Predictable modulation of plant root development using engineered cytokinin regulators

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Abstract

Achieving precise control over quantitative developmental phenotypes is a key objective in plant biology. Recent advances in synthetic biology have enabled tools to reprogram entire developmental pathways; however, the complexity of designing synthetic genetic programs and the inherent interactions between various signaling processes remains a critical challenge. Here, we leverage Type-B response regulators to modulate the expression of genes involved in cytokinin-dependent growth and development processes. We rationally engineered these regulators to modulate their transcriptional activity (*i.e.*, repression or activation), potency, and sensitivity to cytokinin. By localizing the expression of these engineered TFs using tissue-specific promoters, we can precisely tune cytokinin-regulated traits. As a proof of principle, we deployed this synthetic system in *Arabidopsis thaliana* to either decrease or increase the number of lateral roots. The simplicity and modularity of our approach makes it an ideal system for controlling other developmental phenotypes of agronomic interest in plants.

Introduction

Plant development is controlled by a complex interplay of genetic and environmental cues. Central to this process are hormonal signaling networks, which tightly regulate growth, differentiation, and adaptation in response to changing environmental conditions. Key hormones, such as auxin and cytokinin, serve as central regulators of developmental pathways, orchestrating processes like cell division, elongation, and organ formation[1–3]. As plants continuously adjust to environmental stressors and nutrient availability, these hormone-driven signaling pathways become pivotal in regulating plant phenotypes[4]. In recent years, efforts to manipulate plant development through modulation of these hormonal pathways have gained traction, offering the potential to enhance traits such as yield, stress tolerance, and overall growth vigor[4,5]. Such approaches hold promise in addressing global agricultural challenges,

where the ability to fine-tune developmental outcomes can improve plant resilience and productivity under diverse environmental conditions[6].

Among the various developmental processes influenced by plant hormonal networks, root system architecture stands out due to its fundamental role in nutrient acquisition and water uptake. Root configuration is a critical determinant of a plant's ability to efficiently explore and exploit the surrounding soil environment, accessing vital resources such as minerals and water. Among the various components of the root system, lateral roots have garnered particular attention, as they serve as key drivers of root branching and contribute directly to nutrient use efficiency and water absorption[7]. The ability to precisely and predictably modulate lateral root formation holds immense potential for optimizing plant performance under diverse environmental conditions seen with the effects of climate change[8,9]. As such, developing systems capable of tuning important phenotypes, like lateral root count, has become an emerging goal in plant biology research.

Lateral root formation is tightly regulated by hormonal pathways, with cytokinin signaling playing a pivotal role as a negative regulator of this process[10]. A key component of the cytokinin signaling pathway involves Type-B response regulators (Type-B ARRs), a family of TFs that bind cis-regulatory elements in cytokinin-responsive genes. Upon cytokinin sensing, a cascade of phosphotransfer events is initiated, beginning with histidine kinases (AHKs) that phosphorylate histidine phosphotransfer proteins (HPTs) (Fig. 1A). HPTs activate Type-B ARRs by phosphorylating a conserved aspartate residue in their receiver domain. Once activated, Type-B ARRs act as TFs that regulate the expression of cytokinin-responsive genes, including Type-A ARRs, which provide negative feedback regulation to fine-tune the cytokinin response[11–13].

Given the central role of Type-B ARRs in mediating cytokinin responses, we selected this family of TFs as amenable candidates for engineering the cytokinin signaling pathway. The approach of fusing regulatory domains to TFs has a strong precedent as a strategy for modulating their activity. It has been demonstrated that fusing the SRDX EAR repression motif to activator TFs can dampen their activity or even convert them to repressors[14]. In addition, previous studies have demonstrated that the activity of activator TFs can be significantly amplified by fusing them with strong activation domains like VP16[15,16]. Furthermore, it has also been shown that tuning the activity of Type-B ARRs is feasible, as demonstrated by the ARR1-SRDX variant, which can effectively suppress cytokinin signaling when overexpressed in *A. thaliana*[17]. With this foundation, we aimed to employ this strategy by constructing a library of engineered Type-B ARRs, incorporating both activation and repression domains at the TF's C-terminus to generate repressor and activator variants with a range of regulatory strengths capable of tuning cytokinin responses (Fig. 1B).

To overcome the challenges of endogenous regulation, an ideal engineered system requires both orthogonality and dominance over native regulation. Orthogonality is essential to ensure the engineered variants remain active independently of internal cytokinin fluctuations, preventing interference from endogenous signaling. We sought to address this goal by

incorporating phosphomimetic variants such as Type-B ARRs with D→E mutations on a conserved aspartate residue within the receiver domain. Previous studies have established a similar strategy, demonstrating that the D94E ARR1 variant induces cytokinin insensitivity by mimicking cytokinin-dependent phosphorylation, effectively bypassing endogenous regulation and providing a blueprint for engineering constitutively active Type-B ARRs[18]. In regards to dominance, many studies have demonstrated that activator TFs fused with the SRDX repression motif exhibit dominant-negative properties[14]. Dominance would ensure the desired phenotypic outcomes without the need for a specific genetic background.

To achieve precise control over a discrete cytokinin-dependent developmental pathway while minimizing pleiotropic effects, we reasoned that tissue-specific expression of our engineered Type-B ARRs would be critical. For instance, targeting the expression of engineered ARRs to lateral root founder cells could allow direct modulation of lateral root density without disrupting normal functions in other tissues (Fig. 1C). Given the role of cytokinin as a negative regulator of lateral root formation, we anticipate that strong activators expressed in lateral root precursor cells will lead to a diminished lateral phenotype, while strong repressors may increase the number of lateral roots (Fig. 1D).

In this study, we sought to implement this strategy as a proof of principle for the precise and predictable tuning of plant development. We demonstrate that the Type-B ARR ARR12 can be rationally engineered to alter its regulatory function and strength as well as its sensitivity to cytokinin.

Results:

Construction and functional characterization of engineered ARR12 variants with tunable regulatory activity

It was previously shown that overexpression of the Type-B ARR1 TF fused to the SRDX repression motif driven by the strong 35S promoter represses cytokinin signaling in *A. thaliana*[17]. For example, these plants had more lateral roots than WT, indicating that the normal role of ARR1 as a transactivator can be reversed to a repressor. However, because of the strength of SRDX and/or the strong and ubiquitous expression of 35S, this effect was extremely severe and pleiotropic. Building upon these results, we sought to achieve a more tunable system by fusing a Type-B ARR to repression domains of varying potency. Additionally, since Type-B ARRs are generally considered to be activators, we hypothesized that it may be possible to enhance cytokinin signaling by fusing Type-B ARRs to activation domains of different strengths and thereby achieve a larger dynamic range of tunability. ARR12, a member of the Type-B family, was selected as the Type-B of choice due to its well-established role in lateral root formation as well as its function in shoot regeneration, making it an ideal candidate for modulating key developmental processes through the cytokinin pathway[19,20]. ARR12 is typically classified as a weak transcriptional activator, and it contains an acidic activation domain that is sufficient for transactivation in a yeast-based assay[21].

To generate a comprehensive library of ARR12 variants with varying regulatory logic (*i.e.*, repressors or activators) and strength, we fused several repression and activation domains to the C-terminus of the *A. thaliana* ARR12 gene (Fig. 2A). The library included well-characterized domains such as PR[22] and EDLL[23], in addition to novel EAR repression motifs of various strengths that we previously have identified and characterized (Fig. 2A)[24]. To achieve TFs with constitutive activity, we engineered phosphomimetic variants of ARR12, designated as pmARR12, by introducing a D74E substitution in the conserved cytokinin-responsive phosphorylation site[18]. To rapidly characterize these variants *in planta*, we utilized the *Agrobacterium*-mediated transient gene expression system in *Nicotiana benthamiana,* which has previously been employed to study the transactivation of Type-A promoters by Type B ARRs (Fig. 2B)[25]. Specifically, we utilized a two-strain coinfiltration-based system. One strain carried an effector plasmid coding for pmARR12 driven by the strong *A. thaliana* UBQ10 promoter. Our pmARR12 variants were fused to blue fluorescent protein (BFP) at the N-terminus to enable normalization of varying transgene expression levels (Fig. 2B). The second coinfiltrated strain carried a reporter gene composed of either the ARR5 or ARR7[26] promoters from *A. thaliana*, driving the expression of GFP and providing a quantitative readout of transcriptional activity in response to the different pmARR12 constructs (Fig. 2C,D). These ARR5 and ARR7 promoters were defined as 2 kb upstream of the start codon, including the 5' UTR, following a previous approach that was shown to capture sufficient cis-regulatory elements for detectable promoter activity[27]. Notably, these promoters contain the canonical AGATHY cis-elements, a sequence motif directly bound by Type-B ARRs like ARR12, enabling the direct regulation and activation of these reporters by ARR12[28,29].

Our transient expression system revealed significant variation in protein expression levels across the different pmARR12 variants, as measured by BFP fluorescence, despite these constructs being driven by the same UBQ10 promoter, highlighting the importance of considering differences in expression efficiency for characterizing variants (Fig. 2C,D). To account for these differences in expression among variants, we normalized the expression value by dividing the GFP reporter signal by the BFP fluorescence (see Materials and Methods). This strategy yielded more reproducible results compared to other normalization approaches that utilize a second, constitutive red fluorescent protein (RFP) gene for normalization (Supplementary Fig. 1A,B). As a baseline control for engineered ARR12 activity, we coinfiltrated the reporters with an empty vector strain instead of a pmARR12 variant. Consistent with previous studies, we found the pmARR12 variant without an added domain to be a weak activator of both ARR5 and 7 (Fig. 2C,D). For both reporter constructs, we were able to generate a library of pmARR12 constructs with statistically significant differences in functional strength (Fig. 2C,D). In general, the strength of the pmARR12 variants correlated with the previously known activity of the fusion domains. One exception was that of the pmARR12-KMTR162 EAR motif variant which behaved as a weak activator instead of a repressor. Thus, we have established a collection of cytokinin regulator TFs with varying repressive and activating potentials capable of altering the expression profile of native *A. thaliana* promoters.

Modulation of lateral root development in A. thaliana using engineered Type-B ARRs

Next, we sought to test if quantitative differences in regulatory activity across engineered ARR12 variants translate into differences in a quantitative phenotype controlled by cytokinin. To this end, we tested whether transgenes carrying our engineered variants could predictably modulate lateral root density in stably transformed *A. thaliana*. To mitigate the risk of pleiotropic effects that could arise from ectopic expression of engineered TFs, we employed tissue-specific promoters. The cell type-specific promoter *proLBD16* was initially chosen for its established roles in lateral root development. In particular, LBD16 is expressed during the early stages of lateral root primordium initiation[30]. By driving the expression of our engineered pmARR12 variants under this promoter, we aimed to confine the effects to lateral root formation, minimizing off-target effects and ensuring a focused developmental readout. We selected representative constructs from our library, including a strong activator, a strong repressor, and a weak activator pmARR12 variant without an added domain, which served as a baseline control.

Next, we generated at least 10 stable transgenic lines in a WT *A. thaliana* (Col-0) background carrying the selected BFP-pmARR12 variants under the *proLBD16* promoter. We then used T2 seedlings to determine lateral root count and primary root length, with lateral root density defined as the ratio between these two metrics (see Materials and Methods). As cytokinin signaling negatively regulates lateral root formation and growth, we expected the strong repressor to increase lateral root density and the strong activator to diminish lateral root density relative to the control line. Initial results with the *proLBD16* transgenic lines were inconclusive; the engineered regulators did not significantly impact the lateral root phenotype, and the differences in lateral root density among the three variants were statistically insignificant (Fig. 3A). This suggested to us that *proLBD16*-driven expression may not provide sufficient sensitivity to enable detection of subtle phenotypic changes in lateral root formation. Additionally, it is plausible that the *proLBD16* promoter lacks the necessary spatiotemporal expression patterns to influence this phenotype effectively. If LBD16 is primarily expressed in the primordium, its activation may occur too late to impact lateral root initiation.

Motivated by these findings, we hypothesized that targeting an earlier stage in root development might yield more pronounced effects. Therefore, we subsequently employed the *proGATA23* promoter, which natively regulates the expression of GATA23, a key TF known to participate in the specification of lateral root founder cells[31]. In contrast to the *proLBD16* lines, we observed significantly diminished lateral root density in the activator line and a density boost in the repressor line when compared to pmARR12 without an added domain (Fig. 3B,3C). The primary root length did not change between the control line and the repressor line, but the activator line's median primary root length was 30% shorter than the repressor line and 22% shorter than the control line (Fig. 3B,3C). Importantly, these differences in primary root lengths do not explain differences in lateral root density. Thus, the density measurement reflects the true modulation of lateral root formation rather than being an artifact of altered primary root length.

In prior studies, 35S-ARR1-SRDX transgenic lines reported severely stunted growth within the shoot architecture[17]. In contrast, our engineered lines maintained normal shoot morphology, maintaining structural integrity across the shoot regions (Fig. 3C). Notably, the effects of our engineered TFs were largely confined to the intended target tissues, demonstrating effective spatial specificity.

We next took advantage of the BFP fluorophore fused to pmARR12 to determine the protein expression pattern of the engineered TF in the *proGATA23* transgenic lines. We observed clear nuclear BFP fluorescence in lateral root primordiums and emerged lateral roots of the 3xSRDX fusion line but this signal weakened as lateral roots matured (Fig. 3D, S2). This protein expression pattern was somewhat surprising since transcription of the *proGATA23* promoter is known to be restricted to just a few lateral root initial cells. This discrepancy could be the result of post-transcriptional dynamics regulating the accumulation of BFP-pmARR12. Indeed, GUS reporter assays have shown that in WT plants the ARR12 protein accumulates in young emerging lateral roots, even though its promoter is not active in these cells[19,32]. These dynamics may partially explain why this transgene was effective at regulating lateral root formation and growth.

Engineered TFs are insensitive to fluctuations in cytokinin levels

It is well established that exogenous cytokinin addition inhibits lateral formation in WT seedlings[33,34]. Hence, to test whether the variant library exhibited insensitivity to cytokinin, we analyzed lateral root density in WT and the strong repressor line pmARR12-3xSRDX in the presence of an added cytokinin analog (see Materials and Methods). In the WT line, the addition of cytokinin led to a significant reduction in lateral root density, as expected (Fig. 4a). In contrast, the repressor line, pmARR12-3xSRDX, showed no significant difference in lateral root density when treated with cytokinin, demonstrating a disruption in cytokinin responsiveness.

This result aligns with previous findings that phosphomimetic Type-B ARR lines exhibit cytokinin insensitivity. While the precise molecular mechanism remains to be clarified, it is plausible that the phosphomimetic mutation locks the ARR12 protein in a constitutively active state, thereby uncoupling it from the typical cytokinin signaling cascade. This sustained activation likely disrupts normal cytokinin-mediated regulation, leading to a persistent repressive effect on lateral root development, similar to the phenotypes observed in Type-B ARR knockout lines responding to cytokinin.[35]

In addition to lateral root density, we measured primary root length across all conditions to control for potential confounding factors. Similar to our previous lateral root experiments, we observed no significant differences in primary root length between the WT and pmARR12-3xSRDX lines, either with or without cytokinin treatment (Fig. 4a). This consistency in primary root length indicates that the observed differences in lateral root density are not attributable to variations in primary root growth.

The absence of significant changes in lateral root density following cytokinin treatment, coupled with unchanged primary root length, underscores the robust and dominant nature of this engineered repressor. This dominance is a highly desirable trait, as plants naturally adjust endogenous cytokinin levels to environmental stimuli. A cytokinin-insensitive system ensures stable and predictable outcomes, making this variant particularly valuable for further applied studies in other tissue types and broader applications in plant development engineering.

Discussion:

In this study, we engineered a library of phosphomimetic ARR12 variants with tunable regulatory logic and strength to fine-tune a quantitative developmental phenotype in plants. By fusing activation and repression domains to the C-terminus of the ARR12 TF and expressing them with cell type-specific root promoters, we developed a system capable of finely controlling cytokinin signaling pathways *in planta*. Using the modulation of lateral root development in *A. thaliana*, we demonstrated that our system meets two key engineering goals: dominance and insensitivity to cytokinin. Dominance allows the engineered variants to exert their effects independently of the native signaling components, regardless of the genetic background. Additionally, since endogenous cytokinin levels can vary due to internal and external factors, insensitivity to cytokinin is critical for maintaining the overall stability of the platform.

Recent advances have established a robust foundation for a new generation of plant developmental engineering. For instance, Brophy et al. (2022) achieved controlled suppression of root branching through cis-regulatory designs that incorporated buffer logic of varying strengths[36]. In a similar theme, Khakhar et al. (2018) designed hormone-activated Cas9-based repressors (HACRs) capable of regulating hormone-responsive genes, ultimately allowing for the precise control of key developmental traits like shoot branching[37]. Our simple and modular approach extends the scope of plant engineering beyond single-direction tuning to enable more flexible bidirectional manipulation of quantitative developmental phenotypes.

The conservation of cytokinin signaling in plants[38,39], combined with the modular nature of our engineered Type-B ARR system supports its broad applicability across plant species and developmental processes. Our framework could easily be adapted to specifically target other cytokinin-mediated developmental processes by substituting the tissue-specific promoters driving effector expression. This adaptability provides a valuable tool for tailoring plant developmental processes relevant to many other agronomically relevant traits beyond lateral root regulation. For example, cytokinin signaling regulates shoot meristem size via the direct activation of WUSCHEL (WUS) by type B ARRs[40–42]. In turn, the levels and spatial pattern of WUS expression dictate the size and shape of fruits and other reproductive organs. It may thus be possible to fine-tune these phenotypes with engineered type B ARRs.

Regarding these potential applications, we note that different promoters can drastically affect the phenotypic effect of our engineered TFs, even when their expression largely overlaps in space and time such as *proLBD16* and *proGATA23*. Further, in some cases, tissue and cell type-specificity may be dictated by cytokinin signaling itself. This underscores the importance of empirically testing several regulatory regions. Translational fusions to the TF of interest such as our BFP-ARR12 fusion will make it possible to better characterize these expression dynamics.

Random mutagenesis of cis-regulatory regions controlling TF expression has received much attention as a way to quantitatively modulate plant development. We envision that engineering the protein-coding sequence of TFs themselves may represent an equally powerful yet more predictable approach. Realizing this vision will require the characterization of new plant

repression and activation domains as well as better understanding how transcriptional strength in plant cells is encoded at the level of protein sequence.

Materials and Methods:

Plant Material and Growth Conditions

N. benthamiana was grown following a previously published lab protocol[27]. Plants were grown in an indoor growth room at 25°C and 60% humidity using a 16/8 hour light/dark cycle with a daytime PPFD of ~120 µmol/m2s. The soil consisted of Sunshine Mix #4 (Sungro) supplemented with Osmocote 14-14-14 fertilizer (ICL) at 5mL/L and agroinfiltrated 29 days after seed sowing.

Plasmid Construction & Transformation of *A. thaliana*

Plasmids were constructed using Gibson assembly[43] or Golden Gate assembly[44], following established protocols. For every construct containing the ARR12 gene, we used the genomic sequence of ARR12 in which all the introns except for the first one were removed. Retaining the first intron was necessary since constructs carrying the ARR12 cDNA displayed toxicity in E. coli.

For plant transformation, the floral dip technique was followed using an established protocol[45]. Seeds from the transformed plants were screened on plates containing 35 mg/L hygromycin. The plates, sealed with micropore tape, contained a medium composed of 1.5% plant tissue culture agar and half-strength Murashige and Skoog (MS) basal salt mixture with nutrients, adjusted to a pH of 5.7. Seedlings were cultivated for two weeks under axenic conditions in a Percival growth chamber set at 24 °C with constant light. After this period, transformed seedlings could be distinguished from non-transformed ones based on their larger size and successful germination.

N. benthamiana agroinfiltration assay

Binary vectors were introduced into *A. tumefaciens* strain GV3101. Colonies were inoculated in liquid medium with the appropriate antibiotics the evening before the experiment. Cultures were grown to an OD600 of 0.8–1.2 prior to resuspension in infiltration buffer (10 mM MgCl2, 10 mM MES, and 200 µM acetosyringone (added fresh), pH 5.7). Cultures were incubated for 2 hours at room temperature and then combined in equal ODs (final OD600 = 0.15 per strain, 0.45 total OD of bacteria). Each coinfiltration mix included the given reporter strain (Type-A promoter-GFP), an effector strain (Type ARR12 variant), and a normalization RFP strain. For control, an empty vector strain carrying the pCambia1300 backbone was infiltrated at an OD of 0.45. For a baseline of reporter output, each coinfiltration mix included the given reporter strain, an empty vector strain, and the RFP strain each at an OD of 0.15. Leaves 6 and 7 of 4-week-old *N. benthamiana* plants were subsequently syringe infiltrated with the *A. tumefaciens* mixed

suspensions. Following infiltration, *N. benthamiana* plants were kept under the same growth conditions as previously described.

For fluorescence measurements, leaves were harvested three days post-infiltration. For each combination, two leaves per plant and three plants per construct were used, for a total of 36 leaf disks collected per condition. The leaf disks were floated on 300 µL of water in 96 well microtiter plates and GFP (Ex.λ = 488 nm, Em.λ = 520 nm), RFP (Ex.λ = 532 nm, Em.λ = 580 nm), and BFP (Ex.λ = 381, Em.λ = 445) fluorescence measured using a Synergy 4 microplate reader (Bio-tek).

To ensure precise normalization of reporter activity, we employed a BFP-based normalization. For each coinfiltration mix, we divided the GFP signal by the BFP fluorescence to account for any variability in transgene expression levels. This approach enabled us to accurately compare the activity of the pmARR12 library across constructs by correcting for differences in TF expression levels.

Lateral Root Assay

Surface-sterilized T2 seeds were plated on 1.5% agar half-strength Murashige and Skoog (MS) medium containing 35 mg/L hygromycin, adjusted to a pH of 5.7. Plates were sealed with micropore tape and were placed vertically in a Percival growth chamber set at 24 °C with constant light. Seedlings were grown under these conditions for 17 days prior to data acquisition to allow adequate initiation and development of lateral roots.

For the cytokinin insensitivity assays, T2 seedlings of one repressor line (pmARR12-3xSRDX) were initially grown on MS plates under previously described conditions. WT seedlings (Col-0) were cultivated on MS plates without antibiotic selection. Both lines were allowed to germinate under these conditions for 4 days. After successful germination, seedlings were transferred to either MS plates supplemented with 5 µM 6-benzylaminopurine (6-BA) or control MS plates with no antibiotic selection, maintaining the same growth conditions. Following an additional 13 days, seedlings were imaged for subsequent analysis.

Whole plant Imaging & Lateral Root characterization

To visualize and quantity emerged lateral roots, plates and plants were imaged using an Epson Perfection V600 photo scanner to capture high-resolution images. Lateral roots were identified and counted under the conditions that they were clearly visible at the macroscopic level. Measurements of primary root length and lateral root number were conducted using ImageJ software (version 1.53k) to ensure precise analysis and reproducibility.

Fluorescence Microscopy

17 day old T2 seedlings of *proGATA23*::BFP:pmARR12:3xSRDX transgenic lines were grown as described in Materials and Methods: Lateral Root Assay. The seedlings were fixed in 4% paraformaldehyde and cleared for 2 days using ClearSee following a previously published protocol[46]. Cell walls were stained in a 0.1% Direct Red 23 ClearSee solution for 30 minutes

and washed in ClearSee before imaging. For BFP fluorescence 405 nm excitation and 420- 475 nm emission was used, which also captures cell wall autofluorescence. Direct Red 23 was imaged with 560 nm excitation and 580 - 620 nm emission.

Acknowledgements

We thank J. Dalton for the stellar maintenance and management of our growth room. Figure cartoon representations were sourced from BioRender.com. This work was part of the JBEI (https://www.jbei.org) supported by the US Department of Energy (DOE), Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the DOE.

Competing Interests

Author has no competing interests.

Author Contributions

RR, SA, and PMS conceptualized the research. RR, SA, MS, and KM contributed biological research materials. KM contributed preliminary unpublished data. RR performed all experiments except for microscopy which was performed by SA. RR and SA wrote the code to analyze the data. RR analyzed all the data. RR, SA, and PMS wrote the manuscript. PMS provided all research funding.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Figures:

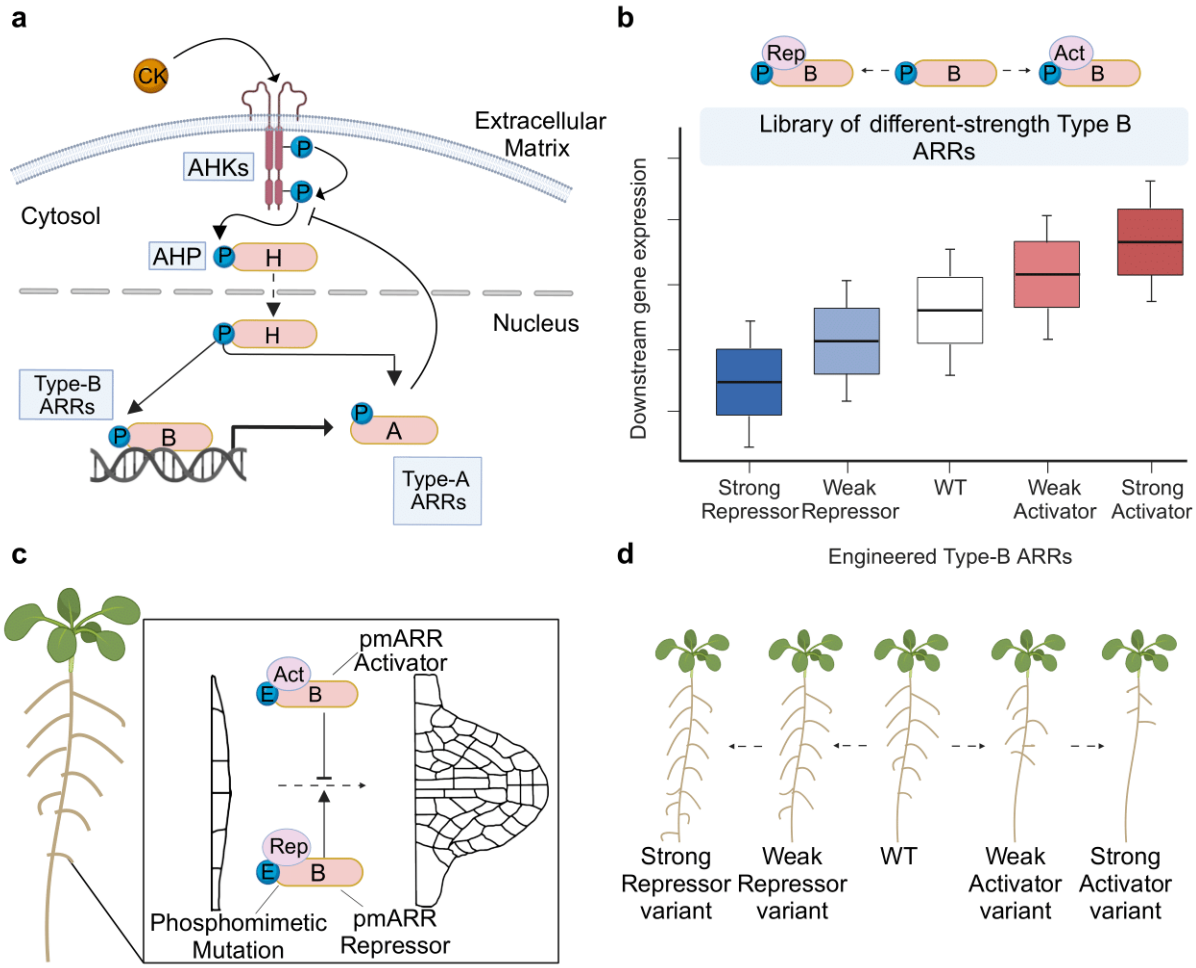
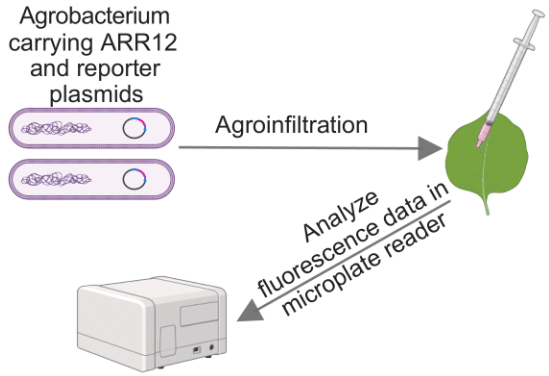


Fig. 1: Synthetic biology approach to modulate plant development using cytokinin signaling machinery. a Diagram of the canonical cytokinin signaling transduction pathway. Cytokinin perception by AHK receptors at the plasma membrane triggers a phosphotransfer cascade, activating Type-B ARRs to modulate transcription. **b** Hypothetical schematic of the characterization process for Type-B ARRs of varying regulatory strength. Regulatory strength is quantified by measuring downstream gene expression and achieved by fusing diverse activation and repression domains to a Type-B ARR. **c** Figure showing the predicted effects of repressor and activator phosphomimetic Type-B ARRs on lateral root initiation, where an activator phosphomimetic ARR acts as a negative regulator and repressor phosphomimetic ARR as a positive regulator of lateral root development. **d** Image illustrating the expected outcomes of deploying an engineered phosphomimetic ARR library to root cells in *A. thaliana*, with the lateral root phenotype scaling in accordance with ARR strength.

a

Fusion	Origin	Size (AA)	Characterized Function
3xSRDX	Synthetic EAR Motif	44	Strong repressor
KMTR141	Synthetic EAR Motif	7	Weak repressor
KMTR182	Synthetic EAR Motif	8	Strong repressor
KMTR151	Synthetic EAR Motif	6	Weak repressor
KMTR162	Synthetic EAR Motif	6	Weak repressor
EDLL	Plant TFs	15	Strong activator
PR	Human TFs	109	Strong activator

b



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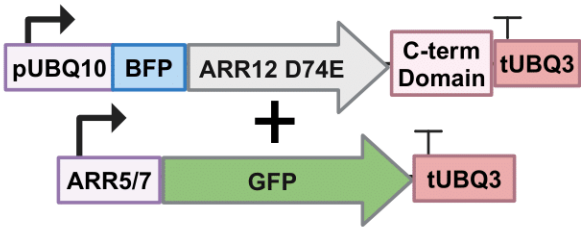

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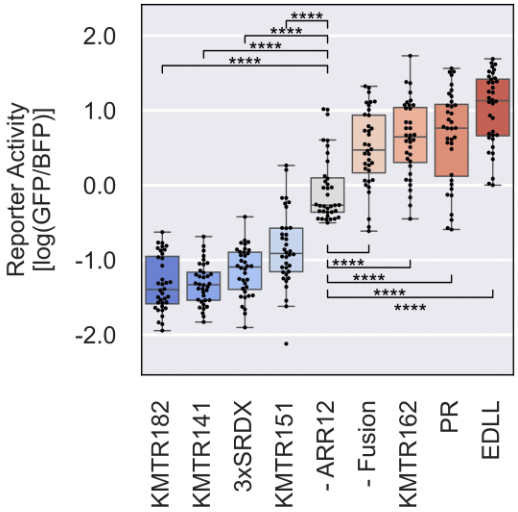

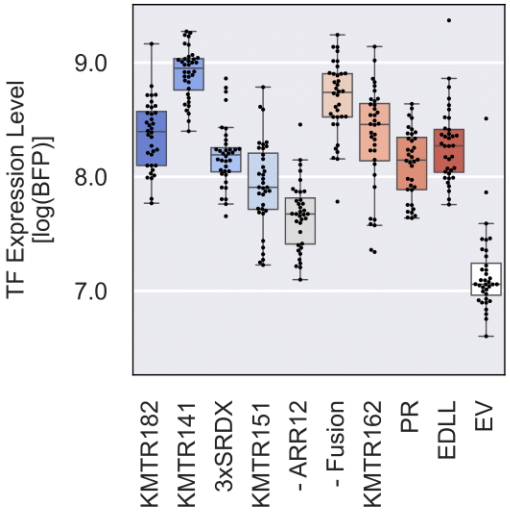

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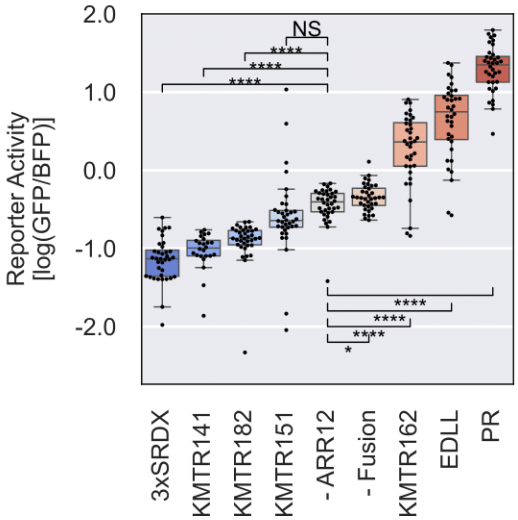

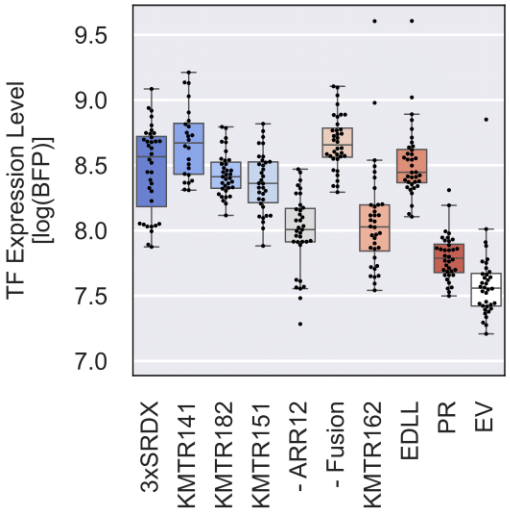

Fig. 2: Characterization of pmARR12 variants for a range of transcriptional strength. a Table detailing the origin, total amino acid size, and the previously established function of the various transcriptional domains used in this study. **b** Schematic depicting the *Agrobacterium*-mediated transgene assay used to characterize engineered Type-B ARRs. The assay involves the coinfiltration into *N. benthamiana* of two *Agrobacterium* strains carrying either a reporter or an effector plasmid, followed by fluorescence measurement using a microplate reader. **c** Figure illustrating our two-strain coinfiltration system, with one strain expressing BFP-pmARR12 under the UBQ10 promoter, and the other strain carrying a GFP reporter under the ARR5 or ARR7 promoters. **d,e** Box plot (left) depicts the measured GFP output in *N.□benthamiana* leaf discs for each BFP-ARR12D74E fusion variant (n□=□36 leaf disks), which normalized values generated as the GFP/BFP ratio for both the ARR5 **(d)** and ARR7 **(e)** reporters. Box plot (right) depicts the corresponding BFP signal for each pmARR12 variant, with empty vector (EV) as a control in white. Boxplots depicted in blue are functional repressors, and plots shaded in red are functional activators. For statistical significance, we ran a Kruskal-Wallis test, where each variant was compared to a -ARR12 control. A significance threshold of P□<□0.05 was applied, where statistical significance was defined as follows: NS (not significant, P□>□0.05), *P□<□0.05, **P□<□0.01, ***P□<□0.001, and ****P□<□0.0001.

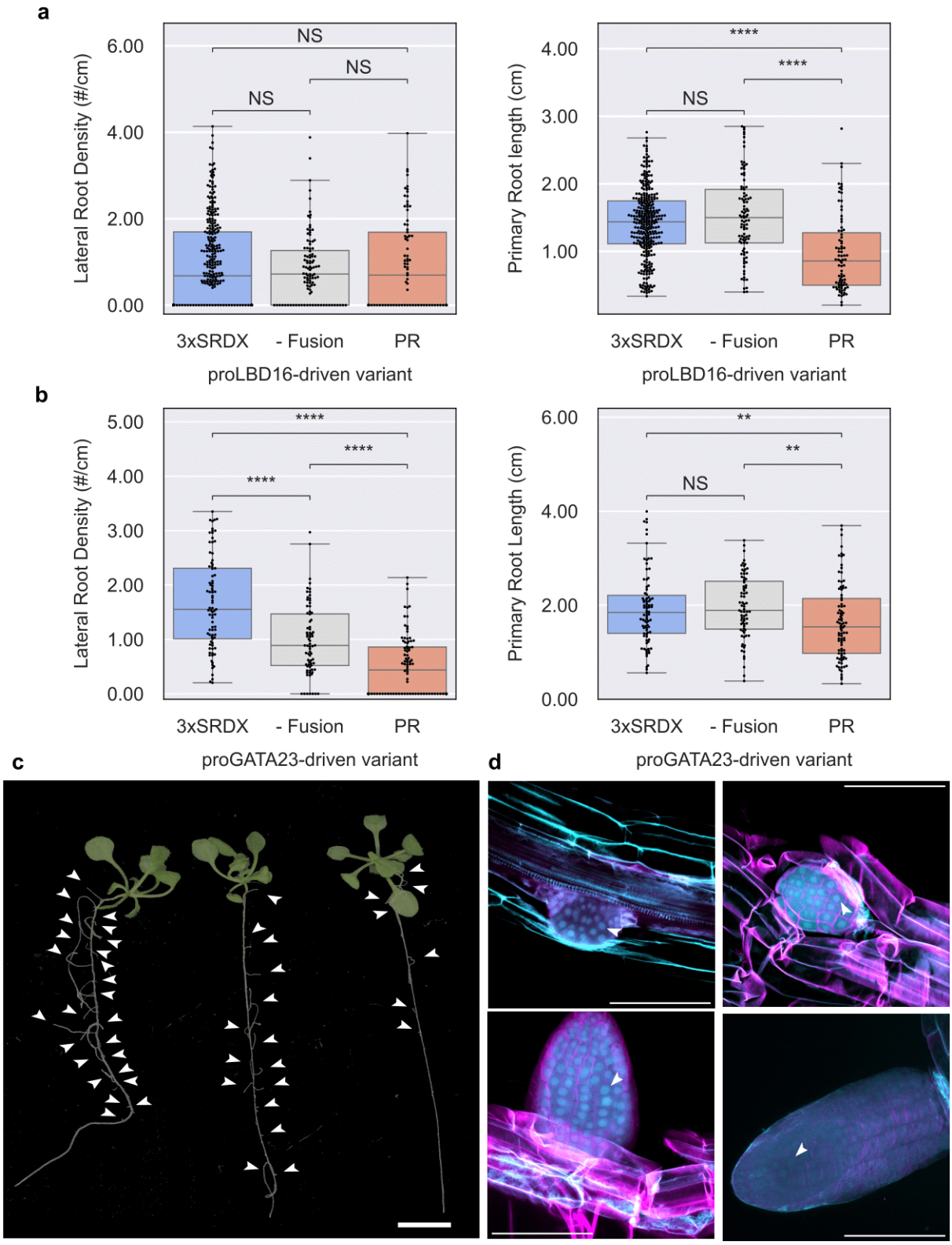

Fig. 3: Bidirectional and predictable modulation of lateral root density by cell-type specific expression of pmARR12 variants. a,b Box plot (left) depicts the lateral root density

(lateral root count / primary root length) of T2 transgenic *A. thaliana* lines with either a repressor, activator, or no fusion pmARR12 variant driven by the root-specific promoters *proLBD16* (**a**) or *proGATA23* (**b**). Box plot (right) depicts the primary root length of the same set of constructs and lines. For statistical significance, we ran a Kruskal-Wallis test, comparing each distinct plant genotype to one another. A significance threshold of P□<□0.05 was applied, where statistical significance was defined as follows: NS (not significant, P□>□0.05), *P□<□0.05, **P□<□0.01, ***P□<□0.001, and ****P□<□0.0001. **c** Image showing the root structure of *A. thaliana* plants engineered to modify root branch density, with a pmARR12-PR activator line (left), a pmARR12 line (middle), and a pmARR12-3xSRDX repressor line (right). Arrowheads indicate visible emerged lateral roots. Scale bar = 1 cm. **d** Maximum intensity projections of confocal fluorescence image stacks of lateral roots at different developmental stages expressing *proGATA23*::BFP:pmARR12:3xSRDX stained with the cell wall dye Direct Red 23. The cyan channel corresponds to BFP fluorescence and cell wall autofluorescence. Magenta corresponds to stained cell walls. Arrowheads indicate lateral root nuclei containing nuclear-localized BFP. Images were obtained from separate individual T2 seedlings derived from one T1 line. Scale bar = 100 µm.

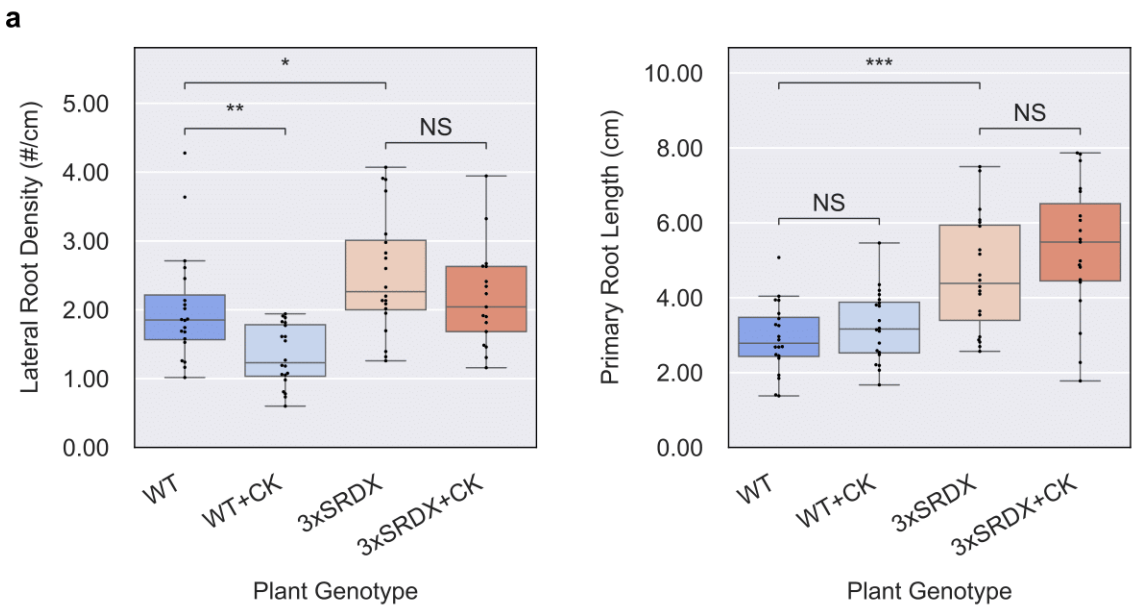

Fig. 4: PmARR12 variant exhibits cytokinin insensitivity. Left: Box plot showing the lateral root density (lateral root count / primary root length) of Arabidopsis seedlings grown with or without cytokinin. Col-0 WT plants have a significantly reduced lateral root density in the presence of cytokinin but the pmARR12-3xSRDX repressor line does not. Right: Box plots showing the primary root length of the plants displayed on the left. T2 descendants from a single T1 transformant were used for pmARR12-3xSRDX. To assess statistical significance, we performed a Kruskal-Wallis test comparing the +/- cytokinin conditions within each group and between WT and pmARR12-3xSRDX under - cytokinin conditions. A significance threshold of P□<□0.05 was applied, where statistical significance was defined as follows: NS (not significant, P□>□0.05), *P□<□0.05, **P□<□0.01, ***P□<□0.001, and ****P□<□0.0001.

Supporting Information for

Predictable modulation of plant root development using engineered cytokinin regulators

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This PDF file includes:

Figures S1, S2.

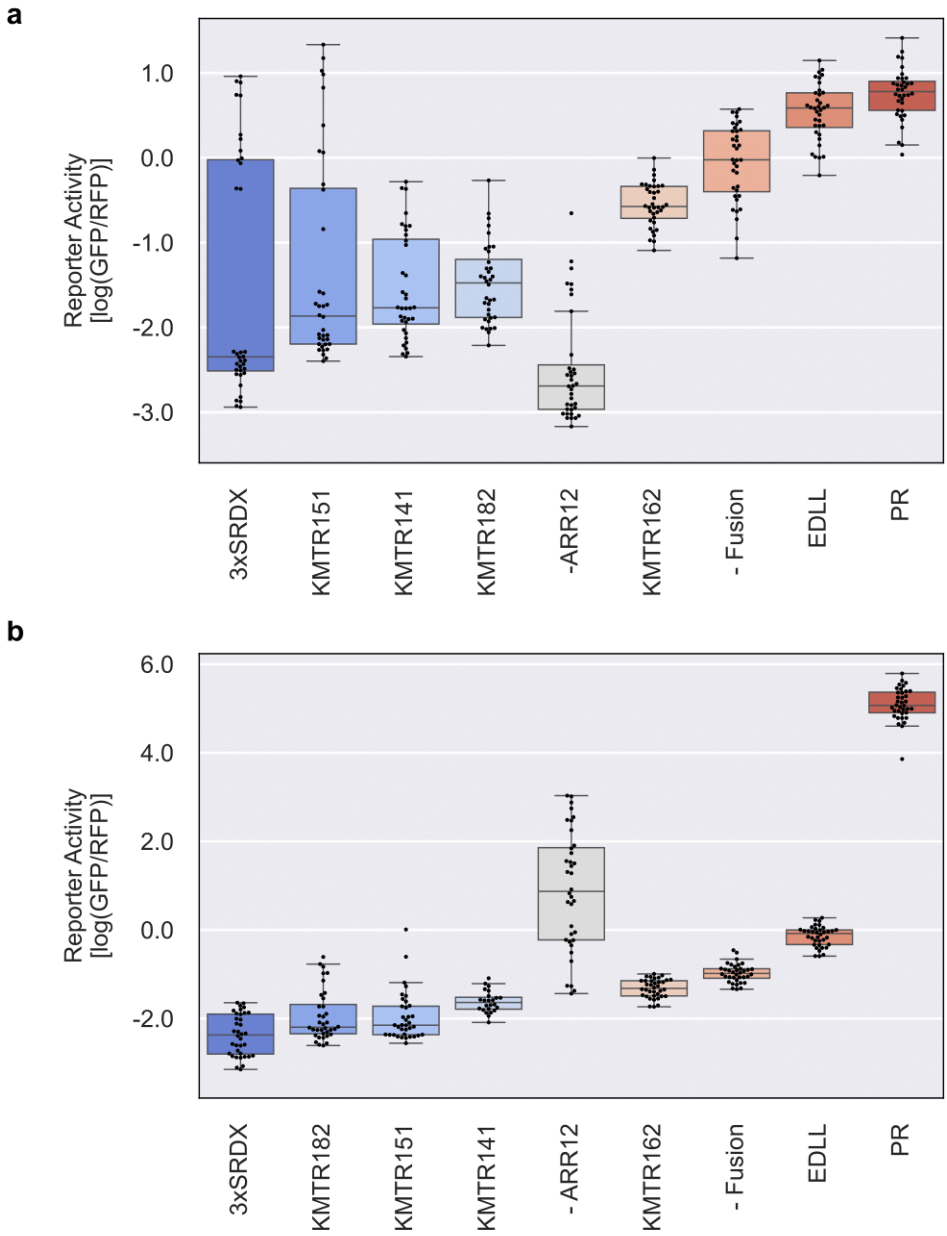

Figure S1: Characterization of pmARR12 variants for a range of transcriptional strength. a,b Box plot (top) depicts the fluorescence of *N.benthamiana* leaf discs normalized by the RFP fluorescence for each BFP-ARR12D74E fusion variant (n=36), which normalized values generated as the GFP/RFP ratio for both the ARR5 (**a**) and ARR7 (**b**) reporters. Boxplots depicted in blue are functional repressors, and plots shaded in red are functional activators.

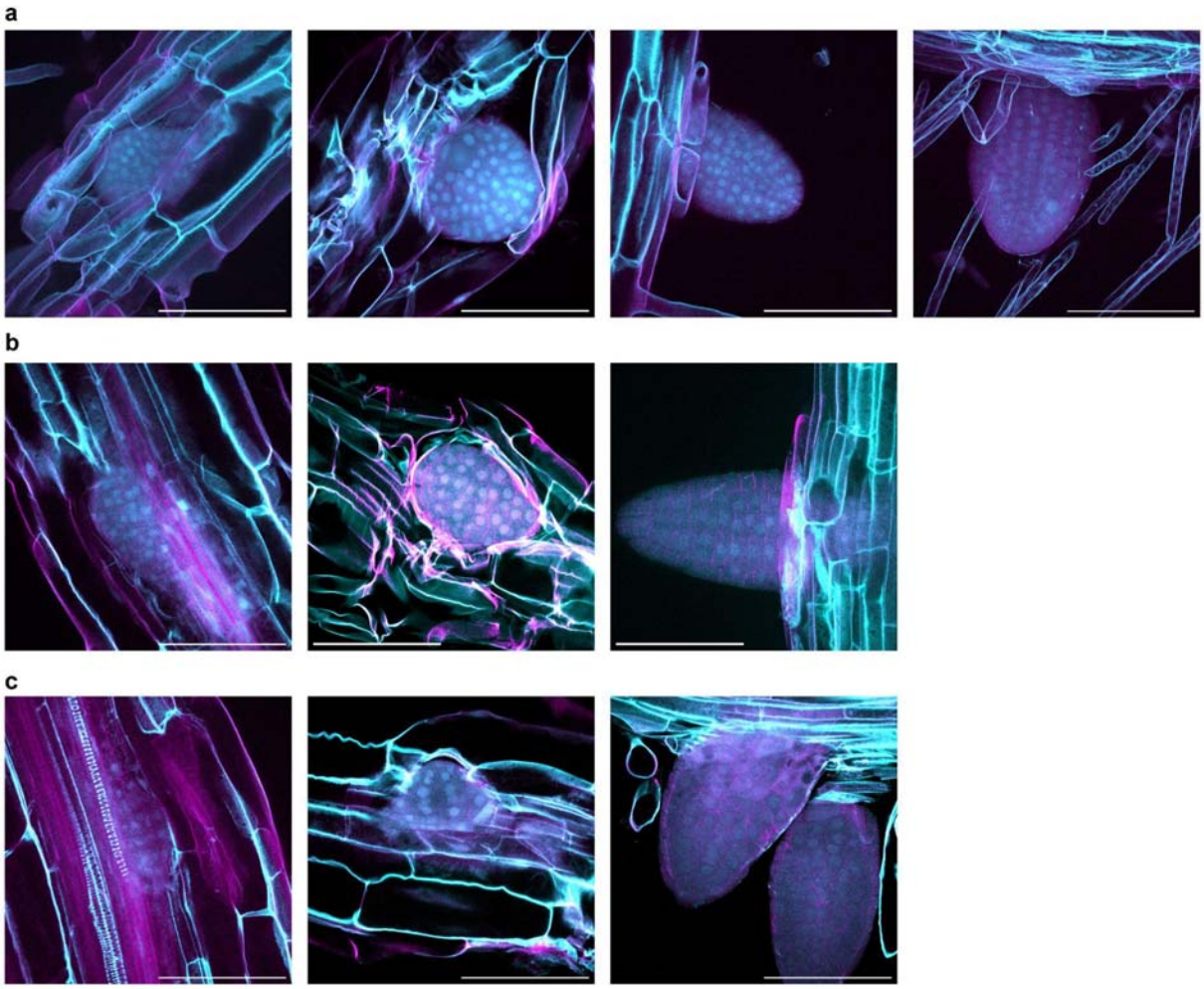

Figure S2: Localization of BFP-pmARR12-3xSRDX driven by the *proGATA23* promoter in Arabidopsis seedlings. Maximum projection confocal microscopy fluorescence images of *proGATA23*::BFP:pmARR12:3xSRDX. Cyan corresponds to BFP and cell wall autofluorescence. Magenta corresponds to the cell wall dye Direct Red 23. Figures a ,b, and c each shows details from T2 seedlings derived from different T1 lines. Scale bar = 100 µm.

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