auxin in wild-type gynoecia (*Col-0*)
446 but not in *tir1/afb* double and triple mutants. The ETT-target genes *HEC1* and *PID* are up-
447 regulated in response to auxin in both wild-type and auxin receptor mutants (**b, c**). This
448 suggests a TIR1/AFB independent regulation of these genes. **d,** Expression of *IAA19*, *HEC1*
449 and *PID* in response to treatment with 100 μM IAA and 100 μM cvxIAA in wild-type (*Col-0*)
450 and *pTIR1:ccvTIR1* gynoecia in the *tir1 afb2* double mutant (*ccvTIR1*). The data confirm
451 TIR1/AFB independent regulation of *HEC1* and *PID* in the gynoecium. ***$p < 0.0001$; Shown
452 are mean ± standard deviation of three biological replicates. See Figure 2-source data 1 for
453 statistical analyses.

454

455 **Figure 3. ETT interacts with members of the TPL/TPR co-repressor family in an auxin-**
456 **sensitive manner.**

457 **a,** Schematic representation of ETT protein highlighting an EAR-like motif in the C-terminal
458 ETT-specific domain. **b,** Y2H showing that ETT interacts with TPL, TPR2 and TPR4. These

459 interactions depend on the identified C-terminal RLFGF motif and are auxin-sensitive. DBD,
460 DNA-binding domain. **c,** Co-IP revealing that ETT interacts with TPL in an auxin-sensitive
461 manner with increasing IAA concentrations weakening the interaction.
462
463 **Figure 4. ETT, TPL/TPR2 and HDA19 co-operatively regulate gene expression to**
464 **facilitate gynoecium development.**
465 **a-d,** Promoter GUS expression analysis of *pETT:GUS* (**a**), *pTPL:GUS* (**b**), *pTPR2:GUS* (**c**)
466 and *pTPR4:GUS* (**d**) revealed that *ETT*, *TPL* and *TPR2* but not *TPR4* are co-expressed in
467 the Arabidopsis style. Scale bar = 300 µm. **e-h,** Gynoecium phenotypes of wild-type (**e**), *ett-*
468 *3* (**f**) *tpl tpr2*^{ge} (**g**) and *hda19-4* (**h**). Scale bar = 100 µm. **i, j,** *HEC1* (**i**) and *PID* (**j**) are
469 constitutively mis-regulated in *ett-3*, *tpl tpr2*^{ge} and *hda19-4* gynoecia. This misregulation is
470 unaffected by treatment with 100 µM IAA. ***p-Values<0.0001; Shown are mean ± standard
471 deviation of three biological replicates. Differences between untreated and IAA-treated
472 mutants are not significant. See Figure 4-source data 1 for statistical analyses.
473
474 **Figure 5. ETT, TPL and HDA19 co-operatively regulate *HEC1* and *PID* by modulation of**
475 **chromatin acetylation.**
476 **A,** Chromatin immunoprecipitation (ChIP) shows ETT, TPL and HDA19 binding to conserved
477 regions of *HEC1* and *PID* loci. *WUS* served as negative control. **b, c,** H3K27ac accumulation
478 (from ChIP analysis) along the *HEC1* (**b**) and *PID* (**c**) loci in wild-type (*Col-0*) and *ett-3* plants
479 ± treatment with 100 µM IAA. Numbers on the x axes are distances to the Transcription Start
480 Site (TSS). The schematic of the loci is shown below each panel. Dashed boxes represent
481 ETT binding regions. **d,** Schematic model illustrating alternative TIR1/AFB independent
482 auxin signaling. Under low auxin conditions an ETT-TPL-HDA19 complex binds to ETT-
483 target genes keeping their chromatin environments repressed, through de-acetylation. High
484 nuclear auxin concentrations abolish the ETT-TPL-HDA19 complex through direct ETT-
485 auxin interaction. This leads to an accumulation of histone acetylation and up-regulation of
486 ETT-target genes.

487 Values in **a**, **b** and **c** are means ± standard deviation of three biological replicates. See
488 Figure 5-source data 1 for statistical analyses.
489
490 **Figure 1-figure supplement 1. Parameters for HSQC NMR experiment.**
491
492 **Figure 2-figure supplement 1. Expression of *HEC1* and *PID* in Col-0 and *ett-3*.**
493 In wild-type gynoecia *HEC1* and *PID* are up-regulated upon auxin treatment while both
494 genes are constitutively up-regulated in *ett-3*. Treatment with 100 µM IAA does not affect
495 *HEC1* and *PID* expression in the *ett-3* mutant suggesting that ETT acts as a transcriptional
496 repressor. Asterisks indicate significant change upon auxin treatment compared to untreated
497 *Col-0* (*** indicating $p < 0.0001$). Shown are mean ± standard deviation of three biological
498 replicates. See Figure 2-source data 1 for statistical analyses.
499
500 **Figure 3-figure supplement 1. ETT can interact with several members of the TPL/TPR**
501 **co-repressor family through a conserved EAR-like motif.**
502 **a,** Alignment of ETT protein sequences of 22 species identified a conserved repressive motif
503 (RLFGF) at its c-terminal domain. **b,** ETT interacts with several members of the TPL/TPR
504 co-repressor family in Y2H. Additionally, controls for Fig. 2 are shown.
505
506 **Figure 3-figure supplement 2. Interaction between ETT and TPL, TPR2 and TPR4 is**
507 **auxin-sensitive and specific to IAA.**
508 **a,** In Y2H increasing concentrations of IAA lead to reduction of yeast growth abolishing the
509 interaction between ETT and its partners. The interactions are, therefore, auxin-sensitive. **b,**
510 Y2H to test specificity of auxin-sensitivity using benzoic acid (BA), NAA, and 2,4D in a yeast
511 growth assay. The data suggest that the auxin-sensitivity observed in (**a**) is IAA-specific. **c,**
512 Y2H based ONPG assay measuring the β-galactosidase activity as a measure of interaction
513 strength. **d,** Co-IP experiments show that the interaction between ETT and TPL cannot be
514 disrupted by NAA. The data support that the ETT TPL/TPR interactions are sensitive to IAA

515 but not to NAA. ***p <0.0001; Shown are mean ± standard deviation of three biological
516 replicates. See Figure 3-source data 1 for statistical analyses.
517
518 **Figure 3-figure supplement 3. Original western blot images.**
519 The red boxes indicate the areas used in Figure 3b and Figure 3-figure supplement 2d.
520
521 **Figure 4-figure supplement 1. Expression of *TPL*, *TPRs* and *HDAs* genes in the**
522 **gynoecium.**
523 Expression analysis using qRT-PCR in wild-type gynoecia showed that *TPL* and *TPR2* are
524 more strongly expressed than *TPR1*,*3* and *4*. Likewise, *HDA19* exhibits higher expression
525 compared to *HDA6*. ***p-Values<0.0001; Shown are mean ± standard deviation of three
526 biological replicates. See Figure 4-source data 1 for statistical analyses.
527
528
529 **Source data**
530 **Figure 1-source data 1. Parameters for HSQC NMR.**
531 **Figure 2-source data 1. Output of statistical tests**
532 **Figure 3-source data 1. Output of statistical tests**
533 **Figure 4-source data 1. Output of statistical tests**
534 **Figure 5-source data 1. Output of statistical tests**
535
536 **Supplementary Data. Oligonucleotides used in this study.**
537
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541

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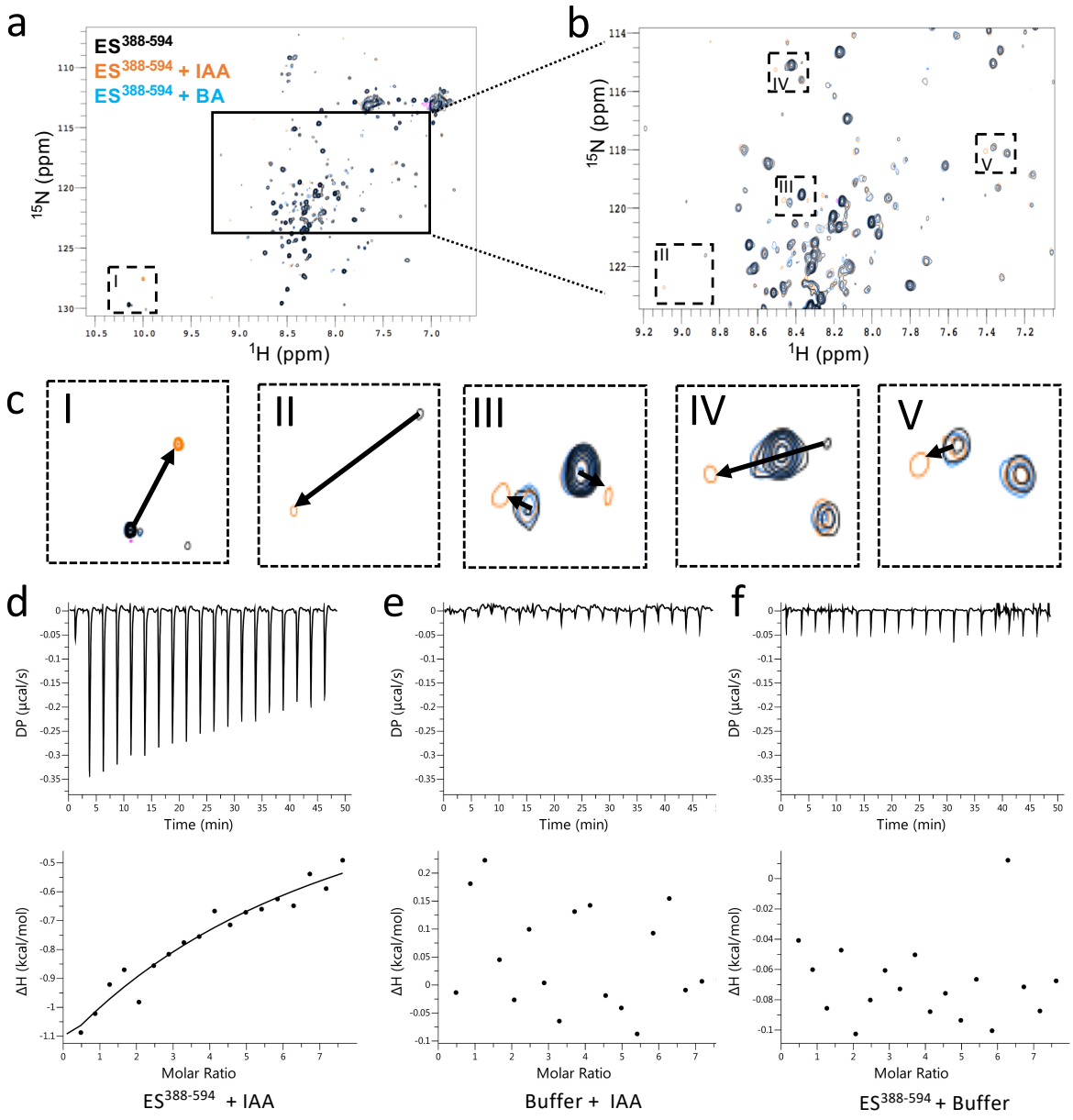
Figure 1. ETT directly binds auxin (IAA).



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a, HSQC-NMR performed with ES$^{388-594}$ protein either alone (black), with indole-3-acetic acid (IAA, orange) or benzoic acid (BA, blue). **b,** zoom-in of the indicated rectangular region in a. **c,** zoom-in of the specific shifts (labelled I-V) in the indicated dotted rectangles in a and b. Changes in chemical shifts are indicated by arrows from control to IAA treatment. **d-f,** ITC spectre showing heat exchange between ES$^{388-594}$ protein and IAA (**d**), but not in controls (**e,f**). See Figure 1-figure supplement 1 for parameters used in the HSQC-NMR experiment.

Figure 2. ETT regulates target gene expression independently of TIR1/AFB auxin receptors.

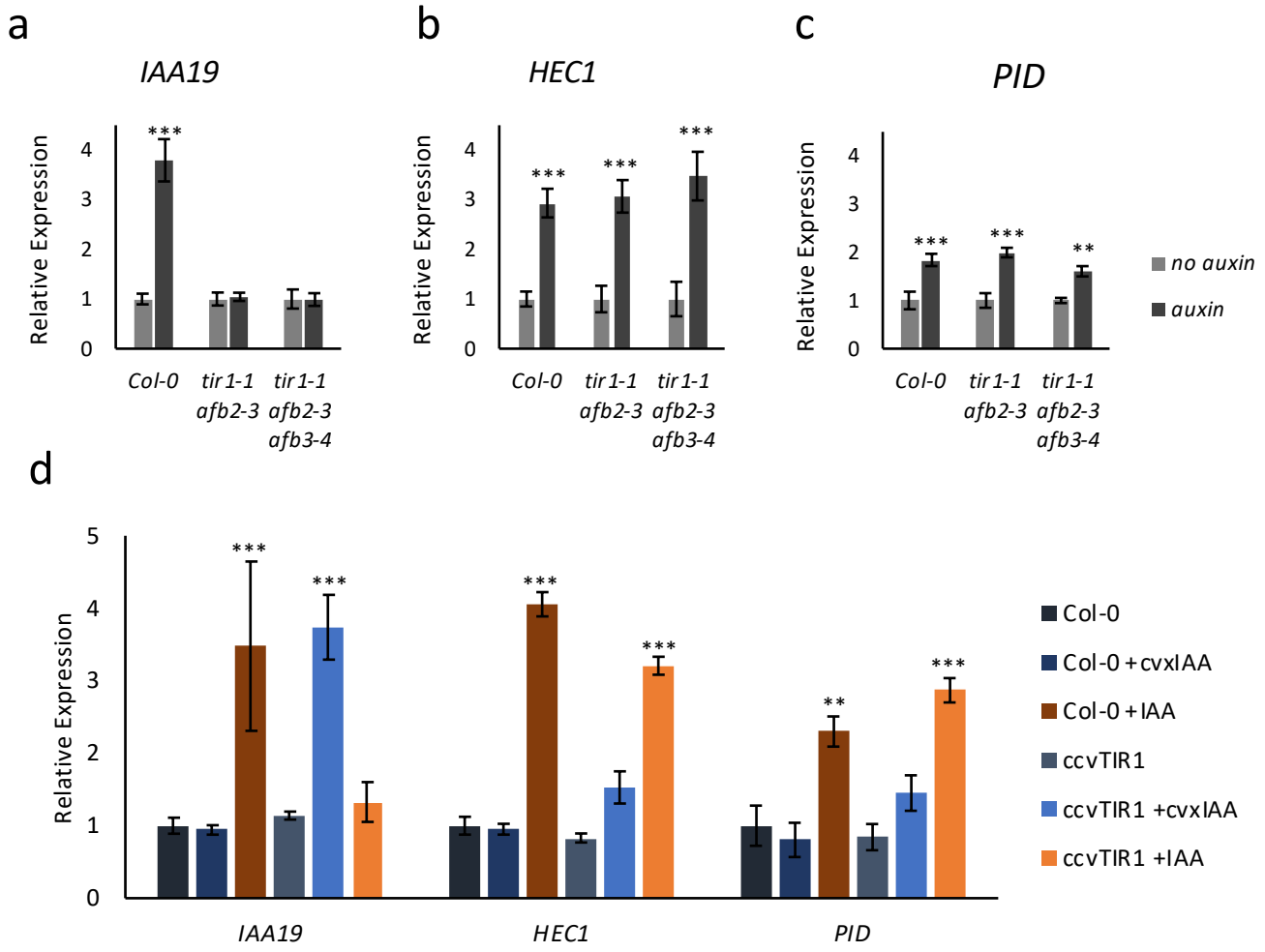

Figure 2. ETT regulates target gene expression independently of TIR1/AFB auxin receptors. Expression of the canonical auxin responsive *IAA19* gene (**a**) and the ETT-target genes *HEC1* (**b**) and *PID* (**c**) in control-treated or 100 µM IAA-treated gynoecia assayed using qRT-PCR. **a,** *IAA19* expression is up-regulated in response to auxin in wild-type gynoecia (*Col-0*) but not in *tir1/afb* double and triple mutants. The ETT-target genes *HEC1* and *PID* are up-regulated in response to auxin in both wild-type and auxin receptor mutants (**b, c**). This suggests a TIR1/AFB independent regulation of these genes. **d,** Expression of *IAA19, HEC1* and *PID* in response to treatment with 100 µM IAA and 100 µM cvxIAA in wild-type (*Col-0*) and *pTIR1:ccvTIR1* gynoecia in the *tir1 afb2* double mutant (*ccvTIR1*). The data confirm TIR1/AFB independent regulation of *HEC1* and *PID* in the gynoecium. ***p <0.0001; Shown are mean ± standard deviation of three biological replicates. See Figure 2-source data 1 for statistical analyses.

Figure 3. ETT interacts with members of the TPL/TPR co-repressor family in an auxin-sensitive manner.

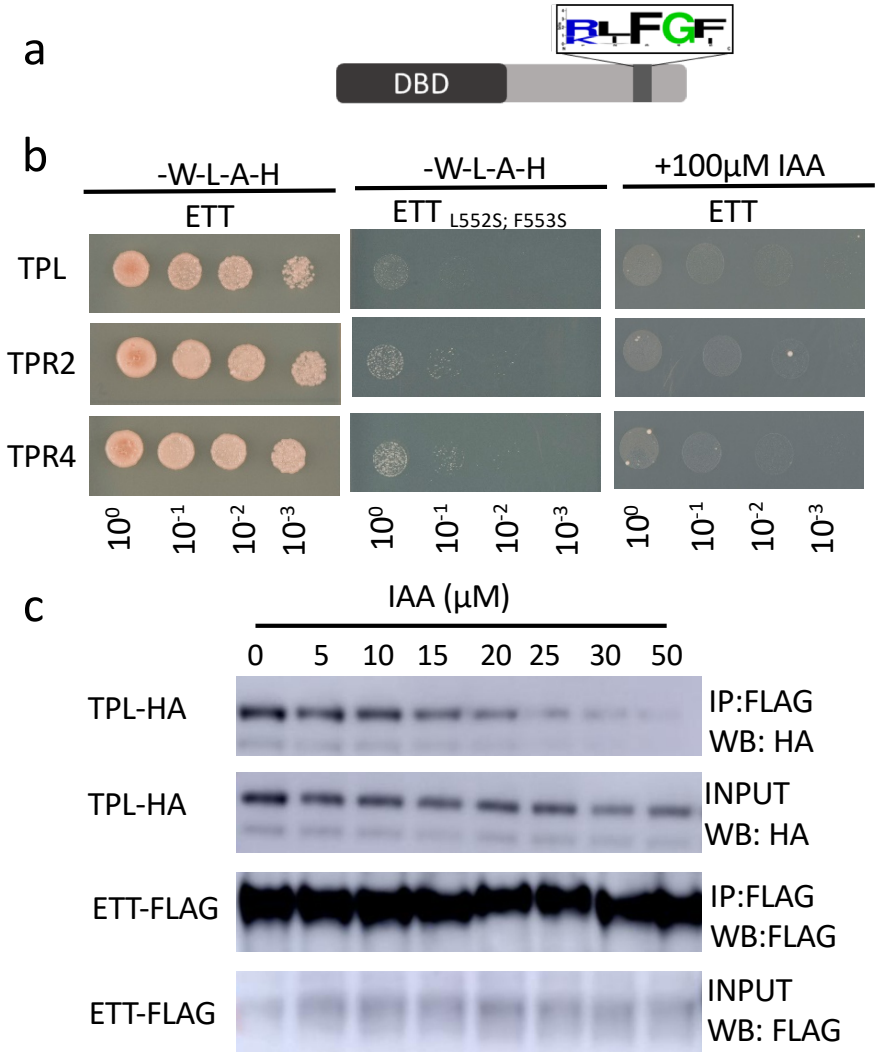

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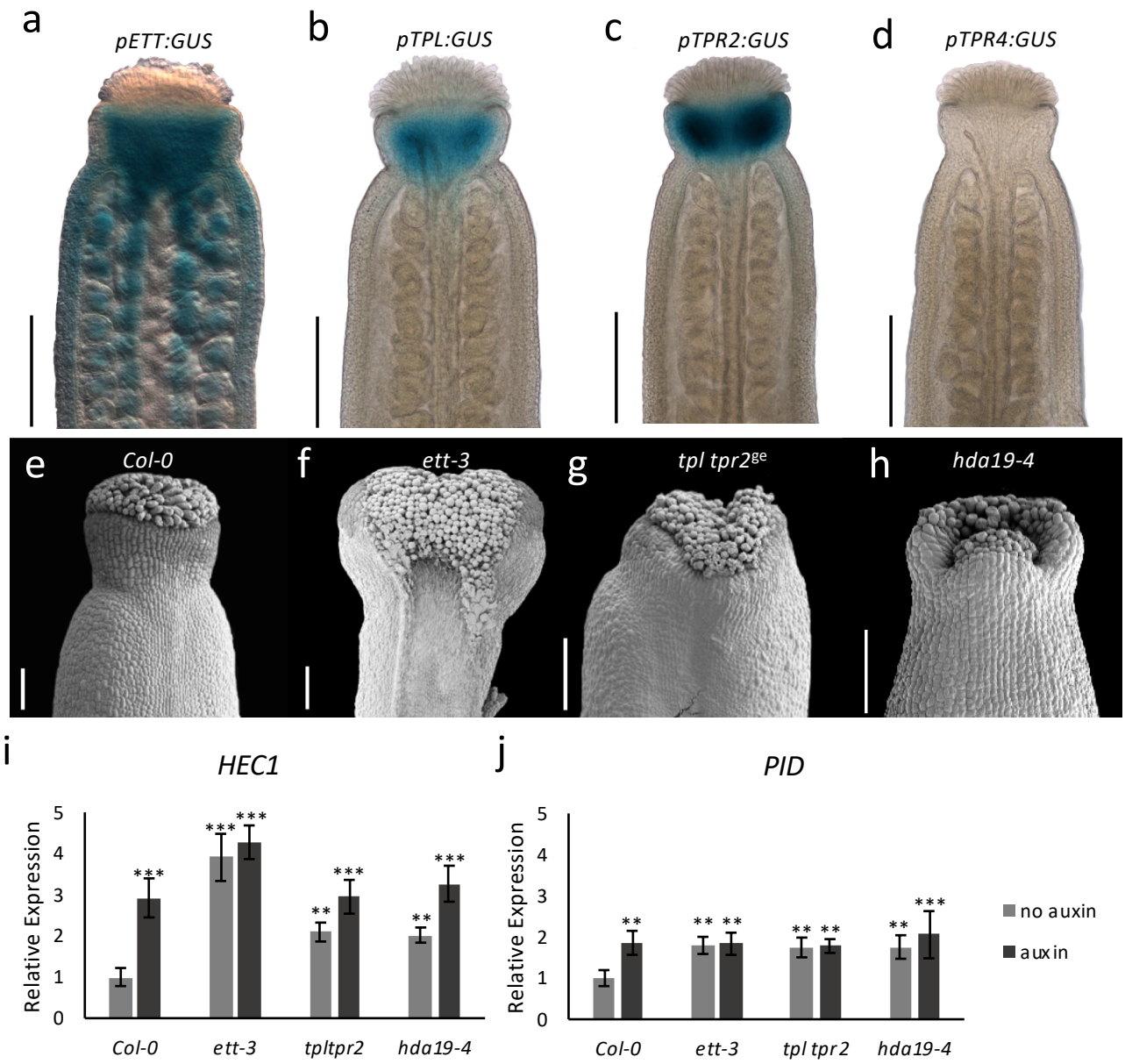
Figure 4. ETT, TPL/TPR2 and HDA19 cooperatively regulate gene expression to facilitate gynoecium development.



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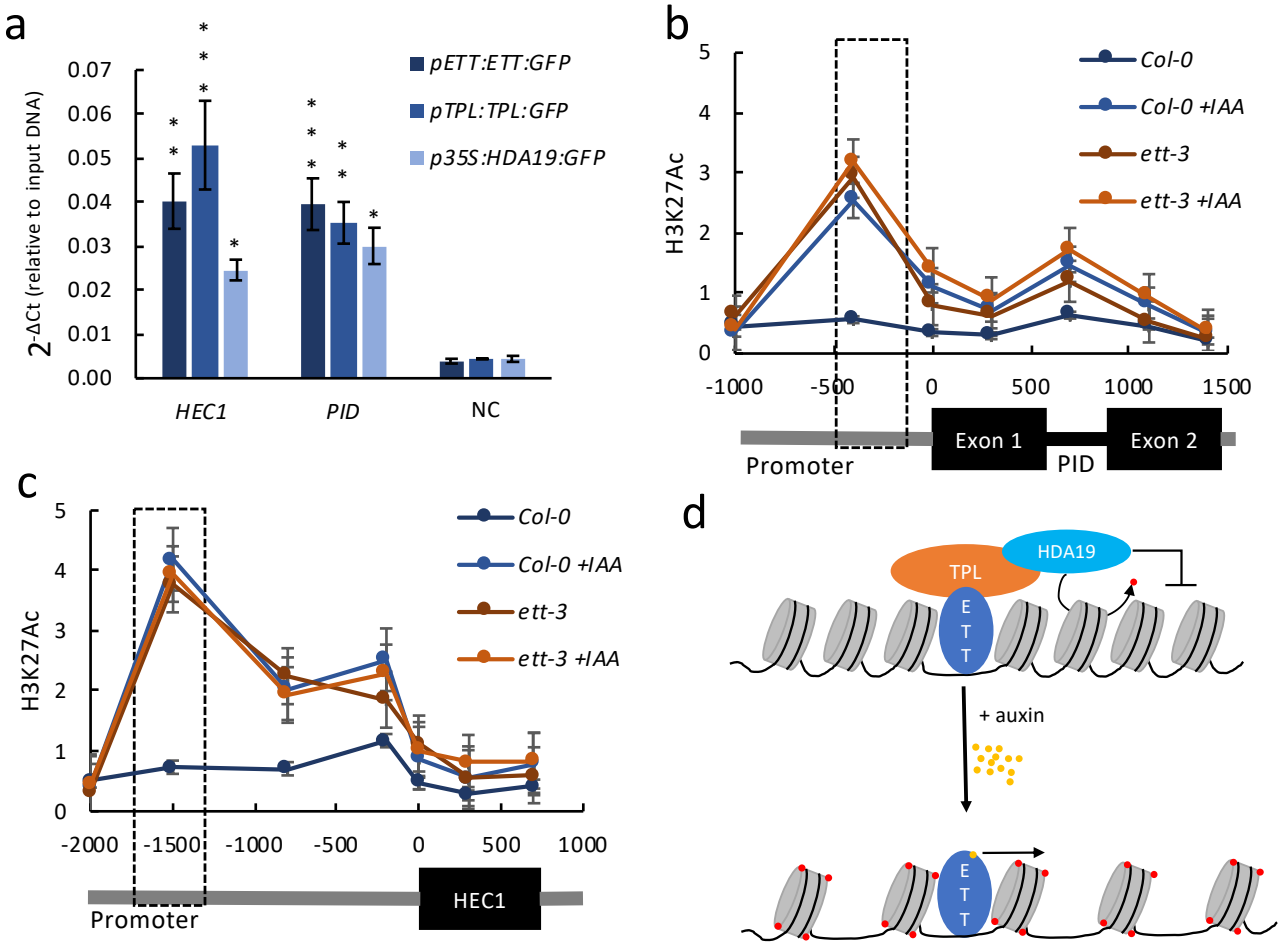
a-d, Promoter GUS expression analysis of *pETT:GUS* (**a**), *pTPL:GUS* (**b**), *pTPR2:GUS* (**c**) and *pTPR4:GUS* (**d**) revealed that *ETT*, *TPL* and *TPR2* but not *TPR4* are co-expressed in the Arabidopsis style. Scale bar = 300 µm. **e-h,** Gynoecium phenotypes of wild-type (**e**), *ett-3* (**f**) *tpl tpr2^{ge}* (**g**) and *hda19-4* (**h**). Scale bar = 100 µm. **i, j,** *HEC1* (**i**) and *PID* (**j**) are constitutively mis-regulated in *ett-3*, *tpl tpr2^{ge}* and *hda19-4* gynoecia. This misregulation is unaffected by treatment with 100 µM IAA. ***p-Values<0.0001; Shown are mean ± standard deviation of three biological replicates. Differences between untreated and IAA-treated mutants are not significant. See Figure 4-source data 1 for statistical analyses.

Fig. 5. ETT, TPL and HDA19 co-operatively regulate *HEC1* and *PID* by modulation of chromatin acetylation.



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A, Chromatin immunoprecipitation (ChIP) shows ETT, TPL and HDA19 binding to conserved regions of *HEC1* and *PID* loci. *WUS* served as negative control. **b, c,** H3K27ac accumulation (from ChIP analysis) along the *HEC1* (**b**) and *PID* (**c**) loci in wild-type (*Col-0*) and *ett-3* plants ± treatment with 100 µM IAA. Numbers on the x axes are distances to the Transcription Start Site (TSS). The schematic of the loci is shown below each panel. Dashed boxes represent ETT binding regions. **d,** Schematic model illustrating alternative TIR1/AFB independent auxin signaling. Under low auxin conditions an ETT-TPL-HDA19 complex binds to ETT-target genes keeping their chromatin environments repressed, through de-acetylation. High nuclear auxin concentrations abolish the ETT-TPL-HDA19 complex through direct ETT-auxin interaction. This leads to an accumulation of histone acetylation and up-regulation of ETT-target genes.

Values in **a**, **b** and **c** are means ± standard deviation of three biological replicates. See Figure 5-source data 1 for statistical analyses.

Figure 1-figure supplement 1. Parameters for HSQC NMR experiment.

Experiment	Recycling Delays (S)	Scans	Nuclei		Spectral width (Hz)		Number of complex points	
			t1	t2	t1	t2	t1	t2
HSQC	1	56	^{15}N	^{1}H	2888.6	15243.9	256	2048

Figure 2-figure supplement 1. Expression *HEC1* and *PID* in Col-0 and *ett-3*.

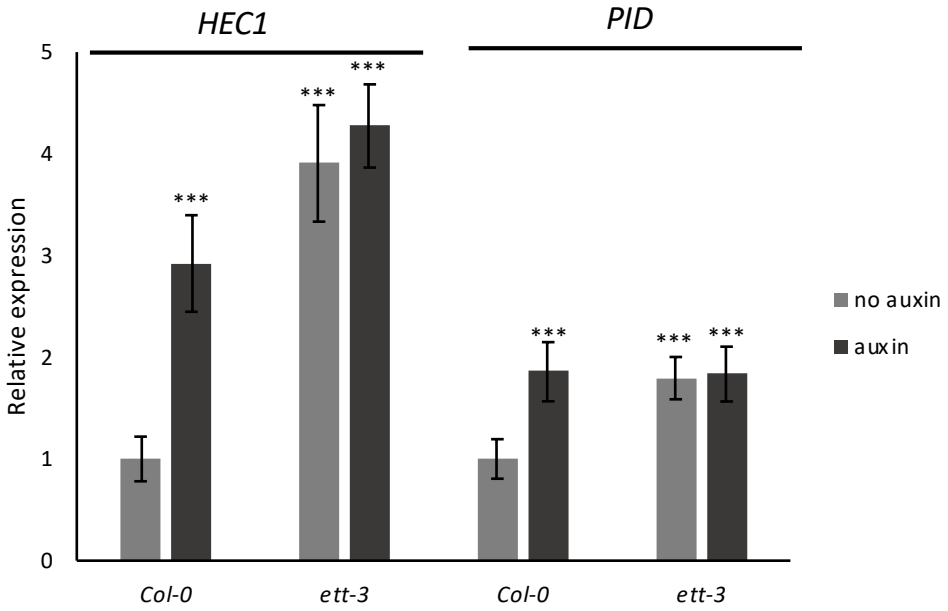
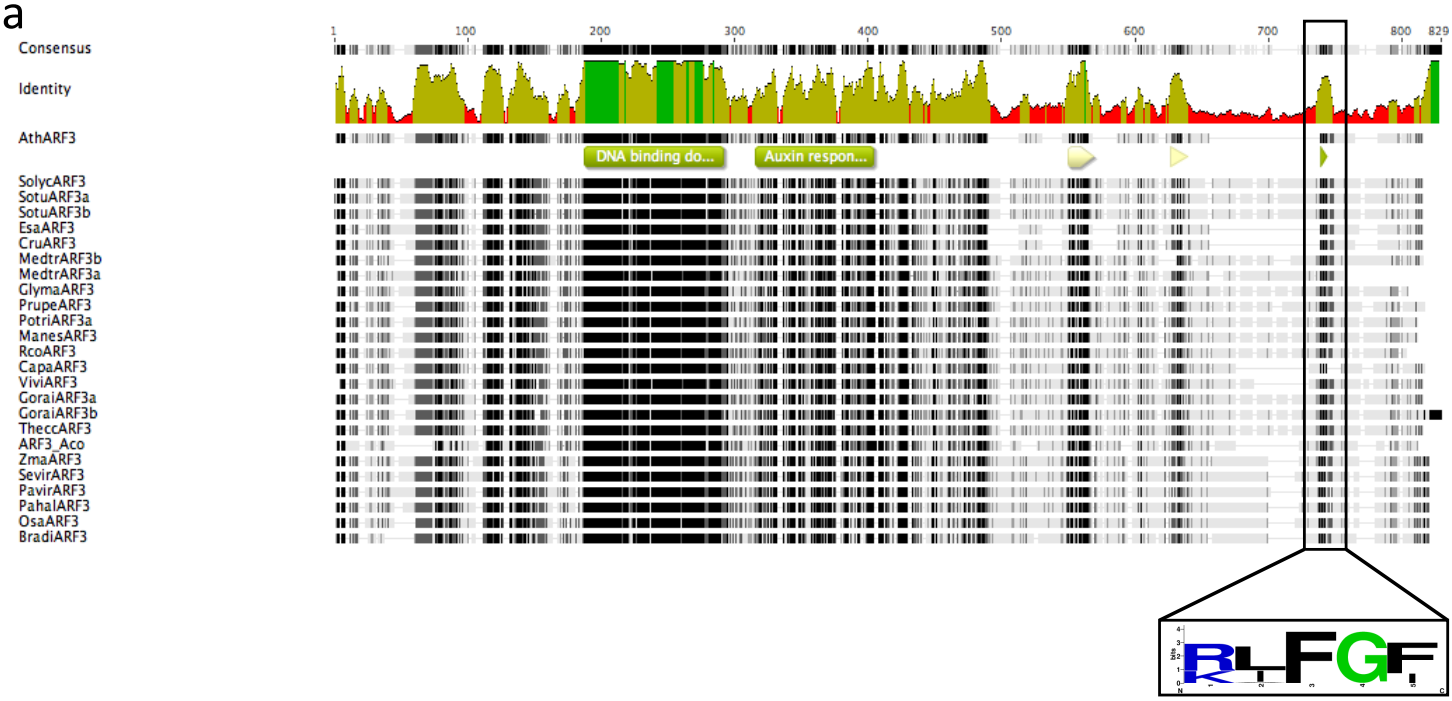


Figure 2-figure supplement 1. Expression of *HEC1* and *PID* in Col-0 and *ett-3*.
In wild-type gynoecia *HEC1* and *PID* are up-regulated upon auxin treatment while both genes are constitutively up-regulated in *ett-3*. Treatment with 100 µM IAA does not affect *HEC1* and *PID* expression in the *ett-3* mutant suggesting that ETT acts as a transcriptional repressor. Asterisks indicate significant change upon auxin treatment compared to untreated *Col-0* (*** indicating $p < 0.0001$). Shown are mean ± standard deviation of three biological replicates. See Figure 2-source data 1 for statistical analyses.

Figure 3-figure supplement 1. ETT can interact with several members of the TPL/TPR co-repressor family through a conserved EAR-like motif.

a



b

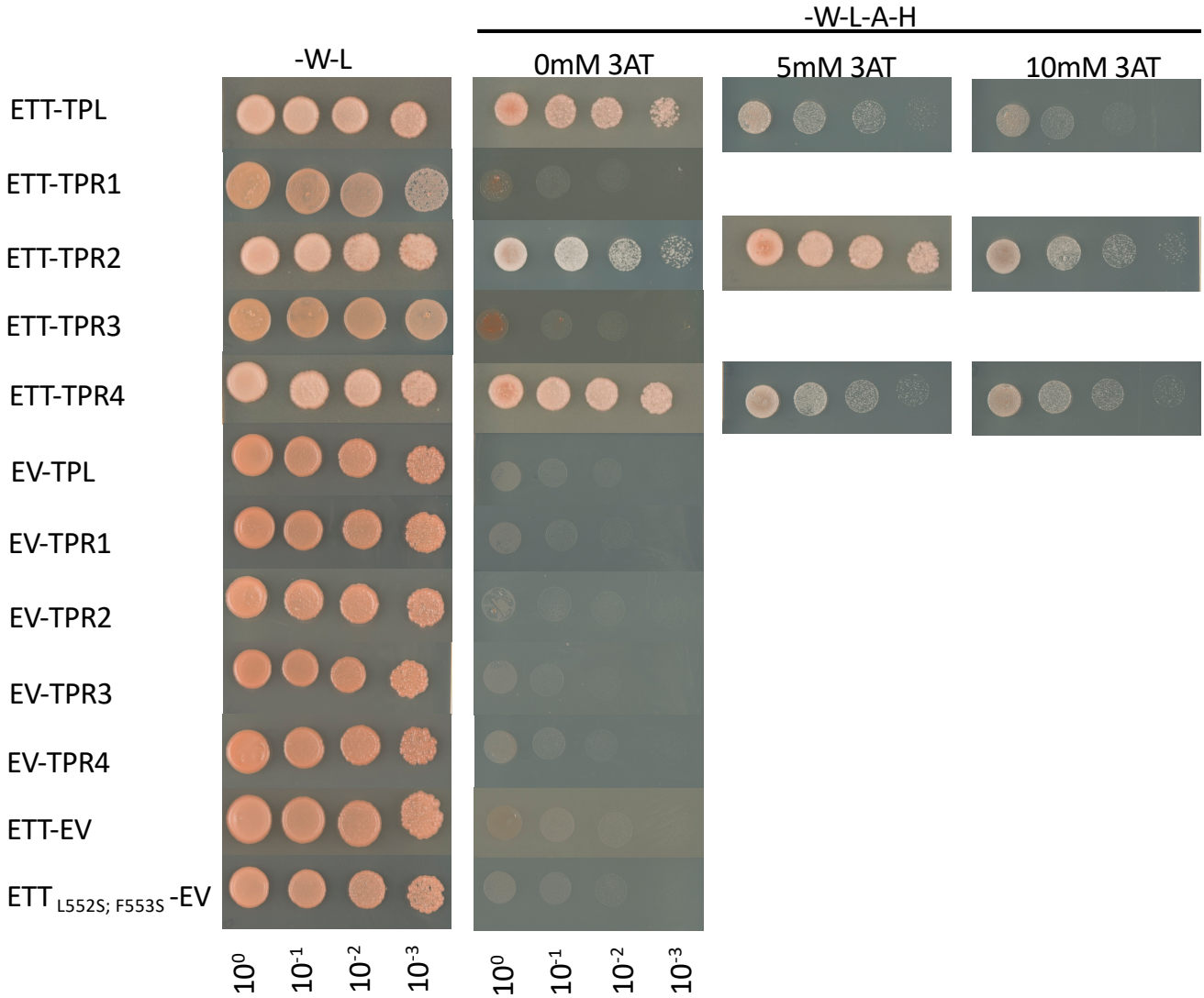


Figure 3-figure supplement 1. ETT can interact with several members of the TPL/TPR co-repressor family through a conserved EAR-like motif.
a, Alignment of ETT protein sequences of 22 species identified a conserved repressive motif (RLFGF) at its c-terminal domain. **b,** ETT interacts with several members of the TPL/TPR co-repressor family in Y2H. Additionally, controls for Fig. 2 are shown.

Figure 3-figure supplement 2. Interaction between ETT and TPL, TPR2 and TPR4 is auxin-sensitive and specific to IAA.

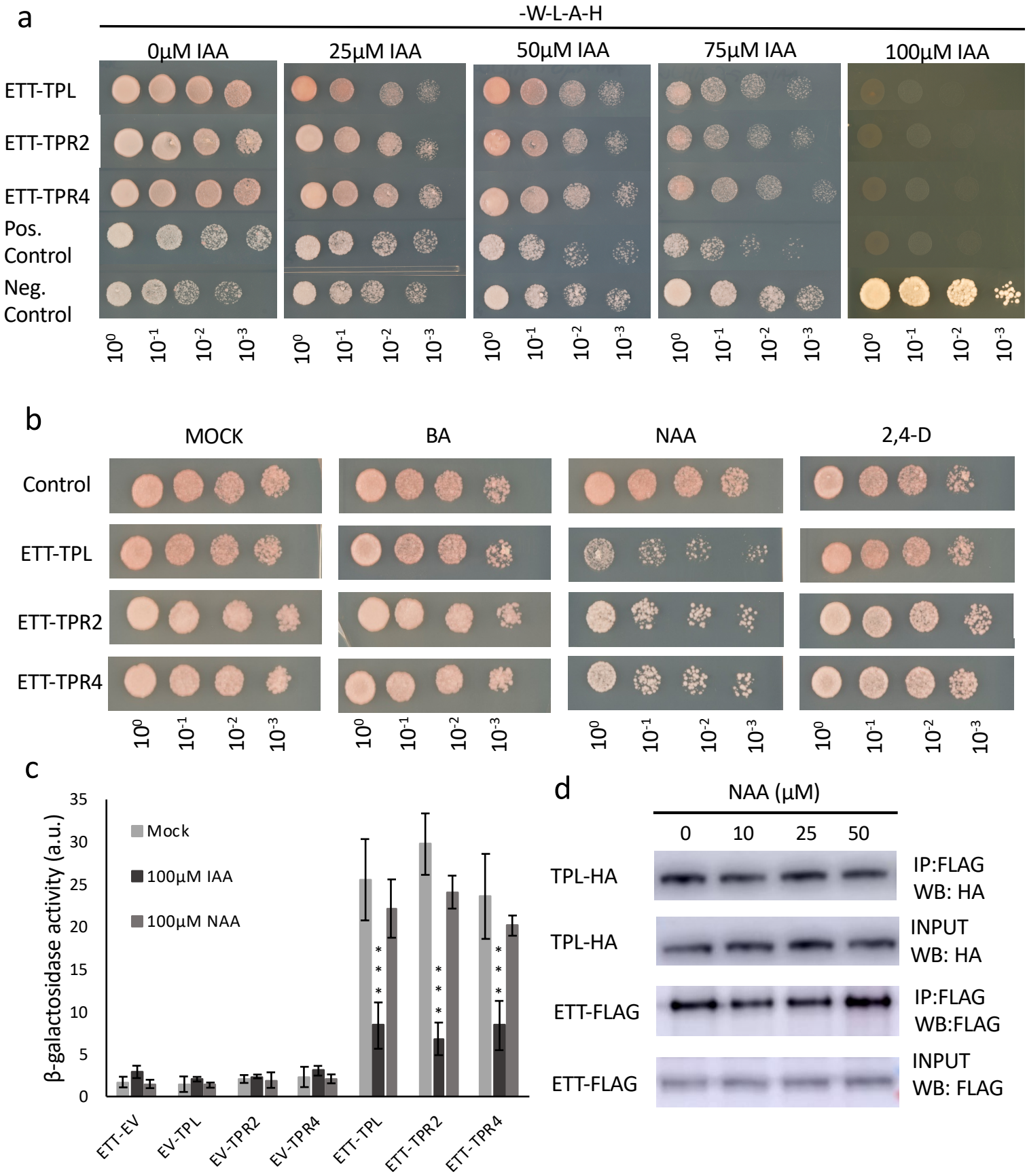
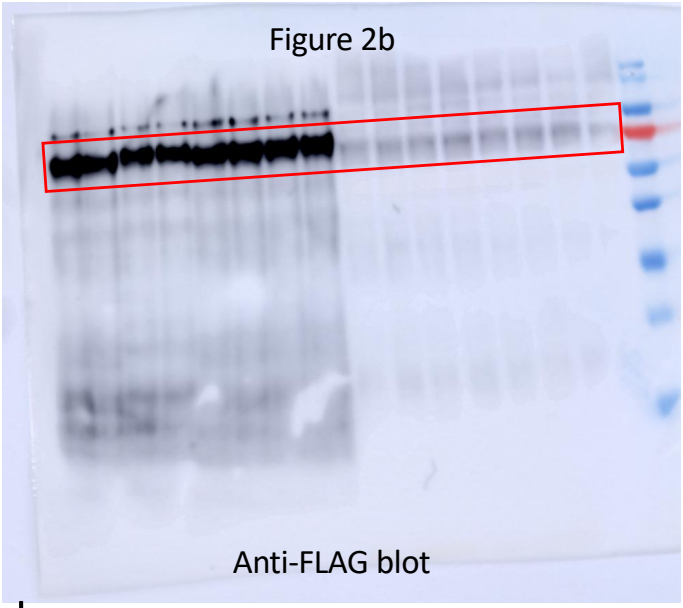


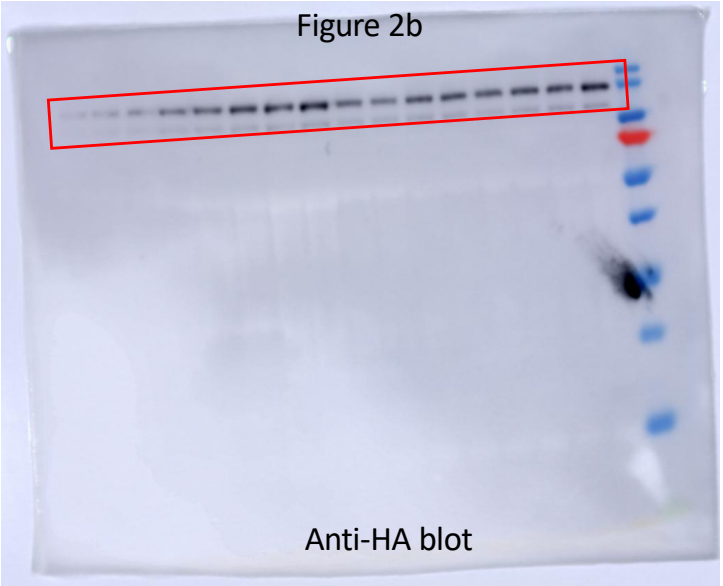
Figure 3-figure supplement 2. Interaction between ETT and TPL, TPR2 and TPR4 is auxin-sensitive and specific to IAA.

a, In Y2H increasing concentrations of IAA lead to reduction of yeast growth abolishing the interaction between ETT and its partners. The interactions are, therefore, auxin-sensitive. **b,** Y2H to test specificity of auxin-sensitivity using benzoic acid (BA), NAA, and 2,4D in a yeast growth assay. The data suggest that the auxin-sensitivity observed in (**a**) is IAA-specific. **c,** Y2H based ONPG assay measuring the β-galactosidase activity as a measure of interaction strength. **d,** Co-IP experiments show that the interaction between ETT and TPL cannot be disrupted by NAA. The data support that the ETT TPL/TPR interactions are sensitive to IAA but not to NAA. ***$p < 0.0001$; Shown are mean ± standard deviation of three biological replicates. See Figure 3-source data 1 for statistical analyses.

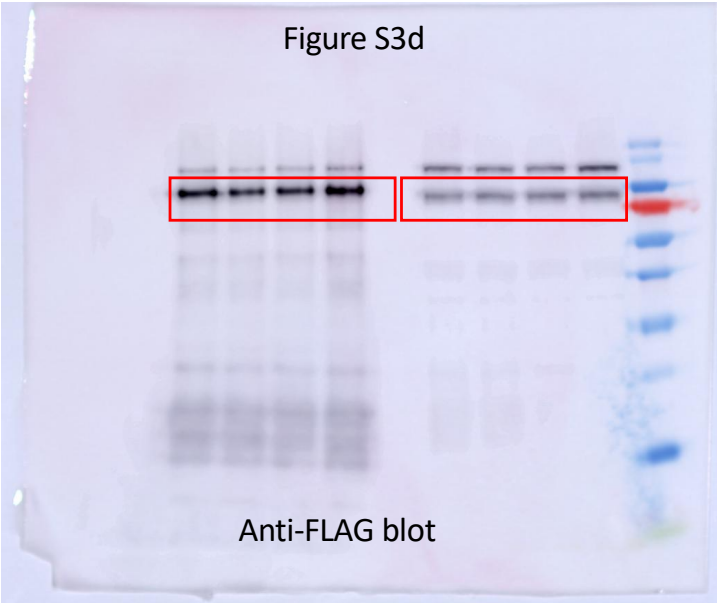
Figure 3-figure supplement 3. Original western blot images.

a





b



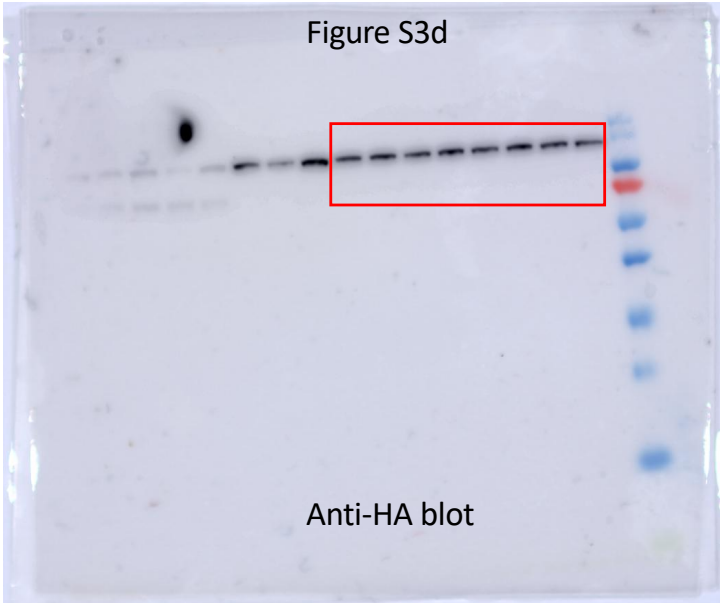


Figure 3-figure supplement 3. Original western blot images.
The red boxes indicate the areas used in Fig. 3b and Figure 3-figure supplement 2.

Figure 4-figure supplement 1. Expression TPL TPRs and HDAs in the gynoecium.

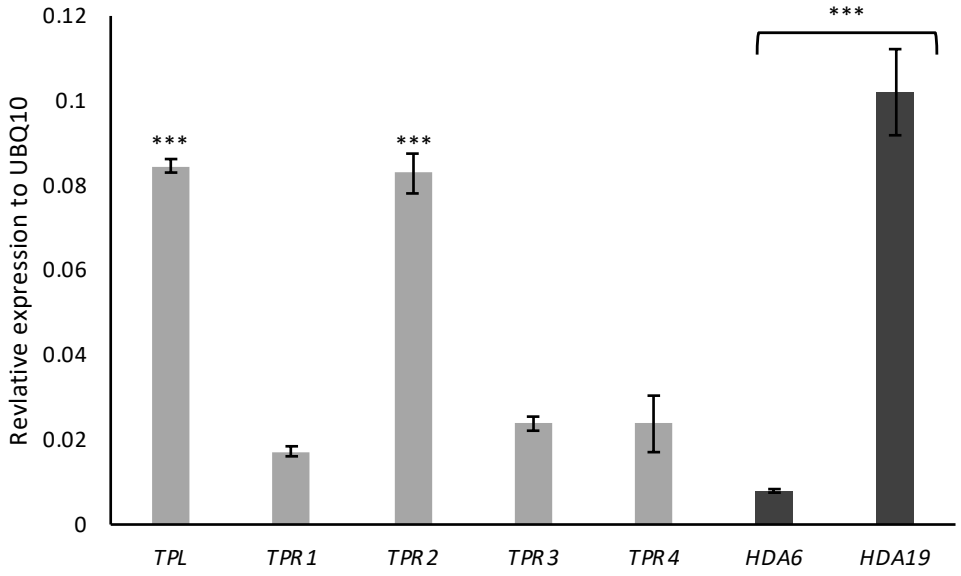


Figure 4-figure supplement 1. Expression of *TPL*, *TPRs* and *HDAs* genes in the gynoecium. Expression analysis using qRT-PCR in wild-type gynoecia showed that *TPL* and *TPR2* are stronger expressed then *TPR1*,*3* and *4*. Likewise, *HDA19* exhibits higher expression compared to *HDA6*. ***p-Values<0.0001; Shown are mean ± standard deviation of three biological replicates. See Figure 4-source data 1 for statistical analyses.