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► **To cite this version:**

Nicolas Max Doll, Eduardo Berengue, Jekaterina Truskina, Gwyneth Ingram. AtEXT3 is not essential for early embryogenesis or plant viability in Arabidopsis. *New Phytologist*, 2022, 236 (5), pp.1629-1633. 10.1111/nph.18452 . hal-03841277

HAL Id: hal-03841277

<https://cnrs.hal.science/hal-03841277v1>

Submitted on 7 Nov 2022

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AtEXT3 is not essential for early embryogenesis or plant viability in Arabidopsis.

Nicolas Max Doll^{*†}, Eduardo Berenguer[†], Jekaterina Truskina and Gwyneth Ingram^{1*}

¹ Laboratoire Reproduction et Développement des Plantes, ENS de Lyon, CNRS, INRAE, UCBL, F-69342, Lyon, France.

[†] Authors contributed equally to the study.

* Authors for correspondence : Nicolas.Doll@ugent.vib.be, Gwyneth.Ingram@ens-lyon.fr

ORCID IDs

N.M. Doll: 0000-0001-5429-8920

E. Berenguer: 0000-0002-0902-1057

G. Ingram: 0000-0002-1425-9545

Running title: *AtEXT3* is a non-essential gene

32 EXTENSINs (EXTs) are a subclass of the very diverse family of cell wall proteins known as
33 hydroxyproline-rich glycoproteins. The EXT subclass is defined by the presence of at least
34 two repeats of the Ser-Pro₃₋₅ motif in which the proline residues can be hydroxylated and,
35 subsequently, O-glycosylated with arabinose containing oligosaccharides leading to the
36 formation of hydrophilic domains (Cannon *et al.*, 2008; Velasquez *et al.*, 2011; Ogawa-
37 Ohnishi *et al.*, 2013). In addition, many EXTs from land-plants also contain distinct and
38 characteristic hydrophobic Tyr-containing motifs (Schnabelrauch *et al.*, 1996; Held *et al.*,
39 2004; Cannon *et al.*, 2008). These have recently been termed cross-linking (CL)-EXTs,
40 because of their ability to undergo peroxidase-mediated covalent cross linking *in vitro*
41 through their Tyr residues, a phenomenon that has been proposed to underlie important
42 structural functions in plant cell walls (Marzol *et al.*, 2018). EXTs have a remarkable ability to
43 self-assemble into dendritic networks *in vitro* (Cannon *et al.*, 2008). This capacity, proposed
44 to be based on self-ordering due to the alternation of hydrophobic and hydrophilic
45 domains (Lampert *et al.*, 2011), may mediate observed interactions with other cell wall
46 components, including pectins (Valentin *et al.*, 2010).

47 Despite an abundance of information regarding the biochemical behavior of EXTs *in*
48 *vitro*, information on their biological functions *in vivo* remains remarkably scarce, possibly
49 due to extensive redundancy between the multiple genes encoding EXTs and EXT-like
50 proteins within plant genomes. Work in Arabidopsis has identified potential roles for AtEXTs
51 in promoting root-hair elongation (Velasquez *et al.*, 2011), pollen development (Choudhary
52 *et al.*, 2015) and early embryogenesis (Hall & Cannon, 2002; Saha *et al.*, 2013; Chen *et al.*,
53 2015). This last function is our focus in this comment.

54 Following an enhancer-trap (transposon)-based mutagenesis screen, Hall and Cannon
55 (Hall & Cannon, 2002) isolated a seedling-lethal mutant called *root-shoot-hypocotyl-*
56 *defective (rsh)* which, when homozygous, produces disorganized embryos that arrest either
57 before (Cannon *et al.*, 2008) or just after (Hall & Cannon, 2002) germination. Mutants show
58 clear defects in cell-plate formation. A transposon insertion in the promoter of the *AtEXT3*
59 gene was stated to be the causal lesion in *rsh* mutants. Consistent with this,
60 complementation of the *rsh* phenotype using an *AtEXT3* genomic DNA fragment was
61 reported. However, in two subsequent studies, “self-rescue” of the *rsh* phenotype to a fully
62 wild-type phenotype was reported in up to 20% of genetically *atext3* homozygous plants
63 (Saha *et al.*, 2013; Chen *et al.*, 2015). No intermediate phenotypes were reported, and
64 “rescue” was transmitted stably to all the progeny in subsequent generations. This calls into
65 question the validity of genetic complementation experiments, since the reported “self-
66 rescue” phenomenon would interfere with this approach. A mechanism involving
67 compensatory regulation of other AtEXT-encoding genes was evoked to explain this
68 phenomenon. Nonetheless it appears difficult, based on the published data, to exclude the
69 possibility that the reported *rsh* phenotype is caused by a lesion linked to the original
70 transposon insertion in *AtEXT3*, but affecting a separate locus, and lost by segregation in
71 “rescued” lines.

72 We became interested in the *AtEXT3* gene as a result of our discovery that the
73 developing embryo of Arabidopsis is covered with an endosperm-derived “sheath” rich in
74 epitopes detected by anti-EXT antibodies (Moussu *et al.*, 2017). *In silico* data (Le *et al.*, 2010)
75 concur with published *in situ* hybridization data (Francoz *et al.*, 2016) in showing that *AtEXT3*
76 is expressed primarily in the embryo-surrounding endosperm during Arabidopsis seed
77 development, rather than in the embryo itself. We were therefore keen to further
78 investigate the function of *AtEXT3* and we generated a series of new alleles in the Col-0

79 background using the CRISPR-Cas9 based technique. We selected 4 alleles containing
80 deletions or insertions within the 5' coding sequence of the *AtEXT3* gene (Figure 1A). All are
81 predicted to produce either strongly truncated or frame-shifted proteins (Figure 1B). Being
82 aware of recent work suggesting that 5' mutations generated by CRISPR can lead to the
83 production of functional proteins due to the use of downstream in-frame ATGs (Smits *et al.*,
84 2019), we verified the *AtEXT3* coding sequence (which lacks introns) for such sequences.
85 None are present. We therefore conclude that all four alleles are null. Homozygous plants
86 were all fully phenotypically wild-type in terms of root growth, plant growth and
87 reproductive development (Figure C-E). No defective seeds or seedlings were detected for
88 any line, in any generation.

89 Our results suggest that loss of AtEXT3 do not lead to detectable defects in development
90 in the Col-0 background. However, the *rsh* mutant originally described by Hall and Cannon
91 was identified in the Landsberg *erecta* (L-*er*) background. To eliminate the possibility that a
92 suppressor exists in Col-0 that is not present in the L-*er* background, we carried out CRISPR
93 experiments in the L-*er* background, this time using guides predicted to interrupt the
94 EXTENSIN 2 domains of the AtEXT3 protein. Of multiple null alleles brought to homozygosity
95 we selected three deletions predicted to produce either strongly truncated or frame-shifted
96 proteins (Figure 2). We were again unable to detect any defects in root growth, plant growth
97 or reproductive development in any of these lines. In addition, we confirmed the absence of
98 phenotypes during seed development, and germination (Figure S1).

99 Taken together, our results indicate that AtEXT3 is not essential for embryogenesis and
100 plant development in Arabidopsis, suggesting that its function is likely redundant with other
101 related proteins. We feel that it is important that our result be communicated to the
102 scientific community, since the role of AtEXT3 has been very widely cited as a proof of the
103 important function of EXTs in plant development and particularly in cytokinesis. Although
104 fully convinced that EXTs are of primary importance in plants, we propose that the roles of
105 AtEXT3 may be almost fully redundant with those of other related proteins. Supporting this
106 hypothesis, phenotypeless *atext3* mutants were shown to have extensive changes in the
107 expression of other AtEXT-encoding genes, or the accumulation of AtEXT proteins (Saha *et al.*,
108 2013; Chen *et al.*, 2015). It is possible that a similar phenomenon occurs in the CRISPR
109 generated *atext3* mutants.

110 Finally, our study does not call into question the presence of AtEXT3 in cell walls post
111 germination, or the beautiful biochemical studies of AtEXT3 behavior *in vitro* that have been
112 published (Cannon *et al.*, 2008).

113

114 **Material and methods**

115

116 **CRISPR/Cas9 allele generation**

117 The CRISPR/Cas9 alleles of *AtEXT3* (*AT1G21310*) were generated through the method
118 described by Wang and co-workers for double guides assembly (Wang *et al.*, 2015). The 2
119 guides were designed on the CRISPR-P v2.0 software and chosen to target the 5' end of the
120 gene (first guide: 5'-GAAGAATAGAAATAGTTAG-3', second guide: 5'-TATTCTTCTCCTCCACCAC-
121 3'). For the cloning, the primers used were 5'-
122 ATATATGGTCTCGATTGGAAGAATAGAAATAGTTAGGTT-3' and 5'-
123 TGGAAGAATAGAAATAGTTAGGTTTTAGAGCTAGAAATAGC -3' for the first guide and 5'-
124 AACGTGGTGGAGGAGAAGAATACAATCTCTTAGTTCGACTCTAC -3' and 5'-

125 ATTATTGGTCTCGAAACGTGGTGGAGGAGAAGAATACAA -3' for the second guide. The
126 pHEE401 plasmid was used as the destination vector.

127

128 For the generation of the CRISPR/Cas9 alleles of *EXT3* in the *L-er* background the method
129 described by Stuttmann and co-workers was used (Stuttmann *et al.*, 2021). Two guides (first
130 guide: 5'-GGTGGGGAGTGGTATACCGG-3', second guide: 5'-ATACAAATCTCCACCTCCAC-3')
131 were inserted into the pDGE332 and pDGE334 shuttle vectors and recombined into the
132 pDGE347 recipient vector.

133

134 **Stable transformation of *Arabidopsis thaliana* (L.) Heynh.**

135 The plasmids generated were first transformed into the C58PMP90 *Agrobacterium* strain by
136 electroporation at 2,2kV in a 1mL cuvette (Eurogenetec). *Agrobacteria* were then grown at
137 28°C for 2h in LB liquid media without antibiotics before being spread on Petri dishes
138 containing YEB solid media with rifampicin (50 mg.L⁻¹), gentamicin (20 mg.L⁻¹) and kanamycin
139 (50 mg.L⁻¹) for the pHEE401 based plasmid or rifampicin (50 mg.L⁻¹), gentamicin (20 mg.L⁻¹)
140 and spectinomycin (100 mg.L⁻¹) for the pDGE347 based plasmid. After 2 days, the
141 *Agrobacteria* were used to transformed Col-0 plants by the floral-dip method (Logemann *et*
142 *al.*, 2006). Selection of transgenic plants was performed on MS plates with 30 mg.L⁻¹ of
143 hygromycin.

144

145 **Identification of the mutant alleles and generation of homozygous plants.**

146 Amplification of the region around the cutting sites was performed by PCR on DNA extracted
147 from transgenic Col-0 plants using the 5'-GGGTGTGAAGGGAAGGCACTAAATC-3' and 5'-
148 GTAAACGTAGTGCTTCTTTGGTGG-3' primers. For the transgenic *L-er* plants the primers used
149 were 5'-GGGTGTGAAGGGAAGGCACTAAATC-3' and 5'-AGGTGGGGGTGGGGAATGGTA-3'.
150 Sequencing was carried out with the 5'-GTAAACGTAGTGCTTCTTTGGTGG-3' (Col-0) or and 5'-
151 GGGTGTGAAGGGAAGGCACTAAATC-3' (*L-er*) primers. Homozygous mutants for *atext3* were
152 selected from the T2 plants. In the Col-0 background the CRISPR/Cas9 cassette was removed
153 by selection of hygromycin sensitive plants and validated by an absence of cassette
154 amplification by PCR with the 5'-TGTCCCAGGATTAGAAATGATTAGGC-3' and 5'-
155 AGCCCTCTTCTTCGATCCATCAAC-3' primers. Homozygous mutants were confirmed by
156 sequencing. In *L-er* transgenic plants non-fluorescent seeds were selected in the T2 and
157 homozygous mutants were confirmed by sequencing.

158

159 **Plant growth**

160 For the selection processes and *in vitro* growth (Figure 1C and 2C), seeds were gas sterilized
161 with Chlorine gas (3mL of HCl (33%) in 100mL of bleach) for at least 3 hours in a hermetic
162 box. Seeds were then sown on MS medium with 0.5% of sucrose and, for the selection of
163 Col-0 mutants, with 30 mg.L⁻¹ of hygromycin. Stratification was carried out 2 days at 4°C in
164 darkness. Plates were then transferred into growth chambers in long day condition (16h
165 light, 21°C). For the *in vitro* growth experiments, plants were grown for 14 days (Col 0) or 10
166 days (*L-er*) and pictures were taken with an EPSON perfection V300 PHOTO scanner. For
167 germination tests, plants were grown for 12 days (100 seeds per plate). In all the other
168 cases, seedlings were transferred after 7 days onto soil (Argile 10 (favorit)) and grown in long
169 day conditions (16h light, 21°C). Images of plants at 29 DAS and 46 DAS (Figure 1D and E) or
170 28 DAS and 46 DAS (Figure 2D and 2E) were taken with a Cannon EOS 450D camera with a

171 Sigma 50 mm f/2,8 DG Macro objective. For analysis of silique filling, individual siliques were
172 opened at the mature green stage, and seeds were counted under a binocular microscope.

173

174 **Seed clearing**

175 To visualize developing seeds, the siliques at 7 DAP were dissected with a needle and forceps
176 on adhesive tape on a microscope slide and mounted in the clearing solution (1 vol glycerol /
177 7 vol chloral hydrate liquid solution, VWR Chemicals). After 48h incubation at 4°C samples
178 were imaged under Zeiss Axio Imager M2 microscope.

179

180 **Acknowledgements:** We thank Alexis Lacroix, Patrice Bolland, Justin Berger, Isabelle
181 Desbouchages and Hervé Leyral for technical assistance, Cindy Vial, Stéphanie Maurin,
182 Laureen Grangier and Nelly Camilleri for administrative assistance.

183

184 **Funding:** The study was financed by the French Agence National de Recherche (ANR- 17-
185 CE20-0027) (supporting J.T. and N.M.D.) and ANR- 20-CE20-CLEANSE (supporting E.B.).
186 N.M.D. was also funded by a PhD fellowship from the Ministère de l'Enseignement Supérieur
187 et de la Recherche.

188

189 **Author contributions:** G.I. and N.M.D. led the study. G.I. obtained funding for the study. G.I.,
190 supervised the work. N.M.D., E.B and J.T carried out the experiments. G.I. and N.M.D. wrote
191 the paper with input from all authors. N.M.D. and E.B. contributed equally.

192

193 **Data availability:** The data that support the findings of this study are available from the
194 corresponding author upon reasonable request.

195

196 **Competing interests:** None declared.

197

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261

262 **Keywords:** cell wall, cytokinesis, development, embryogenesis, EXTENSIN

263

264 **Figure Legends:**

265 **Figure 1: Loss of AtEXT3 function gives no obvious phenotypes in the Arabidopsis Col-0**
266 **background.**

267 A) DNA sequence of the *AtEXTENSIN3 (AT1G21310)* gene between position +60 and +118
268 from the predicted start of translation. The CRISPR/Cas9 alleles generated are represented
269 below and named *atext3-1 to -4*. The protospacer adjacent motif (PAM) sequences of the 2
270 guides used are underlined. The mutations in the mutant alleles sequence are indicated in
271 red. B) Predicted consequences of the mutations on the AtEXT3 protein. The 3 EXTENSIN-2
272 domains predicted by the Protein Families database (Pfam) are represented as well as the
273 position of the 2 guides used. For the predicted mutant proteins, sequences that differ from
274 those of Col-0 due to a frameshift are represented in grey. The amino acid positions from the
275 predicted first methionine are indicated. C) 14 days after stratification (DAS) seedlings,
276 grown *in vitro* for Col-0 and for the four AtEXT3 null alleles. D) 29 DAS plants grown on soil
277 for Col-0 and for the four AtEXT3 null alleles. E) 46 DAS plants grown on soil for Col-0 and for
278 the four AtEXT3 null alleles. Scale bars in C and D = 2cm and in E = 3cm.

279

280 **Figure 2: Loss of AtEXT3 function gives no obvious phenotypes in the Arabidopsis L-er**
281 **background.**

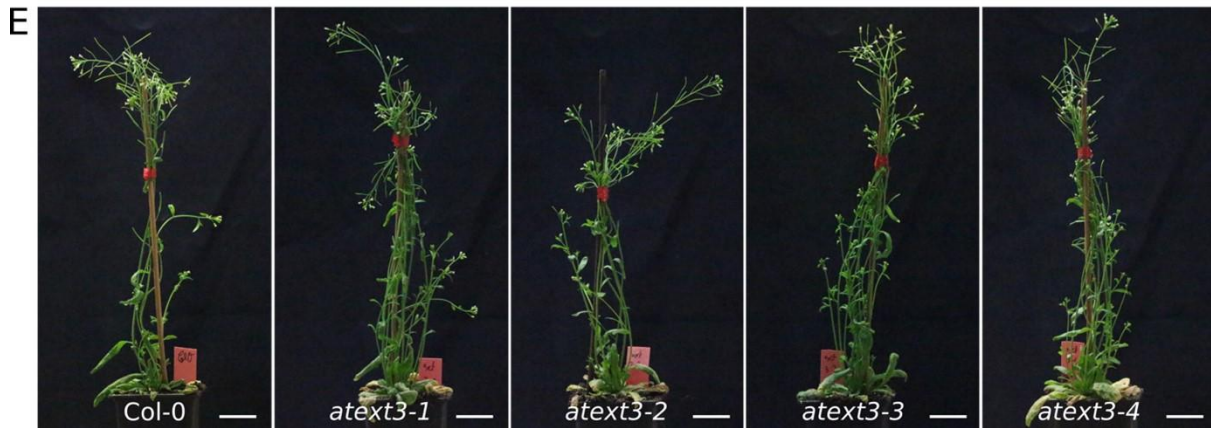
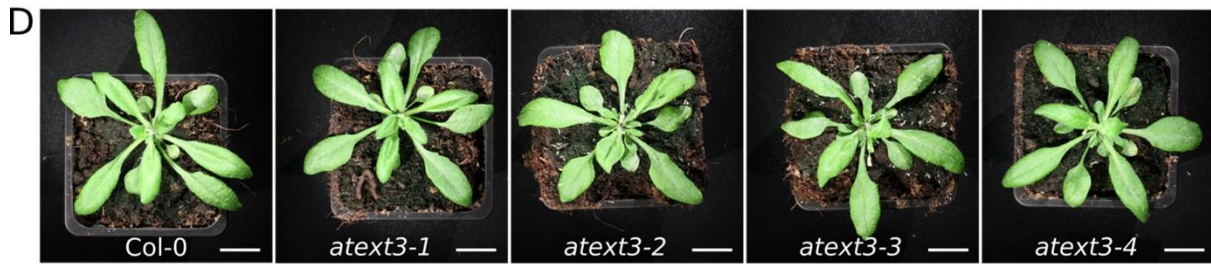
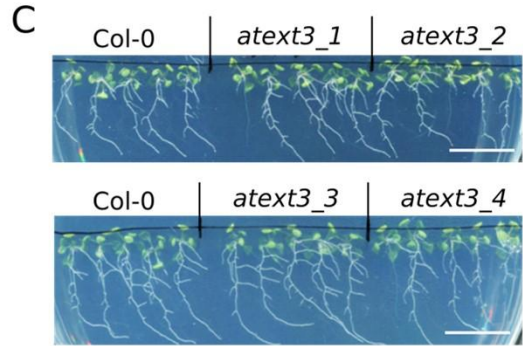
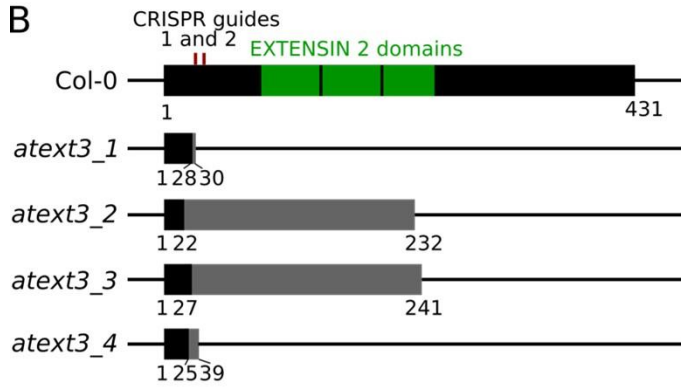
282 A) DNA sequence of the *AtEXTENSIN3 (AT1G21310)* gene between position +145 and +245
283 from the predicted start of translation. The CRISPR/Cas9 alleles generated are represented
284 below and named *atext3-5 to -7*. The protospacer adjacent motif (PAM) sequences of the 2
285 guides used are underlined. The mutations in the mutant alleles are indicated in red. B)
286 Predicted consequence of the mutations on the AtEXT3 protein. The 3 EXTENSIN-2 domains
287 predicted by the Protein Families database (Pfam) are represented as well as the position of
288 the 2 guides used. For the mutant proteins, sequences that differ from L-er due to a shift in
289 the ORF are represented in grey. The amino acid positions from the predicted first
290 methionine are indicated. C) 10 days after stratification (DAS) seedlings, grown *in vitro* for L-
291 er and for the three AtEXT3 null alleles. D) 28 DAS plants grown on soil for L-er and for the
292 three AtEXT3 null alleles. E) 46 DAS plants grown on soil for L-er and for the three AtEXT3

293 null alleles. Scale bars in C and D = 2cm and in E = 3cm.

A

```

+60                                     +118
Col-0  TTTGTATCTCAATCAACCGCTA-ACTATTTCTATTCTTCTCCTCCAC-CACCGGTTAAAC
atext3_1 TTTGTATCTCAATCAACCGCTA-A-----CACCGGTTAAAC
atext3_2 TTTG-----TATTCTTCTCCTCCACACACCGGTTAAAC
atext3_3 TTTGTATCTCAATCAACCGCTAAACTATTTCTATTCTTCTCCTCCACACACCGGTTAAAC
atext3_4 TTTGTATCTCAATCAA-----TCTATTTCTATTCTTCTCCTCCACTCACCGGTTAAAC
  
```



294

A

```

+145                                     +200
L-er   CTCCTCCACC-GGTATACCACTCCCCACCACCACCTAAGAAGCACTACGAATACAA
atext3-5 CTCCTCCACC-GGTTTACCATTCC-----CCACCTAAGAAGCACTACGAATACAA
atext3-6 CTCCTCCACC-GG-----CAA
atext3-7 CTCCTCCACCGGGTATACCACTCCCCACCACCACCTAAGAAGCACTACGAATACAA

+200                                     +245
L-er   ATCTCCACCTCC-ACCGGTTAAGCACTACTCACCTCCTCCGGTTTA
atext3-5 ATCTCCACCTCCCACCGGTTAAGCACTACTCACCTCCTCCGGTTTA
atext3-6 ATCTCCACCTCC-ACCGGTTAAGCACTACTCACCTCCTCCGGTTTA
atext3-7 ATCTCCACCTC-----GTTTA

```

