Selective auxin agonists induce specific AUX/IAA protein degradation to modulate plant development

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Auxin phytohormones control most aspects of plant development through a complex and interconnected signaling network. In the presence of auxin, AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) transcriptional repressors are targeted for degradation by the SKP1-CULLIN1-F-BOX (SCF) ubiquitin-protein ligases containing TRANSPORT INHIBITOR RESISTANT 1/AUXIN SIGNALING F-BOX (TIR1/AFB), CULLIN1-neddlylation is required for SCFTRI/AFB functionality, as exemplified by mutants deficient in the NEDD8-activating enzyme subunit AUXIN-RESISTANT 1 (AUXR1). Here, we report a chemical biology screen that identifies small molecules requiring AXR1 to modulate plant development. We selected four molecules of interest, RubNeddin 1 to 4 (RN1 to -4), among which RN3 and RN4 trigger selective auxin responses at transcriptional, biochemical, and morphological levels. This selective activity is explained by their ability to consistently promote the interaction between TIR1 and a specific subset of AUX/IAA proteins, stimulating the degradation of particular AUX/IAA combinations. Finally, we performed a genetic screen using RN4, the RN with the greatest potential for dissecting auxin perception, which revealed that the chromatin remodeling ATPase BRAHMA is implicated in auxin-mediated apical hook development. These results demonstrate the power of selective auxin agonists to dissect auxin perception for plant developmental functions, as well as offering opportunities to discover new molecular players involved in auxin responses.

Significance

The plant hormone auxin coordinates almost all aspects of plant development. Throughout plant life, the expression of hundreds of genes involved in auxin regulation is orchestrated via several combinatorial and cell-specific auxin perception systems. An effective approach to dissect these complex pathways is the use of synthetic molecules that target specific processes of auxin activity. Here, we describe synthetic auxins, RubNeddins (RNs), which act as selective auxin agonists. The RN with the greatest potential for dissecting auxin perception was RN4, which we used to reveal a role for the chromatinremodeling ATPase BRAHMA in apical hook development. Therefore, the understanding of RN mode of action paves the way to dissecting specific molecular components involved in auxin-regulated developmental processes.

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Data deposition: The whole genome sequencing data of Arabidopsis thaliana is available at the European Nucleotide Archive, https://www.ebi.ac.uk/ena (accession number: PRJEB21529). The RNA sequencing data of Arabidopsis cell suspension culture treated with IAA, RN3, and RN4 have been deposited at the European Nucleotide Archive, http://www.ebi.ac.uk/ena (accession number: PRJEB13149).

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The ubiquitin-proteasome pathway plays an essential role in plant hormone signaling (8–10). Modification of the relevant components by the ubiquitin-like protein, RELATED TO UBQUITIN/NEURAL PRECURSOR CELL EXPRESSED DEVELOPMENTALLY DOWN-REGULATED PROTEIN 8 (RUB/NEEDD8), which is catalyzed by a cascade of enzymatic reactions analogous to ubiquitination, is critical for the full activity of the proteasome complex (11). In plants, the CULLINs (CUL1, CUL3, and CUL4) are NEDD8-modified proteins that form multimeric E3 ubiquitin ligase complexes (12). CUL1 acts as a scaffold within the SCF-type E3 ligases and neddylation states of CUL1 are essential for the ubiquitin ligase activity of the SCF complex (13). Loss of components of the neddylation pathway, such as the NEDD8-activating enzyme subunit AUXIN RESISTANT 1 (AXR1), reduces the response to several phytohormones, including auxin (14–17).

To understand how auxin perception mediates multiple aspects of plant development, we established an AXR1-dependent developmental defect-based chemical biology screen. Using this approach, we identified small synthetic molecules, RubNeddins (RNs), which selectively promote SCF-TIR1/AFB–AUX/IAA coreceptor assembly, allowing local and precise modulation of auxin signaling pathways. Furthermore, these synthetic selective agonists possess the ability to identify and distinguish the molecular players involved in different aspects of auxin-regulated development, thereby dissecting the diversity of auxin action. We demonstrated this by employing these agonists to reveal different roles for specific AUX/IAA proteins during lateral root and apical hook development. In particular, the use of the selective auxin agonist RN4 revealed a role for the chromatin remodeling ATPase BRAhma in apical hook development.

Results
The Rubylation/Neddylation Pathway Is Required for RNs to Alter Seedling Development. To address the complexity of auxin response, we established a chemical biology screen to isolate synthetic molecules targeting the NEDD8-mediated signaling pathway in Arabidopsis (SI Appendix, Fig. S1A and B). We reasoned that some of these molecules might also target the auxin signaling pathway (SI Appendix, Fig. S1A) and we used 1-naphthaleneacetic acid (NAA) as control (SI Appendix, Fig. S1C). This strategy is complementary to previous ones aiming at isolating auxin-related small molecules (18, 19). Compounds affecting auxin-related developmental processes, such as primary root growth, hypocotyl elongation, and gravitropism responses in wild-type but not in axr1-30 seedlings, were selected (SI Appendix, Fig. S1B). This screening strategy, based on differential effects on the two genetic backgrounds (Col-0 wild-type vs. axr1-30), was essential to filter out chemical activities with general impacts on seedling growth. We hypothesized that a small molecule for which activity was dependent on the AXR1 signaling machinery could be recognized by one or several TIR1/AFB–AUX/IAA coreceptor complexes. Of 8,000 diverse compounds (ChemBridge), we identified 34 small molecules (4,259 of which selectivity affected the growth of wild-type compared with axr1-30 seedlings. Four molecules, named RN1 and RN2 and RN3 and RN4), that selectively affected the growth of wild-type compared to several pg the NEDD8-mediated signaling pathway in Arabidopsis (SI Appendix, Fig. S1A and B). We reasoned that some of these molecules might also target the auxin signaling pathway (SI Appendix, Fig. S1A) and we used 1-naphthaleneacetic acid (NAA) as control (SI Appendix, Fig. S1C). This strategy is complementary to previous ones aiming at isolating auxin-related small molecules (18, 19). Compounds affecting auxin-related developmental processes, such as primary root growth, hypocotyl elongation, and gravitropism responses in wild-type but not in axr1-30 seedlings, were selected (SI Appendix, Fig. S1B). This screening strategy, based on differential effects on the two genetic backgrounds (Col-0 wild-type vs. axr1-30), was essential to filter out chemical activities with general impacts on seedling growth. We hypothesized that a small molecule for which activity was dependent on the AXR1 signaling machinery could be recognized by one or several TIR1/AFB–AUX/IAA coreceptor complexes. Of 8,000 diverse compounds (ChemBridge), we identified 34 small molecules (4,259 of which selectivity affected the growth of wild-type compared with axr1-30 seedlings. Four molecules, named RN1 and RN2 and RN3 and RN4), that selectively affected the growth of wild-type compared to several pg the NEDD8-mediated signaling pathway in Arabidopsis (SI Appendix, Fig. S1A and B). We reasoned that some of these molecules might also target the auxin signaling pathway (SI Appendix, Fig. S1A) and we used 1-naphthaleneacetic acid (NAA) as control (SI Appendix, Fig. S1C). This strategy is complementary to previous ones aiming at isolating auxin-related small molecules (18, 19). Compounds affecting auxin-related developmental processes, such as primary root growth, hypocotyl elongation, and gravitropism responses in wild-type but not in axr1-30 seedlings, were selected (SI Appendix, Fig. S1B). This screening strategy, based on differential effects on the two genetic backgrounds (Col-0 wild-type vs. axr1-30), was essential to filter out chemical activities with general impacts on seedling growth. We hypothesized that a small molecule for which activity was dependent on the AXR1 signaling machinery could be recognized by one or several TIR1/AFB–AUX/IAA coreceptor complexes. Of 8,000 diverse compounds (ChemBridge), we identified 34 small molecules (4,259 of which selectivity affected the growth of wild-type compared with axr1-30 seedlings. Four molecules, named RN1 and RN2 and RN3 and RN4), that selectively affected the growth of wild-type compared to several pg the NEDD8-mediated signaling pathway in Arabidopsis (SI Appendix, Fig. S1A and B).

The RNs Act as Developmental Regulators in Several Land Plants. We then analyzed RN effects on Populus (poplar) and Physcomitrella patens (moss). RN1, which induced hypocotyl elongation and promoted adventitious root formation in Arabidopsis, and RN3, which increased lateral root number in Arabidopsis, were applied to three different lines of poplar explants (SI Appendix, Fig. S2 B–D). The poplar lines were selected for their different rooting abilities; T89 is an easy rooting hybrid while SwAsp19 and -35 have a low rooting capacity even when treated with indole-3-butryic acid, an auxin commonly used as a rooting agent. Interestingly, both RN1 and RN3 promoted adventitious root formation preferentially in the SwAsp lines. Next, the effects of the RNs were investigated in moss and compared with those of IAA (SI Appendix, Fig. S3). Similar to IAA, most of the RNs inhibited caulonemal colony outgrowth (SI Appendix, Fig. S3A). The RN-induced effects on shoots were more diverse. At the tested concentrations, while no effect of RN1 was observed, application of RN2 caused a clear increase in shoot length, RN3 treatment resulted in thinner leaves, and RN4 slightly reduced shoot size (SI Appendix, Fig. S3B). At low concentration, IAA increased the number of buds/shoots per colony after 1 wk (SI Appendix, Fig. S3C), while it reduced bud/shoot formation after 2 wk regardless of the concentrations tested (SI Appendix, Fig. S3D). This dual effect of IAA was mimicked by RN4. RN1 and RN3 treatment resulted mainly in an increase of the bud/shoot number per colony after 1 wk and RN2 only reduced bud/shoot formation after 2 wk. These results demonstrate that the activities of the RNs are mediated by pathways present in several species.

The RNs Partly Function as Prohormones. RN1, RN3, and RN4 share structural similarities with previously described prohormones (19, 20). Because prohormones are hydrolyzed in vivo to release the...
active hormone moieties (21), we examined the potential metabolism of the RN compounds in liquid treatment media and *in planta* (SI Appendix, Fig. S4). In RN-supplemented MS media without plants, negligible concentrations of free acids were detected at the 0 h time point, except for 2,4-dichlorophenoxyacetic acid (2,4-D) originating from RN2 and 2,4,5-trichloroacetic acid (2,4,5-T) from RN3 (SI Appendix, Fig. S4D). Importantly, in these plant-free media, no obvious degradation of RN compounds was observed 24 h after treatment. However, in the presence of seedlings, higher levels of the corresponding free acids, 2,4-D, 2,4,5-T, and RN4-1, were found after 24 h in the media treated with RN1, RN3, and RN4, respectively, although the level of 2,4-D in RN2-treated media was not changed (SI Appendix, Fig. S4D).

As expected, in *Arabidopsis* seedlings treated by the RNs for 24 h, all RNs completely disappeared from the media. Like RN2, none of the RN2 analogs visibly altered the number and introduced a strong inhibitory effect on primary root elongation. The RN1 derivative, RN4-2, showed most probably attributable to the release of 2,4-D in the growing plant media. Like RN2, none of the RN2 analogs visibly altered the number and introduced a strong inhibitory effect on primary root elongation. Moreover, the activity of RN2 is most probably due to its in vitro cleavage into 2,4-D and the hydroxymethylphenylamine substructure (analog RN4-11), generated by methylation of RN4 on the amide bond, inhibited primary root elongation without affecting hypocotyl length (SI Appendix, Fig. S6D). Consistent with the SAR results, even though RN4-2 shows a bipartite structure, it was still able to induce pDR5::GUS expression (SI Appendix, Fig. S6D). RN4-10, in which the nonauxinic side chain is also required to induce maximal hypocotyl elongation (SI Appendix, Fig. S6 D and I), further comparison between RN4-2 and RN4, as well as their free acids (RN4-3 and RN4-1, respectively), highlight the key contribution of the bromophenylmethoxylation to the selective activity of RN4 on hypocotyl rather than primary root (SI Appendix, Fig. S6 D, I, and J). Consistent with our results, these analogs could not induce pDR5::GUS expression, indicating that the typical bipartite prohormone structure of RN4 is important for its effect on hypocotyl elongation and that hydrolysis is required to liberate this activity. Moreover, except for RN4-9, these compounds could not induce pDR5::GUS expression. Interestingly, the analog RN4-11, generated by methylation of RN4 on the amide bond, abolished RN4's effect on the hypocotyl in a dose-dependent manner (SI Appendix, Fig. S6D). Because the predicted corresponding free acid RN4-1 did not reduce primary root length, this result indicates that the full, nonhydrolyzed RN4 structure possesses additional auxin-like activity.

Overall, we showed that RN1, RN3, and RN4 function as prohormones, being metabolized in * planta* to release more potent auxin agonists, while the effects of RN2 are most likely due to its degradation to 2,4-D. However, our SAR results also suggest that the nonhydrolyzed forms of RN1, RN3, and RN4 display additional auxin-like effects and therefore might themselves act as selective auxin agonists.

**The RNs Act as Selective Auxin Agonists.** AXR1 is a component of the neddylation pathway targeting, among others, the CUL4-AK1-ARF family members (11). To determine which CUL proteins might be involved in mediating the effects of each RN, we tested their potency on the loss-of-function *cul1-6, cul5a,b*, and *cul4-1* mutants. We limited these tests to RN1, RN3, and RN4 as we showed that RN2 activity is most probably due to its in vitro cleavage into 2,4-D, an already well-described synthetic auxin. All three tested RNs had a lesser effect on the *cul4-1* elongation than other CUL4-AK1-ARF mutants (Fig. 2A), indicating that they function at the level of or upstream of CUL1. Given that signaling pathways mediated by AXR1 and CUL1 converge at the SFB complex, and that the chemical structures and activities of the three RNs are related to auxin, we hypothesized that auxin receptor F-box proteins might
also be required for RN activities. To test this, we examined tir1 single and tir1/afb multiple mutants and found that the RN-induced phenotypes were strongly reduced when the compounds were applied on tir1-1 and tir1-1afb1-3afb3-4 (24, 25) (Fig. 2B). Thus, a functional SCF[TIR1/AFB] complex is essential for the effects of the RNs. To further confirm this result, we tested the effect of cotreatment of the compound auxinole (26), an auxin antagonist specific for SCF[TIR1/AFB], together with each of the three RNs or the endogenous auxin IAA in the wild-type. The RN-induced phenotypes were inhibited by auxinole (Fig. 2C), demonstrating that auxin coreceptor complex formation is essential for RN activities.

Next, we employed a molecular modeling strategy to explore the possible interactions of the RNs with the DII degron of AUX/IAA7 in the auxin-binding pocket of TIR1. Docking experiments validated that the physical property of the auxin-binding pocket was promiscuous enough to accommodate the potential steric hindrance of RN1, RN3, or RN4 (Fig. 3A–C and Movie S1). The calculated free energies (ΔG) of binding also revealed thermodynamic stability for the three RNs inside the auxin pocket of TIR1 (Fig. 3A–C and SI Appendix, Fig. S7A). The positive control IAA was able to bind TIR1 with a ΔG(IAA-TIR1) of −11.68, whereas the negative control Tryptophan (Trp) was not, with a ΔG(Trp-TIR1) of 63.34 (SI Appendix, Fig. S7A). Among the RN analogs, RN4-1 and RN4-2 showed stronger thermodynamic stability compared with IAA. RN2 and the inactive analog RN4-8 could not dock inside the auxin-binding site to stabilize TIR1 (SI Appendix, Fig. S7A). This last result confirmed once again that RN2 activity is most likely due to its cleavage into 2,4-D.

To experimentally confirm the binding of the RNs within the auxin coreceptor complex, we tested their ability to promote the interactions between TIR1 and AUX/IAA proteins using in vitro pull-down assays. First, TIR1-myc protein purified from wheat germ extract and four different GST-AUX/IAA proteins were used (27–29). IAA stimulated the interaction of TIR1-myc with all AUX/IAAs tested (Fig. 3D and SI Appendix, Fig. S7B). All three RNs stimulated the recovery of TIR1-myc in complex with GST-SHY2/IAA3 or GST-AXR2/IAA7 to a similar extent (Fig. 3D and SI Appendix, Fig. S7B). In the case of GST-AXR5/IAA1, RN1 stimulated the interaction with TIR1-myc, while RN3 had little effect and surprisingly, RN4 decreased the basal interaction (Fig. 3D and SI Appendix, Fig. S7B). When GST-AXR3/IAA17 was used as bait, RN1 strongly promoted the interaction with TIR1-myc, while RN3 had little effect and again, RN4 reduced the basal interaction (Fig. 3D and SI Appendix, Fig. S7B). These data imply that RN3 and RN4 are able to selectively promote the interactions between specific TIR1 and AUX/IAA protein combinations in this system, while RN1 and IAA promoted each interaction, as shown previously for IAA (27–29).

To test that these effects on TIR–AUX/IAA complex formation were not dependent on metabolism of the RN compounds in the wheat germ extract, we next performed a complementary pull-down experiment using insect cell-expressed TIR1 (as a His-MBP-FLAG-TIR1 fusion protein) with bacterially expressed GST-AXR2/IAA7 or GST-AXR3/IAA17 in the presence of the RNs or the RN4 degradation product RN4-1 (SI Appendix, Fig. S7 C and D). In this system, the RNs again promoted selective interactions between TIR1 and AXR2/IAA7 or AXR3/IAA17, this time in the absence of potential plant hydrolases (in insect cells). Importantly, the promotion and inhibition of TIR1 interaction with AXR2/IAA7 and AXR3/IAA17, respectively, by RN3 and RN4 were identical in the two in vitro systems. Moreover, the degradation product RN4-1 behaved differently from RN4, by not promoting the interaction between TIR1 and AXR2/IAA7 and slightly reducing the interaction between TIR1 and AXR3/IAA17.
which might explain these compounds’ different activities in vivo. In fact, we were able to confirm that the observed TIR1–AUX/IAA interactions in this system were induced or repressed specifically by the RNs and not by their free-acid degradation products, as no 2,4-D, 2,4,5-T, or RN4-I could be detected at relevant time points in the pull-down reactions treated with RN1, RN3, or RN4, respectively (SI Appendix, Fig. S7E). These data demonstrate that RN3 and RN4 are able to selectively promote the interactions between TIR1 and certain AUX/IAA proteins. Hence, our results suggest that RN3 and RN4 are not just prohormones, but also act consistently as selective auxin agonists in two different in vitro experimental conditions and their effects on plant development may therefore be attributable to selective auxin agonistic activity. To test whether the RNs might also act as selective auxin agonists in planta, we assayed their potency in promoting the in vivo degradation of the AUX/IAA proteins. In a 1-h time course, IAA significantly increased the degradation rate of the four tested AUX/IAA-LUCIFERASE (LUC) proteins, while the RNs had different potency depending on the AUX/IAA proteins used (Fig. 3 E–H and SI Appendix, Fig. S7F). Therefore, the RN molecules act as selective auxin agonists both in vitro and in vivo, but the specificity of the interactions seems to be dependent on the experimental conditions, as the predicted behavior of AUX/IAA proteins based on their selectivity to IAA and RNs and RN4-I does not always match that in our in vitro pull-down assays. While the conditions tested in vivo reflect RNA capacity to enhance the interactions of the different SCF\(^{\text{TIR1/IAA}}\)-AUX/IAA coreceptors within a complex molecular surrounding, those tested in vitro reflect the interactions in much simpler conditions. Nonetheless, our results imply that altering interaction affinity within each co-receptor complex with selective auxin agonists might modulate a multitude of specific plant development aspects.

**RN3 and RN4 Induce Selective Early Transcriptional Responses.** The in vitro assays indicated that RN3 and RN4 are the most selective auxin agonists, showing different effects on different AUX/IAA proteins. Moreover, RN3 and RN4 induced distinct developmental processes, particularly on lateral root development. While RN3 enhanced the density of lateral roots without affecting primary root length in the wild-type, RN4 inhibited lateral root development (Fig. 1). Because these RNs promoted fast degradation of AUX/IAA proteins fused to LUC, we investigated how their activities fine-tuned events downstream of co-receptor complex formation. To this end, we performed transient expression profiling of Arabidopsis cell suspension cultures treated with IAA, RN3, and RN4, to characterize the early transcriptional responses induced by these compounds (Dataset S1). The data have been deposited at the European Nucleotide Archive (www.ebi.ac.uk/ena) under the accession number PRJE31496 (30). Analysis of the differentially expressed genes (DEGs) revealed subsets that were up- or down-regulated specifically by one, two, or all three chemical treatments (SI Appendix, Fig. S4 and Table S1). Among the early auxin-responsive genes identified, AMS/IAA1, IAA2, SHORT HYPOCOTYL 2 (SHY2)/IAA3, and IAA30 were significantly up-regulated by IAA, RN3, and RN4 (Fig. 4A and SI Appendix, Table S1). IAA5 and IAA16 expressions were induced specifically by IAA and RN3, while IAA10 and IAA29 expressions were up-regulated selectively by IAA and RN4, revealing some differences between RN3 and RN4 in their capacity to induce early-responsive AUX/IAA genes. In total, 121 genes were differentially up-regulated by IAA, RN3, and RN4, such as LATERAL ORGAN BOUNDARIES-DOMAIN 16 (LBD16), BASIC HELIX-LOOP-HELIX 32 (BHHL32), PINOID-BINDING PROTEIN 1 (PBP1), and PIN-FORMED 3 (PIN3) (31–34) (Fig. 4A), confirming the potential of the RNs to modulate auxin-related developmental processes. The genes CINNAMATE 4 HYDROXYGENASE (C4H), TRANSPARENT TESTA 4 (TT4), TT5, DEHYDRATION RESPONSE ELEMENT-BINDING PROTEIN 26 (DREB26), and EARLY-RESPONSE TO DEHYDRATION 9 (ERD9) were commonly up-regulated by IAA and RN3 but not by RN4. These five genes are known to be tightly regulated in a tissue-specific and auxin-dependent manner to modulate lateral root density and architecture (35–39). Among the genes commonly regulated by IAA and RN4 but not RN3, we identified MYELOBLASTOSIS 77 (MYB77) and BREVIX RADIX (BRX) transcription factors, which have been shown to control lateral root formation in an auxin-dependent manner (40, 41). These results correlate with the differential effects of RN3 and RN4 on lateral root development. Taken together, these data demonstrate the potential of RN3 and RN4 to specifically identify auxin-responsive genes involved in defined developmental processes, such as lateral root formation. Overall, we showed that RN molecules are able to selectively trigger specific auxin perception machinery, inducing expression of specific sets of genes, and resulting in distinct developmental traits.

**RN3 and RN4 Induce Specific Subsets of Auxin Responsive Promoters.** We further investigated the abilities of RN3 and RN4 to selectively induce later auxin responses using various auxin-responsive reporter line treatments after 45 min, 5 h, or 16 h of RN treatment. We found that neither the auxin-responsive reporter pDR5:GUS nor the indicator of nuclear auxin perception p35S:DII-Venus (42) showed any response to RN treatment in the primary root (Fig. 4B and SI Appendix, Fig. S9 A and D). However, in the root–hypocotyl junction, the expression of pDR5:GUS was promoted by either longer treatment (24 h) or higher concentration (50 µM) of RN3 or RN4 (Fig. 4C and SI Appendix, Fig. S8C). To determine whether these effects were specific to the RNs or rather due to their free-acid degradation products, we first determined the appropriate treatment concentrations of 2,4,5-T and RN4-1 that lead to their accumulation within the roots to similar levels as found after 16-h treatments with RN3 and RN4, respectively (SI Appendix, Fig. S9 A and B). While treatment with 2,4,5-T, similar to RN3 and RN4, had no effect on pMASSUGU2::DII-Venus expression with different patterns compared with that induced by IAA, but did not stimulate pMASSUGU2::DII-Venus expression significantly (SI Appendix, Fig. S9A and D). In contrast to RN3, RN4 induced pDR5:GUS expression in the root (SI Appendix, Fig. S9D). For other auxin-responsive reporter line tested, RN3 and RN4 induced expression patterns that partially overlapped with those induced by IAA (Fig. 4 B and C). In the primary root, the RN compounds induced pSHY2/IAA3::GUS and pBODENLOS (BDL)/IAA12::GUS expression with different patterns compared with that induced by IAA, but did not stimulate pMASSUGU2 (MSG2)/IAA19::GUS expression (Fig. 4B). Both compounds also promoted the expression of pMASSUGU2::GUS in the hypocotyl founder cell identity (43). RN4 additionally induced pSHY2/IAA3::GUS expression in the hypocotyl and the shoot apical meristem (Fig. 4C). In contrast to the primary root, RN3 and RN4 induced pMSG2/IAA19::GUS expression in the hypocotyl (Fig. 4C), although only RN4 induced hypocotyl elongation (Fig. 4B). Treatment of these auxin-responsive reporter line with 2,4,5-T induced similar expression patterns in the primary root as treatment with RN3 (SI Appendix, Fig. S9C), suggesting that the observed effects of RN3 may in fact be due to 2,4,5-T activity. However, as found for the DR5 promoter, RN4-1 induced the expression of most of the other promoters tested more strongly than RN4 in the primary root (SI Appendix, Fig. S9D), suggesting that these two compounds affect auxin-responsive promoter expression rather differently. Despite the release of RN4-1 during RN4 treatment, the effects of RN4 appear to be prominent as this compound did not induce pDR5:GUS despite the presence of RN4-1. Our data indicate that RN3 and RN4 may be able to induce specific auxin-regulated promoters, which might be responsible for their selective activities on plant development. Indeed, these RNs activate some but not all modules of the auxin signaling pathway within the same tissue, confirming their selective auxin agonist activities.

A summary of the results obtained for the four RNs is presented in SI Appendix, Table S2. In particular, RN3 and RN4 behave as auxin agonists, which selectively promote or inhibit AUX/IAA degradation in a reproducible manner, leading to specific transcriptional regulation and developmental outputs.
RN3 and RN4 activate independent auxin responses. (A) Selected sets of up-regulated genes in cell culture representing: IAA-specific induced genes (dark blue); IAA-, RN3-, and RN4-induced genes (light blue); IAA- and RN3-specific induced genes (light lilac); and IAA- and RN4-specific induced genes (green) (see Dataset S1 for the complete list of genes and SI Appendix, Table S1 for fold induction values of the selected genes). (B and C) Five-day-old seedlings expressing pDR5::GUS, pSHY2/IAA3::GUS, pBDL/IAA12::GUS, pMSG2/IAA19::GUS, or pGATA23::GUS transonal fusions treated with IAA, RN3, and RN4 at 10 μM for 16 h. DMSO was used as control. (B) Representative primary roots after GUS staining. (C) Representative hypocotyl-root junctions after GUS staining. (Scale bars, 100 μm in B and 1 mm in C)

Fig. 4. RN3 and RN4 activate independent auxin responses. (A) Selected sets of up-regulated genes in cell culture representing: IAA-specific induced genes (dark blue); IAA-, RN3-, and RN4-induced genes (light blue); IAA- and RN3-specific induced genes (light lilac); and IAA- and RN4-specific induced genes (green) (see Dataset S1 for the complete list of genes and SI Appendix, Table S1 for fold induction values of the selected genes). (B and C) Five-day-old seedlings expressing pDR5::GUS, pSHY2/IAA3::GUS, pBDL/IAA12::GUS, pMSG2/IAA19::GUS, or pGATA23::GUS transonal fusions treated with IAA, RN3, and RN4 at 10 μM for 16 h. DMSO was used as control. (B) Representative primary roots after GUS staining. (C) Representative hypocotyl-root junctions after GUS staining. (Scale bars, 100 μm in B and 1 mm in C)

AUX/IAA Sensitivity to RN3 and RN4 in Planta. We hypothesized that as the RN molecules show selectivity toward the auxin coreceptor complex, they might help to dissect specific functions of individual AUX/IAAs in distinct developmental processes. One approach to achieve this could be to investigate the responses of AUX/IAA gain-of-function mutants to auxin treatment; however, such a genetic approach could prove problematic due to high redundancy among the AUX/IAAs. As a potentially more effective alternative, we challenged such mutants with the specific auxin analogs RN3 and RN4.

We first focused on lateral root development as RN3 and RN4 had opposite effects on this process (Fig. 1 D and E). Furthermore, based on our transcriptomic analysis, RN3 and RN4 induce different sets of IAA-responsive genes that are known to be involved in the regulation of lateral root development (Fig. 4). We therefore investigated the sensitivities of 8-d-old seedlings of AUX/IAA gain-of-function mutants axr5-1/iaa1 (28), axr2-2/iaa7 (44), shy2-2/iaa3 (45, 46), and solitary root (sr-) to RN3 and RN4 with regards to lateral root development. We tested the sensitivities of these gain-of-function mutants to RN3, which increases lateral root density in Col-0 and Ler, with the Col-0 accession interestingly showing much higher sensitivity to this effect (Fig. 5A). We found that most of the mutants were also sensitive to this effect, with the exception of sr-1/iaa4 (Fig. 5A). The mutant shy2-2/iaa3 was more sensitive to this effect of RN3 than the wild-type (Fig. 5A); however, it is important to note that in this mutant, this compound mainly induced the slight emergence of lateral root primordia rather than the emergence of well-developed lateral roots. These data suggest that apart from SLR/IAA14, the AUX/IAAs we tested are not required for the stimulatory activity of RN3 on lateral root density. We next aimed to characterize RN4 activity on lateral root development in these mutants. RN4 reduced lateral root density in Col-0 and Ler (Fig. 5B). Compared with Col-0, axr5-1/iaa1 was resistant to this effect of RN4 at 5 μM, while axr2-2/iaa7 was sensitive at both tested RN4 concentrations (Fig. 5B). Interestingly, shy2-2/iaa3 was more sensitive to RN4 at 5 μM, but resistant at 2 μM, compared with Ler (Fig. 5B). Our results suggest that AXR5/IAA1 and SHY2/IAA3 might be degraded by RN4 to reduce lateral root density.

By using the RN molecules, we revealed potential contributions of specific AUX/IAAs to the complicated process of lateral root development. However, the sensitivities of the aux/iaa gain-of-function mutants to the RNs in terms of lateral root development did not exactly match the RN-induced AUX/IAA degradation/stabilization results found with our binding affinity assays. Lateral root development is a complicated process that requires the formation of a new meristem and emergence through several root layers, suggesting that the specific tissue context may affect RN activity and selectivity. We therefore decided to switch our focus to apical hook development in etiolated seedlings, a
rather simpler process than lateral root, but one also regulated by auxin (48). Apical hook development is characterized by differential growth between the two sides of the apical hypocotyl and comprises the formation, maintenance, and opening phases (49, 50). We first tested the effects of RN3 and RN4 on apical hook development in the wild-type (SI Appendix, Fig. S10A). While 2 μM RN3 did not affect apical hook development, RN4 completely abolished hook formation in a dose-dependent manner (SI Appendix, Fig. S10A and B).

We decided to exploit RN4 to understand whether selected AUX/IAAs play specific roles during apical hook development. We tested the effects of 0.5 μM RN4 on hook development in the gain-of-function mutants axr5-1/iaa1, axr2-2/iaa7, and axr3-1/iaa17 for 6 d in the dark. All three mutants showed altered apical hook development compared with the wild-type in control conditions (Fig. 5 C, E, and G). A detailed analysis of these results indicates that AXR5/IAA1 and AXR3/IAA17 need to be degraded for a proper apical hook to develop, while AXR2/IAA7 is likely stabilized during the formation phase and degraded during the maintenance phase. Similar to the wild-type, axr5-1/iaa1 showed sensitivity to RN4 during the formation phase, with no hook being present at 24 h; however, by 36 h the mutant had attained a slight hook curvature of 50°, which then started opening directly (Fig. 5D). The mutant axr2-2/iaa7 was resistant to RN4 in the formation phase (Fig. 5F) and axr3-1/iaa17 was sensitive to RN4 (Fig. 5H). Taken together, these results indicate that all three AUX/IAAs tested here play a role during apical hook development. In particular, our results suggest that AXR2/IAA7 is stabilized during apical hook formation while AXR2/IAA1 stabilization occurs during the maintenance phase.

The effects of 0.5 μM RN4 on AUX/IAA mutants during the first 24 h of apical hook development (Fig. 5 D, F, and H) correlate strikingly with our in vitro pull-down assay results (Fig. 3D). AXR2/IAA7 proteins strongly interacted with TIR1 in the presence of RN4 (Fig. 3 D and G and SI Appendix, Fig. S7B), suggesting that a stabilized version of this AUX/IAA should confer resistance to the RN4 auxin agonist, which is indeed what we found with the axr2-2/iaa7 gain-of-function mutant (Fig. 5F). In contrast, AXR5/IAA1 and AXR3/IAA17 did not interact with TIR1 when RN4 was present in the pull-down assay (Fig. 3 D, E, and H and SI Appendix, Fig. S7B) and the corresponding gain-of-function mutants were sensitive to the effects of RN4 on hook development (Fig. 5 D and H).

Overall, our study of the effects of RN4 in particular on the AUX/IAA gain-of-function mutants, distinguishes the involvement of specific AUX/IAAs in lateral root and apical hook development. Thus, we demonstrated the potential of such selective auxin agonists in dissecting auxin perception controlling specific developmental processes in vivo.

**Fig. 5.** RN-induced phenotypes require the degradation of specific AUX/IAAs. (A and B) Relative lateral root density (treated/DMSO) was measured for gain-of-function mutants axr5-1/iaa1, axr2-2/iaa7, slr-1/iaa7, and shy2-2/iaa3 and their respective wild-type grown on media supplemented with RN3 (A) and RN4 (B) for 8 d. DMSO was used as control. Statistical analyses were performed using Student’s t test (A), or ANOVA and Tukey’s test (B) to compare the effect of RN3 (A) or RN4 (B) relative to the DMSO control for each genotype, as indicated with triple asterisks and square brackets (A) or different letters (B). The Student’s t test was used to compare the relative effect of RN3 (A) or RN4 (B) on the mutants to that on the relevant wild-type, as indicated with single asterisks. (C–H) Gain-of-function mutants axr5-1/iaa1 (C and D), axr2-2/iaa7 (E and F), and axr3-1/iaa17 (G and H) were grown in the dark on DMSO (C, E, G, and H) and RN4 (D, F, and H) -supplemented media for 6 d. Measurement of apical hook angle was performed every 3 h. Means ± SEM are shown, n > 20 seedlings across three independent replicates; *P < 0.05, **P < 0.01, ***P < 0.001, different letters indicate significant differences at P < 0.05. Concentrations in micromolars are indicated in brackets.

**Mutation in the ATPase Domain of AtBRM Confers Resistance to RN4.** RN4 represents a useful tool to investigate the role of auxin during early stages of skotomorphogenesis. To identify new molecular players involved in apical hook development, we performed a forward genetic screen of sensitivity to RN4, using an ethyl methanesulfonate-mutagenized Col-0 population and selected those mutants that were able to form an apical hook in the presence of 0.5 μM RN4 in the dark, which we named hookback (hkb) mutants. We then further selected only those of the mutants that were sensitive to the effects of 75 nM 2,4-D on seedling phenotype in the light (SI Appendix, Fig. S10C). Using this strategy, we could exclude known auxin resistant mutants that might appear in the screen. Several independent hkb lines, each carrying a single recessive mutation, were isolated from the screen and we focused on characterizing one of these, hkb1. In contrast to Col-0, hkb1 had formed well-curved apical hooks in the presence of RN4 24 h after germination, while under mock-treated conditions there were no major differences between the two genotypes (Fig. 6A). Whole-genome sequencing of hkb1 revealed the presence of one nonsynonymous ethyl methanesulfonate-like mutation (C-to-T...
nucleotide substitution) in the coding region of the *AT2G460020* gene that encodes for the SWITCH/Sucrose Non-Fermentable (SWI/SNF) chromatin remodeling ATPase BRAHMA (BRM). The data have been deposited at the European Nucleotide Archive (www.ebi.ac.uk/ena) under the accession number PRJEB21529 (51). To confirm that the mutation in BRM is responsible for the resistance against the negative effect of RN4 on apical hook formation, we carried out several analyses. First, we checked the phenotypes of available T-DNA mutants for BRM, including *brm-1*, *brm-2*, *brm-4*, and *brm-5* (ectopic expression of seed storage proteins3, esp3) (52, 53). However, we focused our investigations on *brm-5* because both *hkb1* and *brm-5* contain a mutation in the ATPase domain (54) and 4-wk-old plants of the two mutants showed similar phenotypes, including twisted leaves and less siliques than wild-type (Fig. 6B). Importantly, *brm-5* showed similar resistance to the effect of 0.5 μM RN4 on apical hook formation to that shown by *hkb1* (Fig. 6 C and D). These results strongly suggest that the mutation in the ATPase domain of BRM in *hkb1* is responsible for the resistance of this mutant to RN4. Next, we crossed *hkb1* with *brm-5* and the F2 generation was analyzed. The *hkb1*/*brm-5* mutant showed the same apical hook phenotype and similar RN4 resistance as the single *hkb1* and *brm-5* mutants (Fig. 6 C and D), confirming that the mutation that confers resistance against RN4 in *hkb1* is in the *BRM* gene.

Our results suggest that BRM may function as a negative regulator of apical hook formation. Considering the resistance of both the *axr2/iaa7* gain-of-function mutant and *hkb1/brm-5* to the effect of RN4 on apical hook formation, we hypothesize that AXR2/IAA7 might negatively regulate BRM-induced gene transcription. We suggest that RN4 induces degradation of AXR2/IAA7, which may lead to BRM-mediated promotion of transcription of genes negatively regulating apical hook formation, potentially through chromatin remodeling.

Overall, our results show that selective auxin agonists can enable us to dissect the roles of specific AUX/IAAs in developmental processes, leading to the dissection of the molecular mechanisms of these processes.

**Discussion**

Complicated auxin perception modules translate auxin signals into a multitude of developmental responses (55, 56). Several studies have demonstrated that IAA displays different affinities for different SCF*TTIR1/AUX/IAA* coreceptor complex combinations (6, 57) and specific auxin perception modules have even been shown to act sequentially during development (58). In this work, we isolated the RNs as selective auxin agonists and revealed their potential to dissect the complex and redundant mechanisms of auxin perception machinery that control specific aspects of plant development. We employed RN4 in particular as a tool to characterize specific auxin perception modules and their potential targets. Remarkably, we even found variability of RN sensitivity between different accessions in both *Arabidopsis* and poplar, pointing to future challenges toward developing the most suitable auxin agonists for specific species and accessions. However, it is important to emphasize that we identified degradation products released from all four RNs in *plants*, which in some cases also induced plant responses. This finding highlights that it is essential to investigate the stability of any such identified auxin agonists and take into account any degradation products released.

Auxin behaves like molecular glue within the SCF*TTIR1/AUX/ IAA* complex (55) by fitting into a space between the TIR1/AFB receptor and AUX/IAA coreceptor and extending the hydrophobic protein interaction surface. It has long been known that the auxin-binding pocket of SCF*TTIR1/AFB* is promiscuous, a feature that was heavily investigated during the early years of auxin research in the 1940s (59, 60). During this time, several auxinic compounds were discovered, including NAA, 2,4-D, and picolinate auxins, such as picloram (61), which are widely used today for basic research and agricultural applications. The 2,4-D and NAA modes of action are similar to that of IAA, as they also enhance the binding affinity between TIR1 and the AUX/IAAs. Their affinity to the coreceptor complex is lower than that of IAA, but they are more stable metabolically, which explains their robust activity. Although the full details of the mode of action of these synthetic auxins are not yet known, they have been instrumental in the discoveries of crucial auxin signaling components, such as AXR1, AXR3/IAA17, AXR5/IAA1, AFB4, and AFB5 (62–66). Thus, synthetic compounds with auxin-like activities hold the potential to dissect the convoluted mechanisms of auxin signaling. Moreover, our isolation and characterization of RN4 revealed different activity and selectivity compared with most of the currently available synthetic auxins, and thus open up new possibilities to identify novel actors in auxin biological responses.

Here, we have shown that the selective capacity of RN3 and RN4 to promote the interaction of TIR1 with specific AUX/IAA coreceptors, highlighting a strong potential for such auxin agonists in defining AUX/IAA involvement in specific transcriptional responses and developmental traits. This potential was strongly supported by our genetic approach, showing that different AUX/IAA gain-of-function mutants display defined sensitivities to RN3 and RN4 in terms of lateral root development. Importantly, we uncoupled the effects of RN3 and RN4 on TIR1-AUX/IAA interactions and lateral root development from their free acid degradation products, thus confirming the usefulness of these RN compounds as selective auxin agonists. Multiple AUX/IAA-ARF modules act sequentially over time and space to orchestrate lateral root development (58, 67). Our data indicate that RN3 may promote development of lateral roots through SLR/IAA14 degradation and the stabilization of SHY2/IAA3, but we cannot yet conclude whether degradation of additional AUX/IAAs is also required for this effect. On the other hand, the resistance of the *axr5-1/iaa1* mutant to high concentrations of RN4 revealed a role for AXR3/IAA1 as a positive regulator of lateral root development.

Moreover, we used the RN with the greatest potential, RN4, as a tool to identify which of several AUX/IAA proteins are directly involved in apical hook development and revealed the implication of auxin-signaling components, such as the SWI/SNF
chromatin remodeling ATPase BRM. Remarkably, BRM has already been shown to be involved in auxin-dependent floral fate acquisition (68). In the inflorescence, when MONOPTEROS (MP)/ARF5 is free from AUX/IAA repression, it recruits BRM or its homolog SPLAYED (SYD) to remodel chromatin and thus promote gene transcription. Interestingly, in a yeast three-hybrid assay, AXR3/IAA17 and BDL/IAA12 have been shown to prevent the association of MP to BRM (68). According to these results and our data showing the resistance of axr2-1/iaa7 and hkb1/brm-5 to RN4-mediated suppression of apical hook formation, we hypothesize that BRM, by associating with an unknown ARF transcription factor, might promote transcription of genes negatively regulating hook formation. We also hypothesize that AXR3/IAA17 might play a more specific role in the interaction of AUX/IAA with ARF to BRM. Application of RN4 prompts the degradation of AXR2/IAA7, which may facilitate the association of the ARF to BRM, promoting transcription of downstream genes negatively regulating apical hook formation, potentially through chromatin remodeling. However, the hypothesis that stabilization of AXR2/IAA7 during apical hook formation blocks BRM activity raises the question of whether MP plays a role during hook development or whether BRM is recruited by other ARFs.

The different affinities of AUX/IAA proteins for IAA, RNA3, and RN4 might lie in differences in residues within the DII domain. Our study thus brings us a step closer to a better quantitative understanding of the TIR1–AUX/IAA interaction system of auxin perception in a tissue-specific manner. Besides IAA, several other phytohormones including jasmonate-isoleucine, gibberellic, brassinosteroids, and abscisic acid (ABA), also function by modulating the protein–protein interactions of their coreceptors (69). Isolation of novel molecules modulating such interactions could therefore also be useful in uncovering the signaling components of these phytohormones.

Auxins have many uses in agriculture, horticulture, forestry, and plant tissue culture (59). The selective auxin agonists described here may also find niche applications in these fields. RN activities in the low micromolar range and conservation of their specific developmental effects in land plants enforces this possibility. Moreover, the availability of models for ligand-bound coreceptors may allow rational design of a wider array of auxin agonists using RN structures, in particular RN4, as a starting point. Indeed, a rational design approach has already paved the way for developing agrochemicals interacting specifically with a subset of AUX/IAA coreceptors (70). Such an approach might also have the potential to overcome the limitations of some of the RNs, for example by enhancing stability to eliminate the release of degradation products.

Overall, the isolation and characterization of chemical modulators of plant hormone signaling is an effective way to better understand the specificity of hormonal receptors. Because of the availability of genetic and genomic methods, most chemical biology approaches are performed in model species, such as Arabidopsis. However, chemicals that induce well-characterized effects in Arabidopsis can be applied to nonmodel species to improve crop and plant tissue culture (59). The selective auxin agonists described here may allow rational design of a wider array of auxin agonists using RNs, for example by engineering RNs to remodel chromatin and thus promote gene transcription. Interestingly, in a yeast three-hybrid assay, AXR3/IAA17 and BDL/IAA12 have been shown to prevent the association of MP to BRM (68). According to these results and our data showing the resistance of axr2-1/iaa7 and hkb1/brm-5 to RN4-mediated suppression of apical hook formation, we hypothesize that BRM, by associating with an unknown ARF transcription factor, might promote transcription of genes negatively regulating hook formation. We also hypothesize that AXR3/IAA17 might play a more specific role in the interaction of AUX/IAA with ARF to BRM. Application of RN4 prompts the degradation of AXR2/IAA7, which may facilitate the association of the ARF to BRM, promoting transcription of downstream genes negatively regulating apical hook formation, potentially through chromatin remodeling. However, the hypothesis that stabilization of AXR2/IAA7 during apical hook formation blocks BRM activity raises the question of whether MP plays a role during hook development or whether BRM is recruited by other ARFs.

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