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1 **Concerted expression of a cell-cycle regulator and a metabolic enzyme from a**
2 **bicistronic transcript in plants**

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17

18 **Abstract**

19 **Eukaryotic mRNAs frequently contain upstream open reading frames (uORFs),**
20 **encoding small peptides which may control translation of the main ORF (mORF) by**
21 **various mechanisms. Here we report the identification of a bicistronic transcript in**
22 **Arabidopsis, in which the uORF encodes an ortholog of the cell cycle regulator CDC26**
23 **whose genome locus was previously unknown. AtCDC26 is part of the plant anaphase**

24 **promoting complex / cyclosome (APC/C), regulates accumulation of APC/C target**
25 **proteins and controls cell division, growth and embryo development. AtCDC26 is**
26 **translated together with the inorganic polyphosphatase AtTTM3 from a single**
27 **transcript conserved in the plant lineage. While there is no apparent biochemical or**
28 **physiological connection between the two gene products, concerted AtCDC26 and**
29 **AtTTM3 transcription and translation occurs in different plant tissues and organs. Our**
30 **work reveals that uORFs in plants may code for functional proteins not involved in the**
31 **translational regulation of the mORF.**

32

33 **Introduction**

34 uORFs are coding sequences in the 5' untranslated region (UTR) of mRNAs. Many uORFs
35 code for small, non-conserved peptides^{1,2} and regulate expression of the mORF³. While a
36 significant fraction of fungal, animal and plant genes contain uORFs⁴, only few functional
37 uORF-derived peptides have been found in cells and tissues^{1,5}. In plants, several uORFs have
38 been genetically characterized in transcription factors, metabolic enzymes, membrane
39 transporters and signaling proteins⁶. uORFs regulate the expression of the mORF during
40 growth and development⁷, in response to stress^{8,9} or to changes in nutrient availability¹⁰. As
41 in other eukaryotes, plant uORFs are involved in translational repression of the mORF⁷ and
42 in the nonsense mediated decay (NMD) of the respective mRNA¹¹⁻¹³.

43 A fraction of uORFs do not encode small peptides but rather independent, functional
44 proteins, which are transcribed together with the mORF from *bona fide* bicistronic
45 transcripts¹⁴. Eukaryotic transcripts encoding for more than one protein have been initially
46 reported from vertebrates, where the transforming growth factor-beta family ligand GDF1 is
47 transcribed together with ceramide synthase 1 from a single 3 kb transcript¹⁵. Other examples

48 include the p16^{INK4a} gene, which contains two overlapping open reading frames coding for
49 distinct proteins involved in cell cycle regulation¹⁶. A similar gene architecture has been
50 reported for the mammalian *XLas/Gas* gene. Here, the two translation products code for the
51 extra-large G-protein *XLas* and for a sequence-unrelated protein ALEX, which directly binds
52 *XLas*¹⁷. A uORF located in the mammalian A_{2A} adenosine G-protein coupled receptor gene
53 encodes the ~ 15 kDa uORF5 protein, whose expression is regulated by A_{2A} activation¹⁸.
54 These examples may suggest that mORF and uORF protein products are often functionally
55 and/or biochemically linked¹⁴, but mORF/uORF pairs with no apparent biological connection
56 have been described as well¹⁹.

57 **Results**

58 Here we report a novel uORF with unusual properties located in the annotated 5' UTR of
59 *TTM3* in Arabidopsis. *AtTTM3* encodes an inorganic polyphosphatase that releases inorganic
60 phosphate from short-chain linear polyphosphates, a storage form of phosphate in many pro-
61 and eukaryotes^{20,21}. *TTM3* is a conserved single copy gene in Arabidopsis and many other
62 plant species (Extended Data Fig. 1a). To define physiological functions for TTM3, we
63 analyzed different *ttm3* mutant alleles: *ttm3-1* maps to the *TTM3* ORF (Fig. 1a), completely
64 abolishes TTM3 expression and protein accumulation (Fig. 1b) and reduces hypocotyl and
65 root growth, in agreement with an earlier report²⁰ (Fig. 1d, e). A second T-DNA insertion in
66 the 5' UTR of *TTM3* (*ttm3-2*) likely causes a knock-out of TTM3, impairs embryo
67 development and blocks seed germination (Fig. 1c, 3d). *ttm3-4* maps to the 3' UTR, shows
68 reduced TTM3 transcript and protein levels (Fig. 1b) and consistently, a weak growth
69 phenotype (Fig. 1d,e). The observed inconsistencies between the *ttm3-1* and *ttm3-2* mutant
70 lines prompted us to generate an additional CRISPR/Cas9-based mutant. The resulting *ttm3-3*
71 allele harbors a 16 base-pair deletion in the *TTM3* coding sequence (Extended Data Fig. 1b)

72 that abolishes TTM3 protein accumulation and also reduces *TTM3* transcript levels *in planta*
73 (Fig. 1b). To our surprise, *ttm3-3* mutants did neither resemble the root and hypocotyl growth
74 phenotypes of *ttm3-1* plants nor the *ttm3-2* embryo phenotype (Fig. 1d, e).

75 We investigated these phenotypic differences by complementing *ttm3-1* and *ttm3-2* mutant
76 alleles with the fluorescent protein-tagged TTM3-mCITRINE expressed under the control of
77 the *TTM3* promoter, including the annotated 5' UTR. Even though TTM3-mCITRINE
78 protein levels were low compared to endogenous TTM3, we observed full complementation
79 of the different *ttm3* alleles, suggesting that the observed phenotypes were specific to the
80 *TTM3* locus (Fig. 2a, b). We next analyzed TTM3-mCITRINE and TTM3-GUS transgenic
81 reporter lines and found that TTM3 is a cytoplasmic/nuclear localized protein expressed in
82 ovules, roots and hypocotyls, in good agreement with the observed phenotypes (Extended
83 Data Fig. 2a, b). We next complemented the embryo phenotype of *ttm3-2* plants with
84 versions of TTM3 compromised in either substrate binding or catalysis²¹ (Fig. 2c). To our
85 surprise, catalytically inactive versions of TTM3 could fully complement the *ttm3-2* mutant
86 phenotype (Fig. 2d, e), indicating that the enzymatic activity of TTM3 is dispensable for
87 proper embryo development.

88 Close inspection of the *TTM3* locus revealed the presence of a putative uORF in the
89 annotated 5'UTR of TTM3, ending 8 base-pairs upstream of the mORF start codon (Fig. 3a,
90 Extended Fig. 3a). The uORF encodes a putative peptide of 65 amino acids, sharing
91 significant sequence homology with CDC26 superfamily proteins (Fig. 3a). CDC26, whose
92 genome locus was previously unknown^{22,23}, forms a component of the APC/C, an E3-
93 ubiquitin ligase that targets substrates for degradation, allowing for cell cycle progression²⁴.
94 AtCDC26 protein accumulates in different plant tissues throughout development (Fig. 3b).
95 Since *ttm3* phenotypes did not seem to be related to TTM3 protein levels or its catalytic

96 activity, we analyzed AtCDC26 transcript and protein expression levels in *ttm3-1*, *ttm3-3* and
97 *ttm3-4* mutants. Although transcript levels were lower, we found no observable differences
98 for AtCDC26 protein levels in the non-lethal *ttm3-1*, *ttm3-3* and *ttm3-4* mutants (Fig. 3c).

99 Based on these observation, we hypothesized that complementation of our *ttm3-1* and *ttm3-2*
100 mutant lines (Fig. 2a-d) may have been due to the re-introduction of functional AtCDC26,
101 present in what we thought would be the *TTM3* 5' UTR. We could indeed detect wild-type
102 levels of AtCDC26 in our *ttm3-2* complemented lines (Fig. 2f). Ubiquitous expression of the
103 AtCDC26 CDS alone fully rescued *ttm3-2* embryo lethality and *ttm3-1* defective root and
104 hypocotyl growth (Fig. 3d-f), suggesting that the observed and reported²⁰ phenotypes for
105 *ttm3-1* were caused by interference with *CDC26* expression, rather than *TTM3* loss-of-
106 function.

107 We next tested if AtCDC26 is a *bona fide* component of the Arabidopsis APC/C. We
108 performed immunoprecipitation assays followed by mass-spectrometry in wild-type plants
109 expressing epitope-tagged AtCDC26-6xHA. AtCDC26 interactors included APC1, APC5,
110 APC6 and APC7/CDC27B, which have been previously shown to interact with CDC26 in
111 human²⁵, and in addition APC2 and APC8, together forming the APC/C complex (Fig.
112 2g)^{24,26}. The plant APC/C regulates cell division and affects many aspects of plant growth
113 and development^{22,27}. To test whether *ttm3-1* mutant plants have abnormal cell divisions
114 cycles, we quantified GFP levels in *ttm3-1* plants expressing fluorescent tagged Cyclin B1;1
115 (CYCB1;1-GFP²⁸). CYCB1;1 is a marker of cell division and a target of the APC/C^{29,30}. We
116 found that CYCB1;1-GFP expression and protein levels (inferred from the GFP fluorescent
117 signal) are less variable and overall reduced in *ttm3-1* plants vs. wild-type plants, indicating
118 that the mutant is defective in cell division and CYCB1;1-GFP protein stability, respectively
119 (Fig. 3h). Together, our findings suggest that AtCDC26 is plant cell-cycle regulator and part

120 of the *Arabidopsis* APC/C. Importantly, we did not recover TTM3 peptides in our
121 immunoprecipitation assays and recombinant AtTTM3 and AtCDC26 showed no detectable
122 interaction in *in vitro* isothermal titration calorimetry assays, suggesting that TTM3 does not
123 form part of the plant APC/C complex (Extended Data Fig. 4).

124 *CDC26* and *TTM3* are both present in the entire plant lineage and their ORFs are always in
125 close proximity (Fig. 4a, Extended Data Fig. 3a). The *CDC26* stop codon may be spaced
126 ~150 base-pairs apart from the start codon of *TTM3* (e.g. in *Chlamydomonas reinhardtii* and
127 *Marchantia polymorpha*), be separated by only a short sequence (in *Arabidopsis*), or the
128 ORFs may even overlap, as found for example in tomato and maize (Fig. 4a, Extended Data
129 Fig. 3a-c). As *CDC26* and *TTM3* are always in close proximity, we speculated that both
130 proteins could be expressed from a single bicistronic transcript. We performed northern blots
131 with probes against *CDC26* and *TTM3* in wild-type and *ttn3-1* mutant plants. We detected a
132 major transcript of ~1,200 nucleotides using both probes, which is absent in *ttn3-1* plants
133 (Fig. 4b). Next, we performed 5' and 3' RACE experiments with *CDC26* specific primers
134 and recovered a transcript of similar size (Fig. 4c). Sequencing of 5' RACE products
135 confirmed the presence of the *CDC26* and *TTM3* ORFs in a single transcript in wild-type
136 plants (see Supplementary Information). One additional, minor transcript was recovered in
137 the RACE experiments, encoding *CDC26* only (see Supplementary Information). The
138 presence of both transcripts could be confirmed in RT-PCR experiments (Fig. 4d). cDNA
139 clones suggest that *CDC26* and *TTM3* are encoded in a single transcript in *Chlamydomonas*,
140 tomato and maize (Extended Data Fig. 3b). To test if AtCDC26 and AtTTM3 are translated
141 from a single mRNA, we performed *in vitro* translation assays in wheat germ extracts where
142 products were labeled with ³⁵S methionine. Two protein products migrating at the expected
143 size of AtCDC26 and AtTTM3, respectively, were produced from an *in vitro* transcribed

144 *CDC26-TTM3* transcript (Fig. 4e). Mutating the start codon of either *CDC26* or *TTM3*
145 eliminated translation of the respective gene product, but did not affect translation of the
146 other ORF (Fig. 4e). Together our *in vivo* and *in vitro* experiments reveal that *CDC26* and
147 *TTM3* are transcribed and translated from a single transcript, yielding two proteins with
148 different biochemical and physiological functions. The *ttm3-3* allele and the complementation
149 experiments using catalytically inactive versions of *TTM3* together suggest that neither the
150 enzyme itself nor its catalytic function impact the phenotypes described for the *TTM3* locus²⁰.
151 Our loss-of-function phenotypes reveal an essential role for *CDC26* in Arabidopsis, as
152 previously seen in animals³¹. The fact that *CDC26* is expressed from a conserved uORF in
153 the *TTM3* gene suggests that this bicistronic transcript may have important regulatory
154 functions in plant cell cycle control. Translation of different cyclin-dependent kinases from a
155 single transcript occurs via cell-cycle regulated internal ribosome entry sites (IRES) in
156 metazoa³², but it is unknown if this mechanism is present in plants⁶. Notably, expression of
157 At*CDC26* from a strong ubiquitous promoter alone can rescue all observed phenotypes, while
158 expression of At*CDC26* under control of its native promoter requires the endogenous 3'UTR
159 or the presence of the *TTM3* mORF (Extended Data Fig. 5a-e). Complementation of a *ttm3-2*
160 mutant with a construct harboring a mutated *CDC26* start codon (**CDC26-TTM3*) did not
161 rescue the *ttm3-2* embryo lethal phenotype. (Fig. 5a,b). In contrast, we observed stunted-
162 growth and 'broom-head' phenotypes previously seen in *APC6* and *APC10* knock-down
163 mutants³³ or in a *APC8* missense allele³⁴, when complementing *ttm3-2* plants with a construct
164 harboring a mutated *TTM3* start codon (*CDC26-*TTM3*) (Fig. 5a,c). *CDC26* transcript levels
165 are high in plants expressing *CDC26-*TTM3* but protein levels were reduced compared to
166 wild-type, indicating that *CDC26* and *TTM3* may require to be translated in a concerted
167 fashion (Fig. 5d). To investigate this issue further, we performed polysome profiling

168 experiments. We find the *TTM3* transcript associated with polysomes in wild-type seedlings,
169 but to a lesser extent in *CDC26-TTM3* plants, indicating that *TTM3* translation recruits the
170 bicistronic transcript to polysomes (Fig. 5e).

171 It has been previously reported that the Target of rapamycin (TOR) complex can
172 regulate uORF translation and loading of the respective transcripts to polysomes^{10,35}. We thus
173 tested whether *CDC26* and *TTM3* expression is regulated by TOR. As previously reported,
174 the uORF-containing transcript *bZIP11* is shifted to monosomes upon treatment with the
175 TOR-inhibitor AZD8055 (AZD), while the polysome profile of *TTM3* seems unaffected (Fig.
176 5f). In line with this, *TTM3* and *CDC26* protein levels are not significantly reduced in
177 seedling treated with the TOR-inhibitors KU63794 and AZD, when compared to the actin
178 loading control (Fig. 5g). These experiments together indicate that TOR may not have a
179 major role in the translational regulation of *CDC26* and *TTM3*.

180 Finally, we tested if the *CDC26-TTM3* transcript is regulated by NMD, as previously
181 reported for other uORF containing transcripts in Arabidopsis¹¹⁻¹³. We found *CDC26-TTM3*
182 transcript levels to be higher in wild-type plants treated with cycloheximide (CHX), an
183 inhibitor of protein translation that represses NMD³⁶ (Fig. 6a), or in known NMD mutant
184 backgrounds (Fig. 6b). Consistently, also *CDC26* and *TTM3* protein levels were increased in
185 NMD mutants (Fig. 6c). Together, these experiments indicate that the *CDC26-TTM3*
186 bicistronic transcript is regulated by nonsense-mediated decay.

187

188 **Discussion**

189 In Arabidopsis, uORF-containing mRNAs represent more than 30% of the transcriptome and
190 these uORFs may control translation efficiency and mRNA stability of the mORF₆. Here we
191 present a novel uORF in Arabidopsis not involved in the regulation of translation, but rather

192 forming an essential component of the plant APC/C complex. This CDC26 subunit shows a
193 monocistronic gene architecture in other eukaryotes, but is encoded in a bicistronic
194 transcript upstream of the inorganic polyphosphatase TTM3 in the entire green lineage from
195 algae to higher land plants (Extended Data Fig. 3a). Our finding now provides the genome
196 locus for AtCDC26, whose transcript had been previously detected^{22,23}. Our genetic analyses
197 suggest no strong functional connection between AtCDC26 (which our analyses define as an
198 essential gene) and AtTTM3 (whose enzymatic function appears to be dispensable at least in
199 the growth conditions tested, compare Fig. 2), and AtTTM3 does not seem to interact
200 biochemically with stand-alone CDC26 or the plant APC/C (Fig. 3g, Extended Data Fig. 4).
201 It is however of note that inorganic polyphosphates promote cell cycle exit in bacteria³⁷ and
202 fungi³⁸, and we thus cannot exclude a functional connection between TTM3 and CDC26.

203 We did not observe regulation of the *TTM3* mORF by the *CDC26* uORF, but rather we found
204 that translation of the mORF recruited the bicistronic transcript to polysomes, enhancing
205 CDC26 translation, and thus protein levels *in planta*. While the mechanism of concerted
206 CDC26 and TTM3 translation remains to be investigated, we found that in some species both
207 ORFs are located at a very short distance, or even overlap (Extended Data Fig. 3). This
208 makes TTM3 translation by ribosome re-initiation unlikely³⁹. We confirmed that transcripts
209 featuring an overlapping arrangement of *CDC26* and *TTM3* ORFs are translated in wheat
210 germ extracts, leading to full-length CDC26 and TTM3 protein products (Extended Data Fig.
211 6). In addition, we could not detect an internal ribosome entry site (IRES) in the *CDC26*
212 coding sequence, and a synthetic *CDC26-TTM3* transcript with altered codons did still
213 support translation of both proteins (Extended Data Fig. 6). We thus speculate that leaky
214 ribosome scanning may reach the *TTM3* start codon⁴⁰, as it has been previously suggested for
215 viral bicistronic transcripts translated in plants⁴¹. In line with this, we do not observe major

216 changes in CDC26 and TTM3 expression upon treatment with TOR inhibitors, with TOR
217 affecting ribosome re-initiation in plants^{10,35}. We could however confirm that the *CDC26*-
218 *TTM3* transcript is under regulation by nonsense-mediated decay (Fig. 6). As the Arabidopsis
219 NMD pathway counteracts cyclin-dependent kinases activity required for cell cycle
220 progression^{42,43}, NMD of the *CDC26-TTM3* transcript could represent an additional
221 regulatory mechanism of CDC26 function in the control of plant cell cycle.

222 Taken together, we demonstrate that a novel cell cycle regulator in plants is expressed from a
223 conserved uORF in a gene coding for a metabolic enzyme. Our genetic and biochemical
224 characterization of the *CDC26 TTM3* locus now enables the mechanistic dissection of
225 bicistronic transcription and translation in plants.

226 METHODS

227

228 **Plant material and growth conditions.** *ttm3-1* (SALK_133625), *ttm3-2* (FLAG_368E06),
229 and *ttm3-4* (SALK_050319) T-DNA insertion lines were obtained from NASC (Nottingham
230 Arabidopsis Stock Center). The *ttm3-3* mutant was generated using CRISPR/Cas9.
231 Specifically, the *TTM3*-specific sequence 5'-ATTGAGACGGAGATGAGCAGCGG-3'
232 (*sgTTM3*) was cloned into the PTTK352 vector⁴⁴, containing Cas9 in a cassette with
233 hygromycin-resistance and RFP as selection-markers. *Arabidopsis* Col-0 plants were
234 transformed with pTTK352-*sgTTM3* (see *generation of transgenic lines*). T1 generation
235 plants were selected via hygromycin resistance. *ttm3-3* was identified by PCR followed by
236 sequencing. In T2 generation, seeds not expressing RFP (lacking Cas9) were selected. Plants
237 were grown under conditions of 50 % humidity, 22 °C and 16 h of light.

238

239 **Real-time quantitative reverse transcription polymerase chain reaction** RNA was
240 extracted with the Rneasy Plant Mini Kit (Qiagen). 2 µg of RNA was treated with Dnase I
241 (Qiagen), copied to cDNA using an Oligo dT and the SuperScript™ II Reverse Transcriptase
242 (Invitrogen). Transcript levels were estimate using the SYBR Green PCR Master Mix
243 (Applied Biosystems), and transcript abundance was normalized to *ACT8*. Values indicate the
244 mean ± standard deviation of three replicates. Primer sequences can be found in Extended
245 Data Table 1.

246

247 **Protein expression and generation of antibodies** TTM3 was produced and purified as
248 described⁴⁵. For generation of the TTM3 antibody, rabbits were immunized with purified
249 TTM3 dialyzed against phosphate-buffered saline (PBS). The resulting serum was affinity-

250 purified over AtTTM3-coupled Affigel 15 (Biorad, www.bio-rad.com), eluted in 200 mM
251 glycine pH 2.3, 150 mM NaCl and stored in PBS pH 7.5.

252 CDC26 was cloned into pMH-TrxT vector, providing an N-terminal 6xHis-StrepII-
253 Thioredoxin tag (HST) and a tobacco etch virus (TEV) protease cleavage site. CDC26
254 expression was induced in *Escherichia coli* BL21 (DE3) RIL cells with 0.25 mM isopropyl
255 β -D-galactoside (IPTG) at OD₆₀₀ ~0.6, and grown at 16 °C for 16 h. Cells were collected by
256 centrifugation (4,500 g, 30 min), resuspended in lysis buffer (20 mM Tris pH 8, 500 mM
257 NaCl, 1 mM MgCl₂, 5 mM β -mercaptoethanol and cOmplete[™] EDTA-free Protease Inhibitor
258 Cocktail (Merck) and homogenized (Emulsiflex C-3, Avestin). HST-CDC26 was isolated
259 from the lysate via tandem Ni²⁺ and StrepII affinity purification (HisTrap HP 5 ml, GE
260 Healthcare; Strep-Tactin Superflow high capacity, IBA), and purified further by size-
261 exclusion chromatography (Superdex 75 HR10/30, GE Heathcare). The HST-CDC26 fusion
262 protein was incubated with TEV protease (1:100 molar ratio) for 16 h at 4 °C. CDC26 was
263 recovered by a second Ni²⁺ affinity step followed by size exclusion chromatography. The
264 molecular weight of the purified protein was determined to be 7.3 kDa (as predicted) by
265 MALDI-TOF mass spectrometry. A polyclonal CDC26 antibody was generated in rabbit
266 (Eurogentec) and purified as described for AtTTM3.

267

268 **Western blotting** Plant material was snap-frozen in liquid nitrogen and homogenized with
269 mortar and pestle. The material was resuspended in 50 mM Tris pH 8.0, 150 mM NaCl, 0.5
270 % Triton X-100 and cOmplete[™] EDTA-free Protease Inhibitor Cocktail (Merck). 20-50 μ g
271 of total protein extract (estimated by Bradford, Bio-Rad), pre-boiled for 5 min, was run on a
272 10 % SDS-PAGE gel. Proteins were blotted onto nitrocellulose membranes (GE Healthcare),
273 then blocked using TBS buffer containing 0.1 % (v/v) tween 20, 5 % (w/v) powder milk.

274 Membranes were incubated for 1 h at room temperature with CDC26 or TTM3 antibodies,
275 and then with an anti-rabbit peroxidase conjugate ($1 \mu\text{g } \mu\text{l}^{-1}$) antibody (Calbiochem), or with
276 an anti-HA-HRP (Miltenyi Biotec) (dilution 1:5,000). Membranes were then stained with
277 Ponceau (0.1 % (w/v) Ponceau S in 5 % (v/v) acetic acid). Bands corresponding to RuBisCO
278 (~56 kDa) are shown as a loading control.

279

280 **Phenotyping assays** For root length measurements, stratified seeds (2-5 d, 4 °C, in darkness)
281 were germinated on $\frac{1}{2}$ MS medium, containing $\frac{1}{2}$ MS (Duchefa), 0.5 g/L MES, 0.8 % (w/v)
282 agar, 1 % (w/v) sucrose, pH 5.7. After 4 days, seedlings were transferred to new plates and
283 grown for 7 d at 22 °C, 16 h of light. For hypocotyl length measurements, seeds were plated
284 in $\frac{1}{2}$ MS and exposed 3 h to light after 2-5 d of stratification (4 °C, in darkness). Seedlings
285 were grown for 6 d in darkness at 22 °C. Measurements were done using the NeuronJ
286 plugin⁴⁶ in Fiji⁴⁷. The simultaneous comparisons of root and hypocotyl growth against wild
287 type for a fold change was performed for a Dunnett-type procedure ratio-to-control⁴⁸
288 assuming approximate normal distributed variance heterogeneous errors using the package
289 'mratio' in R-CRAN. Adjusted two-sided p-values are reported in figure legends.

290 For germination assays, seeds were plated in $\frac{1}{2}$ MS and stratified for 2-5 d at 4 °C in
291 darkness. Germination rates were determined after 2 d of light exposure. Imaging of *ttm3-2*
292 embryos was performed by opening siliques from *ttm3-2* heterozygous plants. Seeds were
293 mounted on a cover slip and covered by a destaining solution containing 2.7 g/L chloral
294 hydrate, 0.25 % (v/v) glycerol. Samples were destained for 16 h at 4 °C and imaged under a
295 conventional light microscope.

296

297 **Generation of transgenic lines** For constructs cloned in pH7m34GW (pH7) and
298 pB7m34GW (pB7)⁴⁹ vectors (compare Extended Data Table 1), promoters were cloned first
299 into the pDONR P4-P1R vector, coding sequences into pDONR221 or pDONR207 vectors,
300 and C-terminal tags into pDONR P2R-P3 vector with the Gateway™ BP Clonase™ II
301 Enzyme mix (Merck). Constructs were assembled by the Gateway™ LR Clonase™ Enzyme
302 mix (Merck). If avoidance of gateway cloning sites was necessary, , constructs were cloned
303 into the pGreenII vector (pGIIB) (GenBank reference: EF590266.1) or in a modified-version,
304 pGIIH by Gibson assembly⁵⁰. In the pGIIH vector, the gene conferring resistance to Basta
305 was replaced by a hygromycin resistance gene cassette by Gibson ⁵⁰. *Agrobacterium*
306 *tumefaciens*, strain pGV2260, was transformed with pH7, pB7, or with the binary vectors
307 pGIIH or pGIIB (pSOUP was used as a help plasmid, GenBank reference: EU048870.1).
308 *Arabidopsis thaliana* was transformed using the floral dip method⁵¹. T1 plants were selected
309 using hygromycin (pH7, pGIIH) or Basta (pB7, pGIIB), and homozygous plants were
310 analyzed in T3 generation.

311

312 **β-glucuronidase (GUS) reporter assay** Plants or plant organs were fixed in 2 % (v/v)
313 formaldehyde, 50 mM sodium phosphate buffer pH 7.0 for 30 min at room temperature. After
314 two washes with 50 mM sodium phosphate buffer, plants were submerged into a staining
315 solution containing 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide and 0.1
316 mM X-GlcA. Vacuum was applied 3 times, 1 min per pulse. Staining occurred for 2 h at 37
317 °C. After washing samples twice (1 h incubation per wash) with 96 % (v/v) ethanol and 60 %
318 (v/v) ethanol, respectively, plants were stored in 20 % (v/v) ethanol. Pictures were taken with
319 a Canon EOS 1000D SLR digital camera coupled to a stereomicroscope Zeiss SteREO
320 Discovery.V8.

321 **Isothermal titration calorimetry (ITC).** CDC26 and TTM3 interaction was assayed using a
322 Nano ITC (TA Instruments) at 25 °C. Both proteins were gel-filtrated into ITC buffer (20
323 mM Tris pH 8, 500 mM NaCl, 1 mM MgCl₂). 10 µL of CDC26 (at 200 µM) was injected into
324 250 µM TTM3 protein, in 25 injections at 150 s intervals. Data was corrected for the dilution
325 heat and analyzed using the software NanoAnalyze (version 3.5) provided by the
326 manufacturer.

327

328 **Immunoprecipitation followed by LS-MS** Ws-4 wild-type and Ubi10p:CDC26-6xHA
329 seedlings were snap-frozen in liquid nitrogen, homogenized with mortar and pestle and
330 resuspended in protein extraction buffer (PBS buffer pH 7.4, 1 mM EDTA, cOmplete™
331 EDTA-free Protease Inhibitor Cocktail from Merck) and including 0.1 % (v/v) Triton X-100.
332 The lysate was incubated with anti-HA microbeads (µMACS HA Isolation Kit, Miltenyi
333 Biotec) at 4 °C for 2 h. Beads were washed 4 times with protein extraction buffer
334 supplemented with 0.05 % (v/v) Triton X-100, 1 time with protein extraction buffer, and
335 eluted with the denaturing elution buffer provided in the kit. The elution was boiled for 5 min
336 at 95 °C and separated into a 10 % SDS-PAGE gel. Silver staining was performed as
337 previously described⁵². Bands present in the Ubi10p:CDC26-6xHA sample and absent or
338 reduced in the Ws-4 sample were cut and analyzed by LC-MS at the Proteomics Core Facility
339 (Centre Medical Universitaire, CMU, Geneva). Results were analyzed using the software
340 Scaffold (Proteome Software Inc, Portland, Oregon), setting a threshold of 99.9 % for peptide
341 and protein identification.

342

343 **RNA extraction and northern blot** Col-0 wild-type and *ttm3-1* mutant seedlings were snap-
344 frozen in liquid nitrogen and homogenized with mortar and pestle. RNA was isolated using

345 TRIZOL (Gibco BRL, Grand Island, NY, USA) according to the supplier's instructions. 5 µg
346 of total RNA was treated with DnaseI (Qiagen) and recovered with the standard phenol-
347 chloroform purification⁵³ (UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v),
348 Merk). Samples containing 7 µg of RNA, 0.1 % (v/v) formaldehyde and 1x MOPS buffer (20
349 mM MOPS pH 7.0, 5 mM NaOAc, 1 mM EDTA) were heated for 15 min at 60 °C, and
350 loaded into a formaldehyde gel (1 % (w/v) agarose, 1x MOPS, 1 % (v/v) formaldehyde).
351 Electrophoresis was performed for 3 h at 60 V in MOPS buffer. The gel was blotted
352 overnight in a hybond-N membrane (GE Healthcare) in 10x SSC buffer (150 mM sodium
353 citrate, 1.5 M NaCl). The membrane was cross-linked by UV and pre-hybridized with church
354 buffer (0.25 M PBS pH 7.2, 1 mM EDTA, 1 % (w/v) BSA and 7 % (w/v) SDS) for 45 min at
355 65 °C. 50 ng of *CDC26* and *TTM3* probes (compare Extended Table 2), labeled with dCTP[α-
356 ³²P] (PerkinElmer), were hybridized overnight at 65 °C. Membranes were washed with 1x
357 SSC buffer supplemented with 0.1 % SDS, and subsequently exposed to a X-ray film for 3 d
358 at -80 °C.

359

360 **5' and 3' Rapid amplification of cDNA ends (RACE)** 3' RACE was performed using the
361 ThermoFisher 3' RACE kit following the manufacturer's instructions. For the 5'RACE, RNA
362 was copied to cDNA using RACE_SP1 primer (compare Extended Data Table 2). A polyA
363 tail was added artificially with a terminal transferase (NEB) using ATP as substrate. The
364 cDNA was used as a template in a second PCR with an oligo dT fused to an adaptor primer
365 (AP) and RACE_SP2 primer. A third PCR with RACE_SP3 and AUAP gave a 5' RACE
366 specific product. The major band was cut and sent for sequencing with *TTM3_3'_F* and
367 *CDC26_RT_F* primers (compare Extended Data Table 2). Alternatively, RACE products
368 were cloned in pCR™8 (Merck) and sequenced with M13 forward and T7 forward primers.

369

370 ***In vitro* transcription and translation** *CDC26-TTM3* transcript (including the *CDC26* start
371 codon and 1268 down-stream base-pairs) was cloned into pCR_{TM}8 (Merck), under the control
372 of the T7 promoter. Mutations were performed by site-directed mutagenesis (see Extended
373 Data Table 2). Capped RNA was transcribed *in vitro* using the MEGAscriptTM T7
374 Transcription Kit (Merck). 1 µg of RNA was added to wheat germ extract (Promega), and *in*
375 *vitro* translation was performed as recommended by the manufacturer. Products from the *in*
376 *vitro* translation, labeled with ³⁵S methionine, were loaded onto a 12 % polyacrylamide gel
377 and run for ~ 2 h at 100 V. The gel was washed with water and exposed to a X-ray film for 4
378 h at -80 °C.

379

380 **Confocal microscopy** CYCB1;1-GFP seeds were crossed with the *ttm3-1* mutant allele.
381 Selection of CYCB1;1 insertion was done via Basta selection, and the presence of the *ttm3-1*
382 insertion was confirmed by genotyping. Plants were analyzed in F3 generation. 4 d old
383 seedlings were fixed in PBS with 4 % (v/v) paraformaldehyde and 0.01 % (v/v) Triton X-100
384 for 1 h at room temperature after vacuum infiltration. Samples were washed twice in PBS and
385 incubated for one week in the dark at room temperature in ClearSee solution⁵⁴. Next, samples
386 were mounted between slide and coverslip in ClearSee solution and imaged under an SP8
387 confocal microscope (Leica) equipped with a 10x NA 0.3 lens and a HyD detector using 488
388 nm excitation and 492-533 nm emission, a pinhole of 1AU and a pixel size of 180 nm.
389 Number of GFP-expressing cells was quantified⁵⁵ using the software Fiji⁴⁷. The transmission
390 image was used to estimate cell length; the last cortical cell, the length of which was
391 approximately 1.5 times its width, was defined as the last cortical meristematic cell and was
392 used to define the limit of the meristem. Maximal intensity projections of the confocal z

393 stacks were performed and the look-up table “Fire” was used to optimize the visualization of
394 GFP signal. Localization of the TTM3-mCITRINE fusion protein was analyzed in 5 d old
395 seedlings again using the SP8 confocal microscope.

396

397 **Polysome profiling.** Wild-type and two independent transgenic lines expressing CDC26-
398 *TTM3 (in *ttn3-2* background) were grown for 10 days in ½ MS medium. Alternatively, 7-
399 days old wild-type seedlings were treated for 4 h in 1/8 MS liquid medium containing either 1
400 µM AZD or DMSO (mock). Seedlings were snap-frozen and homogenized with mortar and
401 pestle. The material was resuspended in 1 volume of polysome extraction buffer, containing
402 200 mM Tris pH 9.0, 200 mM KCl, 1 % DOC, 1 % PTE, 35 mM MgCl₂, 1 mM DTT and 100
403 µg/mL cycloheximide. After 15 min incubation on ice, the cell extract was centrifuged for 15
404 min at 16000 g, 4 °C. The clarified extract was loaded on top of a 15 % to 60 % sucrose
405 gradient. Polysomal fractions were separated by ultracentrifugation on a SW55 rotor
406 (Beckman), at 50000 rpm, 1 h 15 min, 4 °C, and collected from top to bottom into 10
407 fractions using a gradient holder (Brandel) coupled to a spectrophotometer. RNA from each
408 fraction was extracted using TRIZOL (Gibco BRL, Grand Island, NY, USA), according to
409 the supplier's instructions. After a second RNA precipitation (500 mM ammonium acetate,
410 2.5 volumes of ethanol, 1 h, -20 °C), RNA was reverse transcribed into to cDNA using the
411 M-M-MLV RNase H minus kit (Promega) and oligo dT, and analyzed by qRT-PCR as
412 described before. The fraction 1 was excluded from the analysis since it contained very low
413 amount of both *TTM3* and *ACT* mRNAs. The percentage of TTM3 mRNA in each fraction
414 (relative to *ACT2*) was determined as described⁵⁶.

415

416 **Data availability** Authors confirm that all relevant data has been included in this paper and it
417 is available upon reasonable request.

418 **FIGURE LEGENDS**

419 **Figure 1 | Different *ttm3* mutant alleles show inconsistent phenotypes related to embryo**
420 **development and plant growth. a**, Overview of *ttm3* alleles in the *TTM3* locus. **b**, *TTM3*
421 transcript and protein levels in *ttm3* mutants relative to wild-type, estimated by qPCR (left)
422 and western blot (right), respectively (error bars represent standard deviation (SD) of three
423 replicates). **c**, Developing wild-type and *ttm3-2* embryos in different stages (scale bars are 20
424 μm). Quantification of embryo-developmental phenotypes in seeds from *ttm3-2* heterozygous
425 plants is shown alongside. **d**, Seedling root growth assay (n=13 seedlings per genotype).
426 Representative *ttm3* mutant and wild-type seedlings are shown. Quantification of root length
427 (normalized to wild-type root length average) is represented with box plots (p-values: *ttm3-*
428 *1/wild-type*<0.001, *ttm3-3/WT*=0.693, *ttm3-4/WT*=0.019). **e**, Hypocotyl growth assay (n=62
429 seedlings per genotype). Representative seedlings are shown. Hypocotyl length
430 measurements (normalized to hypocotyl length average in wild-type) are represented with
431 dots (p-values: *ttm3-1/WT*<0.001, *ttm3-3/WT*=<0.001, *ttm3-4/WT*=0.002).
432

433 **Figure 2 | Catalytically inactive TTM3 variants complement *ttm3* phenotypes when**
434 **expressed from the native promoter including the 5' UTR. a**, Complementation of the
435 embryo lethal *ttm3-2* allele with TTM3-mCITRINE restores seed germination. Shown are
436 representative seedlings with the corresponding germination rates (%). Total number of seeds
437 analyzed is shown in brackets. Scale bars correspond to 0.5 cm. **b**, Seedling root growth
438 assay with *ttm3-1* plants expressing TTM3-mCITRINE (n=12). Representative seedlings are
439 shown. Quantification of root length (normalized to wild-type) is represented in box plots

440 (p_values: #8/WT=0.479, #12/WT=0.600). **c**, Ribbon diagram of the AtTTM3 tunnel domain
441 (in yellow) with a triphosphate molecule bound in the center, coordinated by a Mn²⁺ ion (pink
442 sphere). Arg52 involved in substrate binding²¹ is shown in cyan, three glutamate residues
443 required for metal co-factor binding and catalysis²¹ are highlighted in magenta (Glu2, Glu4
444 and Glu169). **d**, AtTTM3 mutant proteins, impaired in either substrate binding or catalysis
445 fully complement the *ttm3-2* mutant phenotype when expressed from the *TTM3* promoter
446 including the annotated 5' UTR, as judged from seed germination assays. Shown are
447 germination rates, with total number of seeds analyzed in brackets. **e**, TTM3-mCITRINE is
448 expressed at lower levels than endogenous TTM3, as judged from western blotting, while **f**,
449 CDC26, translated from the *TTM3* 5'UTR, is expressed at wild-type levels.

450

451 **Figure 3 | A uORF in *TTM3* encodes the cell-cycle regulator AtCDC26.** **a**, Arabidopsis
452 *CDC26* maps to the 5' UTR of *TTM3* and contains the N-terminal CDC26 motif required for
453 APC/C binding. Sequence identity is shown alongside **b**, CDC26 and TTM3 proteins are
454 expressed in different tissues and stages of development. **c**, *CDC26* transcript and protein
455 levels in different *ttm3* mutants relative to wild-type, measured by qPCR (left) and western
456 blot (right), respectively (error bars represent SD, n=3). **d**, Germination assay in *ttm3-2* plants
457 complemented with Ubi10p:CDC26-6xHA. Shown are percentages of germinated seeds in
458 total number of seeds (in brackets, scale bars are 0.5 cm). AtCDC26-6xHA protein levels
459 were detected by western blot using an anti-HA antibody (right). **e**, Seedling root growth
460 assay in *ttm3-1* plants complemented with Ubi10p:CDC26-6xHA vs. *ttm3-1* and normalized
461 to wild-type (n=24) (p-values: #8/WT=0.920, #6/WT=0.017). **f**, Hypocotyl growth assays in
462 *ttm3-1* plants complemented with Ubi10p:CDC26-6xHA in relation to wild-type (n=53) (p-
463 values: #6/WT=0.037, #8/WT=0.447). **g**, APC/C components recovered by IP-MS (arrows

464 indicate bands present in CDC26-6xHA and absent or reduced in wild-type protein extracts).
465 AtCDC26 protein interactors are highlighted in the human APC/C structure (PDB-ID 4ui9₂₄)
466 (bottom). **h**, Root tips of *ttm3-1* plants (n=6) expressing p:CYCB1;1-GFP show less GFP
467 expressing-cells and an overall lower GFP intensity compared to wild-type plants expressing
468 CYCB1;GFP. Quantification of GFP signal is shown alongside. Scale bars correspond to 25
469 μm .

470

471 **Figure 4 | CDC26 is expressed from a bicistronic transcript in plants.** **a**, Phylogenetic tree
472 of *CDC26* and *TTM3* ORFs from different plant species. Distances between the *CDC26*
473 termination codon and the *TTM3* start codon are indicated in base-pairs. **b**, Northern blotting
474 reveals a major transcript detected with *CDC26* and *TTM3* probes and absent in *ttm3-1* plants
475 (indicated with an arrow). **c**, RACE experiments result in cDNA products containing either
476 *CDC26* and *TTM3* ORFs or *CDC26* alone (see Supplementary Information).. *ttm3-1* mutants
477 harbor a truncated transcript containing only the *CDC26* uORF (see Supplementary
478 Information). **d**, RT-PCR using primers binding to *CDC26* and *TTM3* confirm the presence
479 of a single *CDC26-TTM3* transcript (blue), absent in *ttm3-1*, and a *CDC26* transcript (red),
480 present in low levels in *ttm3-1*. **e**, *In vitro*-translation of an *in vitro* transcribed *CDC26-TTM3*
481 bicistronic transcript results in two proteins of the expected molecular weight. Calculated
482 molecular weight is 7,225 Da and 24,162 Da for AtCDC26 and AtTTM3, respectively.
483 Mutation of the *CDC26* or *TTM3* start codon blocks translation of the respective protein.

484

485 **Figure 5 | TTM3 translation recruits the transcript to polysomes in a TOR-independent**
486 **manner.** **a**, Transcripts containing mutations in *CDC26* and *TTM3* start codons (**CDC26* and
487 **TTM3*, respectively) used for complementation of *ttm3-2* plants. **b**, **CDC26* does not

488 complement *ttm3-2* embryo-lethality, as shown in 4 independent transgenic lines. **c**, *TTM3
489 plants show a phenotype reminiscent of *apc* loss-of-function mutants. Bottom picture shows a
490 detail of wild-type and *TTM3 siliques (scale bar is 2 cm). **d**, CDC26 protein levels are
491 reduced in *TTM3 plants (top), while transcript levels are higher than wild-type (bottom).
492 Error bars denote SD for n=3. **e**, Polysome profile (top) reveals that *TTM3* transcript
493 associates with polysomes in wild-type but not in *TTM3 plants (bottom). Error bars denote
494 SD for n=3. **f**, Polysome profiles of wild-type seedlings treated for 4 h with the TOR-
495 inhibitor AZD8055 (AZD) show changes for *bZIP11*, but not for *TTM3*. Error bars denote SD
496 for n=3. **g**, 4-days old seedlings treated for 3 days with 3 μ M TOR-inhibitors show a
497 reduction in CDC26 and TTM3 protein levels comparable to actin (top). *TTM3* mRNA levels
498 are equal or higher upon treatment (bottom).

499

500 **Figure 6 | The CDC26-TTM3 transcript is a target of NMD.** **a**, Treatment of 5-days old
501 wild-type plants with 20 μ M cycloheximide (CHX) for 8 h leads to the accumulation of
502 *SMG7*, a known NMD target, and *CDC26-TTM3* transcript. **b**, *CDC26-TTM3* transcript and
503 protein levels are higher in Arabidopsis *lba1* and *upf3-1* mutants, defective in NMD. Error
504 bars denote SD for n=3.

505

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507

508 **Supplementary Information** is available in the online version of the paper (Extended Data
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510

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518

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520 majority of the experiments and analyzed data. J. W. and A. P. characterized *ttm3* insertion
521 lines, the *ttm3-2* embryo phenotype, performed localization experiments and produced the
522 TTM3 antibody. J. D. and L. L-O designed and performed the polysome profile assays. J. M.
523 together with L. L-O. purified the AtCDC26 protein and antibody. S. L. quantified CYCB1;1

524 levels, Y. J. generated transgenic reporter lines, L. A. H. performed statistical analyses, L. L-
525 O., J. W., S. L., L. A. H. and M. H. analyzed data, and Y. P. and M. H. supervised the study.
526 L. L-O. and M. H. drafted the manuscript and all authors discussed the results, edited and
527 approved the final versions of the manuscript.

528

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531

532

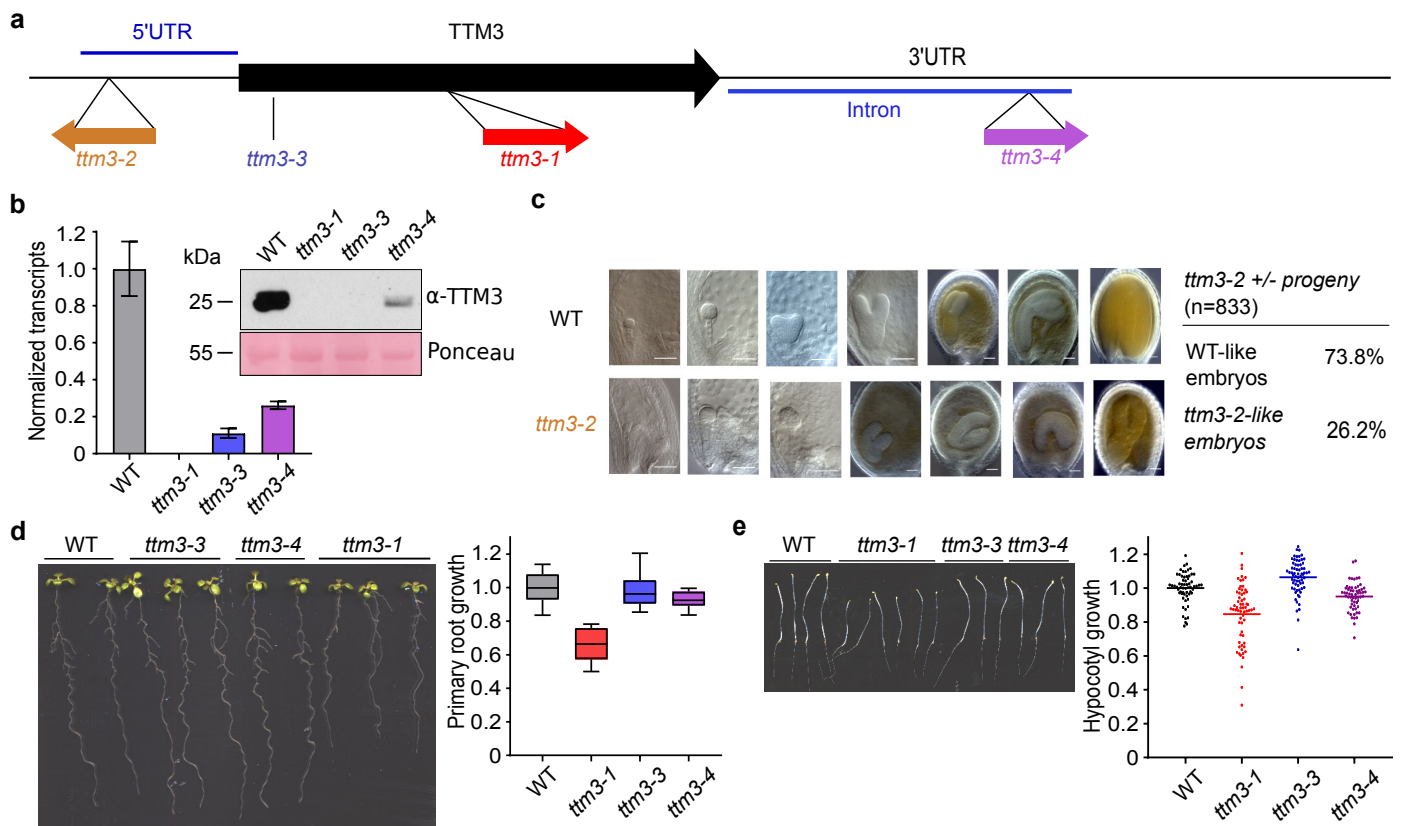


FIG 1

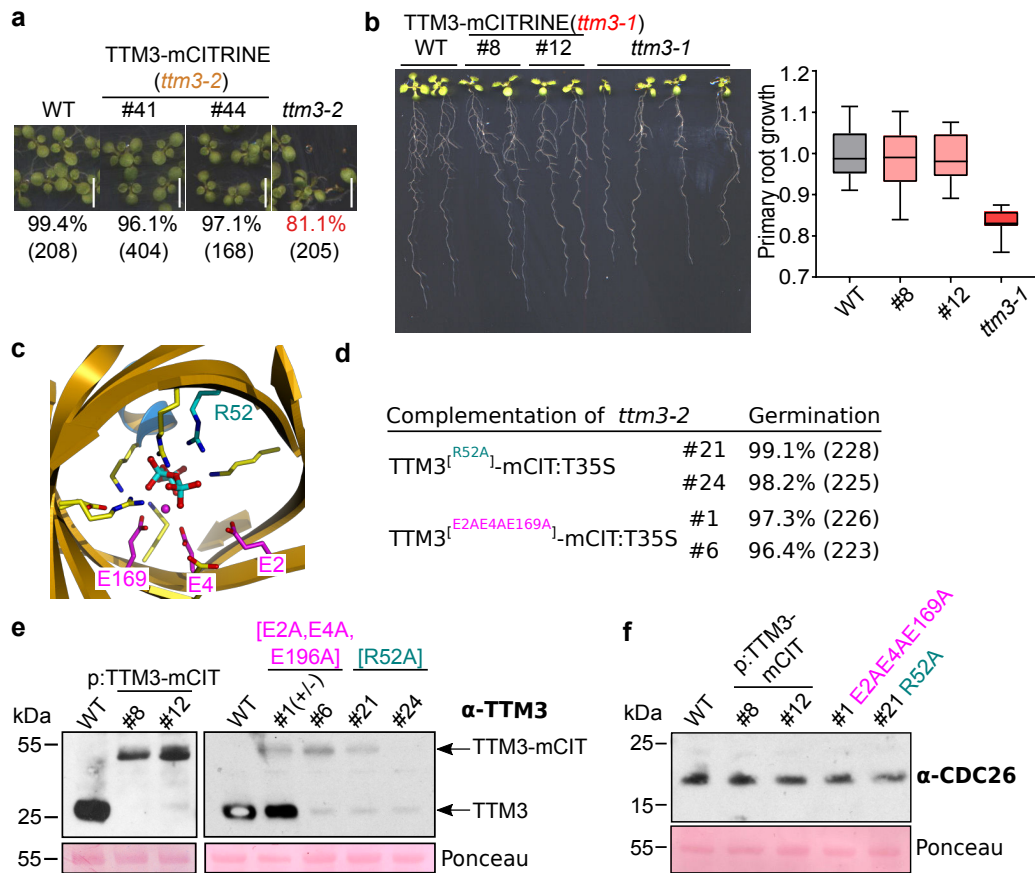


FIG 2

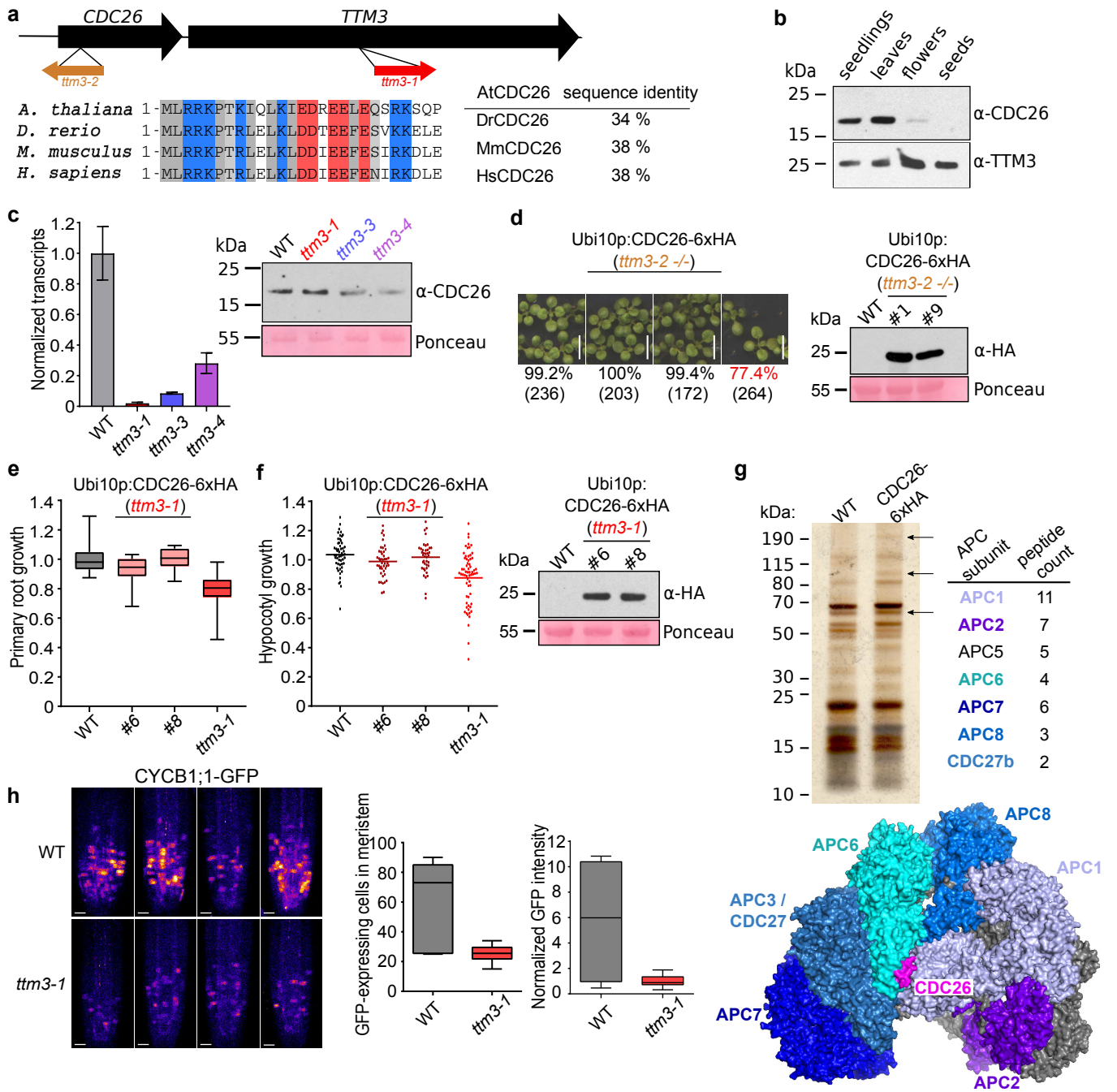


FIG 3

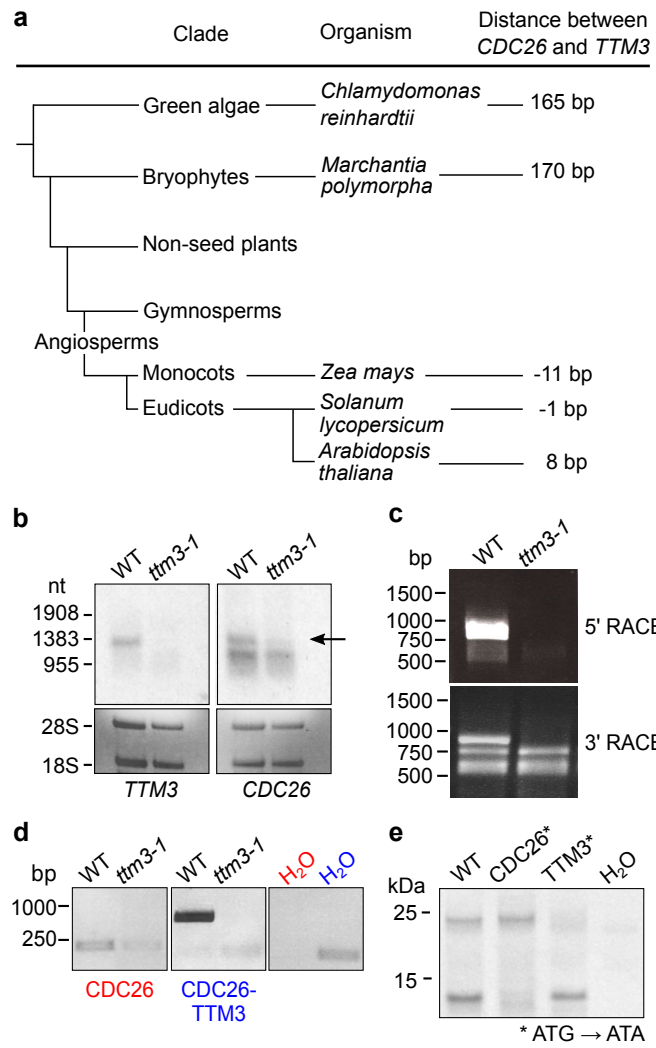


FIG 4

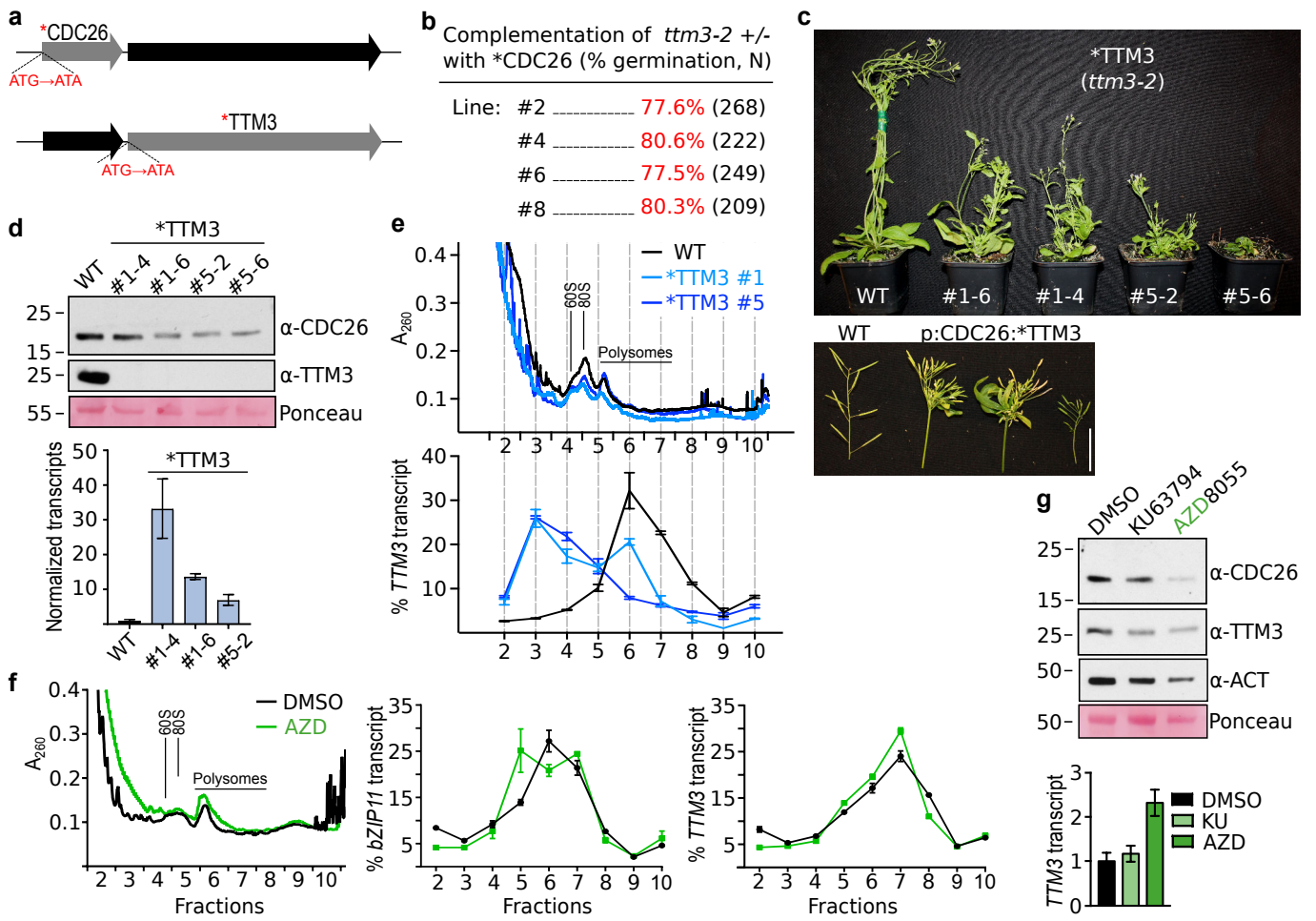


FIG 5

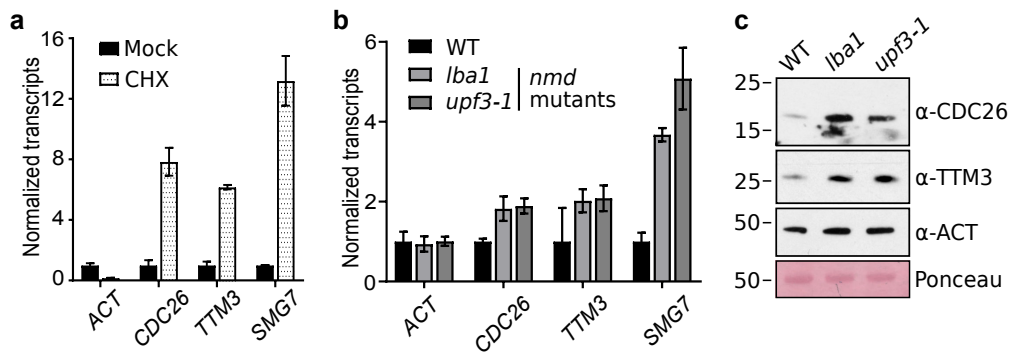


FIG 6

a

TTM3

```
C. reinhardtii 1- MEVEIKIRLPDRAAYEQVAAALAAAPGGKGRLDSHAQGYRMVLVTPTRSRCARPHQANYFFDGPNQELNSRRVLLRVRTYDVD---- -82
M. polymorpha 1- MEVEVKLKLPGREAHEKVASSLKAF-----HEVTHMQENVFFDGANKELSSKRAVLRRLRFYNGD---- -59
Z. mays 1- MEVEIKLRLLPDAAAHRRLSAFLAP-----RLRRTHAQRNLFFDDAARTLGAATAALRVRLYDGPDD-- -61
S. lycopersicum 1- MEVEVKLRLPDSSAHQVLSLFFSS-----HHKKTTHQRNTFFDGAAGELSSRRAVLRRLRFYENSE--- -60
A. thaliana 1- MEVEVKLRLLLTAAAHRLRLLTLLTP-----YHLKTLHQRNFFDTPKNDLSLRRAVLRRLRFQNAAVSA -63

C. reinhardtii 83- ----K KATVTLK GKQILENG IGRASEVEAEVPPAAAAAYLTQPSRML-AEVP IVKDAAEKFGVG---SLVALGGFQNRDVEYEW -158
M. polymorpha 60- ----G KCVVTFK GNAVIVDGISRGELEEDIDVSLGRACVAEPWRLATTTCKLLNKVVADFAC---EDFVCLGGFRNVRTVFNW -136
Z. mays 62- --R GPSRAVLAL KRRPRIEAGVSRVEEIEEPLPALAVACADDPARLGGLDSP IIRLVAAEYGVGGDAAPFLCLGGFNTRAVYEY -145
S. lycopersicum 61- ---KVKCMVCLKAKAVIIDGVS RVEDEEELDPKIGYECVSNPRKLM EVD SRVLKRAREEFHVG-E-EGFIGLGGFKNVRNVFEW -140
A. thaliana 64- ASPSPPRCIVSLKAKPTLANGISRVEDEEEIEYWIGKECVESPAKLSDIGSRVLKRVKKEYGFN-DFLGFVCLGGFENVRNVYEW -148

C. reinhardtii 159- E-----GHTLELDETKFEHGTLYEIEVETE QPEVLRDRLEHWLSGMGVSYSYSQTSKFA NFINKTLL- -220
M. polymorpha 137- E-----GLKIELDETQYDFGTTYEVECESTDPERVREVLGDFL KSKGIEFTYSTKSKFAVFRSGKIE- -198
Z. mays 146- ELEDGGGGLVLELDETRFDGTRYELCE TAEPDRVKEVLERLLTVAGVPYCYCRNSKFA CFMTGKLLP -214
S. lycopersicum 141- C-----GVELEVDETM YDFGT FYEIECESLEPEKVKAMIEAFLKDNDIDYSYSEVSKFATFRAGKLP- -202
A. thaliana 149- R-----GVKLEVD ETKYDFGNCY EIECETE EPERVKTMI EEF LTEEKIEFSNSDMTKFAVFRSGKLP- -210
```

AtTTM3 protein	Identity
CrTTM3	39 %
MpTTM3	47 %
ZmTTM3	45 %
SITTM3	59 %

b

CDS

deleted in *ttn3-3*

```
Ttm3 1 - ATGGAAGTCGAAGTCAAGCTCCGTCCTCCTAACCGCCGCTGCTCATCTCCGTCTCACC ACTCTCCTCACTCC (...) TGA - 633
ttn3-3 1 - ATGGAAGTCGAAGTCAAGCTCC TGCTCATCTCCGTCTCACC ACTCTCCTCACTCC (...) TGA - 617
```

Protein

```
Ttm3 1 - MEVEVKLRLLLTAAAHRLRLLTLLTPYHLKTLHQRNFFDTPKNDLSLRRAVLRRLRFQNAAVSAASPSPPRCIVSLA (...) KLP - 210
ttn3-3 1 - MEVEVKLLLSVSPSSLHTTSKPFTNATPSSSTHPKTTSLSAAPSSVSASFKTPPPRLLLLRRRVSSLLKRSQL - 75
```

FIG S1

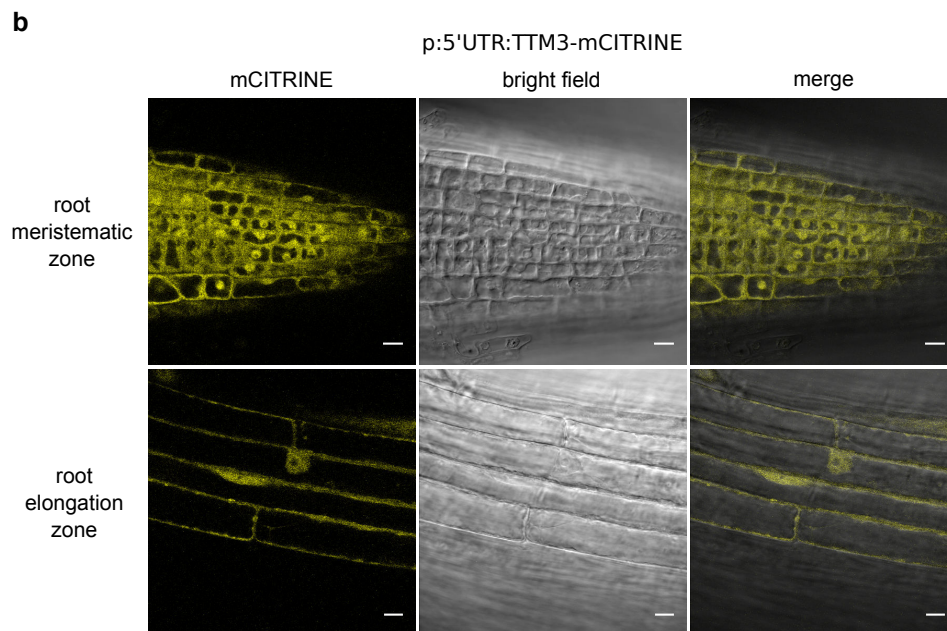
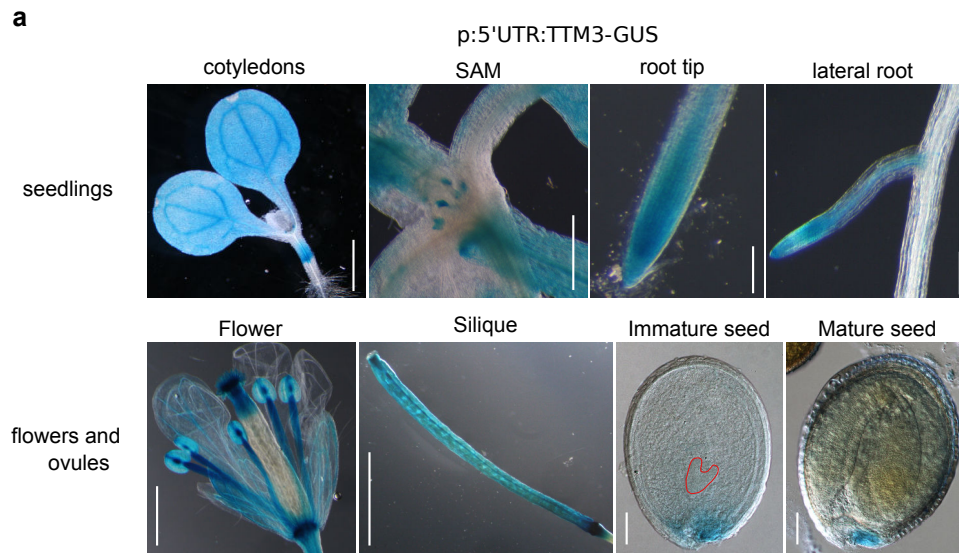


FIG S2

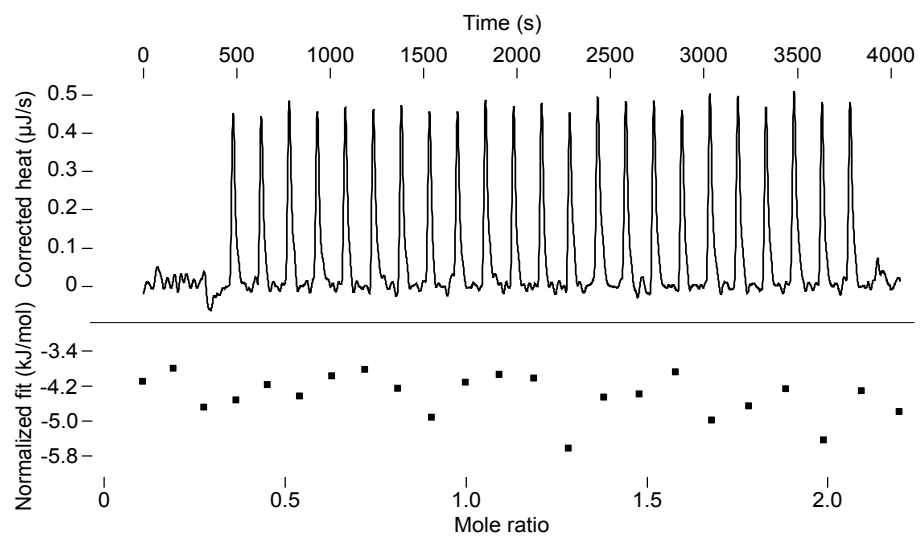


FIG S4

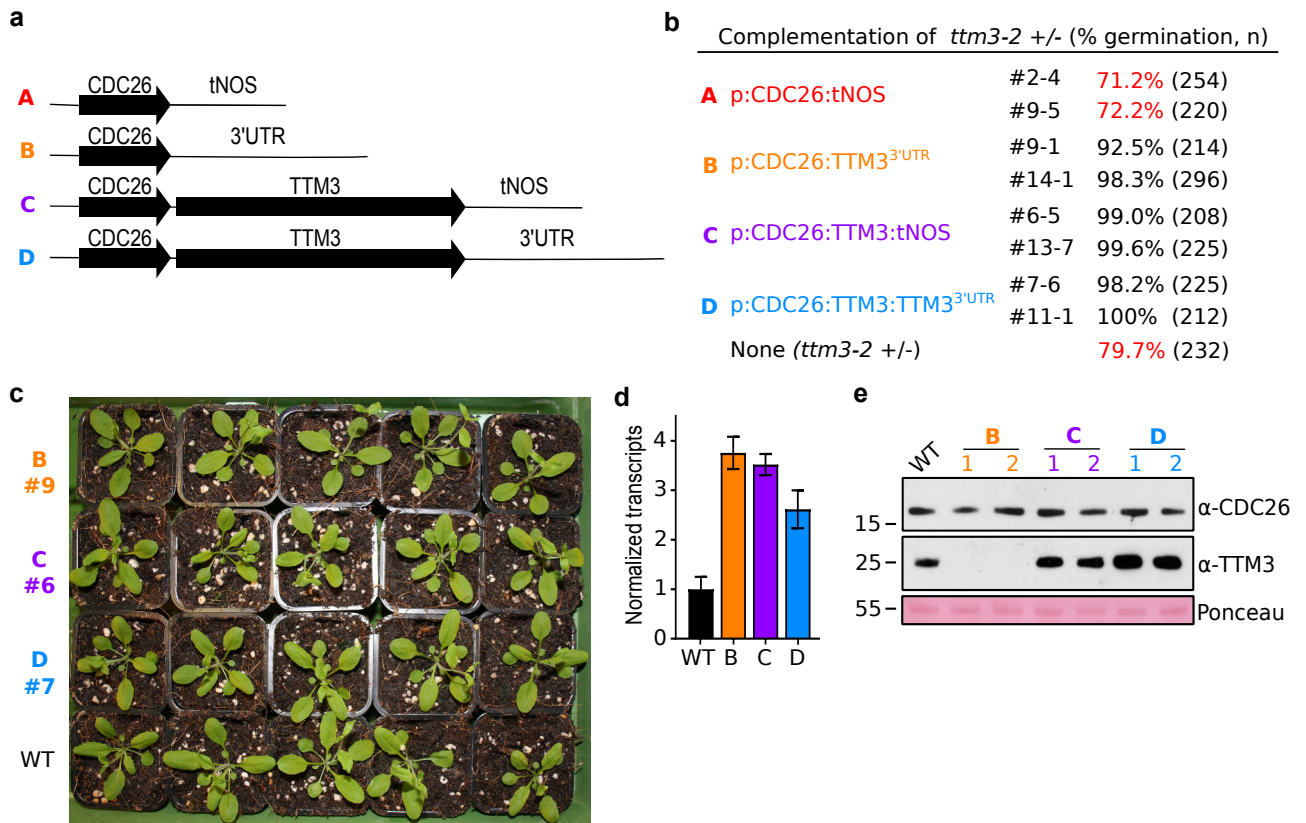


FIG S5

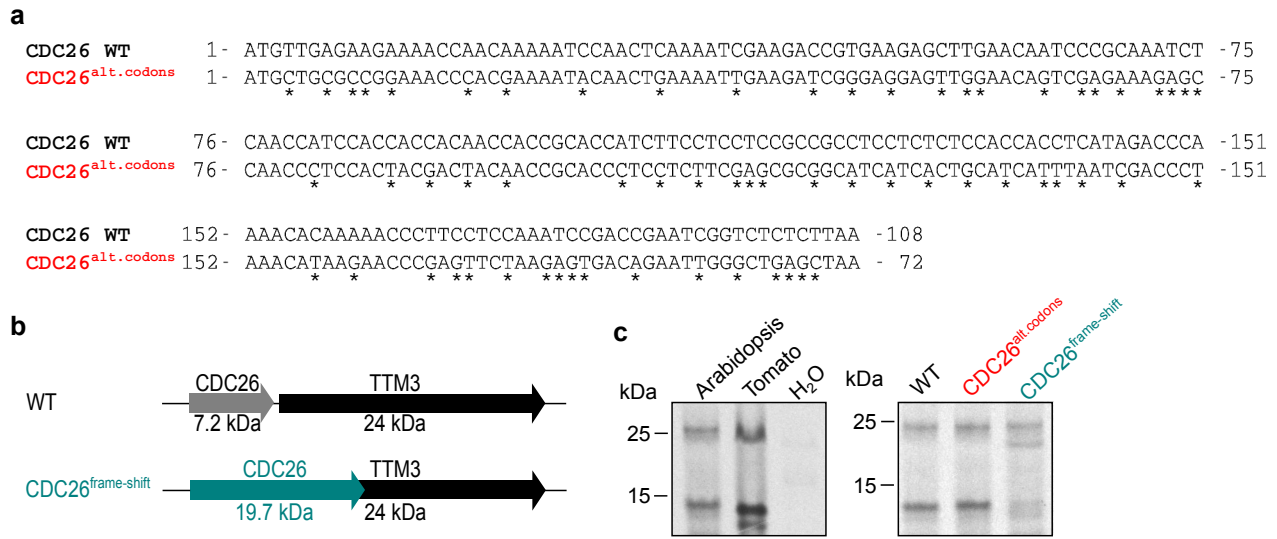


FIG S6