Pharmacological and genetic manipulations of Ca^{2+} signaling have contrasting effects on auxin-regulated trafficking

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Author Contributions

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

ABSTRACT

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

Key words: Auxin, Calcium, Trafficking, Signaling.

INTRODUCTION

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

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

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

80 In contrast to our poor understanding of auxin-induced 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 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

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

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

RESULTS

Characterization of NAA-induced Ca^{2+} signaling.

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

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

Additionally, we modulated the available extracellular Ca^{2+} by washing the seedlings with 0.5xMS medium that lacked $CaCl_2$ (hereafter referred to as CaMIN). This simple treatment was sufficient to reduce the NAA-induced Ca^{2+} response in comparison to normal 0.5xMS medium (≈1.5mM $CaCl_2$, hereafter CaPLUS) (Figure 1C). Jointly, these data suggest that NAA triggers a complex Ca^{2+} response that largely depends on extracellular Ca^{2+}.

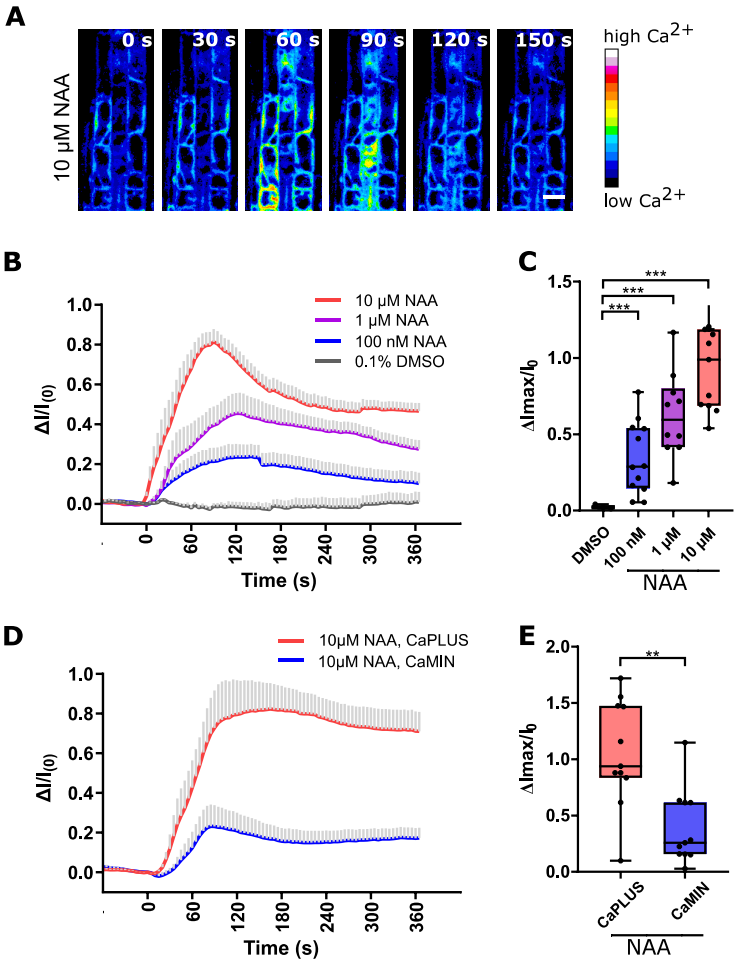


Figure 1. Apoplastic Ca^{2+} determines the amplitude of auxin-induced cytosolic Ca^{2+} dynamics.

A. Single frames of the dynamic response of the Ca^{2+} sensor, R-GECO1, at indicated time points after 10µM NAA treatment. Scale bar = 20 µm.

B. Averaged and normalized R-GECO1 fluorescence intensities over time upon treatment with 0.1% DMSO, 10µM NAA, 1µM NAA, or 100nM NAA. (nr of seedlings = 3, 12, 10, 12 respectively, means +s.e.m.). DMSO and NAA treatments were applied at time point 0.

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

D. The averaged normalized R-GECO1 fluorescence intensities over time upon treatment with 10µM NAA following a 30min pretreatment with CaPLUS or CaMIN (nr of seedlings = 11; 3 repeats; means +s.e.m.). NAA treatments were applied at time point 0s.

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

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

155

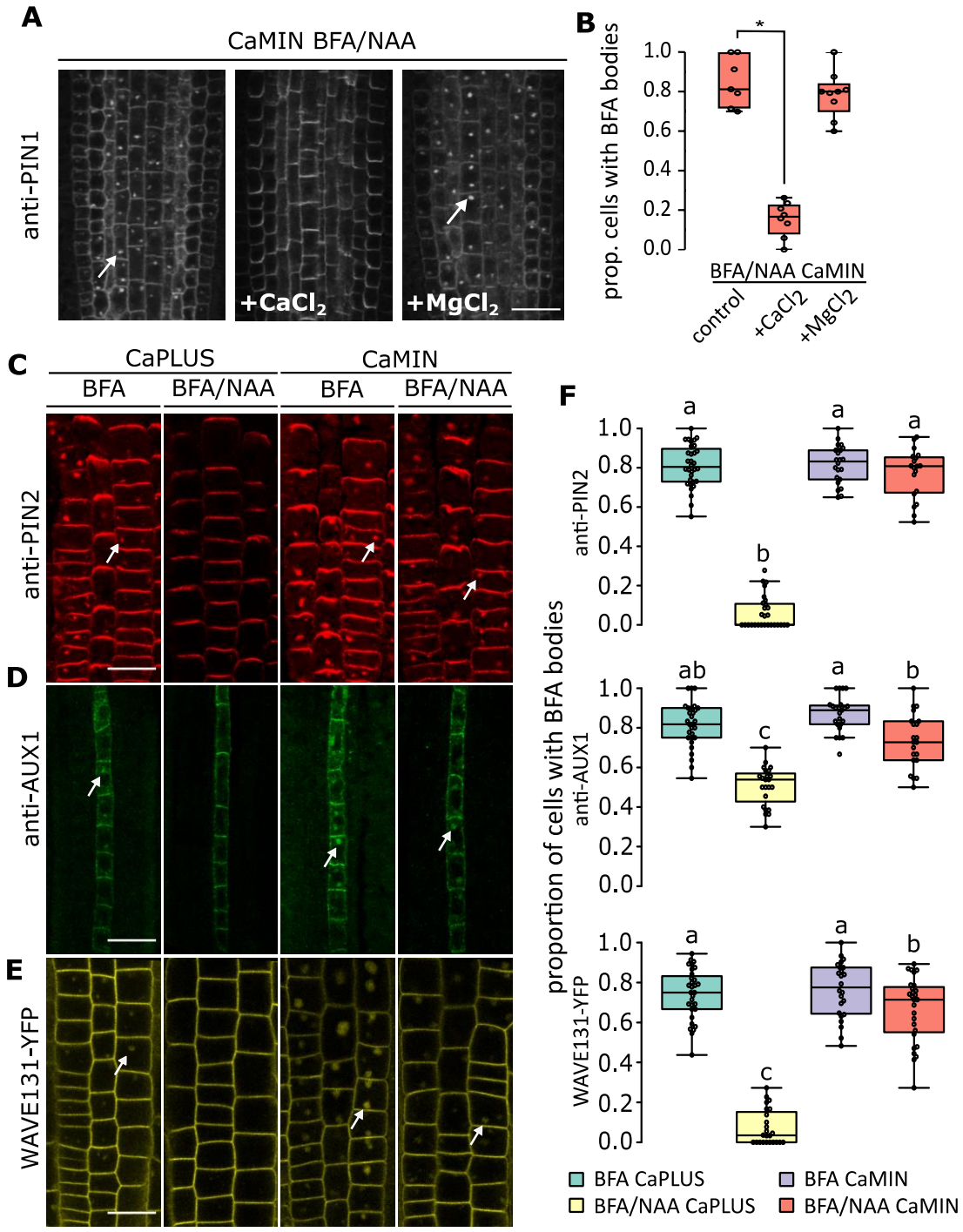
156 **NAA-mediated Inhibition of internalization correlates with Ca^{2+} signaling**

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^{2+} response (Figure 1C,D). Given the immediacy of both processes, we postulated that auxin-induced
163 Ca^{2+} responses reflects an auxin signaling cascade involved in NAA-regulated internalization.

164 Indeed, when we used the organic Ca^{2+} channel blockers Nifedipine and Bepridil at
165 concentrations that interfered with NAA-induced Ca^{2+} 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^{2+} 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^{2+} 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^{2+} availability, we analyzed PIN1 internalization in CaMIN
174 supplemented with either 1.5mM $CaCl_2$ (comparable to 0.5xMS) or 1.5mM $MgCl_2$ (Figure 2A,B). The
175 addition of $CaCl_2$ fully restored the NAA sensitivity of PIN1 internalization. In contrast, the internalization
176 in roots treated with $MgCl_2$ supplemented CaMIN could not restore the NAA sensitivity, indicating the
177 specificity of Ca^{2+} 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.



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

A. Immunolocalisation of PIN1 in 3 day-old seedling roots pretreated with CaMIN (30min), followed by BFA and NAA (co)treatment (1h), in CaMIN, and in CaMIN supplemented with either 1.5 mM $CaCl_2$ and CaMIN supplemented with 1.5mM $MgCl_2$. Scale bar = 20µm.
B. Quantification of the proportion of cells that have PIN1 in BFA bodies for the conditions in **A**, and corresponding controls in CaPLUS.(n ≥ 3 per treatment per repeat; 2 independent repeats). For all box plots, the central line indicates the median, the bottom and top edges of the box the interquartile range, and the box plot whiskers are plotted down to the minimum and up to the maximum value. Data were analyzed using a logistic regression model. * indicates P ≤ 0.05, Wald-type test.
C. Immunolocalization of PIN2 in 3 day-old seedling roots after BFA or BFA/NAA treatments in CaPLUS and CaMIN conditions.
D. Immunolocalization of AUX1 in 3 day-old seedling roots after BFA or BFA/NAA treatments in CaPLUS and CaMIN conditions.
E. WAVE131(NPSN12)-YFP localisation in 3 day-old seedling roots after BFA or BFA/NAA treatments in CaPLUS and CaMIN conditions. Concentrations used for PIN2 and WAVE131-YFP BFA: 25µM, NAA: 10µM, 1h; Concentrations used for AUX1 BFA: 50µM, NAA: 10µM, 90min.
F. Quantification of the proportion of cells that have PIN2 (n=32;28;22;19 roots in total), AUX1 (n=30;26;26;22 roots in total) or WAVE131-YFP (n=31;24;24;27 roots in total) in BFA bodies, corresponding to experiments in Figure 2C-E. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. Different lowercase letters indicate significant differences (P ≤ 0.05, Wald-type test). White arrows in figures indicate proteins accumulated in BFA bodies. Scale bars represents 20 µm.

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

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

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

The discrepancy between the exogenous Ca^{2+} manipulations and the mutant analyses, raised the hypothesis that NAA may induce Ca^{2+} signaling via a TIR1/AFB-CNGC14-independent mechanism. Therefore, we transformed the ratiometric Ca^{2+} indicator NES-YC3.6 in *tir1/afb2,3* and *cngc14-1* and analysed the Ca^{2+} responses to a pulse of NAA in these mutants. We found that the NAA-induced Ca^{2+} response was strongly reduced in *tir1afb2,3* triple mutants and was completely absent from *cngc14-1* (Figure 3A), suggesting that NAA elicits Ca^{2+} signaling through the same mechanism as IAA. Also in the absence of exogenously supplied Ca^{2+}, the NAA-induced Ca^{2+} responses in *tir1afb2,3* and *cngc14-1* mutants were strongly defective (Figure 3B), excluding compensatory Ca^{2+} signaling mechanisms under low Ca^{2+} availability, as reported for pathogen-triggered immunity-associated Ca^{2+} signaling in *cngc2* and *cngc4* mutants (Tian et al., 2019). These data show that NAA-induced Ca^{2+} signaling is fully dependent on the TIR1/AFB-CNGC14 module. Notably, the YC3.6 Ca^{2+} indicator did not allow detecting the obvious differences in the NAA-induced Ca^{2+} response between CaMIN and CaPLUS that we observed when using R-GECO1 (Figure 1D). This probably reflects fundamental differences in Ca^{2+} binding properties and dynamic ranges that exist between both Ca^{2+} indicators' properties (Nagai et al., 2004; Zhao et al., 2011; Keinath et al., 2015; Waadt et al., 2017). This also suggests that the reduction in Ca^{2+} response caused by CaMIN treatment is weaker than the one in *tir1afb2afb3* and *cngc14-1*. The normal NAA sensitivity of PIN internalisation in these mutants, that are strongly defective in auxin-induced Ca^{2+} signaling, thus provides further evidence that auxin-induced Ca^{2+} signaling is not an essential part of the mechanism by which auxin affects trafficking. This is in direct contrast to the conclusions drawn using exogenous Ca^{2+} signaling manipulations, highlighting an important lack of specificity of these treatments.

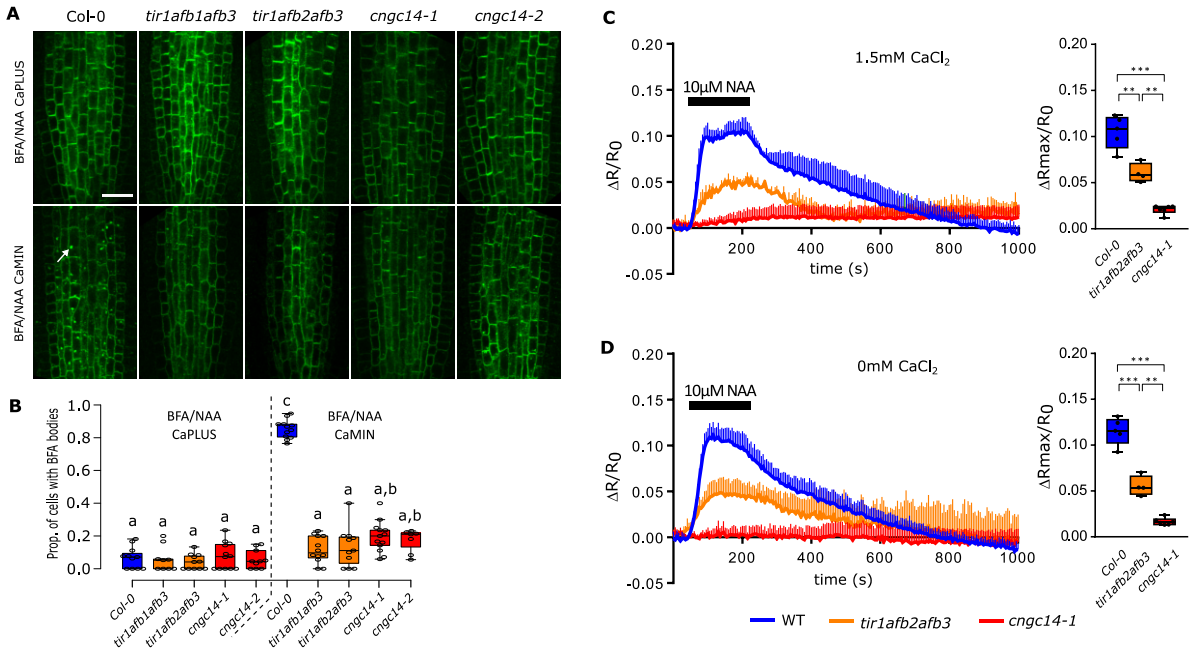

Figure 3. Analysis of inhibition of internalization and auxin-induced Ca^{2+} signaling in *tir1/afb* and *cngc14* mutants

A. Immunolocalization of PIN1 in 3 day-old roots co-treated for BFA and NAA in wild type, *tir1/afb1,3*, *tir1/afb2,3*, *cngc14-1* and *cngc14-2*, in CaPLUS and CaMIN conditions. White arrows indicate PIN1-accumulating BFA bodies. Scale bar is 20 µm.
B. Quantification of the proportion of cells that accumulate PIN1 in BFA bodies visualized by immunolocalisation in 3 day-old roots of indicated genotypes co-treated for BFA and NAA, in CaPLUS, CaMIN as depicted in **c**. Total numbers of roots analyzed in wild type (n=11; n=12 in total), *tir1afb1afb3* (n=9; n=12 in total), *tir1afb2afb3* (n=11; n=11 in total), *cngc14-1* (n=12; n=13 in total) and *cngc14-2* (n=10; n=7 in total), representing the sum of two independent experiments. Significant differences ($P \leq 0.05$, Wald-type test) are indicated by different lowercase letters. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots.
C,D. The Ca^{2+} dynamics (NES-YC3.60 Cameleon) in WT, *tir1afb2afb3*, *cngc14-1* in response to a pulse of 10µM NAA in medium containing (**C**) 1.5mM $CaCl_2$ or (**D**) 0mM $CaCl_2$.

DISCUSSION

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

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

CONCLUSION

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

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis thaliana seeds were sterilized by using bleach gas (8mL concentrated HCl to 150mL bleach) overnight, afterwards the seeds were sown on Petri dishes (12 cm X 12 cm) containing sterile half-strength Murashige and Skoog (½ MS) medium (0.5 x MS salts, 0.8% sucrose, 0.5 g/L 2-(N-morpholino) ethanesulfonic acid, pH 5.7, and 1% w/v agar), and grown under continuous light (21℃, continuous light), after 2 days vernalization at 4℃ in the dark. To prepare 2L liquid CaMIN medium following components were dissolved in MilliQ: 100mL MS basal salt micronutrient solution (catalog Nr), 20 g sucrose, 0.20 g myoinositol, 1.00 g MES, 1.652g NH_4NO_3, 0.180g $MgSO_4$, 1.920g KNO_3, 0.152g KH_2PO_4 and pH 5.7. The liquid CaPLUS medium contains an additional 0.332g $CaCl_2$ per 2L.

Chemicals

The following hormones/chemicals were used: 10µM NAA (catalog Nr N0903; Duchefa Biochemie). 25µM Brefeldin A (BFA, catalog Nr B6542-25MG). All hormones/drugs were dissolved in 100% dimethylsulfoxide (DMSO; catalog Nr D4540-500ML) and obtained from Sigma Aldrich.

Plant Lines Used

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

Immunodetection and Confocal Microscopy

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

Microscopy and image analysis

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

Statistical analysis

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

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

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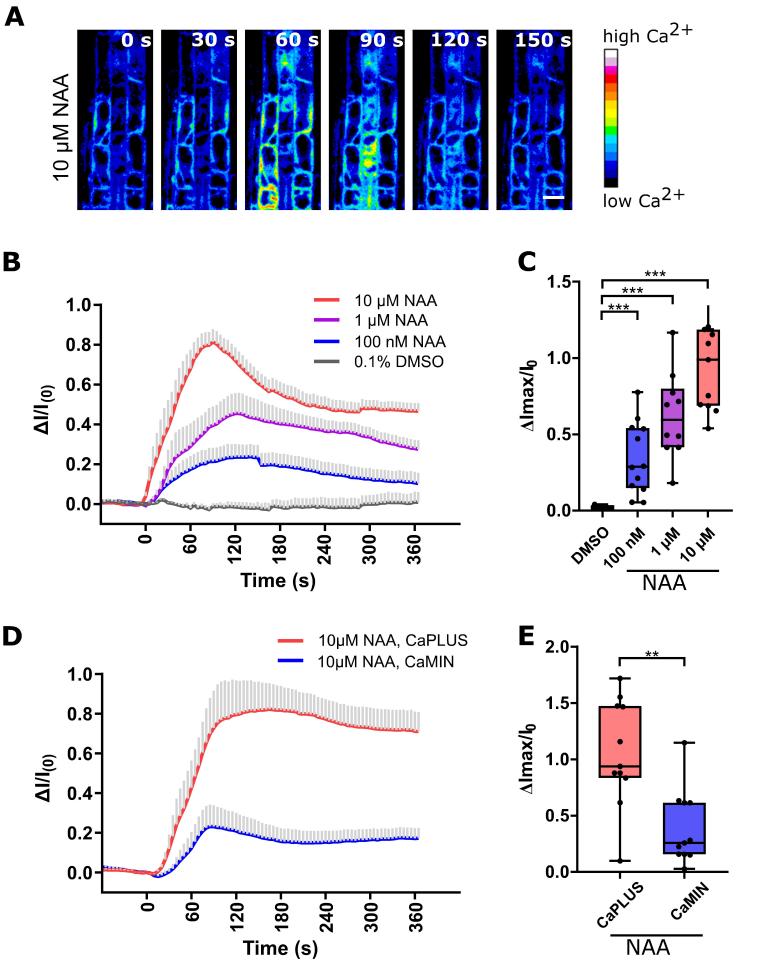
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0 s
30 s
60 s
90 s
120 s
150 s
high Ca2+
low Ca2+
B
10 µM NAA
1 µM NAA
100 nM NAA
0.1% DMSO
ΔI/I(0)
Time (s)
C
ΔImax/I0
***
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***
DMSO
100 nM
1 µM
10 µM
NAA
D
10µM NAA, CaPLUS
10µM NAA, CaMIN
ΔI/I(0)
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E
ΔImax/I0
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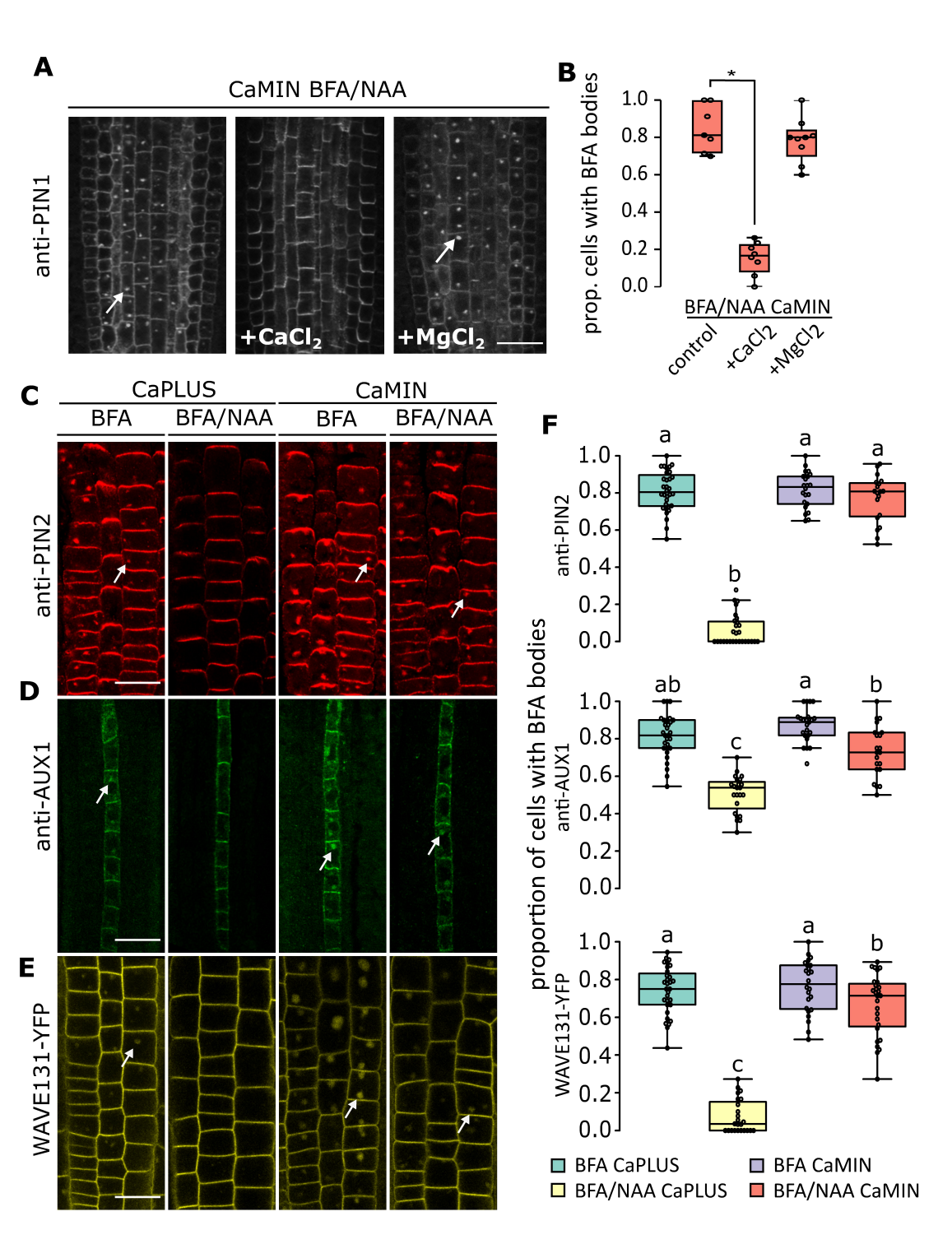
CaPLUS
CaMIN
NAA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CaMIN BFA/NAA
anti-PIN1
+CaCl2
+MgCl2
B
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0.8
0.6
0.4
0.2
0.0
*
BFA/NAA CaMIN
control
+CaCl2
+MgCl2
C
CaPLUS
CaMIN
BFA
BFA/NAA
BFA
BFA/NAA
anti-PIN2
D
anti-AUX1
E
WAVE131-YFP
F
proportion of cells with BFA bodies
anti-PIN2
anti-AUX1
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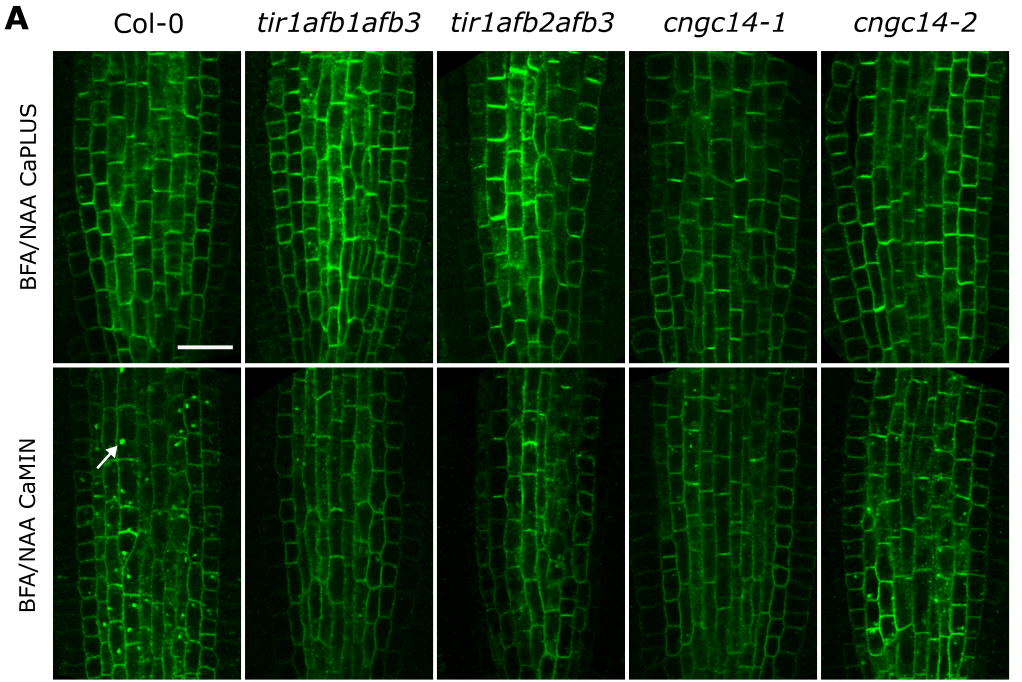
BFA/NAA CaMIN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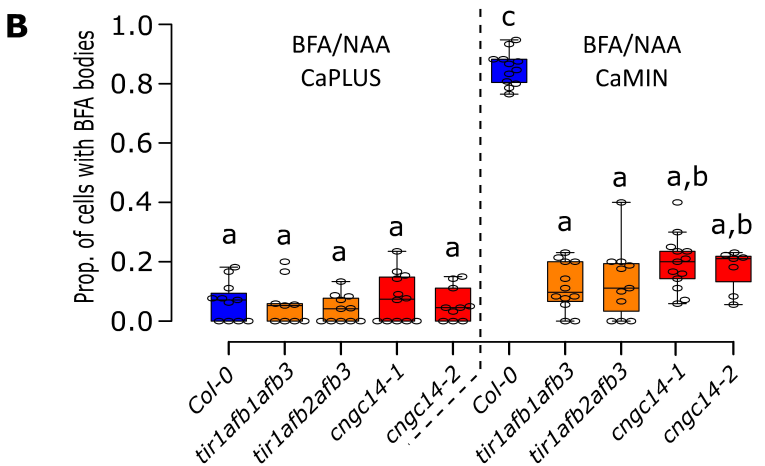
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Prop. of cells with BFA bodies
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a
a
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a
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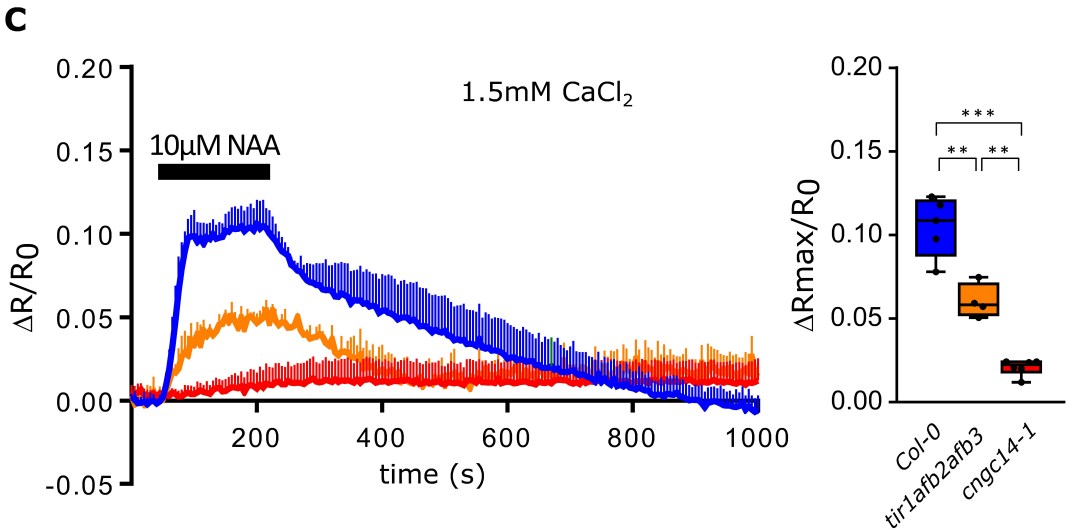
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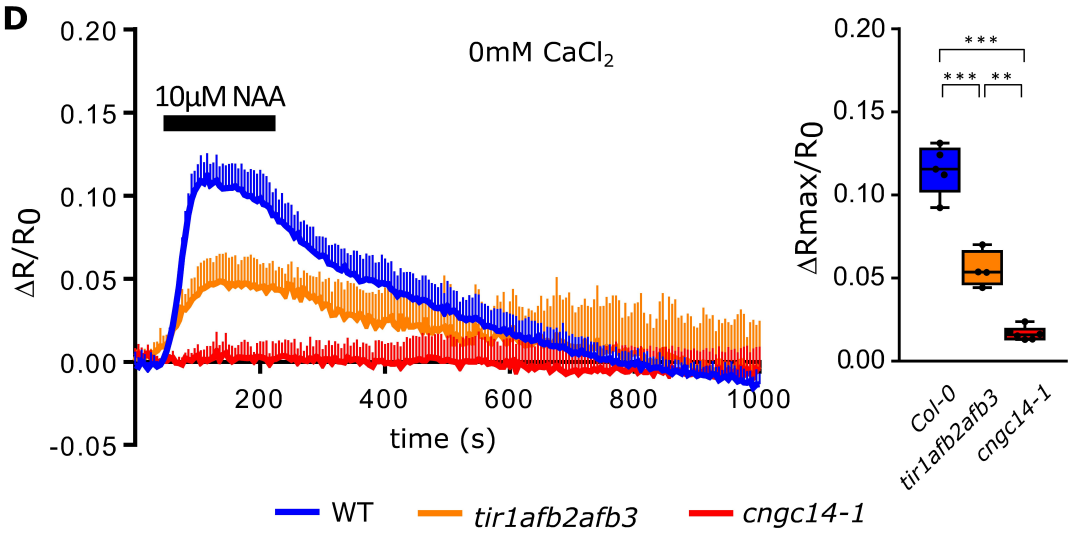
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time (s)
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