

1 **Pharmacological and genetic manipulations of Ca<sup>2+</sup> signaling have contrasting**  
2 **effects on auxin-regulated trafficking**

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18 **Author Contributions**

19 R.W and E.H. performed immunolocalizations and subsequent data analyses according to J.F. methods.  
20 E.H. and M.G. performed the Ca<sup>2+</sup> reporters experiments according to M.K., K.S. and A.C. methods. S.V.  
21 conceived the original screening and research plans and wrote the manuscript with contributions of all  
22 the authors. S.V. agrees to serve as the author responsible for contact and ensures communication.

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24

25 **ABSTRACT**

26

27 **A large part of a plants' developmental plasticity relies on the activities of the phytohormone auxin**  
28 **and the regulation of its own distribution. This process involves a cohort of transcriptional and non-**  
29 **transcriptional effects of auxin on polar auxin transport, regulating the abundancy, biochemical**  
30 **activity and polar localization of the molecular components, predominantly PIN auxin exporters.**  
31 **While the transcriptional auxin signaling cascade has been well characterized, the mechanism and role**  
32 **of non-transcriptional auxin signaling remains largely elusive. Here, we addressed the potential**  
33 **involvement of auxin-induced Ca<sup>2+</sup> signaling in auxin's inhibitory effect on PIN endocytic trafficking.**  
34 **On the one hand, exogenous manipulations of Ca<sup>2+</sup> availability and signaling effectively antagonized**  
35 **auxin effects suggesting that auxin-induced Ca<sup>2+</sup> signaling is required for inhibition of internalization.**  
36 **On the other hand, we addressed the auxin-mediated inhibition of PIN internalization in the auxin**  
37 **signaling (*tir1afb2,3*) or Ca<sup>2+</sup> channel (*cngc14*) mutants. These mutants were strongly defective in**  
38 **auxin-triggered Ca<sup>2+</sup> signaling, but not in auxin-inhibited internalization. These data imply that, while**  
39 **Ca<sup>2+</sup> signaling may be required for normal PIN trafficking, auxin-mediated increase in Ca<sup>2+</sup> signaling is**  
40 **not a direct part of a downstream mechanism that mediates auxin effects on Brefeldin A-visualized**  
41 **PIN intercellular aggregation. These contrasting results obtained by comparing the mutant analysis**  
42 **versus the exogenous manipulations of Ca<sup>2+</sup> availability and signaling illustrate the critical importance**  
43 **of genetics to unravel the role of Ca<sup>2+</sup> in a process of interest.**

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45 **Key words: Auxin, Calcium, Trafficking, Signaling.**

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## 48 INTRODUCTION

49

50 Calcium cross-links pectinate polymers and is therefore an important structural determinant of the cell  
51 wall (Feng et al., 2018; Cosgrove and Anderson, 2020), and inside the cell it directly modulates the  
52 biochemical activities of proteins,  $\text{Ca}^{2+}$  sensors (relays and responders) and phospholipids, impacting on  
53 numerous cellular processes (Himschoot et al., 2017; Kudla et al., 2018). The pleiotropic activities of  $\text{Ca}^{2+}$   
54 necessitate submicromolar range  $\text{Ca}^{2+}$  levels in the cytoplasm, while in the apoplast and in the lumen of  
55 organelles,  $\text{Ca}^{2+}$  levels are several orders of magnitude higher (Stael et al., 2012; Costa et al., 2018). Such  
56 steep concentration gradients allow to trigger a local significant increase in  $\text{Ca}^{2+}$  levels by the simple  
57 opening of a few channels in response to a specific stimulus (Demidchik et al., 2018). In a typical  
58 signaling cascade, the cytoplasmic increase of  $\text{Ca}^{2+}$  is decoded by specialized proteins that translate the  
59  $\text{Ca}^{2+}$  signal into defined cellular responses, such as the modulation of channels or kinases (Kudla et al.,  
60 2018).

61 The plant  $\text{Ca}^{2+}$  signaling toolkit is strongly diversified in comparison to the one in animals (Edel et  
62 al., 2017), most prominently reflected in the existence of plant and animal specific  $\text{Ca}^{2+}$  signaling  
63 components, such as inositol (1,4,5)-triphosphate receptors, and ryanodine receptors. Despite this  
64 important diversification, the current commonly used  $\text{Ca}^{2+}$  pharmacology consists of very general  
65 inhibitors or chelators, or inhibitors that were designed to target mammalian  $\text{Ca}^{2+}$  channels and signaling  
66 components (De Vriese et al., 2018). In most cases, the molecular targets of the inhibitors are not well  
67 enough conserved or even absent in plants, making it difficult to make strong claims based on inhibitor  
68 studies.

69 The signaling function of  $\text{Ca}^{2+}$  is currently best understood in the context of abiotic and biotic  
70 stress responses (Kudla et al., 2018), guard cell movement (Konrad et al., 2018) and in pollen tubes (Guo  
71 and Yang, 2020). In contrast, the function of the since long described auxin-induced  $\text{Ca}^{2+}$  response

72 remains largely elusive (Vanneste and Friml, 2013; Shih et al., 2015; Dindas et al., 2018). Only recently,  
73 this for long overlooked aspect of auxin signaling regained attention with the identification of the non-  
74 selective cation channel CNGC14 as a critical component of auxin-induced  $\text{Ca}^{2+}$  entry (Shih et al., 2015;  
75 Dindas et al., 2018). CNGC14 activity was proposed to participate in root gravitropism (Shih et al., 2015)  
76 and root hair development (Dindas et al., 2018; Brost et al., 2019). Additionally, manipulations of  $\text{Ca}^{2+}$   
77 availability and channels revealed connections to polar auxin transport (Dela Fuente and Leopold, 1973)  
78 and polarization of auxin transporters (Zhang et al., 2011; Li et al., 2019), indicating an important  
79 interplay between auxin and  $\text{Ca}^{2+}$ .

80 In contrast to our poor understanding of auxin-induced  $\text{Ca}^{2+}$  signaling, the mechanism of auxin-  
81 induced transcriptional changes has been characterized in great detail (Lavy and Estelle, 2016; Roosjen  
82 et al., 2018; Powers and Strader, 2020). The canonical pathway for auxin-induced transcription involves  
83 the auxin-stabilized interaction between TIR1/AFB F-box proteins and Aux/IAA transcriptional co-  
84 repressors (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). This results in the ubiquitination and  
85 proteolysis of the latter (Nemhauser, 2018). Consequently, the transcriptional repressive effect imposed  
86 by Aux/IAA on ARF transcription factors is released, and auxin-induced transcription can proceed  
87 (Pierre-Jerome et al., 2016; Roosjen et al., 2018). Recently, a non-transcriptional branch of TIR1-based  
88 auxin signaling was demonstrated to effect acute inhibition of elongation (Fendrych et al., 2018; Gallei  
89 et al., 2020). Moreover, the non-transcriptional repertoire of TIR1/AFBs was recently further expanded  
90 by the observation that *tir1/afb2,3* mutants are defective in auxin-induced  $\text{Ca}^{2+}$  signaling (Dindas et al.,  
91 2018). Additionally, auxin signals converging on pavement cell morphogenesis (Xu et al., 2010), lipid  
92 composition and distribution (Pan et al., 2009; Li et al., 2015; Platre et al., 2019), cell division during  
93 lateral root formation (Huang et al., 2019), suppression of auxin biosynthesis (Wang et al., 2020),  
94 stability and polarity of PIN proteins (Abas et al., 2006; Sauer et al., 2006; Baster et al., 2013; Prat et al.,  
95 2018; Mazur et al., 2020), and their internalization (Paciorek et al., 2005; Robert et al., 2010; Platre et

96 al., 2019), possibly act via alternative auxin perception mechanisms, such as the receptor like kinase  
97 family TRANSMEMBRANE KINASE1-4 (Cao et al., 2019; Huang et al., 2019; Platre et al., 2019).

98 Based on the non-transcriptional character of the inhibition of internalization by NAA (Robert et  
99 al., 2010; Zhang et al., 2020), we postulated that auxin-induced  $\text{Ca}^{2+}$  signaling could be a signaling  
100 component in this response to NAA. Therefore, followed two strategies. On the one hand, we  
101 manipulated NAA-induced  $\text{Ca}^{2+}$  signaling using inhibitors or washing seedlings in  $\text{Ca}^{2+}$  free medium. We  
102 validated their effects on NAA-induced  $\text{Ca}^{2+}$  signaling, and assessed their impact on NAA-inhibited  
103 internalization. This approach provided a convincing and tight correlation between NAA-induced  $\text{Ca}^{2+}$   
104 signaling and NAA's ability to inhibit internalization. However, this correlation between  $\text{Ca}^{2+}$  signaling  
105 and inhibition of internalization could not be confirmed in *tir1/afb* and *cngc14*, two mutants that are  
106 specifically defective auxin-induced  $\text{Ca}^{2+}$  signaling. This discrepancy in outcome between both  
107 approaches calls for extreme caution when analyzing the role of  $\text{Ca}^{2+}$  in a process of interest as current  
108 pharmacology or manipulations of  $\text{Ca}^{2+}$  availability are prone to pleiotropic, misleading effects.

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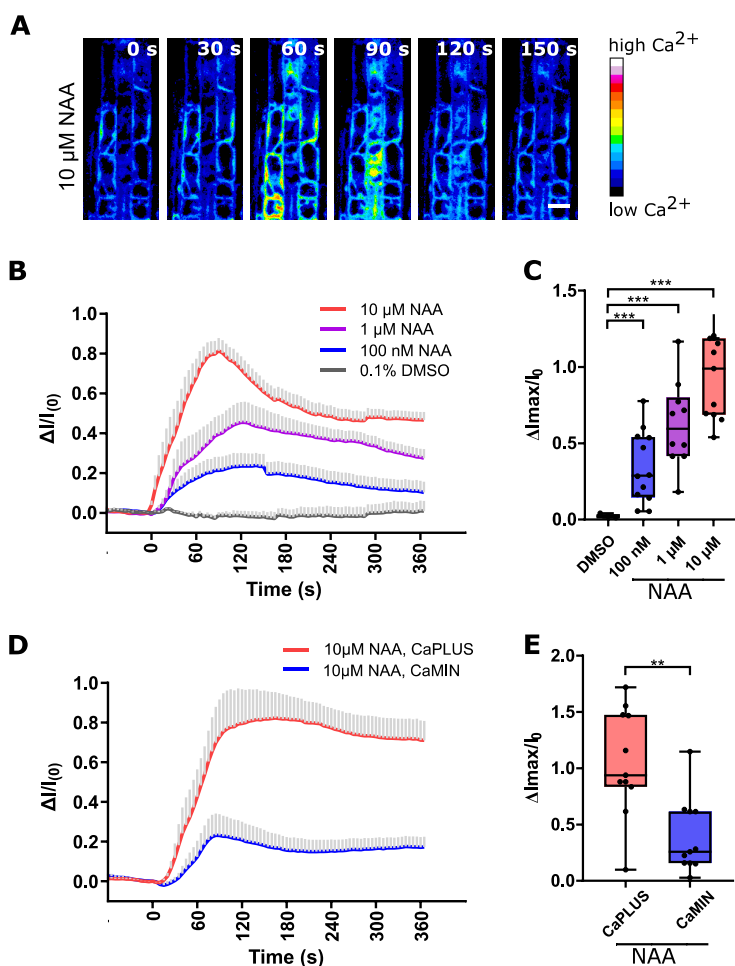
## 110 RESULTS

### 111 Characterization of NAA-induced $\text{Ca}^{2+}$ signaling.

112 The synthetic auxin, 1-naphthaleneacetic acid (NAA) is widely used in auxin biology as a proxy for the  
113 endogenous auxin indole-3-acetic acid (IAA) and is a more potent inhibitor of internalization than IAA  
114 (Paciorek et al., 2005). Because we wanted to evaluate the role of  $\text{Ca}^{2+}$  signaling in inhibition of  
115 internalization, we set out to characterize the NAA-induced  $\text{Ca}^{2+}$  response in Arabidopsis root meristems  
116 in more detail. The cytoplasmic, intensimetric  $\text{Ca}^{2+}$  indicator R-GECO1 (Keinath et al., 2015) reported an  
117 instant cytosolic  $\text{Ca}^{2+}$  elevation in response to 10 $\mu\text{M}$  NAA application (Figure 1A,B; Supplemental Movie  
118 S1). Also at lower concentrations (1 $\mu\text{M}$  and 0.1 $\mu\text{M}$ ), NAA triggered rapid  $\text{Ca}^{2+}$  signaling, albeit with  
119 smaller amplitude (Figure 1B), illustrating a dose-dependence of the maximal response, similar to the

120 one reported for the natural auxin indole-3-acetic acid (IAA) (Dindas et al., 2018). The onset of the  $\text{Ca}^{2+}$   
121 increase started within seconds after NAA application, and reached a maximum after  $\pm 70$  sec, followed  
122 by a gradual attenuation response. Similarly, subcellular targeting of ratiometric  $\text{Ca}^{2+}$  sensors revealed a  
123 rise in  $\text{Ca}^{2+}$  concentrations in the cytoplasm (visualized with NES-YC3.6 (Krebs et al., 2012)), in the  
124 cytosol near the plasma membrane (visualized with PM-YC3.6-Lti6b (Krebs et al., 2012)), in the  
125 endoplasmic reticulum lumen (visualized with CRT-D4ER (Bonza et al., 2013)) and in mitochondria  
126 (visualized with 4mt-YC3.6 (Loro et al., 2012)) (Supplemental Figure S1A-D). The signals in the ER and  
127 mitochondria were slightly delayed compared to the other reporters (Supplemental Figure S1C,D),  
128 suggesting that these organelles may act as  $\text{Ca}^{2+}$  sinks for attenuation of the cytoplasmic  $\text{Ca}^{2+}$  signal. The  
129 lack of  $\text{Ca}^{2+}$  response after benzoic acid (BA) treatment (Supplemental Figure S1E,G), shows that the  $\text{Ca}^{2+}$   
130 response to NAA was not a response to acidification associated with NAA treatment. The NAA-induced  
131  $\text{Ca}^{2+}$  response could be inhibited pharmacologically by the  $\text{Ca}^{2+}$  channel inhibitors Bepridil and Nifedipine  
132 (De Vriese et al., 2018; De Vriese et al., 2019) (Supplemental Figure S1F,G; Supplementary Movies  
133 S2,S3).

134 Additionally, we modulated the available extracellular  $\text{Ca}^{2+}$  by washing the seedlings with  
135 0.5xMS medium that lacked  $\text{CaCl}_2$  (hereafter referred to as CaMIN). This simple treatment was sufficient  
136 to reduce the NAA-induced  $\text{Ca}^{2+}$  response in comparison to normal 0.5xMS medium ( $\approx 1.5\text{mM}$   $\text{CaCl}_2$ ,  
137 hereafter CaPLUS) (Figure 1C). Jointly, these data suggest that NAA triggers a complex  $\text{Ca}^{2+}$  response that  
138 largely depends on extracellular  $\text{Ca}^{2+}$ .



139

140 **Figure 1. Apoplastic  $\text{Ca}^{2+}$  determines the amplitude of auxin-induced cytosolic  $\text{Ca}^{2+}$  dynamics.**

141 **A.** Single frames of the dynamic response of the  $\text{Ca}^{2+}$  sensor, R-GECO1, at indicated time points after  
 142  $10\mu\text{M}$  NAA treatment. Scale bar =  $20\mu\text{m}$ .

143 **B.** Averaged and normalized R-GECO1 fluorescence intensities over time upon treatment with 0.1%  
 144 DMSO,  $10\mu\text{M}$  NAA,  $1\mu\text{M}$  NAA, or  $100\text{nM}$  NAA. (nr of seedlings = 3, 12, 10, 12 respectively,  
 145 means  $\pm$  s.e.m.). DMSO and NAA treatments were applied at time point 0.

146 **C.** Boxplot representation of the maximal amplitude of the treatments described in **B**.

147 **D.** The averaged normalized R-GECO1 fluorescence intensities over time upon treatment with  $10\mu\text{M}$   
 148 NAA following a 30min pretreatment with CaPLUS or CaMIN (nr of seedlings = 11; 3 repeats;  
 149 means  $\pm$  s.e.m.). NAA treatments were applied at time point 0s.

150 **E.** Boxplot representation of the maximal amplitude of the treatments described in **D**.

151 For all box plots, the central line indicates the median, the bottom and top edges of the box indicate the  
 152 interquartile range. The box plot whiskers are plotted down to the minimum and up to the maximum  
 153 value. Data were analyzed by an unpaired two-tailed t-test with Welch correction.  $**P < 0.01$ ,  $***P <$   
 154  $0.001$ .

155

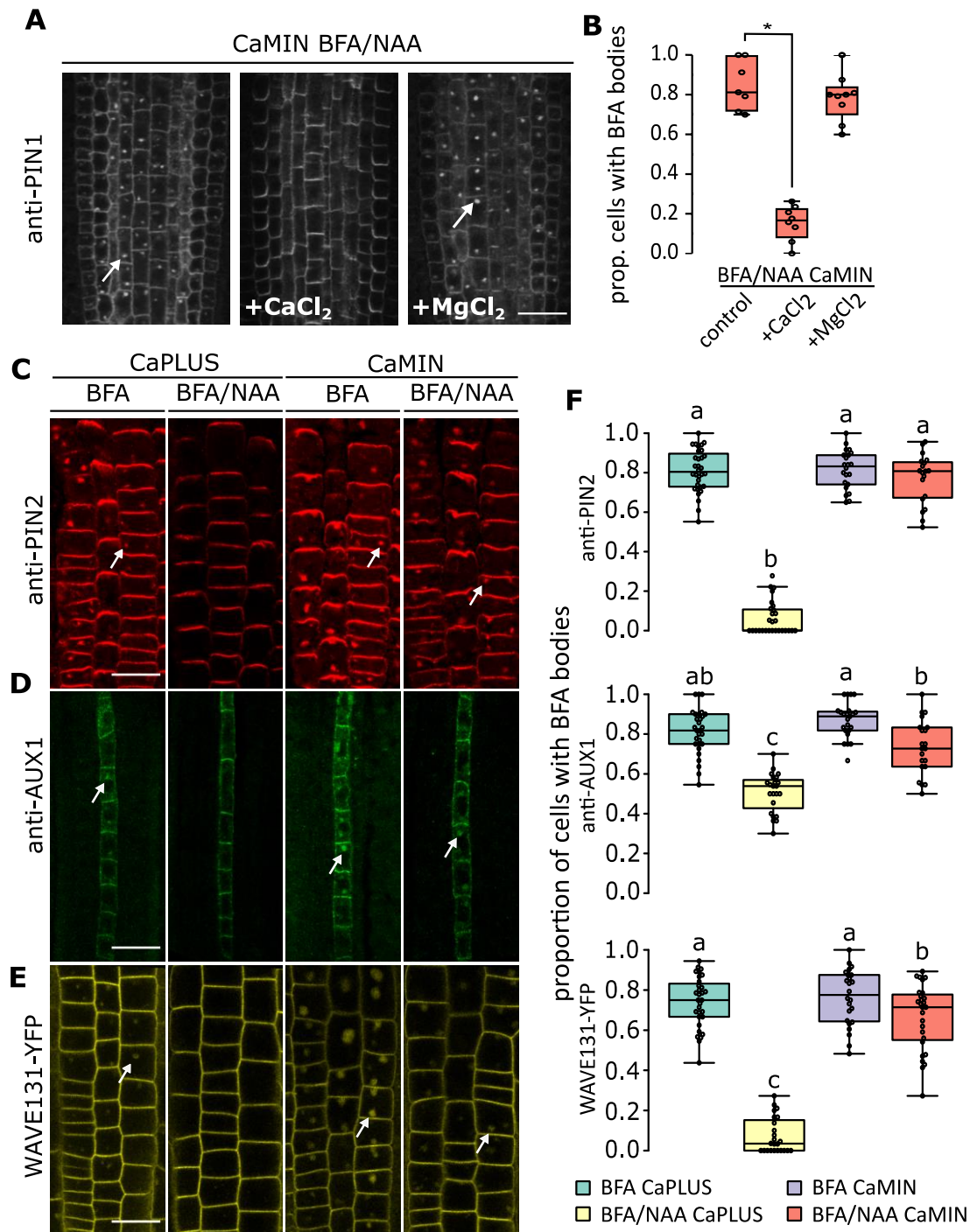
156 **NAA-mediated inhibition of internalization correlates with Ca<sup>2+</sup> signaling**

157 The synthetic auxin NAA interferes rapidly via a non-transcriptional pathway with the internal  
158 accumulation of plasma membrane proteins (hereafter referred to as internalization) in Brefeldin A  
159 (BFA)-induced intracellular endosomal aggregates (so-called BFA bodies) (Paciorek et al., 2005; Robert et  
160 al., 2010). This effect of NAA was concentration dependent, showing the maximum inhibitory effect at  
161 10µM (Supplemental Figure S2A,B), correlating with the dose-dependence of the maximal NAA-induced  
162 Ca<sup>2+</sup> response (Figure 1C,D). Given the immediacy of both processes, we postulated that auxin-induced  
163 Ca<sup>2+</sup> responses reflects an auxin signaling cascade involved in NAA-regulated internalization.

164 Indeed, when we used the organic Ca<sup>2+</sup> channel blockers Nifedipine and Bepridil at  
165 concentrations that interfered with NAA-induced Ca<sup>2+</sup> entry (Supplemental Figure 1F,G), PIN1  
166 internalization was restored in BFA/NAA co-treated roots (Supplemental Figure S2C). A similar  
167 nullification on NAA-inhibited PIN1 internalization was achieved using the membrane-permeable  
168 calmodulin inhibitor W-7 (Supplemental Figure S2C). This suggests that Ca<sup>2+</sup> increase and its  
169 downstream signaling is required for NAA's inhibitory effect on PIN1 internalization. Given the potential  
170 off-target effects of the Ca<sup>2+</sup> channel inhibitors (De Vriese et al., 2018), we also evaluated the effect of  
171 CaMIN on NAA-inhibited PIN1 internalization. Similarly to Bepridil, Nifedipine and W-7, a 30min CaMIN  
172 pretreatment was sufficient to restore PIN internalization in BFA/NAA co-treated roots (Figure 2A,B). To  
173 evaluate the specificity of the treatment to Ca<sup>2+</sup> availability, we analyzed PIN1 internalization in CaMIN  
174 supplemented with either 1.5mM CaCl<sub>2</sub> (comparable to 0.5xMS) or 1.5mM MgCl<sub>2</sub> (Figure 2A,B). The  
175 addition of CaCl<sub>2</sub> fully restored the NAA sensitivity of PIN1 internalization. In contrast, the internalization  
176 in roots treated with MgCl<sub>2</sub> supplemented CaMIN could not restore the NAA sensitivity, indicating the  
177 specificity of Ca<sup>2+</sup> in this process. This effect of CaMIN on NAA-inhibited internalization could also be  
178 observed for other plasma membrane cargoes such as PIN2, AUX1 in the protophloem and NPSN12



179 (WAVE131-YFP) (Figure 2C-F). Jointly, these findings strongly support a notion that  $Ca^{2+}$  is required for  
 180 NAA's inhibitory effect on internalization of plasma membrane proteins.



181

182 **Figure 2. NAA inhibition of internalization requires extracellular  $Ca^{2+}$**

183 **A.** Immunolocalisation of PIN1 in 3 day-old seedling roots pretreated with CaMIN (30min), followed by  
184 BFA and NAA (co)treatment (1h), in CaMIN, and in CaMIN supplemented with either 1.5 mM CaCl<sub>2</sub> and  
185 CaMIN supplemented with 1.5mM MgCl<sub>2</sub>. Scale bar = 20µm.  
186 **B.** Quantification of the proportion of cells that have PIN1 in BFA bodies for the conditions in **A**, and  
187 corresponding controls in CaPLUS.(n ≥ 3 per treatment per repeat; 2 independent repeats). For all box  
188 plots, the central line indicates the median, the bottom and top edges of the box the interquartile range,  
189 and the box plot whiskers are plotted down to the minimum and up to the maximum value. Data were  
190 analyzed using a logistic regression model. \* indicates P ≤ 0.05, Wald-type test.  
191 **C.** Immunolocalization of PIN2 in 3 day-old seedling roots after BFA or BFA/NAA treatments in CaPLUS  
192 and CaMIN conditions.  
193 **D.** Immunolocalization of AUX1 in 3 day-old seedling roots after BFA or BFA/NAA treatments in CaPLUS  
194 and CaMIN conditions.  
195 **E.** WAVE131(NPSN12)-YFP localisation in 3 day-old seedling roots after BFA or BFA/NAA treatments in  
196 CaPLUS and CaMIN conditions. Concentrations used for PIN2 and WAVE131-YFP BFA: 25µM, NAA:  
197 10µM, 1h; Concentrations used for AUX1 BFA: 50µM, NAA: 10µM, 90min.  
198 **F.** Quantification of the proportion of cells that have PIN2 (n=32;28;22;19 roots in total), AUX1  
199 (n=30;26;26;22 roots in total) or WAVE131-YFP (n=31;24;24;27 roots in total) in BFA bodies,  
200 corresponding to experiments in Figure 2C-E. Center lines show the medians; box limits indicate the  
201 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th  
202 percentiles, outliers are represented by dots. Different lowercase letters indicate significant differences  
203 (P ≤ 0.05, Wald-type test). White arrows in figures indicate proteins accumulated in BFA bodies. Scale  
204 bars represents 20 µm.  
205

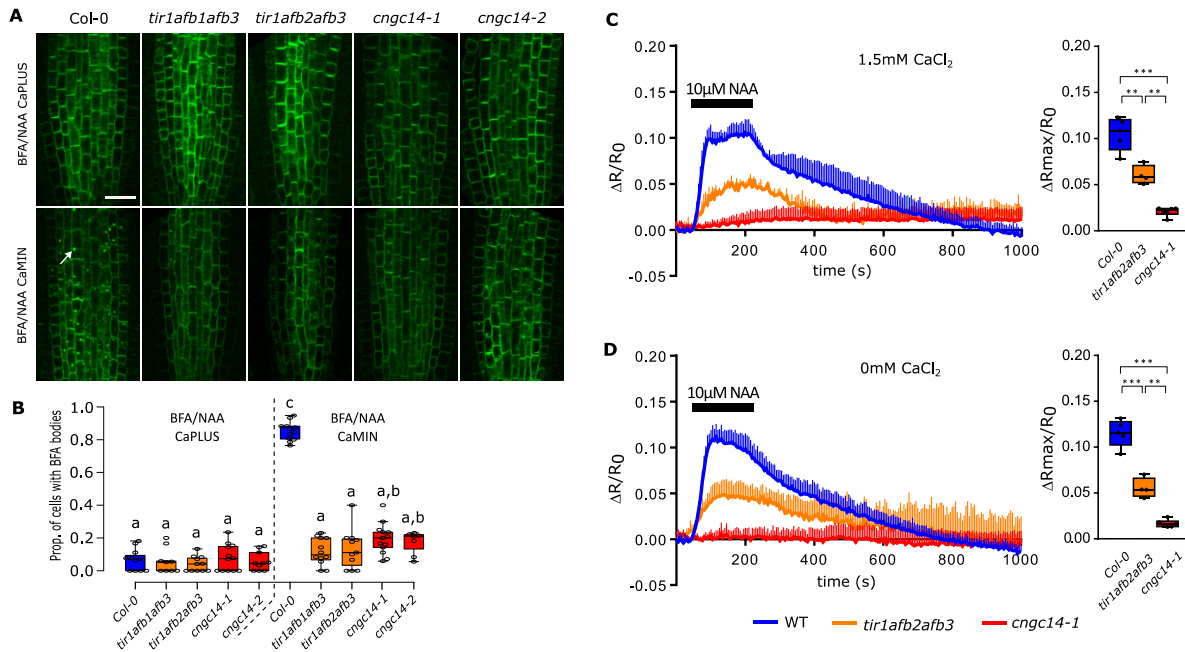
## 206 **The TIR1/AFB-CNGC14 module is not required for NAA's inhibitory effect on internalization**

207 TIR1/AFB-based auxin perception was demonstrated to be required for IAA-induced Ca<sup>2+</sup> signaling via  
208 the Ca<sup>2+</sup> permeable cation channel CNGC14 (Shih et al., 2015; Dindas et al., 2018). Assuming an auxin-  
209 triggered Ca<sup>2+</sup> signal being part of the mechanism for regulation of PIN internalization, we predicted that  
210 the defective auxin-induced Ca<sup>2+</sup> signaling in *tir1/afb* or *cngc14* mutants would result in NAA-insensitive  
211 PIN internalisation. Contrary to expectations based on CaMIN and Ca<sup>2+</sup> signaling inhibitors, none of the  
212 tested mutants showed NAA-insensitive internalisation in CaPLUS, suggesting that the TIR1/AFB-CNGC14  
213 Ca<sup>2+</sup> signaling module is not essential for this effect. Surprisingly, PIN1 internalization was inhibited by  
214 NAA in *tir1afb1afb3*, *tir1afb2afb3* and three different *cngc14* alleles also in CaMIN conditions, unlike in  
215 WT and other *tir1/afb* mutants (Figure 3B-E; Supplemental Figure S3A-E). In contrast to *tir1/afb* and  
216 *cngc14* mutants, the NAA-sensitive internalization in CaMIN was not observed in the auxin biosynthesis  
217 defective mutant *yuc3,5,7,8,9* (*yucQ*) (Chen et al., 2014) (Supplemental Figure S3F). Thus, the restored

218 NAA sensitivity in CaMIN-treated *tir1/afb* and *cncg14* mutants does not seem to be related to changes in  
219 auxin levels but is rather specific to TIR1/AFB-CNGC14-mediated auxin signaling. Jointly, these data  
220 demonstrate that in CaPLUS, NAA inhibits internalisation independently of TIR1/AFB-CNGC14-mediated  
221 auxin signaling.

222 The discrepancy between the exogenous Ca<sup>2+</sup> manipulations and the mutant analyses, raised the  
223 hypothesis that NAA may induce Ca<sup>2+</sup> signaling via a TIR1/AFB-CNGC14-independent mechanism.  
224 Therefore, we transformed the ratiometric Ca<sup>2+</sup> indicator NES-YC3.6 in *tir1/afb2,3* and *cncg14-1* and  
225 analysed the Ca<sup>2+</sup> responses to a pulse of NAA in these mutants. We found that the NAA-induced Ca<sup>2+</sup>  
226 response was strongly reduced in *tir1afb2,3* triple mutants and was completely absent from *cncg14-1*  
227 (Figure 3A), suggesting that NAA elicits Ca<sup>2+</sup> signaling through the same mechanism as IAA. Also in the  
228 absence of exogenously supplied Ca<sup>2+</sup>, the NAA-induced Ca<sup>2+</sup> responses in *tir1afb2,3* and *cncg14-1*  
229 mutants were strongly defective (Figure 3B), excluding compensatory Ca<sup>2+</sup> signaling mechanisms under  
230 low Ca<sup>2+</sup> availability, as reported for pathogen-triggered immunity-associated Ca<sup>2+</sup> signaling in *cngc2* and  
231 *cngc4* mutants (Tian et al., 2019). These data show that NAA-induced Ca<sup>2+</sup> signaling is fully dependent  
232 on the TIR1/AFB-CNGC14 module. Notably, the YC3.6 Ca<sup>2+</sup> indicator did not allow detecting the obvious  
233 differences in the NAA-induced Ca<sup>2+</sup> response between CaMIN and CaPLUS that we observed when using  
234 R-GECO1 (Figure 1D). This probably reflects fundamental differences in Ca<sup>2+</sup> binding properties and  
235 dynamic ranges that exist between both Ca<sup>2+</sup> indicators' properties (Nagai et al., 2004; Zhao et al., 2011;  
236 Keinath et al., 2015; Waadt et al., 2017). This also suggests that the reduction in Ca<sup>2+</sup> response caused by  
237 CaMIN treatment is weaker than the one in *tir1afb2afb3* and *cncg14-1*. The normal NAA sensitivity of  
238 PIN internalisation in these mutants, that are strongly defective in auxin-induced Ca<sup>2+</sup> signaling, thus  
239 provides further evidence that auxin-induced Ca<sup>2+</sup> signaling is not an essential part of the mechanism by  
240 which auxin affects trafficking. This is in direct contrast to the conclusions drawn using exogenous Ca<sup>2+</sup>  
241 signaling manipulations, highlighting an important lack of specificity of these treatments.

242



243

244 **Figure 3. Analysis of inhibition of internalization and auxin-induced Ca<sup>2+</sup> signaling in *tir1/afb* and**  
 245 ***cngc14* mutants**

246 **A.** Immunolocalization of PIN1 in 3 day-old roots co-treated for BFA and NAA in wild type, *tir1/afb1,3*,  
 247 *tir1/afb2,3*, *cngc14-1* and *cngc14-2*, in CaPLUS and CaMIN conditions. White arrows indicate PIN1-  
 248 accumulating BFA bodies. Scale bar is 20 μm.

249 **B.** Quantification of the proportion of cells that accumulate PIN1 in BFA bodies visualized by  
 250 immunolocalisation in 3 day-old roots of indicated genotypes co-treated for BFA and NAA, in CaPLUS,  
 251 CaMIN as depicted in **c**. Total numbers of roots analyzed in wild type (n=11; n=12 in total), *tir1afb1afb3*  
 252 (n=9; n=12 in total), *tir1afb2afb3* (n=11; n=11 in total), *cngc14-1* (n=12; n=13 in total) and *cngc14-2*  
 253 (n=10; n=7 in total), representing the sum of two independent experiments. Significant differences ( $P \leq$   
 254 0.05, Wald-type test) are indicated by different lowercase letters. Center lines show the medians; box  
 255 limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the  
 256 25th and 75th percentiles, outliers are represented by dots.

257 **C,D.** The Ca<sup>2+</sup> dynamics (NES-YC3.60 Cameleon) in WT, *tir1afb2afb3*, *cngc14-1* in response to a pulse of  
 258 10μM NAA in medium containing (C) 1.5mM CaCl<sub>2</sub> or (D) 0mM CaCl<sub>2</sub>.  
 259

## 260 DISCUSSION

261 Auxin-mediated regulation of trafficking is considered an important aspect of auxin's self-regulating  
 262 properties in plant growth and development. However, the underlying molecular mechanisms remain  
 263 largely unclear. The synthetic auxins such as NAA displays a strong effect on trafficking, as illustrated by

264 strong interference with accumulation of plasma membrane cargoes in BFA bodies (Paciorek et al.,  
265 2005). This effect was reported to be very fast, not requiring transcriptional changes and independent of  
266 canonical auxin signaling (Robert et al., 2010). Instead, an extracellular auxin perception mechanism was  
267 proposed based on the activities of AUXIN BINDING PROTEIN1 (Robert et al., 2010). Using updated  
268 genetic tools, however, the latter was put into question (Gao et al., 2015). Given the immediacy of the  
269 response, we hypothesized that auxin-induced  $\text{Ca}^{2+}$  signaling could be involved in auxin's inhibitory  
270 effect on internalization. We established that NAA activates  $\text{Ca}^{2+}$  responses at the plasma membrane via  
271 a TIR1/AFB-CNGC14-dependent mechanism, similarly as was recently described for the endogenous  
272 auxin IAA (Shih et al., 2015; Dindas et al., 2018). Using mutants and pharmacology we interfered with  
273 auxin-induced  $\text{Ca}^{2+}$  signaling and evaluated of the impact on internalization. The pharmacological  
274 interference revealed a good correlation between intensity of auxin-induced  $\text{Ca}^{2+}$  signaling and NAA's  
275 ability to inhibit internalization, supporting our original notion. In striking contrast, genetic disruption of  
276 auxin-induced  $\text{Ca}^{2+}$  signaling did not affect NAA's ability to inhibit internalization. These data  
277 unequivocally show that auxin-induced  $\text{Ca}^{2+}$  signaling does not inhibit internalization. It does however,  
278 not exclude roles for TIR1/AFB-CNGC14-mediated  $\text{Ca}^{2+}$  signaling in the auxin-regulated vacuolar  
279 trafficking of PIN proteins, that depends on TIR1/AFB function (Baster et al., 2013), or PIN polarization  
280 (Sauer et al., 2006; Prat et al., 2018; Mazur et al., 2020; Mazur et al., 2020). The contrasting results  
281 obtained using mutants versus the currently available  $\text{Ca}^{2+}$  pharmacology or manipulating  $\text{Ca}^{2+}$   
282 availability illustrate that  $\text{Ca}^{2+}$  signaling in plants is a highly interconnected system, not allowing for easy,  
283 specific manipulations. Instead, the current lack of highly specific pharmacology imply that the most  
284 reliable conclusions can be drawn through genetic analysis.

285

286 **CONCLUSION**

287 Auxin is well-known to trigger a  $\text{Ca}^{2+}$  response. However, its physiological role remains poorly  
288 understood. Here, we evaluated auxin-induced  $\text{Ca}^{2+}$  signaling in the context of regulating internalization  
289 of plasma membrane cargoes. Pharmacological evidence indicated its requirement. However, genetics  
290 demonstrated unequivocally that NAA's effect on internalization is independent of auxin-induced  $\text{Ca}^{2+}$   
291 signaling.

292

## 293 **MATERIALS AND METHODS**

### 294 **Plant Growth Conditions**

295 *Arabidopsis thaliana* seeds were sterilized by using bleach gas (8mL concentrated HCl to 150mL bleach)  
296 overnight, afterwards the seeds were sown on Petri dishes (12 cm X 12 cm) containing sterile half-  
297 strength Murashige and Skoog ( $\frac{1}{2}$  MS) medium (0.5 x MS salts, 0.8% sucrose, 0.5 g/L 2-(N-morpholino)  
298 ethanesulfonic acid, pH 5.7, and 1% w/v agar), and grown under continuous light (21°C, continuous  
299 light), after 2 days vernalization at 4°C in the dark. To prepare 2L liquid CaMIN medium following  
300 components were dissolved in MilliQ: 100mL MS basal salt micronutrient solution (catalog Nr), 20 g  
301 sucrose, 0.20 g myoinositol, 1.00 g MES, 1.652g  $\text{NH}_4\text{NO}_3$ , 0.180g  $\text{MgSO}_4$ , 1.920g  $\text{KNO}_3$ , 0.152g  $\text{KH}_2\text{PO}_4$   
302 and pH 5.7. The liquid CaPLUS medium contains an additional 0.332g  $\text{CaCl}_2$  per 2L.

### 303 **Chemicals**

304 The following hormones/chemicals were used: 10 $\mu\text{M}$  NAA (catalog Nr N0903; Duchefa Biochemie).  
305 25 $\mu\text{M}$  Brefeldin A (BFA, catalog Nr B6542-25MG). All hormones/drugs were dissolved in 100%  
306 dimethylsulfoxide (DMSO; catalog Nr D4540-500ML) and obtained from Sigma Aldrich.

### 307 **Plant Lines Used**

308 The Arabidopsis used as control in this study was Columbia (Col-0) ecotype. We used the following  
309 mutants and transgenic lines were described previously: *tir1-1* (Ruegger et al., 1998), *afb2-3* (Parry et al.,  
310 2009) , *afb3-4* (Parry et al., 2009), *tir1-1afb2-3* (Parry et al., 2009), *tir1-1afb3-4* (Parry et al., 2009), *tir1-1afb1-3afb3-4* (Parry et al., 2009), *tir1-1afb2-3afb3-4* (Parry et al., 2009), *yucQ* (Chen et al., 2014),  
312 *cngc14-1* and *cngc14-1* (Shih et al., 2015), *cngc6,9*, *cngc6,14*, *cngc9,14* and *cngc6,9,14* (Brost et al.,  
313 2019), WAVE131-YFP (Geldner et al., 2009), R-GECO1 (Keinath et al., 2015), NES-YC3.6 (Krebs et al.,  
314 2012), PM-YC3.6 (Krebs et al., 2012), CRT-D4ER (Bonza et al., 2013), 4mt-YC3.6 (Loro et al., 2012). The  
315 NES-YC3.60 (Krebs et al., 2012) reporter was transformed directly into *tir1-1afb2-3afb3-4* (Parry et al.,  
316 2009) and *cngc14-1* (Shih et al., 2015). Transformants were selected based on uniform expression, and  
317 expression levels comparable to the control NES-YC3.60. Analyses of the Ca<sup>2+</sup> response was done on T2  
318 generation seedling roots showing strong and uniform expression.

### 319 **Immunodetection and Confocal Microscopy**

320 The seedlings used for Immunodetection are 3-day-old and pre-treated in liquid CaPLUS, CaMIN in  
321 presence or absence of chemicals as indicated for 30 minutes. The samples were fixed by  
322 paraformaldehyde (4%) in PBS for 1 hour in vacuum. The following steps of the immunostaining were  
323 performed by the immuno-robot InsituProll, as described by Sauer et al. (2006). The dilutions of the  
324 primary antibodies were: goat anti-PIN1 (1:600) (sc-27163, SantaCruz), rabbit anti-PIN2 (1:600) (Abas et  
325 al., 2006) and anti-AUX1 (1:400) (AS 12 1868, Agrisera). The secondary antibodies used were  
326 AlexaFluor488 donkey anti-goat (1:600) (A-11055, ThermoFisher) and AlexaFluor555 donkey anti-rabbit  
327 (1:600) (A-31572, ThermoFisher).

328

### 329 **Microscopy and image analysis**

330 R-GECO1- based Ca<sup>2+</sup> imaging experiments and analysis were performed as described (Himschoot et al.,  
331 2018). Yellow Cameleon-based experiments were performed and analyzed as described (Behera et al.,  
332 2013). Confocal laser scanning microscopes Leica SP2 (Leica) or Zeiss 710 CLSM microscopes were used  
333 to analyze the immunolocalisations, and imaging of R-GECO1. Fluorescence emission of Alexa488 (ex  
334 488 nm/em 500-545nm), Alexa555 (ex 561nm/em 555-610nm), YFP (ex 514nm/em 520-565nm), was  
335 detected using a 63x water objective. Images were analyzed using Fiji (Schindelin et al., 2015). Fiji was  
336 used to rotate, crop images and label the region of all the roots for quantification. The proportion of  
337 cells with BFA bodies was scored manually and calculated by using Excel. The BoxPlotR was used to  
338 generate the box plots figures (Spitzer et al., 2014).

### 339 **Statistical analysis**

340 For statistical analysis of the Ca<sup>2+</sup> imaging data, unpaired two-tailed t-tests with Welch correction for  
341 unequal standard deviations between populations where performed using GraphPad Prism (GraphPad  
342 Prism 8 for Windows 64-bit, version 8.4.1).

343 For statistical analysis of the immunolocalization experiments, a logistic regression was performed to  
344 compare the presence of BFA bodies in root cells of treated versus untreated roots or wild type versus  
345 mutant. A random effect was added to the model for the experiments with multiple repeats to take into  
346 account the correlation between measurements done at the same time. The analysis was performed  
347 with the glimmix procedure from SAS (Version 9.4 of the SAS System for windows 7 64bit. Copyright  
348 2002-2012 SAS Institute Inc. Cary, NC, USA ([www.sas.com](http://www.sas.com))). Maximum likelihood estimation was done  
349 with the default estimation method. A Wald-type test was performed to estimate the  
350 treatment/genotype effect on the presence of BFA bodies in the root cells.

351

352

353



354 **Acknowledgements**

355 We thank Yunde Zhao (UC San Diego, USA), Marc Estelle (UC San Diego, USA), Petra Dietrich (F.A.  
356 University of Erlangen-Nurnberg, Germany) and Niko Geldner (University of Lausanne, Switzerland) for  
357 sharing published materials, and NASC for providing seeds. We thank Veronique Storme for help with  
358 the statistical analyses. This work was supported by grants of the China Scholarship Council (CSC) (to  
359 R.W. and J.C.), the special research fund Ghent University (to E.H.), the European Research Council (ERC)  
360 (to D.V.D and J.F.), the Deutsche Forschungsgemeinschaft (DFG) through Grants within FOR964 (M.K.  
361 and K.S.), Piano di Sviluppo di Ateneo 2019 (University of Milan) (to A.C.), and by a PhD fellowship from the  
362 University of Milan (to MG).

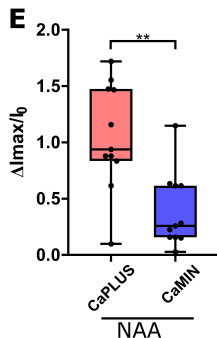
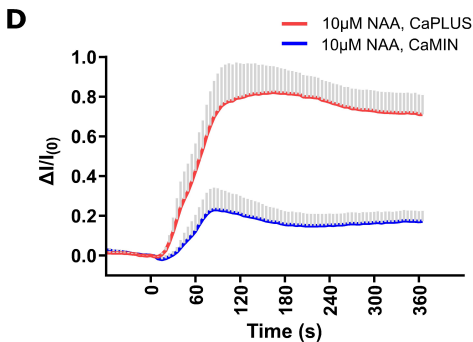
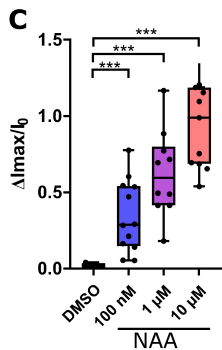
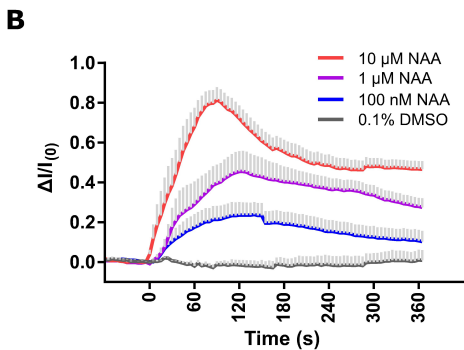
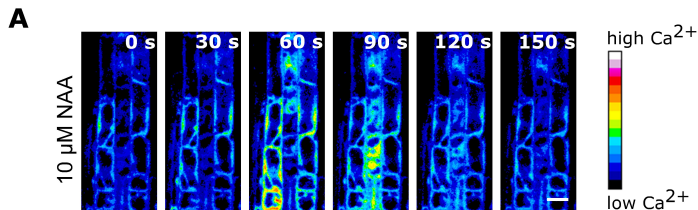
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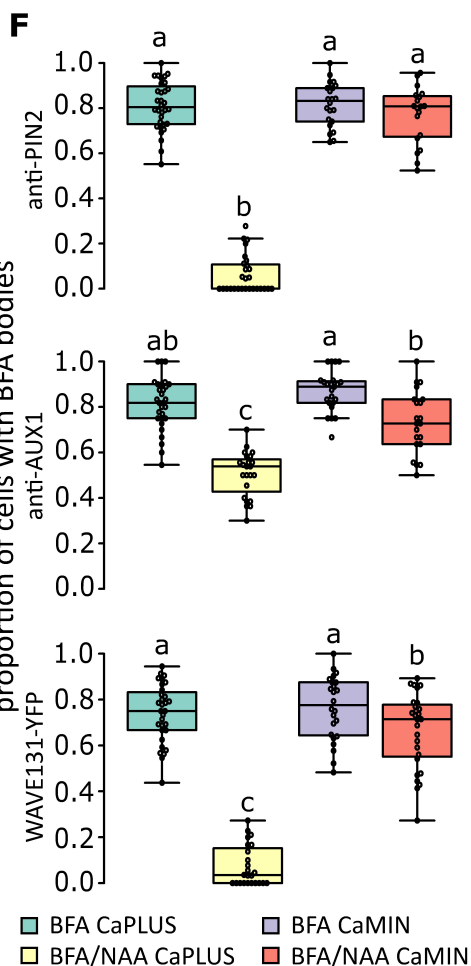
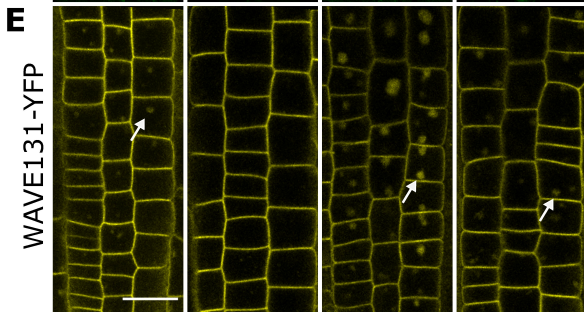
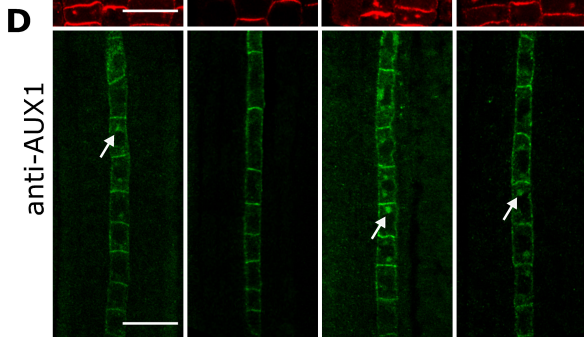
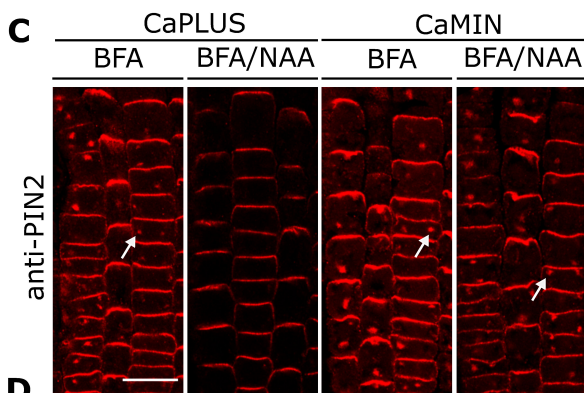
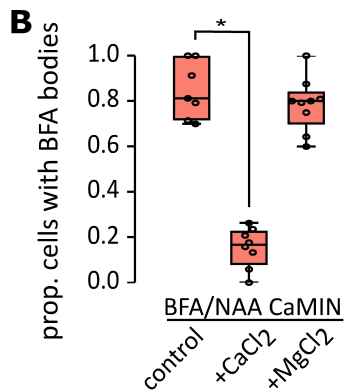
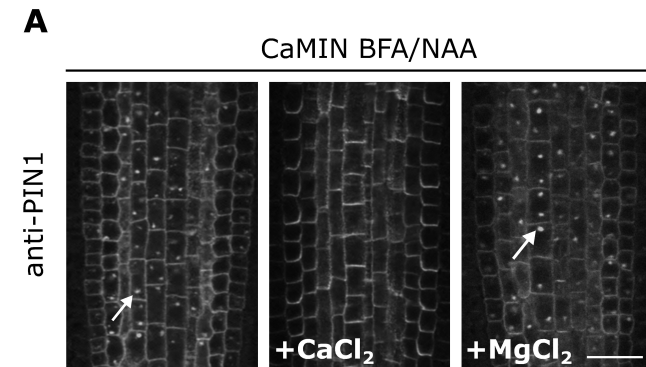
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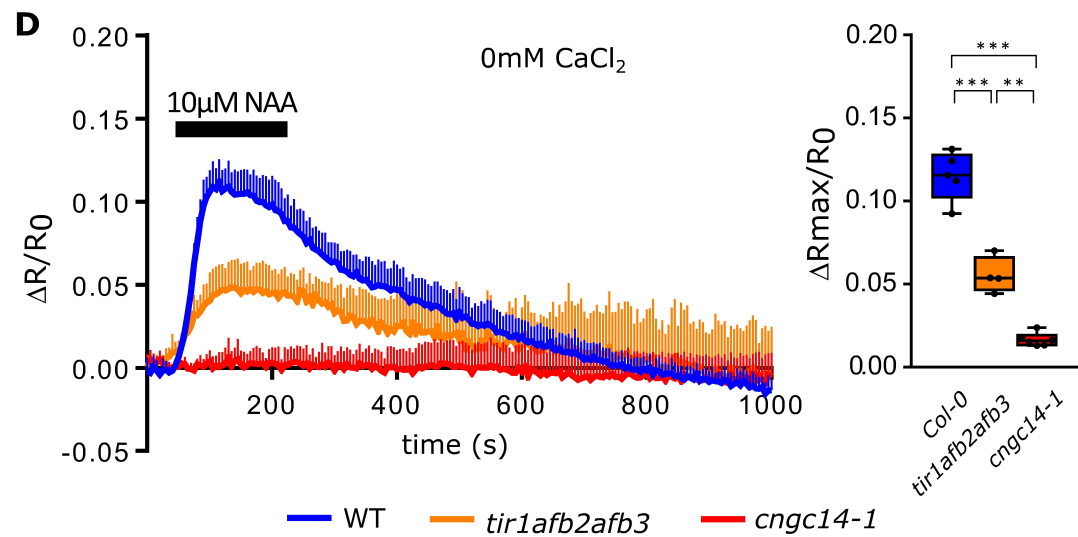
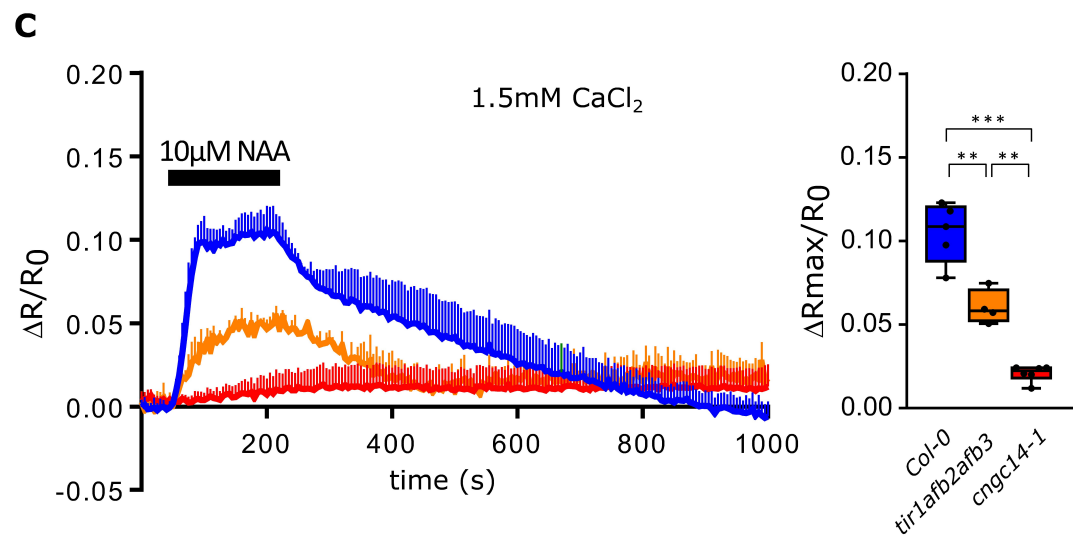
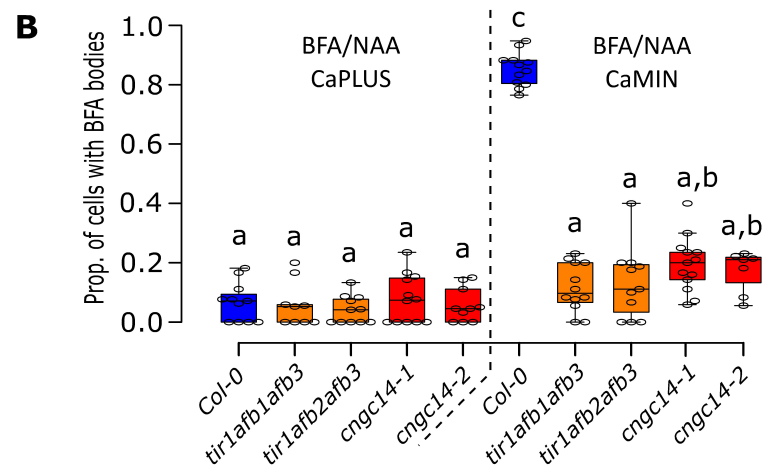
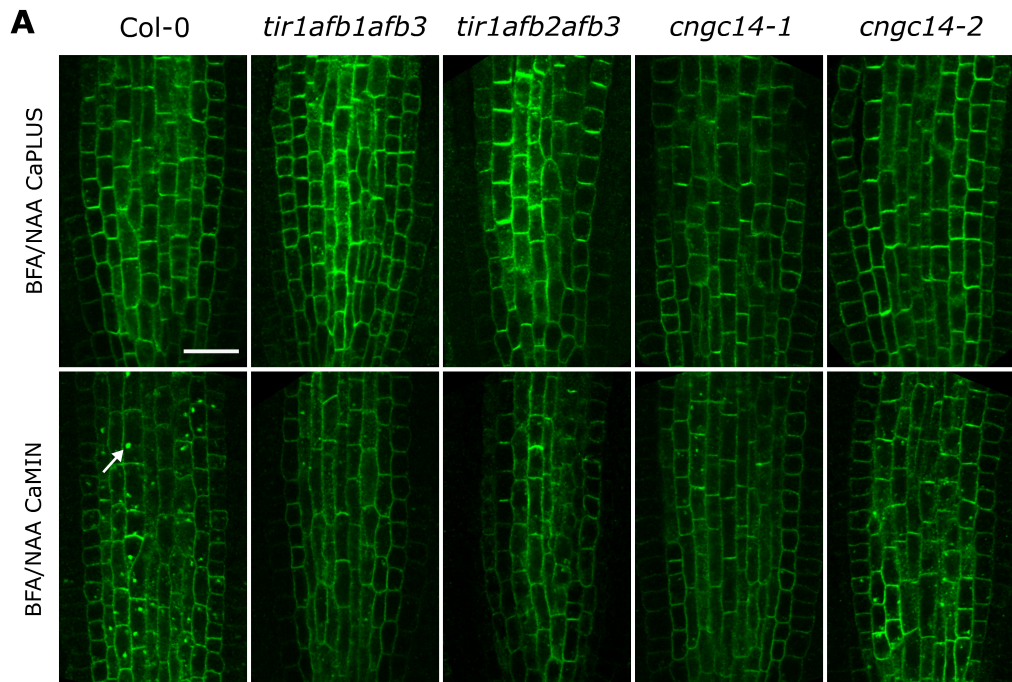
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- Abas L, Benjamins R, Malenica N, Paciorek T, Wisniewska J, Moulinier-Anzola JC, Sieberer T, Friml J, Luschign C (2006) Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. Nat Cell Biol 8: 249-256**  
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