Title: Guanylate cyclase activity of TIR1/AFB auxin receptors in rapid auxin responses

Authors: Linlin Qi[1,2,3], Mateusz Kwiatkowski[4], Ivan Kulich[1], Huihuang Chen[1], Yongqiang Gao[5], Ping Yun[6,7], Lanxin Li[8], Sergey Shabala[6,7,9], Edward E. Farmer[5], Krzysztof Jaworski[4], Jiří Friml[1,*]

Affiliations:

[1]Institute of Science and Technology Austria (ISTA), 3400 Klosterneuburg, Austria.

[2]Faculty of Synthetic Biology, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, China.

[3]Key Laboratory of Quantitative Synthetic Biology, Shenzhen Institute of Synthetic Biology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, China.

[4]Department of Plant Physiology and Biotechnology, Nicolaus Copernicus University in Toruń, 87-100 Toruń, Poland.

[5]Department of Plant Molecular Biology, University of Lausanne, 1015 Lausanne, Switzerland.

[6]School of Biological Science, University of Western Australia, Crawley, WA 6009, Australia.

[7]Tasmanian Institute of Agriculture, University of Tasmania, Hobart, TAS 7001, Australia.

[8]Beijing Key Laboratory of Development and Quality Control of Ornamental Crops, Department of Ornamental Horticulture, College of Horticulture, China Agricultural University, 100193 Beijing, China.

[9]International Research Centre for Environmental Membrane Biology, Foshan University, 528000 Foshan, China.

*Corresponding author. Email: jiri.friml@ist.ac.at

Abstract: The major developmental signal in plants, auxin is perceived by TIR1/AFB receptors. It triggers transcriptional reprogramming via well-established canonical mechanism but also elusive rapid, non-transcriptional responses. Here we demonstrate that TIR1/AFB receptors have, next to recently identified adenylate cyclase, also guanylate cyclase activity. Auxin perception activates independently the cAMP and cGMP production by TIR1/AFBs *in vitro* and increases cAMP and cGMP levels *in planta* with a slow and fast dynamics, respectively. Exogenous cGMP but not cAMP application induces rapid cytosolic Ca^{2+} transients and root growth inhibition, suggesting that TIR1/AFB-derived cGMP mediates rapid auxin responses. This unprecedented combination of adenylate and guanylate cyclase activities in a hormone receptor provides a new paradigm for how a single perception mechanism can mediate a multitude of diverse downstream responses.

One-Sentence Summary: TIR1/AFB auxin receptors have guanylate cyclase activity, whose product, cGMP mediates rapid non-transcriptional auxin responses.

Main Text:

Auxin is one of the most prominent plant hormones and much of its action on growth and development has been attributed to its regulation of transcription (*1-3*). The TIR1/AFB auxin receptors are F-box subunits of the E3 ubiquitin ligase complex, which after auxin perception associates with the Aux/IAAs co-receptors leading to the ubiquitination and degradation of the latter. Since Aux/IAAs act also as transcriptional repressors, by interacting with and inhibiting ARF transcription factors, their auxin-triggered degradation releases ARFs to promote transcription of auxin-regulated genes (*4-8*). This well-established canonical auxin signaling pathway explains most of the auxin developmental roles. Accumulating evidence of recent years suggests that TIR1/AFB signaling mediates also rapid, non-transcriptional cellular responses including cytosolic Ca^{2+} spikes, apoplastic alkalinization (*9-13*) and membrane depolarization (*14*), which are all crucial for rapid, auxin-induced root growth inhibition and root gravitropism.

Recently, adenylate cyclase activity (AC) has been identified in TIR1/AFB auxin receptors, which generates cAMP after auxin perception, however, this activity is not important for rapid responses but for transcriptional regulations (*15*). Thus, mechanism underlying rapid auxin responses remains elusive.

Here we show that TIR1/AFB auxin receptors, next to AC, also have guanylate cyclase (GC) activity, which is stimulated by auxin perception and produces another important second messenger, cGMP. In contrast to cAMP, cGMP triggers rapid root responses suggesting that auxin-induced cGMP production by TIR1/AFB mediates rapid cellular responses.

Results

TIR1/AFBs from angiosperms have guanylate cyclase activity

The AC activity of TIR1/AFBs is not essential for the rapid non-transcriptional auxin responses (*15*), thus prompting us to search for additional TIR1/AFB functionalities. Besides cAMP, a similar small molecule cGMP, the product of GC activity, is another key second messenger in animals (*16*). By a scrutinized sequence analysis, we found a relatively conserved GC motif (*17*) adjacent to the previously characterized AC motif (*15*) in the C-terminal region of TIR1/AFBs (Fig. 1A). To test the potential GC activity of TIR1/AFB auxin receptors, we used the His-GFP-Flag-TIR1 purified from Sf9 insect cells, and GST-AFB1 as well as GST-AFB5 proteins purified from *E. coli* (*15*). We performed the *in vitro* GC activity assay, followed by cGMP detection using liquid chromatography–mass spectrometry (LC–MS/MS) method. cGMP was reliably detected in all reactions with different TIR1/AFB proteins tested (Fig. 1B and fig. S1) and typical Michaelis-Menten kinetics were obtained (Fig. 1, C to E) with parameters of the maximum velocity (V_{max}) and the Michaelis constant (K_M) similar to other reported plant GCs (*18-22*). The GC motif is less conserved compared to the AC motif and it was originally identified only in TIR1 and AFB1sequences. The confirmation of the GC activity also for AFB5 indicated that the originally reported GC motif could be more relaxed.

To evaluate the evolutionary conservation of the TIR1/AFBs GC activity, we compared the TIR1/AFB orthologous sequences from different representative species during the evolution of land plants. There are four key amino acids (a1-a4) in the GC motif (fig. S2A). Whereas a1 and a4 are highly conserved across all the orthologous sequences analyzed, a3 is divergent everywhere. The a2 residue diverged before angiosperms, and became fixed in the angiosperm GC motif, suggesting that GC activity may have evolved only in this lineage. To test this

hypothesis, we purified the GST-PaAFB1 and GST-PaAFB2, which are the only two closest TIR1/AFB orthologues from the gymnosperm *Picea abies*, and GST-AmTrAFB2 and GST-AmTrAFB3 which are two out of the three closest TIR1/AFB orthologues from the ancestral angiosperm *Amborella trichopoda* (fig. S3A), and tested their potential AC and GC activities. As expected, all of them had AC activity (fig. S2B), indicating the proteins purified are biochemically functional. In contrast, only AmTrAFB2 showed clear GC activity (fig. S2C), suggesting that GC activity of TIR1/AFBs indeed evolved in the earliest angiosperm. Hence, presumably most, if not all TIR1/AFB orthologues from flowering plants have GC activity.

GC and AC activities of TIR1/AFBs are independent

The GC and AC motifs are adjacent in sequence and also spatially close to each other in the protein structure (fig. S4). To validate the importance of the C-terminal GC motif for the GC activity of TIR1/AFBs and further test the specificity of the GC and AC motifs for their corresponding activities, we mutated separately three key residues in the C-terminal GC motif of AFB5 (GCm1, GCm2, GCm3 in Fig. 1A) to alanine. Similar mutations had already been generated for the AC motif of AFB5 (ACm1, ACm2, ACm3) (*15*). We purified all the mutated variants of GST-AFB5 (fig. S3B), and performed the *in vitro* GC and AC activity assays. Clearly, GCms but not ACms decreased the GC activity of AFB5 (fig. S5A). Similarly, ACms abolished the AC activity of AFB5 as shown before (*15*), whereas GCms did not show any significant effects on it (fig. S5B). In other words, GCms specifically interfered with the GC activity but did not affect the AC activity, and vice versa for ACms. To further confirm this, we utilized a previously characterized N-terminal deleted TIR1 version - TIR1ΔNT (*15*), and purified the similarly mutated proteins (fig. S3C). Again, all three GCms specifically interfered with the GC activity of TIR1ΔNT, while all three ACms specifically abolished the AC activity (Fig. 2, A and B). Similar results were obtained for the AFB1-GCm1 and AFB1-ACm1 (Fig. 2, C and D, and fig. S3D). Taken together, these results clearly demonstrate that the C-terminal GC motif within TIR1/AFBs is responsible for the GC activity and that GC and AC activities are independent.

It had been shown before that one of the ACms — ACm2 also interferes with auxin perception — the auxin-induced TIR1/AFB interaction with Aux/IAAs (*15*). To clarify the similar problem for GCms, we introduced the three GCms into full length TIR1, and performed the pull-down experiments with *in vitro*-translated TIR1-HA variants and purified GST-IAA7 in the presence of IAA. The results showed that whereas GCm3 abolished the auxin-induced interaction between TIR1 and IAA7, GCm1 and GCm2 did not have any visible effects (Fig. 2E), indicating these two mutations can be used to specifically remove the GC activity without interfering with AC activity or Aux/IAA interaction.

Overall, this shows that TIR1/AFB auxin receptors have independent AC and GC activities. Thus, TIR1/AFBs represent, to our knowledge, the first identified example of protein combining these two enzymatic activities.

Auxin perception rapidly stimulates the GC activity

Next, we tested an impact of auxin perception on TIR1/AFB GC activity. We used the His-GFP-Flag-TIR1 protein purified from Sf9 insect cells, and performed the *in vitro* GC activity assay in the presence of auxin (IAA) with or without IAA7 or IAA17 co-receptors. IAA or IAA7/17 alone did not have any significant effect on the TIR1 GC activity, but auxin together with IAA7 or IAA17 clearly enhanced the GC activity (Fig. 3A). To confirm this observation, we performed

similar experiments for AFB1 purified from *E. coli* with a similar result (Fig. 3B). Together we demonstrate that auxin perception enhances the GC activity of TIR1/AFBs *in vitro*.

Next, we tested how auxin regulates the GC activity *in planta*. We treated Arabidopsis seedlings with 100 nM IAA and harvested root tissues at different time points to measure cAMP and cGMP in the same samples using LC-MS/MS. In contrast to the cAMP dynamics, which showed a slight decrease at 1 min and then steady increase after 1 h (*15*), cGMP level rapidly increased at 1 min after auxin treatment and then decreased after 1h (Fig. 3C). The differential dynamics of cAMP and cGMP after auxin treatment implies that they may mediate different downstream processes. Similar experiments in the *tir1-1 afb2-1 afb3-1* (*tir* triple) and *afb1-3* mutants showed that the auxin-induced rapid increase of cGMP was almost completely abolished in *tir* triple and largely reduced in *afb1-3* (Fig. 3D).

These data show that auxin treatment induces cAMP production in roots with a slow dynamic but rapidly increases the cGMP levels via the TIR1/AFB receptors.

AFB1 but not TIR1 is involved in rapid non-transcriptional auxin responses

The rapid and transient increase of cGMP level in roots after auxin treatment suggests that the GC activity may be important for the so far elusive rapid, non-transcriptional auxin responses. Accumulating evidence indicates that among the 6 TIR1/AFBs auxin receptors in *Arabidopsis*, the predominantly cytosol-localized AFB1 plays major roles in rapid auxin responses (*14*, *23-25*). To further test this, we used the synthetic biology approach and generated ccvAFB1 with mutated IAA binding site, which does not bind natural IAA but its analogue cvxIAA (*26*). To this end, we prepared the *pAFB1::ccvAFB1-HA* line to be specifically activated by the cvxIAA. As expected, cvxIAA triggered strong root growth inhibition in *pTIR1::ccvTIR1* but not in the control *pTIR1::TIR1* line, when the seeds were directly sown on medium containing cvxIAA and were grown for 6 days, but failed to inhibit root growth in either *pAFB1::AFB1-HA* or *pAFB1::ccvAFB1-HA* line (fig. S6A). This shows that AFB1 cannot mediate sustained root growth inhibition in response to auxin as TIR1 does. In contrast, when we tested rapid root growth inhibition response using the vRootchip with the *ccvTIR1* and *ccvAFB1* lines, cvxIAA in *ccvTIR1* line inhibited root growth gradually without affecting the apoplastic pH, whereas in ccvAFB1 line, cvxIAA rapidly alkalinized the apoplast and rapidly but transiently inhibited root growth (fig. S6, B and C). Accordingly, the *afb1-3* mutant was resistant to auxin-induced rapid root growth inhibition within the first 10 min (Fig. 4A).

Besides apoplast alkalinisation, classical rapid cellular effects include a transient increase in the cytosolic Ca^{2+} (*10*), and membrane depolarization (*14*). Indeed, the *afb1-3* mutant was also defective in auxin-induced Ca^{2+} flux and electrical signal in root tips (Fig. 4B, and fig. S7, A and B). This confirms that AFB1 is the major auxin receptor mediating rapid, non-transcriptional responses including root growth inhibition, apoplast alkalinization, membrane depolarization and cytosolic Ca^{2+} transients.

cGMP triggers rapid Ca^{2+} transients via CNGC14 and root growth inhibition

To test a notion that AFB1-produced cGMP mediates the rapid auxin responses, we used exogenous treatment with cAMP and cGMP. We applied the cell-permeable dibutyryl-cGMP to roots using vRootchip. This strongly induced cytosolic Ca^{2+} spikes as monitored by Ca^{2+} fluorescent sensor GCaMP3 (*27*) (Fig. 4C), whereas dibutyryl-cAMP did not have any effect (fig. S8). Moreover, application of dibutyryl-cGMP but not dibutyryl-cAMP also triggered rapid

and reversible root growth inhibition, which became more pronounced after several rounds of treatment and washout (Fig. 4D).

Next we addressed, by which mechanism cGMP may induce Ca^{2+} transients. Previously, one of the putative cyclic nucleotide-gated channel (CNGC) family (*28*), CNGC14 has been shown to be important for both auxin-induced Ca^{2+} transients and rapid root growth inhibition (*10*, *25*). Given that CNGC homologues in animals are established targets of cAMP or cGMP (*16*), this suggested that CNGC14 may be targeted by AFB1-generated cGMP. First, we crossed the Ca^{2+} sensor GCaMP3 into *cngc14-2* and performed the vRootchip experiment. IAA treatment induced normal cytosolic Ca^{2+} increase and root growth inhibition in the WT but not in the *cngc14-2* mutants, which confirmed that CNGC14 is indeed crucial for auxin-induced rapid root growth inhibition (Fig. 4E) and cytosolic Ca^{2+} increase (Fig. 4F). Dibutyryl-cGMP treatment in *cngc14-2* mutants also did not induce Ca^{2+} increase (Fig. 4C) consistent with CNGC14 being targeted by cGMP.

Thus, cGMP is sufficient to induce effects similar to non-transcriptional auxin effects downstream of AFB1, including cytosolic Ca^{2+} transients and rapid, reversible root growth inhibition. Taken together with ability of AFB1 to generate cGMP following auxin perception and requirement of CNGC14, this implies that cGMP produced by AFB1 acts as a second messenger downstream of auxin perception, possibly regulating the activity of CNGC14 and thus inducing cytosolic Ca^{2+} spikes relevant for rapid root growth regulation.

Discussion

AFB1-cGMP-CNGC14 signaling mechanism for rapid responses

TIR1/AFB signaling has been typically associated with transcriptional reprogramming (*4*, *8*). However, recently it became clear that it can mediate also very rapid cellular responses, including cytosolic Ca^{2+} spikes (*13*), apoplastic alkalinization (*12*) and membrane depolarization (*14*); all linked to rapid, auxin-induced root growth inhibition and root gravitropism (*29*). However, how TIR/AFBs as F-box proteins mediate those cellular responses has been a mystery and remained so even after the identification of the TIR1/AFBs AC activity (*15*).

Here we show that TIR1/AFBs have additional GC activity and that its product, cGMP is sufficient to trigger rapid auxin responses including cytosolic Ca^{2+} increase and reversible root growth inhibition. First, auxin treatment rapidly and transiently increases cGMP accumulation in roots (Fig. 3, C and D), in a similar temporal pattern as those rapid auxin responses; Second, auxin-induced Ca^{2+} spike and rapid root growth inhibition are dependent on the predominantly cytosolic localized AFB1 and the putative cAMP/cGMP-gated Ca^{2+} channel CNGC14 (Fig. 4, A, B, E and F); Third, application of cell-permeable cGMP triggers similar cytosolic Ca^{2+} increase in a CNGC14-dependent manner (Fig. 4C) and rapid root growth inhibition (Fig. 4D) *in planta*. These observations imply a linear AFB1 – cGMP - CNGC14 - Ca^{2+} non-transcriptional signaling pathway, which explains well at least part of the mysterious rapid auxin responses.

Tri-functional TIR1/AFB auxin receptors

The classical models of the TIR1/AFBs-mediated signaling mechanism has assumed the degradation of Aux/IAA repressors following TIR1/AFB-mediated ubiquitination as the only signaling output from the TIR1/AFB receptors. Now with a recent discovery of their AC (*15*) and GC activities, we show that TIR1/AFBs have three separate functionalities: E3 ubiquitin ligase as well as AC and GC. Notably, those activities can be mapped to distinct domains within TIR1/AFB proteins and can be abolished by specific mutations, showing that they are

independent (Fig. 2 and fig. S5). *In vitro*, auxin perception enhances the AC and GC activities with similar dynamics, however, in roots, auxin stimulates cGMP accumulation rapidly and transiently, whereas a sustained cAMP increase occurs later (*15*) (Fig. 3, A to C). Hence, following auxin perception, TIR1/AFBs receptors directly generate three independent signaling outputs: Aux/IAA degradation, cAMP and cGMP production. While Aux/IAA degradation and cAMP contribute to transcriptional regulation (*15*), cGMP seems to mediate specifically the rapid responses (Fig. 4 and fig. S8).

Such a multiple functionality at the level of receptors is unique throughout the eukaryotic kingdoms and provides a mechanical explanation how the same signal – auxin – can induce such a variety of diverse rapid and sustained cellular and developmental responses.

Potential role of cGMP as second messenger in plants

Similar to cAMP, cGMP is also a well-known second messenger in mammalian research, acting in peptide and NO signaling (*16*, *30*). However, whether cGMP acts as second messenger in plants has remained controversial, even though the list of plant proteins potentially having GC activity *in vitro* keeps growing in recent years (*17*, *31*) and includes prominent candidates such as brassinosteroid and phytosulfokine receptors (*19*, *20*). This is mainly due to the lack of convincing genetic evidence and failure in identifying the downstream effectors. The identification of the GC activity in TIR1/AFBs receptors with CNGC14 as a likely downstream cGMP target provides a new avenue into studies of cGMP as a second messenger in plants.

In animal cells, cAMP and cGMP are ubiquitously present second messengers involved in dozens of different signaling pathways. They are produced separately by specialized enzymes downstream of signal perception (*16*). In contrast, in plants, TIR1/AFBs receptors directly produce both cAMP and cGMP by independent enzymatic activities in parallel to mediate different subsets of responses, presumably via distinct downstream targets. This makes TIR1/AFBs the only known example in any organism of a protein combining both AC and GC activities allowing this auxin pathway to mediate broad range of responses so typical for auxin. Thus, this work contributes to establishing the roles of cAMP and cGMP as second messengers in plant signaling and highlights the unique, plant-specific aspects of their production and action as compared to other eukaryotes.

References and Notes

1. J. Friml, Fourteen Stations of Auxin. *Cold Spring Harb. Perspect. Biol.* **14**, a039859 (2021).
2. M. Gallei, C. Luschnig, J. Friml, Auxin signalling in growth: Schrodinger's cat out of the bag. *Curr. Opin. Plant Biol.* **53**, 43-49 (2020).
3. D. Weijers, J. Nemhauser, Z. Yang, Auxin: small molecule, big impact. *J. Exp. Bot.* **69**, 133-136 (2018).
4. M. Quint, W. M. Gray, Auxin signaling. *Curr. Opin. Plant Biol.* **9**, 448-453 (2006).
5. W. M. Gray *et al*., Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. *Nature* **414**, 271-276 (2001).
6. N. Dharmasiri, S. Dharmasiri, M. Estelle, The F-box protein TIR1 is an auxin receptor. *Nature* **435**, 441-445 (2005).

7. S. Kepinski, O. Leyser, The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature* **435**, 446-451 (2005).

8. G. Parry, M. Estelle, Auxin receptors: a new role for F-box proteins. *Curr. Opin. Cell Biol.* **18**, 152-156 (2006).

9. G. B. Monshausen *et al*., Dynamics of auxin-dependent Ca^{2+} and pH signaling in root growth revealed by integrating high-resolution imaging with automated computer vision-based analysis. *Plant J.* **65**, 309-318 (2011).

10. H. W. Shih *et al*., The Cyclic Nucleotide-Gated Channel CNGC14 regulates root gravitropism in *Arabidopsis thaliana*. *Curr. Biol.* **25**, 3119-3125 (2015).

11. E. Barbez *et al*., Auxin steers root cell expansion via apoplastic pH regulation in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E4884-E4893 (2017).

12. L. Li *et al*., Cell surface and intracellular auxin signalling for H(+) fluxes in root growth. *Nature* **599**, 273-277 (2021).

13. J. Dindas et al., AUX1-mediated root hair auxin influx governs SCF(TIR1/AFB)-type Ca(2+) signaling. *Nat. Commun.* **9**, 1174 (2018).

14. N. B. C. Serre *et al*., AFB1 controls rapid auxin signalling through membrane depolarization in *Arabidopsis thaliana* root. *Nat. Plants* **7**, 1229-1238 (2021).

15. L. Qi, *et al*., Adenylate cyclase activity of TIR1/AFB auxin receptors in plants. *Nature* **611**, 133-138 (2022).

16. J. A. Beavo, L. L. Brunton, Cyclic nucleotide research -- still expanding after half a century. *Nat. Rev. Mol. Cell Biol.* **3**, 710-718 (2002).

17. A. Wong, C. Gehring, The *Arabidopsis thaliana* proteome harbors undiscovered multi-domain molecules with functional guanylyl cyclase catalytic centers. *Cell Commun. Signal.* **11**, 48 (2013).

18. N. Ludidi, C. Gehring, Identification of a novel protein with guanylyl cyclase activity in *Arabidopsis thaliana*. *J. Biol. Chem.* **278**, 6490-6494 (2003).

19. L. Kwezi *et al*., The phytosulfokine (PSK) receptor is capable of guanylate cyclase activity and enabling cyclic GMP-dependent signaling in plants. *J. Biol. Chem.* **286**, 22580-22588 (2011).

20. J. I. Wheeler *et al*., The brassinosteroid receptor BRI1 can generate cGMP enabling cGMP-dependent downstream signaling. *Plant J.* **91**, 590-600 (2017).

21. T. Mulaudzi *et al*., Identification of a novel *Arabidopsis thaliana* nitric oxide-binding molecule with guanylate cyclase activity *in vitro*. *FEBS Lett.* **585**, 2693-2697 (2011).

22. S. Meier *et al*., The Arabidopsis wall associated kinase-like 10 gene encodes a functional guanylyl cyclase and is co-expressed with pathogen defense related genes. *PLoS One* **5**, e8904 (2010).

23. M. J. Prigge *et al*., Genetic analysis of the Arabidopsis TIR1/AFB auxin receptors reveals both overlapping and specialized functions. *Elife* **9**, e54740 (2020).

24. H. Chen *et al*., Distinct functions of TIR1 and AFB1 receptors in auxin signaling. *Mol. Plant* **16**, 1117-1119 (2023).

25. S. M. Dubey *et al*., The AFB1 auxin receptor controls the cytoplasmic auxin response pathway in *Arabidopsis thaliana*. *Mol. Plant* **16**, 1120-1130 (2023).

26. N. Uchida *et al*., Chemical hijacking of auxin signaling with an engineered auxin-TIR1 pair. *Nat. Chem. Biol.* **14**, 299-305 (2018).

27. M. Toyota *et al*., Glutamate triggers long-distance, calcium-based plant defense signaling. *Science* **361**, 1112-1115 (2018).

28. E. Jarratt-Barnham *et al*., The complex story of plant cyclic nucleotide-gated channels. *Int. J. Mol. Sci.* **22**, 874 (2021).

29. M. Fendrych *et al*., Rapid and reversible root growth inhibition by TIR1 auxin signalling. *Nat. Plants* **4**, 453-459 (2018).

30. L. R. Potter, Guanylyl cyclase structure, function and regulation. *Cell Signal.* **23**, 1921-1926 (2011).

31. A. Wong, C. Gehring, H. R. Irving, Conserved functional motifs and homology modeling to predict hidden moonlighting functional sites. *Front. Bioeng. Biotechnol.* **3**, 82 (2015).

32. N. Dharmasiri *et al*., Plant development is regulated by a family of auxin receptor F box proteins. *Dev. Cell* **9**, 109-119 (2005).

33. M. H. Longair, D. A. Baker, J. D. Armstrong, Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics* **27**, 2453-2454 (2011).

34. D. von Wangenheim *et al*., Live tracking of moving samples in confocal microscopy for vertically grown roots. *Elife* **6**, e26792 (2017).

35. S. N. Shabala, I. A. Newman, J. Morris, Oscillations in H+ and Ca2+ ion fluxes around the elongation region of corn roots and effects of external pH. *Plant Physiol.* **113**, 111-118 (1997).

36. S. Shabala *et al*., Extracellular Ca2+ ameliorates NaCl-induced K+ loss from Arabidopsis root and leaf cells by controlling plasma membrane K+ -permeable channels. *Plant Physiol.* **141**, 1653-1665 (2006).

37. A. M. Waterhouse *et al*., Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**, 1189-1191 (2009).

38. X. Tan *et al*., Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**, 640-645 (2007).

Acknowledgments: This research was supported by the Lab Support Facility (LSF), the Imaging & Optics Facility (IOF) and the Nanofabrication Facility (NFF) of IST Austria (ISTA). We are grateful for the seeds from Mark Estelle and Gabriele B. Monshausen.

Funding:

European Research Council Advanced Grant ETAP-742985 (JF)

Austrian Science Fund (FWF) I 6123-B (JF)

Author contributions:

Conceptualization: LQ, JF

Methodology: LQ, MK, KJ, IK, CH, LL, YG, EEF, PY, SS

Investigation: LQ, MK, IK, CH, YG, PY

Visualization: LQ, MK, IK, CH, YG, PY

Funding acquisition: JF

Project administration: LQ, JF

Supervision: JF, KJ, EEF, SS

Writing – original draft: LQ, JF

Writing – review & editing: LQ, JF

Competing interests: Authors declare that they have no competing interests.

Data and materials availability: All data are available in the main text or the supplementary materials.

Supplementary Materials

Materials and Methods

Figs. S1 to S8

Table S1

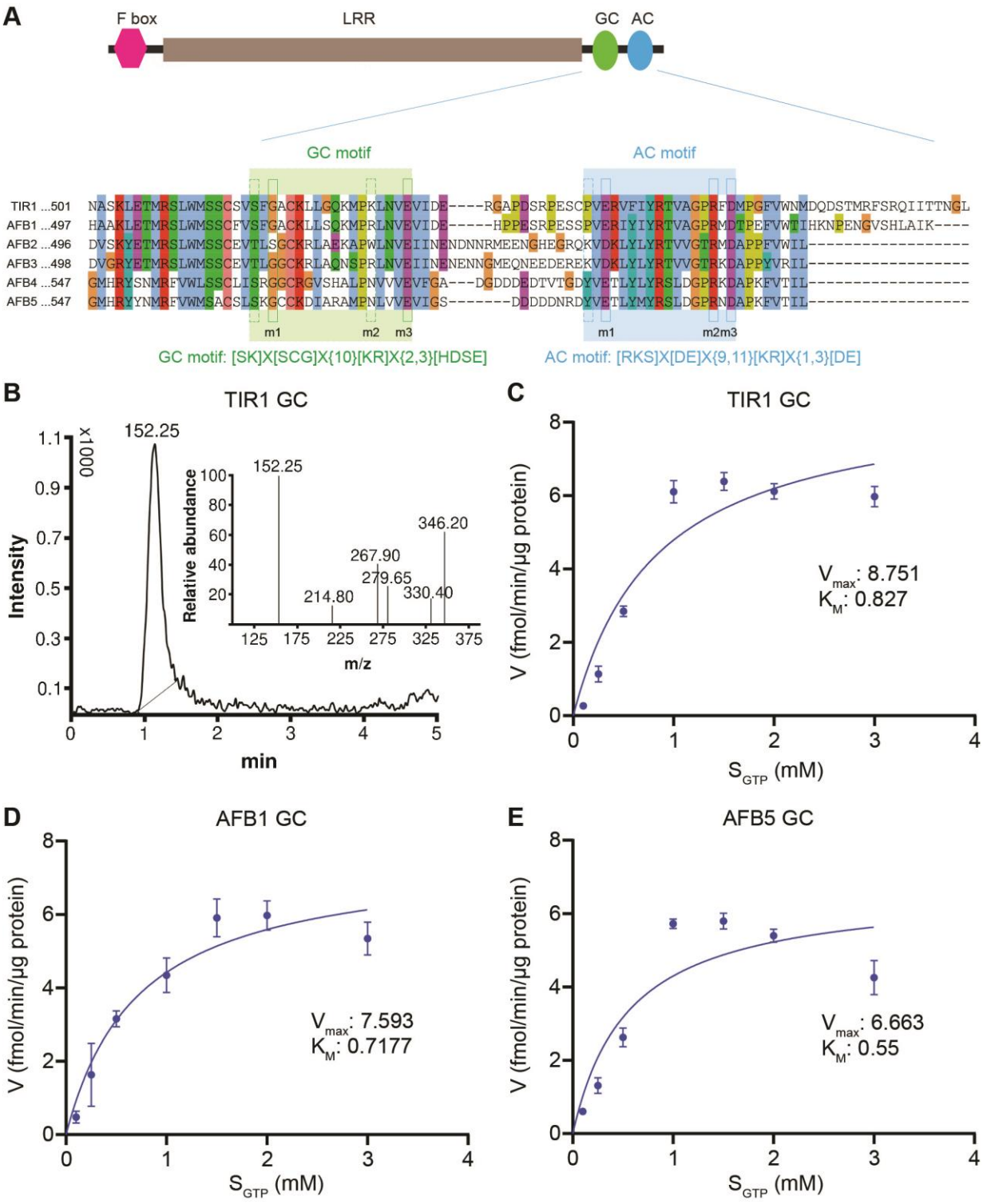

Fig. 1. TIR1/AFB auxin receptors have GC activity. (**A**) Simplified scheme showing the domain structure of TIR1/AFB auxin receptors and a multiple sequence alignment showing the conserved GC and AC motif in the C-terminal region. LRR, leucine-rich repeat. m1, m2 and m3 are conserved key amino acids that were mutated to alanine to disrupt the GC or AC activity in subsequent experiments. (**B**) A representative LC-MS/MS spectrum showing cGMP detection in the GC reaction of His–GFP–Flag–TIR1, with the characteristic peak used for quantification. (**C**)

GC activity of His–GFP–Flag–TIR1. (**D**) GC activity of GST-AFB1. (**E**) GC activity of GST-AFB5. His–GFP–Flag–TIR1 was purified from Sf9 insect cells. GST-AFB1 and GST-AFB5 were purified from E. coli. *In vitro* GC activity assays were performed with the purified proteins followed by cGMP quantification using LC-MS/MS. Michaelis–Menten kinetics were generated using non-linear regression in GraphPad Prism 8. S_{ATP}, substrate (ATP) concentration; V, velocity. Data are mean ± s.d. of three biological replicates.

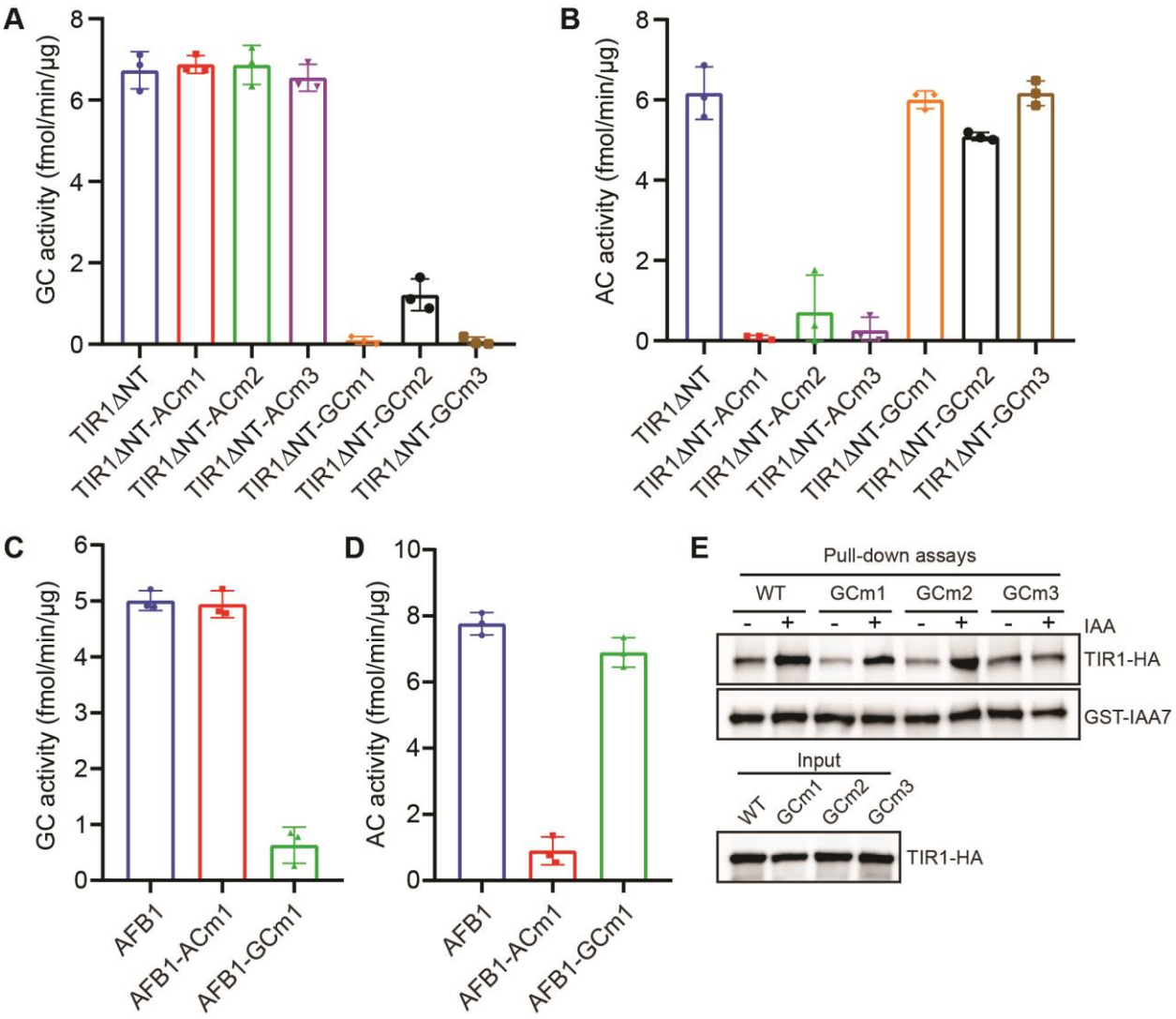

Fig. 2. The C-terminal GC and AC motifs are specifically responsible for the GC or AC activity of TIR1/AFBs respectively. (**A**) The C-terminal GC motif but not AC motif is essential and specific for the GC activity of TIR1ΔNT. (**B**) The C-terminal AC motif but not GC motif is essential and specific for the AC activity of TIR1-ΔNT. (**C**) The GC motif is specifically essential for the GC activity of AFB1. (**D**) The AC motif is specifically essential for the AC activity of AFB1. The indicated GST-tagged proteins were purified from E. coli. *In vitro* GC/AC activity assay was performed and cGMP/cAMP was quantified by LC-MS/MS. Data are mean ± s.d. of three biological replicates. Data for the AC activity of TIR1ΔNT and TIR1ΔNT-ACms have been shown previously (*15*). (**E**) Pull-down assay showing differential effects of TIR1-GCms mutations on the IAA-induced TIR1-Aux/IAA interaction. The TIR1-HA and its GCms variants were translated *in vitro* using wheat germ extracts. GST-IAA7 was purified from E. coli. Pull-down assays were performed in the presence or absence of 10 μM IAA as indicated. The experiment was repeated twice with similar results.

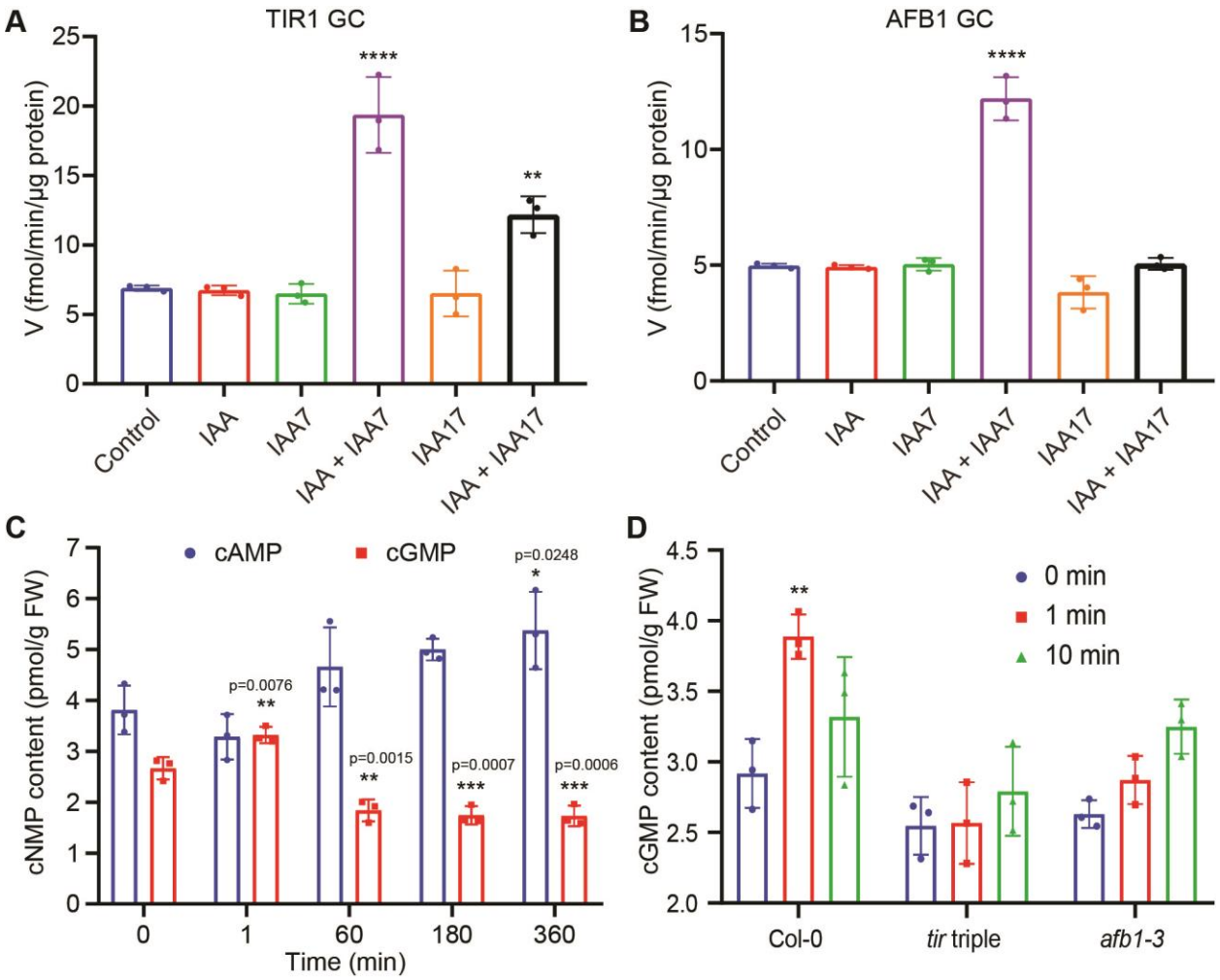

Fig. 3. Auxin perception rapidly stimulates the GC activity of TIR1/AFB receptors. (**A**) Auxin together with Aux/IAA stimulates the GC activity of TIR1. (**B**) Auxin together with Aux/IAA stimulates the GC activity of AFB1. *In vitro* GC activity assay was performed for His–GFP–Flag–TIR1 and GST tag-cleaved AFB1 respectively, in the presence of 10 µM IAA, 5 µg IAA7, 5 µg IAA17, and the indicated combinations. cAMP was quantified by LC–MS/MS after the reactions. Data are mean ± s.d. of three biological replicates. One-way ANOVA and Dunnett's multiple comparisons test. ** $P \leq 0.01$ ($P = 0.0032$); **** $P \leq 0.0001$. (**C**) Differential dynamics of cAMP and cGMP content in root tissues after auxin treatment. Data for cAMP content have been published previously (*15*), and is shown here for comparison. One-way ANOVA and Dunnett's multiple comparisons test. Asterisks indicate significant difference between the corresponding group and the control (0 min). (**D**) The auxin-induced rapid increase of cGMP levels in roots is dependent on TIR1/AFB receptors. Two-way ANOVA and Tukey's multiple comparisons test. ** $P \leq 0.01$ ($P = 0.004$). Data are mean ± s.d. of three biological replicates.

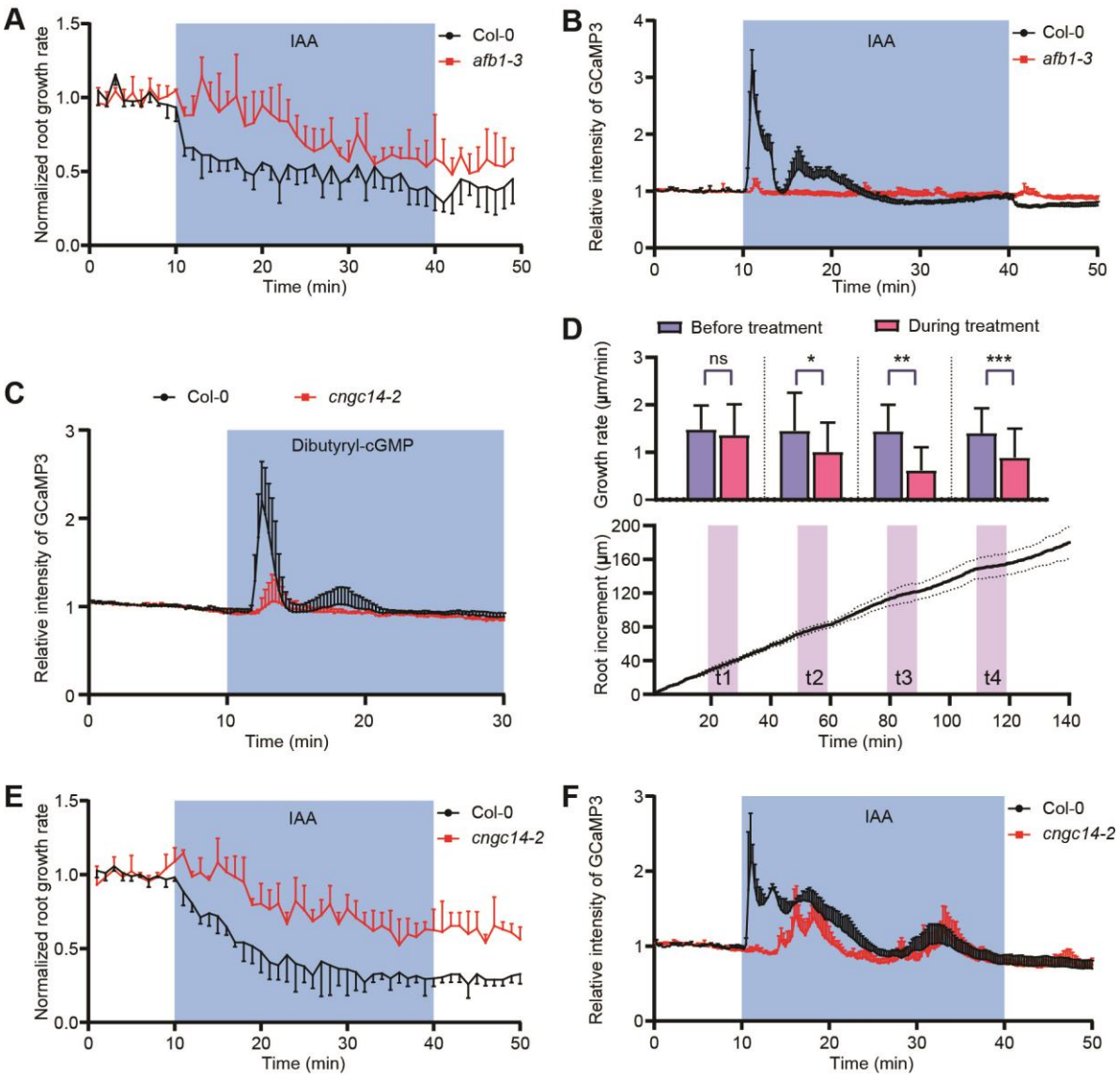

Fig. 4. AFB1 GC activity is involved in rapid auxin responses. (**A**) AFB1 is important for auxin-induced rapid root growth inhibition. (**B**) AFB1 is important for auxin-induced Ca^{2+} spikes. (**C**) Cell permeable dibutyryl-cGMP triggers Ca^{2+} spikes in CNGC14-dependent manner. (**D**) Cell permeable dibutyryl-cGMP triggers rapid and reversible root growth inhibition. (**E**) CNGC14 is important for auxin-induced rapid root growth inhibition. (**F**) CNGC14 is important for auxin-induced Ca^{2+} spikes. For vRootchip experiments with just one treatment, mock medium was changed to medium containing 10 nM IAA or 1 mM dibutyryl-cGMP at 10 min. Normalized root growth rate and relative fluorescence intensity of GCaMP3 in elongation zone were quantified. Data shown are means +/- s.d. n = 4 seedlings. For the vRootchip experiment in (**D**), multiple rounds of treatment with 200 μM dibutyryl-cGMP for 10 min and washout with mock medium for 20 min were applied. Root increment was shown in the lower panel. The black line shows the average and the dotted lines indicate s.d. Root growth rate was quantified and shown in the upper panel. n = 4 seedlings. Two-tailed Student's t-test was used. ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.