Auxin analog-induced Ca²⁺ signaling is not involved in inhibition of endosomal aggregation

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Highlight

The effect of synthetic auxin analogs on non-transcriptional responses is poorly understood. Here, we characterize auxin analog-induced Ca²⁺ signaling, and show that this is independent of inhibition of endosomal aggregation.

ABSTRACT

Much of what we know about the role of auxin in plant development derives from exogenous manipulations of auxin distribution and signaling, using inhibitors, auxins and auxin analogs. In this context, synthetic auxin analogs, such as 1-Naphtalene Acetic Acid (1-NAA), are often favored over the endogenous auxin indole-3-acetic acid (IAA), in part due to their higher stability. While such auxin analogs have proven to be instrumental to reveal the various faces of auxin, they displays in some cases distinct bioactivities compared to IAA. Here, we focused on the effect of auxin analogs on the accumulation of PIN proteins in Brefeldin A (BFA)-sensitive endosomal aggregations (BFA bodies), and the correlation with the ability to elicit Ca^{2+} responses. For a set of commonly used auxin analogs, we evaluated if auxin-analog induced Ca^{2+} signaling inhibits PIN accumulation in. We found that not all auxin analogs elicited a Ca^{2+} response, and that their differential ability to elicit Ca^{2+} responses correlated partially with their ability to inhibit BFA-body formation. However, in *tir1/afb* and *cngc14*, 1-NAA-induced Ca^{2+} signaling was strongly impaired, while 1-NAA's still could inhibit PIN accumulation in BFA bodies. This demonstrates that TIR1/AFB-CNGC14-dependent Ca^{2+} signaling does not inhibit BFA body formation.

Key words: Arabidopsis, Auxin-analogs Calcium, endosomes, root, signaling , trafficking.

INTRODUCTION

The natural auxin, indole-3-acetic acid (IAA) is a potent regulator of plant development. Therefore, plants have developed rigorous mechanisms to control IAA activity. In many cases, auxin itself activates a feedback regulation on its own activity, via activation of auxin transport, compartmentalisation and conjugation (Adamowski and Friml, 2015; Casanova-Saez *et al.*, 2021; Rosquete *et al.*, 2012). This, and a low stability in medium, has led to a preferential use of more stable, synthetic auxin to study long-term auxin effects on plant development. Despite obvious drawbacks, the use of auxin analogues has been very successful for the identification of multiple components of auxin signaling and transport via forward genetic screens based on resistance to 2,4-D (Estelle and Somerville, 1987; Lincoln *et al.*, 1990; Maher and Martindale, 1980) and 1-NAA (Ashton *et al.*, 1979). This illustrates how synthetic auxins have been used to drive auxin research forward.

The auxin analogues can thus activate what is now known as the canonical pathway for auxin-induced transcription. Auxin or a synthetic analog stabilizes interaction between TIR1/AFB F-box proteins and Aux/IAA transcriptional co-repressors (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005), to trigger the proteolysis of the latter (Nemhauser, 2018), resulting in derepression of ARF transcription factors (Pierre-Jerome *et al.*, 2016; Roosjen *et al.*, 2018). TIR1/AFB-based auxin sensing has also recently emerged in the context of non-transcriptional auxin signaling, explaining auxin-induced membrane depolarization, apoplast alkalinisation, Ca^{2+} signaling and root growth inhibition (Dindas *et al.*, 2018; Fendrych *et al.*, 2018; Gallei *et al.*, 2020; Li *et al.*, 2021; Serre *et al.*, 2021).

Arabidopsis contains 6 TIR1/AFB family members with overlapping and specific functions (Prigge *et al.*, 2020). Different auxins and auxin analogs have distinct affinities and binding specificities towards either TIR1-type (TIR1, AFB1,2 & 3) or AFB5-type (AFB4 & 5) AFBs (Lee *et al.*, 2014), and can thus be used to selectively activate a subset of them. A well-known example of this is picloram, that selectively activates AFB4 and AFB5, a biochemical activity that is reflected in the picloram resistance of the corresponding mutants (Prigge *et al.*, 2016). In contrast, most other commonly used auxins and auxin analogs, such as IAA, 1-NAA and 2,4-D are promiscuous activators of all TIR1/AFBs (Calderon Villalobos *et al.*, 2012; Kepinski and Leyser, 2005; Lee *et al.*, 2014). In many cases, 2-NAA is used as inactive 1-NAA analog, despite displaying a weak affinity for TIR1-type AFBs (Lee *et al.*, 2014).

The use of auxin analogs also identified effects of auxin on pavement cell morphogenesis (Xu *et al.*, 2010), lipid composition and distribution (Li *et al.*, 2015; Pan *et al.*, 2009; Platre *et al.*, 2019), cell division during lateral root formation (Huang *et al.*, 2019), suppression of auxin biosynthesis (Wang *et al.*,

2020), stability and polarity of PIN proteins (Abas *et al.*, 2006; Baster *et al.*, 2013; Mazur *et al.*, 2020a; Prat *et al.*, 2018; Sauer *et al.*, 2006), and complex effects on the endomembrane system (Narasimhan *et al.*, 2021; Paciorek *et al.*, 2005; Platre *et al.*, 2019; Robert *et al.*, 2010), apoplast acidification (Li *et al.*, 2021; Lin *et al.*, 2021). In most cases, only the auxin perception mechanism has been identified, TIR1/AFB and/or the receptor-like kinase family TRANSMEMBRANE KINASE1-4 (Cao *et al.*, 2019; Huang *et al.*, 2019; Li *et al.*, 2021; Lin *et al.*, 2021; Platre *et al.*, 2019).

An inhibitory effect of auxin on internalization has been proposed in part based on experiments using the synthetic auxin 1-naphtalene acetic acid (1-NAA) in combination with the fungal toxin Brefeldin A (BFA) (Paciorek *et al.*, 2005; Robert *et al.*, 2010). This inhibitor (BFA) is a commonly used cell biological tool to inhibit secretion. It stabilizes the interaction between ADP-Ribosylation Factor (ARF) small GTPases, and their activators, ARF-Guanine-nucleotide Exchange Factors (ARF-GEF), thereby trapping the complex at the membrane surface (Brumm *et al.*, 2020; Renault *et al.*, 2003), and impairing ARF-mediated membrane trafficking. A side-effect of BFA treatment is the formation of so-called BFA-bodies, large endosomal aggregates that are surrounded by Golgi stacks (Robinson *et al.*, 2008), in which endocytosed, as well as *de novo* synthesized cargoes are concentrated (Jasik and Schmelzer, 2014; Narasimhan *et al.*, 2021). The synthetic auxin, 1-NAA, has a profound effect on the accumulation of PINs in BFA bodies (Paciorek *et al.*, 2005), an effect that is much weaker when using the endogenous auxin IAA (Paciorek *et al.*, 2005; Paponov *et al.*, 2019; Simon *et al.*, 2013). Side by side comparison of the effects of 1-NAA and IAA on the endomembrane system organization and dynamics has revealed that both auxinic molecules interfere with BFA-induced endosomal aggregation, albeit with different efficiency, and neither of them interfered with the incidence and dynamics of clathrin-mediated endocytic events (Narasimhan *et al.*, 2021). On the other hand, unlike IAA, 1-NAA affects the uptake of the endocytic tracer dye FM4-64, and impacted on the organization and identity of endosomal compartments (Narasimhan *et al.*, 2021). These examples indicate that IAA and 1-NAA have differential activities in the cell. A good understanding of the mechanisms underlying the differential activities of both molecules is essential to interpret the physiological relevance of the responses to either molecule.

Calcium is the universal second messenger in living cells, and has a key roles in many cellular processes, including endomembrane trafficking (Himschoot *et al.*, 2017). However, its ubiquitous activity makes it difficult to isolate any specific function. Indeed, auxin-induced Ca^{2+} signaling has been known for many years, but its physiological role remains poorly understood (Vanneste and Friml, 2013). In recent years, however, critical progress has been made in unraveling auxin-induced Ca^{2+} signaling. The reduced IAA-induced Ca^{2+} fluxes *tir1/afb2,3* mutants (Dindas *et al.*, 2018), and induction of Ca^{2+} signals by via

orthogonal activation of an engineered TIR1 variant (Li *et al.*, 2021), identified TIR1/AFB family members as auxin sensors for auxin-induced Ca^{2+} signaling. Two non-selective cation channel subunits CNGC14 and CNGC2 have been identified as critical components of auxin-induced Ca^{2+} entry (Chakraborty *et al.*, 2021; Dindas *et al.*, 2018; Shih *et al.*, 2015). CNGC14 activity was proposed to participate in root gravitropism (Shih *et al.*, 2015) and root hair development (Brost *et al.*, 2019; Dindas *et al.*, 2018). CNGC2 is well characterized in defense related Ca^{2+} signaling, and was recently found to control auxin biosynthesis and signaling, and showing auxin-resistance (Chakraborty *et al.*, 2021). While the interrelationship between these components remains to be resolved, these observations provide 3 independent approaches to specifically interfere with auxin-induced Ca^{2+} signaling, and assess the impact on cellular processes. Here, we used these genetic tools and auxin analogs to assess the role of auxin-analog induced Ca^{2+} signaling in regulation of PIN aggregation in BFA bodies.

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 (0.5 x 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°C, continuous light), after 2 days stratification at 4°C in the dark.

Chemicals

The following hormones/chemicals were used: 1-NAA (catalog Nr N0903; Duchefa Biochemie). Brefeldin A (BFA, catalog Nr B6542-25MG-Sigma Aldrich). 2-NAA (catalog Nr N4002-5G-A-Sigma Aldrich). Quinclorac (catalog Nr 36521-250MG-Sigma Aldrich). 2,4-dichlorophenoxy acetic acid (2,4-D, catalog Nr D7299-100G-Sigma Aldrich). Dicamba (catalog Nr 45430-250MG-Sigma Aldrich) and Picloram (catalog Nr 36774-250MG-R-Sigma Aldrich). α -(phenyl ethyl-2-one)-indole-3-acetic acid (PEO-IAA, catalog Nr TA9H97BAEB94-5MG-Sigma Aldrich). Auxinole (catalog Nr BL3H160B7E0C-50MG-Sigma Aldrich). The natural auxin PhenylAcetic Acid (PAA, catalog Nr P16621-5G-Sigma Aldrich) and the non-specific control Benzoic Acid (BA, catalog Nr 242381-25G-Sigma Aldrich). All hormones/drugs were dissolved in 100% dimethylsulfoxide (DMSO; catalog Nr D4540-500ML-Sigma Aldrich).

Plant Lines Used

The Arabidopsis line 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), *cngc14-1* and *cngc14-2* (Shih *et al.*, 2015), *cngc6-1/9-3*, *cngc6-1,14-4*, *cngc9-3,14-4* and *cngc6-1,9-3,14-14* (Brost *et al.*, 2019), NES-YC3.60 (Krebs *et al.*, 2012), PM-YC3.60-Lti6b (Krebs *et al.*, 2012), CRT-D4ER (Bonza *et al.*, 2013), 4mt-YC3.60 (Loro *et al.*, 2012). The NES-YC3.60 (Krebs *et al.*, 2012) reporter construct 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 (Fig. 4A). Analyses of the Ca²⁺ response was done on T2 generation seedling roots showing strong and uniform expression.

Immunodetection

The seedlings used for immunodetection are 3-day-old seedlings, pre-treated in liquid 0.5 x MS for 30 minutes, followed by a 1h (co)treatment with BFA (25μM) or BFA (25μM) /NAA (10μM) by transfer to fresh 0.5 x MS with the respective chemicals. 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 InsituPro Vsi II (Intavis), as described by Sauer *et al.* (2006). In brief, immediately after fixation, the samples were transferred to the 30-well rack of the robot and subjected to 6 washes (3 in PBS pH 7.4+ 0.1%Triton X-100 (PBS-T); 3 in distilled water + 0.1%Triton X-100; 5 min each), cell wall digestion (1.5% Driselase in PBS, 30min at 37°C;), 3 washes (PBS-T, 5min), permeabilization (3% NP-40, 10% DMSO in PBS; 30min room temperature), 3 washes (PBS-T, 5min), blocking solution (3% BSA in PBS, 1h 37°C), primary antibody solution (primary antibodies diluted in blocking solution ,4h 37°C), 5 washes (PBS-T, 5min), secondary antibody solution (secondary antibodies diluted in blocking solution, 3h, 37°C), 5 washes (PBS-T, 5min) and 5 washes (distilled water, 5 min). The dilutions of the primary antibodies used are: goat anti-PIN1 (1:600) (sc-27163, SantaCruz). The dilutions of the secondary antibodies used are: AlexaFluor488 donkey anti-goat (1:600) (A-11055, ThermoFisher). Seedlings were mounted in AF1 antifadant (Citifluor). A Leica SP2 or a Zeiss LSM 710 confocal laser scanning microscope equipped with a 63x water-corrected objective was used for detection of Alexa488 (ex 488 nm/em 500-545 nm), Alexa555 (ex 561 nm/em 555-610 nm).

Microscopy and image analysis

Yellow Cameleon-based experiments were performed and analyzed as described (Behera *et al.*, 2018). Confocal microscopy calcium imaging analyses of Arabidopsis seedlings expressing NES-YC3.60 were

performed using a Nikon Eclipse Ti2 inverted microscope, equipped with a Nikon A1R+ laser scanning device (Nikon). Images were acquired by a CFI Plan Apo Lambda 20X (N.A. 0.75). Cameleon was excited with the 445 nm laser and the emissions of CFP and cpVenus were collected at 482/35 nm and 540/30 nm respectively. Pinhole was set to 35.76 μm and the images were acquired at 1024 x 1024 pixels resolution with scan speed of 0.5 frame per seconds. NIS-Elements (Nikon; <http://www.nis-elements.com/>) was used as a platform to control the microscope. Images were analysed using NIS-Elements to generate the ratio images and FIJI (<https://imagej.net/Fiji>) to quantify the fluorescence. Kymographs were generated on denoised imaged obtained with the NIS-Element Denoise.ai plugin (https://www.microscope.healthcare.nikon.com/en_EU/products/confocal-microscopes/a1hd25-a1rhd25/nis-elements-ai). By using the Fiji "Dynamic Reslice" function we created a new image, which depicts the temporal evolution of the Ca^{2+} signal through the spatial path previously defined.

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 and crop images and label the region of interest of all the roots for quantification. The proportion of cells with BFA bodies was scored manually and calculated by using Excel. BoxPlotR was used to generate the box plots (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 were 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.

RESULTS

Characteristics of 1-NAA-induced Ca^{2+} signaling

The synthetic auxin 1-NAA interferes via a non-transcriptional pathway with the accumulation of plasma membrane proteins in Brefeldin A (BFA)-induced intracellular endosomal aggregates (hereafter referred to as BFA bodies) (Paciorek *et al.*, 2005; Robert *et al.*, 2010), and is most prominent at high 1-NAA concentrations (Fig. 1A,B). This effect was proposed to reflect a combination of specific and non-specific effects of 1-NAA on protein biosynthesis and endosomal aggregation (Jasik and Schmelzer, 2014; Narasimhan *et al.*, 2021; Paponov *et al.*, 2019). The molecular mechanisms of these effects remain unknown, but are proposed to involve non-transcriptional responses (Robert *et al.*, 2010). Therefore, we set out to determine the involvement of auxin-induced Ca^{2+} signaling, a poorly understood, non-transcriptional auxin response (Dindas *et al.*, 2018; Shih *et al.*, 2015; Vanneste and Friml, 2013).

Using the ratiometric NES-YC3.60 (Krebs *et al.*, 2012), we observed an instant cytosolic Ca^{2+} elevation in response to 10 μM 1-NAA. The onset of the Ca^{2+} increase started within seconds after 1-NAA application, and rapidly reached a maximum, followed by a gradual response attenuation in the presence of 1-NAA, and a rapid return to baseline levels upon 1-NAA washout (Fig. 1C). Subcellular targeting of ratiometric Ca^{2+} sensors revealed a rise in Ca^{2+} concentrations in response to 1-NAA, besides the cytosol near the plasma membrane (visualized with PM-YC3.60-Lti6b (Krebs *et al.*, 2012)), in the endoplasmic reticulum lumen (visualized with CRT-D4ER (Bonza *et al.*, 2013)) and in mitochondria (visualized with 4mt-YC3.60 (Loro *et al.*, 2012)) (Supplementary Fig. S1A-D). The signals in the ER and mitochondria were slightly delayed compared to the other reporters, suggesting that these organelles may act as Ca^{2+} sinks for attenuation of the cytoplasmic Ca^{2+} signal (Resentini *et al.*, 2021b), and is consistent with the proposal that the periplasm is the main source of auxin-induced Ca^{2+} (Lamport *et al.*, 2021).

Also at lower concentrations (1 μM and 0.1 μM), 1-NAA triggered rapid Ca^{2+} signaling, albeit with smaller amplitudes (Fig. 1C,D), illustrating a dose-dependence of the maximal response. These analyses show that the potent inhibition of PIN1 BFA body formation by 1-NAA coincides with intense Ca^{2+} signaling.

Fig1. The dose-response of BFA body formation and Ca^{2+} response

(A) Immunolocalization of PIN1 in 3 day-old roots in 1/2MS (co)treated for 1h with 25 μM BFA, 25 μM BFA and 0.1 μM 1-NAA, 25 μM BFA and 1 μM 1-NAA, and 25 μM BFA and 10 μM 1-NAA. White arrows indicate PIN1-accumulating.

(B) Quantification of the proportion of cell accumulating PIN1 in BFA bodies in (A) (For the respective treatments $n=18$; 22; 21; 17 roots). n is the bulked number of quantified roots from 3 independent experiments. For the box plots, significant differences ($P \leq 0.05$, Wald-type test) are indicated by different lowercase letters. 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.

(C-D). Averaged and normalized NES-YC3.60 fluorescence ratios over time using wide-field illumination upon treatment with 0.1, 1 or 10 μM 1-NAA. ($n \geq 5$) respectively, means + S.D.). 1-NAA treatments were applied as indicated by the black line. The schematic root indicates the region of interest (ROI) used for measurements (C). The maximal NES-YC3.60 responses were quantified and plotted as box-and-whisker plots (D). Experimental points are plotted and their distribution represented as a box that extends from the 25th to 75th percentiles. The central line in the boxes are at the respective medians. (Unpaired Student's t- test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

The ability of auxin analogues to modulate PIN BFA body formation does not correlate with Ca²⁺ signaling

Different auxin analogues and anti-auxins have been shown to inhibit PIN accumulation in BFA bodies at different concentrations (Paciorek *et al.*, 2005; Paponov *et al.*, 2019; Robert *et al.*, 2010; Simon *et al.*, 2013). Therefore, we asked if this effect on trafficking would correlate with Ca²⁺ responses elicited by such molecules. We collected representatives of the most important classes of auxin analogues: 1-NAA, Quinclorac, 2,4-dichlorophenoxy acetic acid (2,4-D), Dicamba and Picloram, the anti-auxins α -(phenyl ethyl-2-one)-indole-3-acetic acid (PEO-IAA), Auxinole, the natural auxin Phenylacetic Acid (PAA), the presumed inactive 1-NAA analog, 2-NAA, and the non-auxinic, weak acid control Benzoic Acid (BA) (Supplementary Fig. S2). Each of these molecules was tested at 10 μ M to allow for comparison of the effects.

Consistently with previous reports (Paponov *et al.*, 2019; Robert *et al.*, 2010), 1-NAA, 2-NAA, and both anti-auxins strongly inhibited PIN1 accumulation in BFA bodies (Fig. 2A,B). Of all other tested molecules, only 2,4-D caused a significant reduction in the proportion of cells with PIN1 in BFA bodies (Fig. 2A,B). It is known that 2,4-D exerts stronger trafficking effects at higher concentrations (Simon *et al.*, 2013). Similar effects of these chemicals were observed for PIN2 accumulation in BFA bodies (Supplementary Fig. S3A,B).

Fig2. Distinct effects of different auxin analogs on PIN1 BFA body formation

(A) Analysis of PIN1 internalization through whole-mount immunolocalization in 3 day-old Col-0 seedling root meristems. Seedlings were treated with 25 μ M BFA and 10 μ M of 1-NAA, 2-NAA, Quinclorac, 2,4-D, Phenylacetic acid, Benzoic acid, Dicamba, Picloram, PEO-IAA, Auxinole. Scale bar=10 μ m.

(B) Quantification of the proportion of cell accumulating PIN1 in BFA bodies for different co-treatments described in (A) (n=18; 18; 16; 13; 17; 14; 14; 11; 14; 16; 17 roots). n is the bulked number of quantified roots from 3 independent experiments. For the box plots, significant differences ($P \leq 0.05$, Wald-type test) are indicated by different lowercase letters. 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.

Next, we analyzed the effects of pulse of the respective molecules on the Ca^{2+} response. Surprisingly, the “inactive” 1-NAA analog, 2-NAA triggered a Ca^{2+} response that was a bit slower and had a lower amplitude than 1-NAA (Fig. 3A,B), indicating 2-NAA is not completely inactive (Fig. 3A,B). In contrast to 1-NAA, the acute Ca^{2+} response to 2,4-D did not return to basal levels after wash-out (Fig. 3D). This could be a consequence of 2,4-D being a poor substrate for efflux machinery and being trapped inside the cell after uptake (Delbarre et al., 1996). In contrast, neither quinclorac, PAA, BA, dicamba, picloram, PEO-IAA nor auxinole activated an acute Ca^{2+} response (Fig. 3C, E-H). The slow linear induction of Ca^{2+} signalling during PAA treatment is somewhat surprising, as this natural auxin analog is also promiscuously sensed via TIR1/AFBs (Lee et al., 2014; Sugawara et al., 2015).

Together with the BFA body formation data, these data suggests that the ability of auxin analogues to inhibit PIN BFA body formation roughly correlates with their ability to induce a Ca^{2+} response. However, this correlation does not hold true for the anti-auxins, as none of them induced an acute Ca^{2+} response (Fig. 3 I-K).

Fig3. Distinct Ca^{2+} dynamics in response to different auxin analogs

(A-J) Averaged and normalized NES-YC3.60 fluorescence ratios over time using wide-field illumination upon treatment with $10\mu\text{M}$ 1-NAA (A), 2-NAA (B), Quinclorac (C), 2,4-D (D), Phenylacetic acid (E), Benzoic acid (F), Dicamba (G), Picloram (H), PEO-IAA (I) and Auxinole (J). Dashed lines indicate the interval of pulse treatment. (K) Quantification of the maximal NES-YC3.60 responses plotted as box-and-whisker plots ($n=5$ for each treatment described in (A-J), means + S.D. (STATISTICS)

1-NAA-induced Ca^{2+} signaling depends on TIR1/AFB-CNGC14

While we observed a correlation between the ability to inhibit PIN BFA body formation and Ca^{2+} signaling for the tested auxin analogues (Fig. 2B, 3K), this correlation does not hold true for the endogenous auxin IAA: IAA triggers rapid Ca^{2+} signals (Dindas *et al.*, 2018), but does not strongly interfere with BFA body formation (Narasimhan *et al.*, 2021; Paponov *et al.*, 2019). This could signify that 1-NAA employs a Ca^{2+} signaling mechanism, that is independent of IAA-induced Ca^{2+} signaling, to inhibit BFA body formation.

Therefore, we tested the involvement of IAA-induced Ca^{2+} signaling components in 1-NAA-induced Ca^{2+} signaling (Dindas *et al.*, 2018; Li *et al.*, 2021; Shih *et al.*, 2015). We transformed the ratiometric NES-YC3.60 construct into *tir1afb2afb3* and *cngc14-1* mutants. Transformants with expression levels similar to our WT control were used to monitor NAA-induced Ca^{2+} signals (Fig. 4A). The 1-NAA-induced Ca^{2+} response was strongly reduced in the *tir1afb2,3* triple mutants and was completely absent from *cngc14-1* (Fig. 4B,C). This corroborates that 1-NAA activates Ca^{2+} signaling via a TIR1/AFB-CNGC14-dependent mechanism, and is consistent with the measured reductions of extracellular Ca^{2+} fluxes in these mutants (Dindas *et al.*, 2018).

Fig. 4. 1-NAA-induced Ca^{2+} responses involve TIR1/AFB and CNGC14

(A) Comparison of the raw cpVenus/CFP ratios of the off-spring of the selected NES-YC3.60 x *tir1afb2afb3* and NES-YC3.60 x *cngc14-1* lines used in this study, relative to the original NES-YC3.60 line (WT). The ratios were quantified and plotted as box-and-whisker plots.

(B-C) The Ca^{2+} dynamics (NES-YC3.60) in WT, *tir1afb2afb3*, *cngc14-1* in response to a pulse of 10 μM 1-NAA using wide-field illumination. Data are presented as means + S.D. n=5 (B). The maximal NES-YC3.60 responses were quantified and plotted as box-and-whisker plots (C).

In (A) and (C) all experimental points are plotted and their distribution represented as a box that extends from the 25th to 75th percentiles. The central line in the boxes are at the respective medians. (Unpaired Student's *t*-test; non-significant (ns) $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

(D) Expression pattern of *CNGC14* in the meristem. Screenshot from <https://bioit3.irc.ugent.be/plant-sc-atlas/root> using *CNGC14* as input. Corresponding annotation of the tissue-specific cell clusters was implemented as outlined in the figure.

(E) Analysis of the 1-NAA-induced Ca^{2+} response of NES-YC3.60 in WT and *cngc14-1* via laser scanning confocal microscopy, in the same ROI as taken in the wide field illumination experiments. The focus was set to capture all root tissues with a z-depth of 3.77 μm .

(F) Kymograph analysis of NAA-induced Ca^{2+} signaling over in WT and in *cngc14-1* based on the movies that were used to construct the graph in Fig. 4E. Fluorescence ratios across the indicated dotted line of a central confocal section (a to b; 100 μm) were plotted over time. A white line in the kymographs indicates the pulse of 10 μM 1-NAA. The color-coded scale indicates NES-YC3.60 fluorescence ratios from low to high.

Transcriptional reporters and single cell expression analyses indicate that *CNGC14* expression is largely restricted to the root epidermis (Fig. 4D)(Brost *et al.*, 2019; Wendrich *et al.*, 2020). Therefore, we explored the contributions of the different root tissues to 1-NAA-induced Ca^{2+} signaling by also analyzing the NES-YC3.60 signal via confocal scanning microscopy (Fig. 4E), instead of wide-field illumination (Fig. 4B). To visualize the response dynamics in all tissues we defined a single line across the root segment of interest and plotted the NES-YC3.60 ratios over time to generate a kymograph (Fig. 4F). In contrast to the epidermis-specific expression of *CNGC14*, the kymograph revealed a near simultaneous 1-NAA-induced Ca^{2+} response in all root tissues (Fig. 4F). What is more, the 1-NAA-induced Ca^{2+} response was *CNGC14*-dependent in all tissues (Fig. 4F). This indicates that *CNGC14* indirectly controls auxin-induced Ca^{2+} responses in all tissues of the root meristem, possibly involving complex signal propagation and amplification mechanisms based on plasmodesmata and electrical signaling (Fichman *et al.*, 2021; Nguyen *et al.*, 2018; Resentini *et al.*, 2021a). Together, these data demonstrate that 1-NAA-induced Ca^{2+} signaling depends on TIR1/AFB and *CNGC14* activities.

Therefore, we could now address the requirement for 1-NAA-induced Ca^{2+} signalling in the inhibition of PIN1 BFA body formation. We tested a series of single, double and triple *tir1/afb* mutants, as well as three *cngc14* alleles and double and triple mutant combination with mutants in related *CNGCs* with demonstrated functional redundancies (*CNGC6*, and *CNGC9*) (Brost *et al.*, 2019). All mutants formed BFA bodies (Fig. 5A,C; Supplementary Fig. S4A-C), that could be inhibited by 1-NAA (Fig. 5B,C; Supplementary Fig. S4A-C), suggesting that the TIR1/AFB-*CNGC14* module is not required for this effect of 1-NAA. We also tested the sensitivity of the *cngc2-3* mutant, which was recently found to be defective in IAA-induced Ca^{2+} signaling (Chakraborty *et al.*, 2021). Similarly to *tir1/afb* and *cngc14* mutants, the 1-NAA sensitivity of BFA body formation was not changed in *cngc2-3* compared to WT (Supplementary Fig. 5).

Jointly, these data demonstrate that 1-NAA inhibits BFA body formation independently of TIR1/AFB-CNGC14-mediated Ca^{2+} signaling.

Fig. 5. Analysis of 1-NAA sensitive BFA body formation in *tir1/afb* and *cngc14* mutants

(A-B) Immunolocalization of PIN1 in 3 day-old roots treated with BFA (A) or co-treated for BFA and 1-NAA (B) in wild type, *tir1afb1afb3*, *tir1afb2afb3*, *cngc14-1* and *cngc14-2*. White arrows indicate PIN1-accumulating BFA bodies. Scale bar is 20 μ m.

(C) Quantification of the proportion of cells that accumulate PIN1 in BFA bodies visualized by immunolocalization as illustrated in (A) and (B) for wild type ($n_A=11$; $n_B=11$), *tir1afb1afb3* ($n_A=10$; $n_B=12$), *tir1afb2afb3* ($n_A=11$; $n_B=11$), *cngc14-1* ($n_A=11$; $n_B=12$) and *cngc14-2* ($n_A=11$; $n_B=12$). n is the pooled number of roots derived from three independent replicates.

For the box plots, significant differences ($P \leq 0.05$, Wald-type test) are indicated by different lowercase letters. 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.

DISCUSSION

CNGC14 is required for 1-NAA-induced Ca²⁺ signaling in all root tissues

In root hairs, CNGC14 acts partially redundant with other CNGCs to generate and maintain tip-focused Ca²⁺ oscillations (Brost *et al.*, 2019; Zhang *et al.*, 2017). In the root meristem, however, a single mutation in *CNGC14* is sufficient to abrogate IAA-induced Ca²⁺ signaling (Dindas *et al.*, 2018; Shih *et al.*, 2015). Similarly, we found that *cngc14* mutants are defective in 1-NAA-induced Ca²⁺ signaling, suggesting that 1-NAA and IAA activate similar Ca²⁺ signaling mechanisms.

In performing our analyses, we found that the 1-NAA-induced Ca²⁺ response is near instantaneous in all tissues of the root. Notably, single cell expression datasets showed that CNGC14 is predominantly expressed in the epidermis, and is possibly absent in the inner root tissues. Yet, the entire 1-NAA-induced Ca²⁺ response was defective in the *cngc14* mutant. This discrepancy between the expression domain and the observed effects suggests non-cell autonomous coordination of auxin-induced Ca²⁺ signaling in the root meristem. Such systemic Ca²⁺ responses are emerging as a prevalent theme in plant Ca²⁺ signaling, in which different Ca²⁺ channel types, in conjunction with electrical signals, reactive oxygen species and plasmodesmata, are involved in initiation and propagation of intercellular Ca²⁺ signaling (Choi *et al.*, 2014; Fichman and Mittler, 2020; Fichman *et al.*, 2021; Resentini *et al.*, 2021a; Toyota *et al.*, 2018). Intercellular coordination of auxin-induced Ca²⁺ via similar mechanism can be expected, given the requirement of coordination of growth of different tissues during gravitropic bending. Moreover, ROS production in response to a mechanical stimulus has been connected to Ca²⁺ signaling in the root (Monshausen *et al.*, 2009). Plasmodesmata are also emerging as regulators of auxin distribution and activity (Gao *et al.*, 2020; Mellor *et al.*, 2020; Sager *et al.*, 2020), indicating that these auxin-regulated plasmodesmata could modulate intracellular ROS movement for amplification of auxin-regulated Ca²⁺ signaling.

Does AFB1 contribute to auxin-induced Ca²⁺ signaling?

Recent efforts identified TIR1/AFBs as auxin sensors for auxin-induced Ca²⁺ signaling. This was demonstrated by the induction of rapid Ca²⁺ signaling by the orthogonal activation of an engineered TIR1 variant (ccvTIR1) (Li *et al.*, 2021). In contrast to *tir1* single mutants, auxin-induced Ca²⁺ signaling is strongly reduced *tir1afb2,3* triple mutants (Dindas *et al.*, 2018; Monshausen *et al.*, 2011), demonstrating functional redundancy among TIR1/AFBs. The fact that we could still detect an important auxin-induced Ca²⁺ signaling in *tir1afb2,3* triple mutants, but not in *cngc14* (Fig. 4B,C), indicates that additional TIR1/AFBs are involved in auxin-induced Ca²⁺ signaling.

Two of the used auxin analogues, picloram and quinclorac, have a strong biochemical preferences for AFB4/AFB5 over TIR1-type AFBs (Calderon Villalobos *et al.*, 2012; Lee *et al.*, 2014; Prigge *et al.*, 2016), suggesting they selectively activate AFB4 and AFB5. As neither of these compounds activated a detectable Ca^{2+} response (Fig. 3), we propose that these AFBs have minor, if any, roles in auxin-induced Ca^{2+} signaling. By excluding AFB4 and AFB5, we suggest that AFB1 auxin sensing activities could explain the majority of residual auxin-induced Ca^{2+} signaling in *tir1afb2,3* triple mutants. Consistently with a role in membrane associated auxin signaling, AFB1 is most closely related to TIR1, has a non-nuclear localization (Prigge *et al.*, 2020), and was recently highlighted as an auxin sensor for non-transcriptional membrane depolarization and initiation of root growth inhibition (Serre *et al.*, 2021).

1-NAA inhibits BFA body formation independently of TIR1/AFB-CNGC14-dependent Ca^{2+} signaling

Auxin-mediated regulation of trafficking is considered as an important aspect of auxin's self-regulating properties in plant growth and development. However, the underlying molecular mechanisms remain largely unclear. Synthetic auxins, such as 1-NAA, display non-specific effects on the endomembrane system, such as a strong interference with aggregation of endosomes in BFA bodies (Narasimhan *et al.*, 2021; Paponov *et al.*, 2019). This effect was reported to be very fast, not requiring transcriptional changes and independent of canonical auxin signaling (Robert *et al.*, 2010). Given the immediacy of the response, we explored if Ca^{2+} signaling could be involved. We observed some correlation between the ability to inhibit PIN1 accumulation in BFA bodies and to stimulate Ca^{2+} signaling at high 1-NAA concentrations (Fig. 1) and for different auxin analogs (Fig. 2, 3). Interestingly, the presumed "inactive" 2-NAA (Paponov *et al.*, 2019), also triggered a pronounced Ca^{2+} response, possibly via its weak affinity for TIR1 (Lee *et al.*, 2014), indicating 2-NAA is not an ideal negative control for fast auxinic responses. However, this correlation did not hold true for anti-auxins (Fig. 2,3), and IAA (Dindas *et al.*, 2018; Paponov *et al.*, 2019), indicating that different auxin signaling pathways could be involved.

We established that 1-NAA activates Ca^{2+} responses at the plasma membrane via a TIR1/AFB-CNGC14-dependent mechanism, similarly as was previously described for the endogenous auxin IAA (Dindas *et al.*, 2018; Shih *et al.*, 2015). This shows that 1-NAA-induced Ca^{2+} signaling is a specific auxinic response. However, this genetic disruption of 1-NAA-induced Ca^{2+} signaling in *tir1afb2,3* and *cngc14* mutants did not affect 1-NAA's ability to inhibit PIN accumulation in BFA bodies. Moreover, the *cngc2* mutant that is defective in auxin signaling and homeostasis {Chakraborty, 2021 #929}, also did not alter the 1-NAA sensitivity of BFA body formation. These data unequivocally show that BFA body formation is not inhibited by auxin-induced Ca^{2+} signaling, and further support the notion that the synthetic auxin 1-

NAA differentially affects the endomembrane system compared to IAA. 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 (Mazur *et al.*, 2020a; Mazur *et al.*, 2020b; Prat *et al.*, 2018; Sauer *et al.*, 2006). The use of genetic tools will be imperative to resolve which parts of the responses to auxin analogs can be attributed to a particular auxin signaling mechanism.

Despite its limitations, 1-NAA's antagonism towards endomembrane aggregation and BFA body formation has been a convenient pharmacological tool to identify and characterize new regulators of endomembrane trafficking (Feraru *et al.*, 2012; Tanaka *et al.*, 2009; Zhang *et al.*, 2020).

Supplementary data

The following supplementary data are available at JXB online.

Fig. S1. Characterization of the subcellular NAA-induced Ca^{2+} response

Fig. S2. Chemical structures of used auxin analogs and anti-auxins

Fig. S3 Distinct effects of different auxin analogs on PIN2 BFA body formation

Fig. S4 Analysis of 1-NAA-dependent BFA body formation in *tir1/afb*, and *cngc14*

Fig. S5 Analysis of 1-NAA-dependent BFA body formation in *cngc2-3*

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Author Contributions

R.W and E.H. performed immunolocalizations and subsequent data analyses according to J.F. methodology and resources. E.H. and M.G. performed the Ca^{2+} reporters experiments according to M.K., K.S. and A.C. methodology. S.V. and A.C. conceived the original screening and research plans and wrote the manuscript with contributions of all the authors. All authors were involved in discussing and analyzing the data. S.V. and A.C agree to serve as the authors responsible for contact and ensure communication.

Conflict of interest

The authors declare no conflict of interest

Data Availability

All data generated or using in the submitted article are available from the corresponding authors upon request.

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Figure legends

Fig. 1. The NAA-induced Ca²⁺ response is an auxinic response

A-B. Averaged and normalized NES-YC3.60 fluorescence ratios over time using wide-field illumination upon treatment with 0.1, 1 or 10 μ M NAA ($n \geq 5$) respectively, means + S.D.). NAA treatments were applied as indicated by the black line. Root scheme indicates ROI used for measurements (**A**). The maximal NES-YC3.60 responses were quantified and plotted as box-and-whisker plots (**B**).

C-D. The Ca²⁺ dynamics (NES-YC3.60) in WT, *tir1afb2afb3*, *cngc14-1* in response to a pulse of 10 μ M NAA using wide-field illumination. Data are presented as means + S.D. $n=5$ (**C**). The maximal NES-YC3.60 responses were quantified and plotted as box-and-whisker plots (**D**). In (**B**) and (**D**) all experimental points are plotted and their distribution represented as a box that extends from the 25th to 75th percentiles. The central line in the boxes are at the respective medians. (Unpaired Student's t- test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

E. Kymograph analysis of NAA-induced Ca²⁺ signaling over in WT and in *cngc14-1* using NES-YC3.60 via confocal laser scanning microscopy (z-section = 3.77 μ m). Fluorescence ratios across the dotted line of a central confocal section (a to b; 100 μ m) were plotted over time. A white line indicates the pulse of 10 μ M NAA. The color-coded scale indicates NES-YC3.60 fluorescence ratios from low to high.

Fig. 2. Analysis of inhibition of BFA body formation and auxin-induced Ca²⁺ signaling in *tir1/afb* and *cngc14* mutants

A-B. Immunolocalization of PIN1 in 3 day-old roots treated with BFA (**A**) or co-treated for BFA and NAA (**B**) in wild type, *tir1afb1afb3*, *tir1afb2afb3*, *cngc14-1* and *cngc14-2*, White arrows indicate PIN1-accumulating BFA bodies. Scale bar is 20 μ m.

C. Quantification of the proportion of cells that accumulate PIN1 in BFA bodies visualized by immunolocalization in 3 day -old roots of indicated genotypes treated with BFA or BFA and NAA, for wild type (n=11; n=11), *tir1afb1afb3* (n=10; n=12), *tir1afb2afb3* (n=11; n=11), *cngc14-1* (n=11; n=12) and *cngc14-2* (n=11;n=12). n is the pooled number of roots derived from three independent replicates. 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.

Fig. 1. The NAA-induced Ca^{2+} response is an auxinic response

A-B. Averaged and normalized NES-YC3.60 fluorescence ratios over time using wide-field illumination upon treatment with 0.1, 1 or 10 μM NAA. ($n \geq 5$) respectively, means + S.D.). NAA treatments were applied as indicated by the black line. Root scheme indicates ROI used for measurements (**A**).

The maximal NES-YC3.60 responses were quantified and plotted as box-and-whisker plots (**B**). **C-D.** The Ca^{2+} dynamics (NES-YC3.60) in WT, *tir1afb2afb3*, *cngc14-1* in response to a pulse of 10 μM NAA using wide-field illumination. Data are presented as means + S.D. $n=5$ (**C**). The maximal NES-YC3.60 responses were quantified and plotted as box-and-whisker plots (**D**). In (**B**) and (**D**) all experimental points are plotted and their distribution represented as a box that extends from the 25th to 75th percentiles. The central line in the boxes are at the respective medians. (Unpaired Student's t-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

E. Kymograph analysis of NAA-induced Ca^{2+} signaling over in WT and in *cngc14-1* using NES-YC3.60 via confocal laser scanning microscopy (z-section = 3.77 μm). A line was drawn across the root segment of interest (a to b; 100 μm) and the NES-YC3.60 fluorescence ratios across this line were plotted over time. A white line indicates the pulse of 10 μM NAA.

The color-coded scale indicates NES-YC3.60 fluorescence ratios from low to high.

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