Autoregulation of *RCO* by Low-Affinity Binding Modulates Cytokinin Action and Shapes Leaf Diversity

**Graphical Abstract**

**Highlights**
- Identification of genome-wide target genes for the *RCO* transcription factor
- *RCO* delimits its own expression through autorepression by low-affinity binding
- *RCO* represses local leaf growth via regulating multiple cytokinin (CK)-related genes
- *RCO* negative autorepression fine-tunes CK activity and regulates leaf shape

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**In Brief**
Hajheidari et al. identify target genes for the *RCO* homeodomain protein that drove leaf shape diversity. They show that *RCO* regulates growth via orchestrating homeostasis for the hormone cytokinin and that it also represses its own transcription via low-affinity binding sites. This autorepression helps delimit *RCO* expression and shape leaf form.

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Autoregulation of RCO by Low-Affinity Binding Modulates Cytokinin Action and Shapes Leaf Diversity

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SUMMARY

Mechanisms through which the evolution of gene regulation causes morphological diversity are largely unclear. The tremendous shape variation among plant leaves offers attractive opportunities to address this question. In cruciferous plants, the REDUCED COMPLEXITY (RCO) homeodomain protein evolved via gene duplication and acquired a novel expression domain that contributed to leaf shape diversity. However, the molecular pathways through which RCO regulates leaf growth are unknown. A key question is to identify genome-wide transcriptional targets of RCO and the DNA sequences to which RCO binds. We investigate this question using Cardamine hirsuta, which has complex leaves, and its relative Arabidopsis thaliana, which evolved simple leaves through loss of RCO. We demonstrate that RCO directly regulates genes controlling homeostasis of the hormone cytokinin to repress growth at the leaf base. Elevating cytokinin signaling in the RCO expression domain is sufficient to both transform A. thaliana simple leaves into complex simple leaves through loss of RCO. We also identify RCO as its own target gene. RCO directly represses its own transcription via an array of low-affinity binding sites, which evolved after RCO duplicated from its progenitor sequence. This autorepression is required to limit RCO expression. Thus, evolution of low-affinity binding sites created a negative autoregulatory loop that facilitated leaf shape evolution by defining RCO expression and fine-tuning cytokinin activity. In summary, we identify a transcriptional mechanism through which conflicts between novelty and pleiotropy are resolved during evolution and lead to morphological differences between species.

INTRODUCTION

cis-regulatory variation of developmental genes plays a pivotal role in morphological evolution of plants and animals and often involves diversification of transcriptional enhancers [1–8]. Regulatory sequence variation is believed to facilitate morphological change while minimizing the potentially adverse effects of pleiotropy—the phenomenon by which a single gene influences multiple aspects of development [1, 9, 10]. However, the precise mechanisms that link cis-regulatory changes to morphological diversity remain poorly understood [11, 12]. For example, do cis-regulatory changes at transcription factor loci cause specific effects on downstream gene expression or global transcriptome remodeling? Do these transcriptional changes affect few genes with large effects on development or a multitude of downstream processes with small effect? And how do cis-regulatory changes circumvent pleiotropy, given that transcriptional enhancers can show considerable pleiotropy despite their modularity [13]?
Recent work indicates that low-affinity transcription factor binding sites play a vital role in fine-tuning developmental gene expression, thus ensuring its specificity and robustness [14]. These sites can evolve rapidly and therefore might also have important yet undiscovered roles in morphological evolution [15].

Plant leaves provide a powerful model in which to explore such questions on the mechanistic basis of evolutionary change because they show substantial, heritable morphological variation at different evolutionary scales and are ecophysiologically important because they fix CO₂ in terrestrial ecosystems [16, 17]. Considerable insights into the genetic basis for diversification of leaf shape have come from comparative studies of simple leaves of the reference plant A. thaliana versus complex leaves of its relative Cardamine hirsuta, where leaves are divided into distinct units called leaflets. The RCO gene was discovered in C. hirsuta on the basis of its simplified mutant phenotype, and RCO-type genes support development of leaf marginal outgrowths of different sizes in different crucifer species. RCO arose by duplication of its ancestral paralog LATE MERISTEM IDENTITY 1 (LMI1), which is conserved in seed plants, and its emergence promoted evolution of leaf complexity in crucifers [18]. Neofunctionalization of an RCO enhancer element (RCOnon) altered leaf shape by changing RCO expression from the distal leaf blade to the leaf base [19]. In this domain, RCO represses growth in a series of foci along the leaf margin, allowing the outgrowth of lobes or leaflets between flanking regions of RCO expression [20]. RCO was secondarily lost from the A. thaliana genome, leading to leaf simplification. However, its reintroduction in A. thaliana as a transgene was sufficient to increase leaf complexity, demonstrating that RCO is a large-effect gene underlying morphological evolution. However, the downstream effector genes through which RCO acts to repress growth and the upstream transcriptional inputs that delimit RCO expression are both unknown.

To address these issues, we identified genome-wide RCO target genes by combining chromatin immunoprecipitation sequencing (ChIP-seq) and RNA sequencing (RNA-seq) experiments. We further conducted functional validation of our findings using genetics, molecular biology, microscopy, and hormone measurements. In this way, we show that RCO coordinates homeostasis of the hormone cytokinin (CK) through direct regulation of multiple genes involved in CK biosynthesis and catabolism and provide evidence that this RCO/CK module is required for complex leaf development. In parallel, we show that RCO directly delimits its own expression through binding to clusters of low-affinity repressive sites in its 5' upstream regulatory region and gene body and that this autoregulatory loop also shapes CK activity in the leaf. Thus, a paradigm emerges whereby low-affinity binding sites facilitated morphological evolution by dampening the effects of cis-regulatory divergence in a potent transcription factor. We propose that this regulatory architecture allowed regulatory evolution to fine-tune levels of hormonal homeostasis and circumvent pleiotropy.

RESULTS AND DISCUSSION

**Genome-wide Identification of Molecular Targets of RCO Reveals It Is Subject to Negative Autoregulation**

To understand the molecular basis of RCO’s action in C. hirsuta, we performed ChIP-seq profiling in three biological replicates of C. hirsuta rco mutant plants expressing pRCO::RCOgenomicVENUS using an anti-GFP antibody (see STAR Methods for details). We identified 598 binding peaks for 592 potential target genes, which showed a consistent binding pattern across all biological replicates (Data S1A). RCO is among the top 10 potential target genes of RCO, according to ranking of peaks by p value, and RCO associates with chromatin at its own promoter and gene body in a single broad peak (Figures 1A and S1; Data S1A). By contrast, a single narrow peak was detected at the 3’ end of LMI1—the ancestral paralog of RCO (Figures 1A and S1; Data S1A). To test whether RCO regulates its own expression, we compared RCO expression in transgenic rco mutant lines complemented with either the native version of RCO or the stabilized form of RCO (RCOA48D) [19]. In the RCOA48D lines where protein levels are elevated relative to native RCO [19], we found reduced RCO transcript levels compared to rco mutant lines expressing the native RCO gene.

**Figure 1. Genome-wide Analysis of RCO-Binding Peaks in C. hirsuta Reveals RCO Autoregulation**

(A) RCO ChIP-seq profile (blue) along the RCO locus in three biological replicates. Numbers on the y axis represent read coverage. Brown-filled rectangles indicate the peak regions of RCO binding from MACS2 [21]. The asterisk indicates the un-mapped gap regions.

(B) qRT-PCR comparing RCO and RCOA48D transcript levels in seedlings. Black dots depict the mean of three biological replicates of qRT-PCR, each in three independent lines expressing RCO-3HA and RCOA48D-3HA. Blue and orange dots represent individual data points. Error bars represent ±1 SD.

(C and C’) Light microscopy images of GUS staining in leaves showing the expression of pRCO::GUS in C. hirsuta wild-type (WT) (Oxford; C) and rco mutant (C’) carrying a single copy of the pRCO::GUS transgene. Note an increased GUS staining signal in the leaves of rco mutants relative to WT plants. Scale bars, 500 μm (C and C’).

See also Figure S1 and Data S1A.
This indicates that RCO might undergo negative autoregulation, an idea further supported by the observation that a single-copy RCO::GUS reporter gene showed broader and stronger expression in rco leaves compared to wild type (Figures 1C and 1C). Together, these results suggest that RCO represses its own expression.

**Negative Autoregulation of RCO Requires Low-Affinity Binding Sites to Delimit RCO Expression and Regulate Leaf Shape**

If RCO binds to the RCO locus to negatively regulate its own expression, then deletion or randomization of these binding sites should increase RCO expression. To test this prediction, we selected 4 binding candidate fragments (BCFs); three fragments with the highest read coverage in the promoter region and the first intron and another fragment covering the whole second intron (Figure S2A). We engineered RCO promoter fragments in which the BCF1 and BCF2 were deleted, randomized, or replaced by the corresponding LMI1 sequence, where RCO is not expected to bind. Each modified RCO promoter was fused to both RCO-coding sequence (RCO-CDS) lacking BCF3 and BCF4 and the genomic sequence (including BCF3 and BCF4), including introns that harbor RCO-binding sites (RCOgenomic), from the ATG-start to the stop codon (Figures S2A and S2G). All constructs, including controls bearing the native promoter sequence, were transformed into the rco mutant background. We observed that removing peaks or modifying them, as described above, increased RCO expression (Figure S2C). In rco mutant plants containing the modified RCO promoters fused to RCO-CDS, which caused higher RCO expression, leaf shape was significantly altered—leaf dissection increased while leaf area and seed mass decreased (Figures S2B–S2F). Notably, transgenic rco mutant plants that expressed RCOgenomic (ATG-stop) driven by modified RCO promoters produced either wild-type or partially rescued leaves (Figures S2G and S2H). This indicates that deleting or modifying BCFs 1 and 2 within the RCO promoter sequence is insufficient to alter leaf development if BCFs 3 and 4 are still present in the gene body. Nevertheless, these constructs still rescued the rco mutant phenotype more effectively than the control construct (Figures S2G and S2H). In summary, RCO autorepression depends on multiple sites in its genomic locus and perturbing this autorepression has detrimental effects on leaf development and plant performance.

We next sought to understand the cis-regulatory logic that underlies RCO autorepression. We first used protein-binding microarrays (PBMs) to identify the DNA-binding motifs that RCO binds to with high affinity in vitro (Figure S3A; Data S1B). We then constructed position weight matrices (PWMs) using these sequences, which we refer to as canonical binding sites [22]. However, when we scanned the peak sequences at the RCO locus with the PBM-derived PWMs using FIMO (default settings), we did not find canonical RCO-binding sites [23, 24]. Yet we found canonical sites in 64 of the genome-wide identified RCO-binding peaks. These observations suggest that RCO-DNA binding may be influenced by protein interactions and/or the chromatin context, as is the case for other transcription factors [25–27]. To identify RCO-binding sites within the RCO locus, we used electrophoretic mobility shift assays (EMSAs) with native and mutantized oligonucleotides to screen the four BCFs selected previously. In this way, we identified 11 RCO-binding sites residing on nine 55-bp fragments (Figure 2). These sites are AT-rich, a feature shared with both the canonical binding sites identified in the PBM and a motif enriched in RCO-bound ChiP-seq peaks (Figures 2A, 2B, S3B, and S3C; Data S1B). These 11 RCO-binding sites in the RCO locus showed concentration-dependent binding to RCO (Figures 2A and 2B) with 2 of the 11 sites located within RCOenh500 (Figures 2A and S4A). All the 11 binding sites show considerable conservation in crucifers (Figure S4B). Statistical analysis (STAR Methods) indicated that evolution of an array of low-affinity, but not high-affinity, binding sites in RCO is unlikely to reflect chance ($P_{\text{null}}(X = 0) = 0.024$, where $P_{\text{null}}$ is the probability of observing X high-affinity binding sites under a neutral evolutionary model for the RCO/LMI1 duplication). In turn, this suggests that selective processes likely prevented accumulation of high-affinity binding sites in RCO while permitting emergence of low-affinity ones. Overall, these findings suggest that RCOenh500 co-evolved with an array of low-affinity RCO-binding sites to provide specificity to RCO expression via negative autoregulation.

To test this hypothesis and understand the mechanism of action of these native RCO-binding sites, we compared their binding affinity to that of the canonical RCO binding sites defined by PBMs. We did this by performing EMSAs with native oligonucleotide sequences and oligos containing the canonical binding site (CAATAATT). Oligos with the native sites were outcompeted and failed to bind to RCO in the presence of an equal amount of oligos containing the canonical binding sites, indicating that native sites bind to RCO with a lower affinity than does the canonical RCO-binding site (Figures 2C and S3D). We thus hypothesized that these low-affinity native binding sites may play a crucial role in shaping the distinctive expression pattern of RCO around emerging leaflets. To test this idea, we made an RCOg construct that contains the high-affinity canonical binding sites in place of the native sites and introduced it into the rco mutant. This construct could not rescue the rco mutant phenotype, likely due to low levels of RCO expression reflecting autorepression (Figure S3E). To assay the functionality of low-affinity RCO-binding sites in vivo, we engineered an RCO promoter fragment in which the low-affinity binding sites were mutated (termed pmutRCO). This mutated promoter was then used to drive the expression of RCOgenomic-VENUS (pmutRCO::RCOg-VENUS) and RCOcds-VENUS (pmutRCO::R- COcds-VENUS) (Figure 3A). The resulting constructs and the control constructs comprising the native, non-mutagenized RCO promoter fused to RCOgenomic-VENUS (pRCO::RCOg-VENUS) and RCOcds-VENUS (pRCO::RCOcds-VENUS) were transformed into the rco mutant. In transgenic plants expressing pmutRCO::RCOcds-VENUS (no RCO-binding sites), RCO-VENUS levels and leaf complexity were significantly increased, relative to transgenic plants that contain either the native construct pRCO::RCOg-VENUS, which has all the RCO-binding sites, or the pmutRCO::RCOg-VENUS construct, which also includes the introns and consequently additional RCO-binding sites (Figures 3B–3E). In addition, ectopic RCO expression was detectable at the boundaries of terminal leaflets in pmutRCO::R- COcds-VENUS-expressing transgenic plants (white arrow in Figure 3E). RCO expression was also higher in rco mutant lines expressing pmutRCO::RCOg-VENUS than in rco mutant plants.
Figure 2. RCO Regulates Its Own Expression through Binding to Clusters of Low-Affinity Binding Sites

(A and B) Schematic representation of the RCO promoter features (A) and RCO gene body features (B) showing introns (black lines) and exon (green boxes) regions. The labeled bars under the schematics represent the oligos with corresponding DNA sequences that were tested for their ability to bind with the RCO protein in EMSA. Numbered white bars (1–5, 7–10, 12–14, 16–18, 20, 22–26, 28 to 29, 30, and 34) represent oligos to which RCO did not bind (RCO non-shifted, EMSA gel images). Labeled gray bars (A1 to I9) represent oligos bound by RCO (RCO shifted, EMSA gel images). The small blue and red boxes on the bars represent the mutagenesis of 2 to 3 T nucleotides to G nucleotides in the AT-rich clusters within the putative RCO binding sites (blue) or outside the putative RCO binding sites (red; see Table S3 for oligos). The mutations within the putative RCO-binding sites could suppress the RCO binding (no shifts in the gel, blue arrowheads), but mutations outside the putative RCO-binding sites did not suppress RCO binding (red arrowheads). Note that, for oligo position H8, there were three putative RCO-binding sites and binding was inhibited only upon mutagenizing all the three sites (asterisks and blue arrowhead in B). The thickness of turquoise bars above each gel image represents RCO protein concentration in the increments of 20 (minimum thickness), 80, and 160 ng (maximum thickness).

(C) EMSA showing the low-affinity binding behavior of RCO using the binding site B2 as an example (also shown in Figure S3). Top: schematic representation of pRCO::RCOg construct; promoter (orange line), exon (green boxes), intron (black line), and low-affinity RCO binding sites (black boxes). Middle: core sequences of the oligos used for EMSA with WT binding sequences (B2-WT; the same as B2 in A), mutated binding sequences (B2-MU; marked in magenta, the same as B2a in A), and binding sequences replaced with the canonical sequence (CAATAATT in B2-HA; marked in green). (C1) EMSA showing the binding of RCO to B2-WT and not to B2-MU. (C2 and C3) Competition test with B2-WT (C1) and B2-HA (C2) oligos. A constant amount of in vitro synthesized RCO protein and IRDye-800-labeled B2-WT oligos were mixed with unlabeled B2-WT or unlabeled B2-HA oligos. Note that 20x concentration of unlabeled B2-WT reduced the binding of labeled WT oligos, while 1x concentration of unlabeled B2-HA oligos was sufficient to reduce the binding of labeled WT oligos (compare lanes marked by black arrowheads in C2 and C3).

See also Figures S2–S4 and Data S1B.
expressing the pRCO::RCOg-VENUS construct (compare RCO-VENUS fluorescence in [1] and [2] in Figures 3D and 3E). However, we did not observe a significant difference in their leaf complexity (compare [1] and [2] in Figures 3B and 3C). Our results demonstrate that low-affinity RCO-binding sites act redundantly with each other to shape leaf development by defining the correct RCO gene expression pattern and dose, thereby preventing pleiotropic RCO effects.

**RCO Directly Regulates Genes Involved in CK Biosynthesis, Catabolism, and Signaling**

Our findings suggest that a cluster of low-affinity repressive binding sites evolved in concert with regulatory neofunctionalization of RCO to delimit its expression. However, it is not known through which target genes RCO represses growth to generate complex leaves and how RCO autorepression influences such RCO-dependent growth regulation. To help identify RCO target genes, we used RNA-seq in combination with hierarchical clustering to assay RCO-dependent gene expression 2–10 h after dexamethasone-induced RCO activation in the rco mutant at 2-h time intervals (Figures S5 A and S5B). Several Gene Ontology (GO) terms were enriched in RCO-responsive genes that relate to response to stimulus and hormones, including cytokinin (CK), and to hormone-mediated signaling (Data S1C–S1E). For example, RCO induction was accompanied by increased expression of LONELY GUY 7.
Taken together, these results suggest that RCO might enhance cytokinin biosynthesis and/or signaling. To test this hypothesis, we measured endogenous CK levels in wild-type C. hirsuta, the rco mutant, wild-type A. thaliana, ChRCO-g-expressing A. thaliana, and in transgenic C. hirsuta rco mutant and A. thaliana expressing DEX-inducible RCO (pRPS5a::RCO). The presence or induction of the RCO gene increased endogenous CK levels (Figures 4B and S6A). We also used the CK-response markers TCSn::GFP (TCS) [30] to examine CK signaling activity in wild-type C. hirsuta, the rco mutant, ChRCO-g-expressing A. thaliana, and wild-type A. thaliana, which lacks the RCO gene (Figures 4C–4F). In agreement with the above results, we found that presence of the RCO gene leads to increased TCS expression in leaves, especially adjacent to leaflet primordia (C. hirsuta) or serrations (A. thaliana), indicating higher CK activity. We also found that higher RCO expression levels, caused by mutation of RCO-binding sites, resulted in increased LOG7, AHP4, and ARR5 expression (Figure S6B). Together, these results indicate that RCO promotes CK activity and that RCO autorepression attenuates this function.

Genetically Elevating CK Activity in the RCO Domain Is Sufficient to Promote Leaf Complexity and Partially Bypass the Requirement for RCO in Leaf Development

Based on these results, we hypothesized that RCO increased leaf complexity by stimulating CK activity at the flanks of developing leaflets, thus contributing to their separation. If RCO is
required for local CK activity, and if the reduced complexity of leaves in the rco mutant reflects decreased CK activity, we should be able to partially bypass the requirement of RCO for leaflet separation by activating CK signaling in the RCO domain. To test this idea, we enhanced CK signaling by expressing ARR1DDK, a constitutively active form of the type-B cytokinin response regulator ARR1 [31], from the RCO promoter in the rco mutant. Consistent with our hypothesis, this transgene suppressed the rco mutant leaf phenotype, making it more similar, although not identical, to wild type (Figures 4G and 4H). In addition, the expression of pRCO::ARR1DDK in A. thaliana converted simple A. thaliana leaves into complex ones, mimicking the effect of expressing RCOg in A. thaliana (Figures 5A–5C) and indicating that locally elevated CK signaling in the RCO domain is sufficient to dramatically increase complexity in A. thaliana leaves.

We next investigated whether similar cell-level effects underlie the increased leaf complexity caused by elevating either CK signaling or expressing RCO in the RCO domain in A. thaliana leaves, which should be the case if CK mediates RCO function (Figures 5 and 6). Consistent with this idea, we observed that both cell area and cell lobeyness—a differentiation measure—are reduced to a similar level relative to wild-type in pRCO::ARR1DDK and pRCO::RCOg-VENUS expressing plants (Figures 6A–6D). These observations indicate that the repressive effect of RCO and CK on local leaf growth involves

Figure 5. Increased CK Signaling Mimics the Action of RCO in Promoting Arabidopsis Leaf Complexity
Rosette leaves of A. thaliana WT plants (A), Arabidopsis plants expressing pRCO::ARR1DDK (J) (n = 10 T1 lines; B), and pRCO::RCOg-VENUS (C). Note the increased complexity in the leaves of Arabidopsis plants expressing pRCO::ARR1DDK, resembling the effects of RCO (compare B and C). Scale bars, 1 cm (A–C).

Figure 6. Increased CK Signaling in the RCO Domain Reduces Cell Size and Delays Cell Differentiation
(A) Heatmaps of quantification of cell area in the sinuses of Arabidopsis WT plants and Arabidopsis plants transformed with pRCO::ARR1DDK and pRCO::RCOg-VENUS.
(B) Heatmaps of quantification of cell lobeyness—a feature that leaf epidermal cells acquire when they differentiate—in the sinuses of Arabidopsis WT plants and Arabidopsis plants transformed with pRCO::ARR1DDK and pRCO::RCOg-VENUS. Scale bars, 100 μm (A and B).
(C and D) Quantifications of cell area (C) and cell lobeyness (D) in Arabidopsis plants transformed with pRCO::ARR1DDK and pRCO::RCOg-VENUS relative to WT plants (three biological replicates each). Note the average cell area and lobeyness in both transgenic lines are significantly reduced compared to WT. The measurements and statistical analysis were restricted to all cells with a neighborhood of 5 cells centered on a focal cell at the center of the sinuses in between the lobes (also see STAR Methods). For statistical validation, two independent nested ANOVAs with genotype as a fixed factor and replicates as nested random factor were used. Significance of all pairwise comparisons between genotypes was performed using Tukey’s all-pair comparison. Cell area: p < 1e−04 (WT versus pRCO::ARR1DDK); p < 1e−04 (WT versus pRCO::RCOg-VENUS). Cell lobeyness: p = 0.0279 (WT versus pRCO::ARR1DDK); p < 0.001 (WT versus pRCO::RCOg-VENUS).
(E) A conceptual diagram summarizing RCO function and its mechanism of action at the base of developing leaflets.
reduced cell size and is associated with slowing down differentiation. These findings are also in line with observations that increased cytokinin levels can cause a reduction in leaf cell size [32, 33]. In conclusion, RCO regulates leaf shape, at least in part, by reprogramming local CK homeostasis, thereby reducing cell growth (Figure 6E).

Finally, we asked whether the large effects of the RCO/CK module on leaf form are mirrored by large effects on the transcriptome that distinguishes A. thaliana and C. hirsuta leaves. An alternative would be that this module alters morphology via more restricted effects on this evolutionary divergent transcriptome. We found that RCO-responsive genes, including CK-related ones, are strongly overrepresented in the transcriptome that is differentially expressed between A. thaliana and C. hirsuta leaf primordia [34] (Data S1G and S1H), supporting the above “large effect on both transcriptome and morphology” hypothesis. Taken together, our findings suggest that the effect of RCO in leaf shape evolution likely reflects the integration of two opposing processes: the first creates the possibility for morphological change through re-shaping the developing leaf transcriptome and CK response, and the second limits the potentially pleiotropic effects of RCO by negative autoregulation via low-affinity binding sites. It is of note that this regulatory logic is in line with RCO acting both as an activator (e.g., of CK-related genes) and a repressor of its own transcription. This dual action may have put it in a favored position for contributing to evolutionary change by allowing fine-grained control of organ development. In the future, it will be interesting to explore the precise significance of RCO autoregulation for CK-dependent cell growth control. For example, from a theoretical perspective, negative autoregulation can reduce the effects of noise on genetic network readouts, so it is possible that this RCO/CK regulatory module allows tighter control of growth during leaf primordium development [35, 36]. CK is also important for leaf complexity in tomato [37], where leaflets evolved without a contribution of RCO [18]. It will thus be interesting to determine which genes fulfill a role similar to RCO to locally regulate CK in that system. It will also be important to understand how RCO effects on CK are integrated with those of KNOX proteins [16, 20]. These transcription factors also promote leaf complexity in a CK-related pathway that appears distinct from RCO [16, 38] as it has much broader effects on leaf primordium growth [20]. CK effects on leaf cell development are multifaceted and include modulation of the rate of cell proliferation, the timing of its cessation, as well as regulation of post-mitotic growth [33, 39]. Consequently, answering this question will require cell-level dissection of CK pathways at different developmental stages.

Conclusions

Previous studies demonstrated that low-affinity transcription factor binding sites play a crucial role in activating developmental genes, thereby ensuring developmental robustness and precise patterns of tissue development [14, 40, 41]. Here, using plant leaves and regulation of CK homeostasis as an example, we show that low-affinity repressive binding sites played a major role in the generation of morphological diversity. Specifically, our work indicates that these sites can help resolve conflicts between pleiotropy and novelty that emerge during evolution by dampening the effects of cis-regulatory changes that underlie novel gene expression patterns [1, 9, 15].

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.10.040.

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AUTHOR CONTRIBUTIONS

M.H. performed the majority of the experiments and analyses and prepared figures. M.H., F.V., Y.W., and M.T. designed experiments. F.V. and Y.W.
conducted CK-related gene expression analyses and verified data and strains. N.B. performed imaging and analysis for cellular morphology quantifications and verified strains. J.M.F.-Z. conducted PBM analyses, M.K. and K.L. conducted ChIP-seq, B.M., B.R.O., R.D.I., and A.W. contributed materials. R.A.M. contributed to RNA-seq. M.T. conducted imaging work and figure organization. M.T. designed and directed the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| GFP-Trap Magnetic Agarose affinity beads | Chromotek | Cat# gtma; RRID: AB_2631358 |
| IgG                 | Diagenode | Cat# C15410206; RRID: AB_2722554 |
| Rabbit Anti-Maltose Binding Protein antibody | abcam | Cat# ab9084; RRID: AB_306992 |
| goat anti-rabbit IgG DyLight 550 conjugated | Thermo Fisher Scientific | Cat# 84541; RRID: AB_10942173 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| MS basal salt mixture with vitamin | Duchefa | N/A |
| MES                 | Sigma | M2933 |
| BAP (6-benzylamino purine) | Sigma | B3274 |
| Silwet L77          | Obermeier | 7060-10 |
| SuperScript VILO    | Invitrogen | 11754050 |
| Isopropyl-β-d-thiogalactopyranoside (IPTG) | Roth | 2316 |
| Phenylmethylsulfonylfluoride (PMSF) | Sigma | P7626 |
| Benzamidine         | Roth | CN38 |
| Aprotinin           | Roth | A162 |
| Leupeptin           | Roth | CN33 |
| Glutathione-Sepharose 4B | Amersham Biosciences | 17075605 |
| HiTrap DEAE-Sepharose FF anion exchange column | GE Healthcare | 17505501 |
| BSA                 | Pharmacia | 27-8915-01 |
| Coomassie Blue      | Roth | 3862 |
| Denatured salmon sperm DNA (ssDNA) | Roth | 5434 |
| HEPES               | BIOMOL | 05288 |
| DTT                 | Carl Roth | 6908 |
| Poly(dI-dC)         | Thermo | 20148E |
| IRDye 800-labeled Dann | Metabion | N/A |
| Novex 6% DNA retardation gels | Invitrogen | EC6365BOX |
| 5-bromo-4-chloro-3-indoly-β-D-glucuronic acid (X-Gluc) | Roth | 0018 |
| Dexamethasone       | Sigma | D4902 |
| DMSO                | Roth | 4720 |
| Igepal              | Sigma | I3021 |
| Protease inhibitor cocktail | Sigma | P9599 |
| Polyvinylidene fluoride membranes | Millipore | N/A |
| Propidium Iodide (PI) | Sigma | 87-51-4 |
| MS basal salt mixture with vitamin | Duchefa | N/A |
| MES                 | Sigma | M2933 |
| BAP (6-benzylamino purine) | Sigma | B3274 |
| Silwet L77          | Obermeier | 7060-10 |
| SuperScript VILO    | Invitrogen | 11754050 |
| Isopropyl-β-d-thiogalactopyranoside (IPTG) | Roth | 2316 |
| Phenylmethylsulfonylfluoride (PMSF) | Sigma | P7626 |
| Benzamidine         | Roth | CN38 |
| Aprotinin           | Roth | A162 |
| Leupeptin           | Roth | CN33 |
| Propidium Iodide (PI) | Sigma-Aldrich | 87-51-4 |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources (plasmids and transgenic lines generated in this study, listed in the Key Resources Table) should be directed to and will be fulfilled by the Lead Contact, Miltos Tsiantis (tsiantis@mpipz.mpg.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Arabidopsis thaliana Col-0, Cardamine hirsuta Ox [5] and Cardamine hirsuta rco [18] backgrounds were used for experiments and for generation of transgenic lines.
Plant Growth conditions
Plant seeds were germinated and grown on soil in MPIPZ greenhouses under long day conditions (16 h of light, 8 h dark) at 20 ± 2°C, with 65 ± 10% relative humidity. For dexamethasone treatment (described below), seeds were germinated and grown on half-MS-medium (Duchefa Biochem, M0222.0050 including vitamins, supplemented with 1% sucrose) containing plates in short days (8 h light: 16 h dark).

METHOD DETAILS

Construction of binary and bacterial expression vectors
To create a Dexamethasone-mediated inducible system, the RCO-coding sequence (RCO-CDS) fused to VENUS was cloned into binary vector pOPin2-AtRPS5a-LhGR (kind gift from Ian Moore). This vector was derived from the inducible RNAi vector pOpOff2 RNAi [42] by introducing the AtRPS5a::LhGR cassette from a pBIN-LhGR vector [32]. It confers BASTA resistance for selection in plants. To perform ChiP-Seq, the synthesized RCOg-VENUS sequence fused to the RCO terminator was cloned downstream of the RCO promoter in the intermediate vector pBJ36 using XmaI and BamHI. The whole cassette was then cloned as a NotI fragment into the binary vector pMLBART for plant transformation. To perform the functional analysis of RCO-binding sites, synthesized modified RCO promoters were transferred into the pBJ36 intermediate vector harboring either the RCO-CDS fused to the RCO terminator or the RCO genomic sequence fused to the RCO terminator using Sall and Xmal sites. Cassettes were transferred as NotI fragments into pMLBART for plant transformation. To understand the role of cytokinin signaling in the regulation of growth, the coding sequence of ARR1::DDK, (synthesized by Genscript Ltd, Hong Kong), was cloned downstream of the RCO promoter and upstream of the RCO terminator in the pBJ36 intermediate vector using XmaI and BamHI sites. Cassette was transferred as NotI fragments into pMLBART for plant transformation. To construct a bacterial expression vector, the coding sequence of RCO was obtained by PCR amplification from seedling cDNAs using RCOFBamHI (GAACAATAAGGATCCCAAATGGAATGGTCAACCA-CAAGCAAC) and RCO-product 1 was then cloned in the polylinker of the bacterial expression vector pGEX-6p-3, using BamHI and Sall. Finally, the construct was verified by sequencing.

Agrobacterium-mediated Transformation
C. hirsuta plants were transformed by Agrobacterium using the floral dip method. Agrobacterium cultures grown in 1000 mL YEB medium at 28 C to an OD600 of 0.8 to 1.0 were harvested by centrifugation and resuspended in infiltration medium containing 4 g/L MS basal salt mixture with vitamin (Duchefa), 3% sucrose, 5 mM MES pH 5.6, 0.05 μM BAP (6-benzylamino purine), and 0.05% (v/v) Silwet L77. Plant inflorescences were submerged in the Agrobacterium suspension for 1 minute and then covered with plastic bags for one day adaptation. Seeds were collected in nylon bags, dried and then selected.

Cytokinin reporter constructs and lines
To monitor the transcriptional output of the cytokinin network in Arabidopsis, Two Component Signaling Sensor TCSn::GFP was used [30]. To visualize this response in C. hirsuta, GFP was replaced with nuclear localized td-Tomato (TCSn::tdTomato) and the resulting construct was transformed into C. hirsuta.

Quantitative RT-PCR
Total RNA was extracted using the RNeasy Mini kit supplied with RNase-Free DNase (QIAGEN). Two micrograms of total RNA template were reverse-transcribed using a first-strand cDNA synthesis kit (SuperScript VILO, Invitrogen). The reaction mixture was diluted to 100 μL, and 4 to 5 μl aliquots were used for real-time PCR assays performed with IQ Supermix (Bio-Rad) in a Bio-Rad iCycler iQ5. All quantitative RT-PCR measurements were performed in triplicate from three independent experiments. The expression of UBQ10 was used to normalize the expression of CT values. The obtained standard curves with R² ≥ 99 confirmed the PCR efficiency equal to or more than 87% and equal to or less than 95% (87 ≥ E ≤ 95) for all reactions. Nonspecific amplification was examined via melting-curve analysis of the qPCR products [43]. Oligos used for quantitative RT-PCR measurements are provided in Table S2 (related to STAR Methods).

Purification of RCO Protein Expressed in Escherichia coli
E. coli BL21 (DE3) pLyS cells carrying pGEX-RCO were grown at 28°C to OD600 of 0.4 to 0.6. Expression of the GST-RCO protein was induced by 1 mM isopropyl-β-d-thiogalactopyranoside for 4 h at 37°C. Cells were harvested by centrifugation and then resuspended and sonicated in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 10 μg/mL aprotinin, and 10 μg/mL leupeptin, pH 8.0). The cell lysate was centrifuged (Servall HB-4 rotor; 16,500g for 30 min at 4°C), and the cleared extract used for affinity purification on glutathione-Sepharose 4B (Amersham Biosciences). The eluted GST-RCO protein fraction was further purified by anion-exchange chromatography on a 1 mL pre-packed HiTrap DEAE-Sepharose FF anion exchange column (GE Healthcare). Finally, protein concentrations were adjusted based on Bradford assays (Bio-Rad) using a BSA standard curve and then confirmed by SDS/PAGE and Coomassie Blue analysis.
**Protein binding microarray assay**

The coding sequence of RCO was recombined in a Gateway LR reaction into pDEST-TH1 vector, yielding a translational fusion with maltose binding protein (MBP). The resulting plasmid was introduced into the BL21 strain of E. coli and the recombinant protein expressed as indicated above. DNA-binding assays were performed as follows [22]. An oligonucleotide nPBM11 array containing all the possible 11 bp sequences, was synthesized by Agilent Technologies. A soluble protein extract was obtained from a bacterial pellet corresponding to 25 mL of an induced culture by resuspending in 1 mL binding buffer (10 mM Tris-HCl pH 8, 60 mM KCl, 4 mM MgCl₂, 0.1 mM EDTA pH 8, 10% glycerol, 0.2% NP40, 200 mg/mL, 1mM Phenylmethylsulfonyl fluoride), then sonicated twice for 30 s, and centrifuged twice at 20,000 g to obtain cleared extracts. The binding mixture was adjusted to 175 μL containing 2% milk and 0.89 μg of denatured salmon sperm DNA. In parallel, the PBM array was converted to double-strand by subjecting it to a primer extension reaction containing at 1.17 μM oligonucleotide 5′-ACAGCAGGGACACGGAAACAGAC-3′, 163 μM dNTPs, 1.63 μM Cy3-dUTP (GE Healthcare PA55022), 1x Thermo Sequenase Buffer and 40 U Thermo Sequenase DNA Polymerase (GE Healthcare E79000Y). This mixture was applied to the pre-heated array and incubated for 10 min at 85°C, 10 min at 75°C, 10 min at 65°C and 90 min at 60°C. After the incubation, the slide was washed in phosphate-buffered saline (PBS), 0.01% Triton X-100 at 37°C for 10 min, followed by three washes in PBS at room temperature. The double-stranded PBM was incubated for 2.5 hours at room temperature with the protein-containing binding mixture to allow DNA-protein interactions and washed 5 times, 3 min in PBS-1% Tween 20 and 3x 5 min in PBS-0.01% Triton X-100. DNA–protein complexes were detected with sequential incubations with primary rabbit polyclonal antibody to MBP (Abcam ab9084) for 16 hours at room temperature and goat anti-rabbit IgG DyLight 550 conjugated (Thermo Fisher Scientific, 84541) in PBS–2% milk for 3 h at room temperature. After each antibody incubation, the slide was washed 3x 5 min PBS-0.05% Tween 20 and 3x 5 min PBS-0.01% Triton X-100. Finally, the array was scanned in a DNA Microarray Scanner at 2 μm resolution and quantified with Feature Extraction 9.0 software (Agilent Technologies). Normalization of probe intensities and calculation of E- and Z-scores of all the possible 8-mers were carried out with the PBM Analysis Suite [44] (also see Data S1B).

**EMSA**

Electrophoretic Mobility Shift Assays (EMSAs) were performed using 20-160 ng of purified GST-RCO fusion protein incubated in binding buffer [25 mM HEPES pH 7.8, 50 mM KCl, 5 mM MgCl₂, 10% glycerol, 1 μg/μl BSA, 1 mM DTT, 1 mM EDTA, 0.05% Tween 20, 2% milk, and 0.05% Triton X-100]. DNA-protein complexes were detected with sequential incubations with primary rabbit polyclonal antibody to MBP (Abcam ab9084) for 16 hours at room temperature and goat anti-rabbit IgG DyLight 550 conjugated (Thermo Fisher Scientific, 84541) in PBS–2% milk for 3 h at room temperature. After each antibody incubation, the slide was washed 3x 5 min PBS-0.05% Tween 20 and 3x 5 min PBS-0.01% Triton X-100. Finally, the array was scanned in a DNA Microarray Scanner at 2 μm resolution and quantified with Feature Extraction 9.0 software (Agilent Technologies). Normalization of probe intensities and calculation of E- and Z-scores of all the possible 8-mers were carried out with the PBM Analysis Suite [44] (also see Data S1B).

**β-Glucoronidase (GUS) staining**

GUS activity staining assays were performed as previously described [5]. Plants after fixation in 90% acetone were incubated overnight at 37°C with 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc, Roth), supplemented with 2 mM ferricyanide and ferrocyanide salts. Signals were documented using a Zeiss Axiohot light microscope and Zeiss Axiocam.

**Confocal microscopy**

Confocal imaging was performed using a Leica SP8 microscope with 20x (HCX APO, numerical aperture 0.8). Excitation was achieved using an argon laser with 514 nm. Fluorescent signal was collected from 519 to 550 nm emission spectrum for VENUS and 675 to 743 nm filter for the chlorophyll autofluorescence. To preserve the transgene signal against the auto-fluorescent background, it was subtracted from the auto-fluorescent background before merging both in different color channels for visual display.

**Leaf shape analysis**

All phenotypic measurements were performed using at least 20 independent T1 lines grown under the same conditions. To obtain leaf silhouettes, leaf 5 samples were flattened onto white paper using a clear adhesive and then digitally scanned. Leaf area and perimeter were calculated from silhouettes using ImageJ software (ImageJ 1.51). National Institutes of Health, USA). Leaf dissection index was then determined by using the formula ([perimeter squared]/[4π x area]) [45].

**Cellular measurements**

Leaf 9 of 19-days old wild-type Arabidopsis plants and Arabidopsis plants transformed with pRCo::ARR1.3DDK and pRCo::RCoqVENUS, grown in long day conditions (16h light, 8 hour dark) on MS medium (1% sucrose, 1X Murashige and Skoog basal salt mixture(Sigma, M5524), 0.05% MES 2-(N-morpholino)-ethane sulfonic acid, 0.8% Bacto Agar, 1% MS vitamins (Sigma, M3900), pH 5.7 adjusted with 1M KOH) were used for analysis. Three biological replicates (leaf 9 from 3 independent plants at the same stage for each genotype) were used. Leaves were dissected off the main plant body and stained with Propidium iodide (1mg/ml solution in water) for 60 minutes. Cells in the sinus regions in between the lobes 1 and 2 or serrations 1and 2 from the proximal end of the leaf were then imaged on a Leica TCS-SP8 upright confocal microscope. To detect fluorescence signal from the cell walls, PI-stained leaves were excited with 561nm white light laser and signal was collected from 565 nm to 580nm. Z stacks were acquired using HyD detectors and a 20x NA 0.8 objective in a 1024x1024 scan format with a step size of 1 μm. Leaves from all the three genotypes were imaged with the same magnification. Quantitative analysis of cell area and cell lobeiness was performed using MorphographX [20, 46]. Briefly, to extract the cell shapes, confocal stacks were loaded into MorphographX and processed to create a mesh with subsequently the signal projected onto it. Cells in the sinus regions in between the lobes or serrations were
manually segmented. For measurements shown in Figures 6C and 6D, cells within 5 cell distances from the center of the notches were included in the analysis. Cell area and cell lobeyness were calculated using the MorphographX plugin as described previously [20]. Matured stomata cells were excluded from the analysis. For statistical validation, two independent nested ANOVA with genotype as a fixed factor and replicates as nested random factor were used. Significance of all pairwise comparisons between genotypes was performed using Tukeys all-pair comparison. We found that significance levels for difference in cell area and cell lobeyness did not change up to measurements performed on 7–cell distance from the center of the sinus in between the lobes. The R script implementing the statistical analyses and the data for individual biological replicates can be found at https://gitlab.mpcdf.mpg.de/slaurent/statistical_analyses_for_hajheidari_et_al.

Dexamethasone treatment
Fourteen-day old rco mutant seedlings harboring a Dexamethasone (DEX)-inducible RCO construct (pOpIn2 -pOp8::RCO-VENUS) were grown on MS plates in short days (8 h light: 16 h dark) and incubated in liquid MS medium with either 0.1% DMSO or 10 μM DEX. Seedlings were harvested at multiple two-hour interval time-points (2, 4, 6, 8, and 10 h) by flash freezing in liquid nitrogen. RNA samples were extracted and sequenced using the HiSeq 2500 sequencing system ( Illumina). For each time point, comparative analysis was done between DEX- and DMSO-treated samples. DEX-responsive genes were identified by comparative RNA-Seq analysis between DEX- and DMSO-treated wild-type samples treated as above and excluded from downstream analyses.

Transcriptome sequence data analysis
RNA for RNA-Seq experiments was obtained from three biological replicates, and total RNA was isolated by using an RNeasy Plant Mini Kit (Qiagen) and subsequently digested with RNase-free DNase, according to the manufacturer’s protocol. A total of 1 μg of RNA was used for library preparation using TruSeq RNA Sample Preparation Kit v2 RS-122-2002 (Illumina). Library quality was validated using 2100 Bioanalyzer (Agilent), and libraries then underwent single-end sequencing (100 bp reads) using the Illumina HiSeq2500 Sequencing System. Reads were aligned to the C. hirsuta reference genome (CHIV1, http://chi.mpipz.mpg.de/) using STAR v2.4.2a with default parameters. Raw read counts per gene were quantified with HTSeq v0.5.4p1 [https://www-huber.embl.de/users/anders/HTSeq/] using the ‘‘-stranded=no–type=CDS’’ option. Differential expression was determined using DESeq2 v1.10.1 [47]. We found the most sensitive parameter settings for the function estimateDispersions were method = “blind,” and sharingMode = “fit-only” [48]. RNA-Seq data are available at the European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena/data/view/PRJEB29757). (Also see Data S1 C, S1D, S1G, and S1H)

ChIP-Seq
Chromatin immunoprecipitations were performed, as in [43] with some modifications. Fourteen-day old seedlings grown on soil were cross-linked with 1% formaldehyde at 4 °C for 20 min, then ground with liquid nitrogen. 5–6 g homogenized tissue was resuspended in 45 mL of nuclei isolation buffer (50 mM HEPES pH 7.4, 25 mM NaCl, 5 mM MgCl2, 5% sucrose, 30% glycerol, 0.25% Triton X-100, 0.1% β-mercaptoethanol, and 0.1% protease inhibitor cocktail Sigma P9599) and centrifuged at 1600 g for 20 min at 4 °C. The pellet was resuspended and washed in Nuclei washing buffer (17 mM HEPES (pH 7.4), 7 mM MgCl2, 33 mM NaCl, 13% sucrose, 13% glycerol, 0.25% Triton X-100, 0.1% β-mercaptoethanol, and 0.1% protease inhibitor cocktail) three times. After centrifugation at 1600 g for 10 min, the pellet was resuspended in 500 μL of TE buffer pH = 8.0 supplemented with 1% SDS and mixed on a rotator for 60 min. The DNA was sheared by sonication in a Bioruptor (Diagenode) to 300–500 bp fragments. The chromatin solution, carrying the same amount of DNA for different samples, was diluted by IP buffer (10 mM Tris pH = 7.5, 0.5 mM EDTA, 150 mM NaCl) and incubated with GFP-Trap magnetic beads (Chromotek) or IgG coated magnetic beads. After overnight incubation with rotation at 4 °C, the samples were cleared on a magnetic stand. The magnetic beads were then washed 4x2 times with washing buffers in the order as follows. Wash buffer 1: 0.1% SDS, 1% Triton-X, 2mM EDTA, 20 mM Tris-HCl pH 7.5, 150 mM NaCl. Wash buffer 2: 0.1% SDS, 1% Triton-X, 2mM EDTA, 20 mM Tris-HCl pH 7.5, 500 mM NaCl. Wash buffer 3: 0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholate, 1mM EDTA, 10 mM Tris-HCl pH 7.5. Wash buffer 4: 10 mM Tris pH 7.5, 0.5 mM EDTA. Immunocomplexes were then eluted from the beads with 500 μL of elution buffer (50 mM Tris pH = 8.0, 10 mM EDTA, 1% SDS). Cross-linking was reversed by incubation at 37°C in the presence of 25 μg/mL proteinase K followed by at least 8 h of incubation at 65°C. The DNA was purified by two successive phenol/TE and phenol/chloroform/iso-amylalcohol extractions and ethanol-precipitated overnight at −20°C. The pellet was washed with 70% ethanol and resuspended in 30 μL of H2O. A similar amount of untreated sonicated chromatin was phenol/chloroform purified, precipitated with ethanol overnight and resuspended in 30 μL of H2O and used as total input DNA sample. To ensure experimental reproducibility, three biological replicates of template, mock control, and input were sequenced to a depth of 22-24 million reads. ChIP-Seq data have been deposited at the European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena/data/view/PRJEB29759).

ChIP-Seq data analysis
Illumina short reads were mapped to the reference genome using BWA version 0.7.15-r1140 with WA-MEM algorithm (command mem) with option “-M” [49]. Reads with mapping quality MAPQ < 30 or those identified as PCR duplicates by PICARD (command Markduplicates) were excluded from subsequent analyses. Stringent QC were performed using ENCODE (phase III) transcription
factor and histone ChIP-Seq pipeline specifications (by Anshul Kundaje) in https://docs.google.com/document/d/1lG_Rd7hnYgRpsSlqrFvIAz2dW1YaSGThzk836Db99c/edit# to access the reproducibility among biological replicates [50], with MACS2 version 2.1.0.20150420 [21] selected as peak caller and P value cutoff of 1e-3.

To make sure both broad peaks and narrow peaks are detected, we followed the principle of DiffBind with minor revisions to enhance the performance for our plant studies. Reads from biological replicates were merged into two pools, case and control, for peak calling using MACS2 with "--nomodel -p 1e-3." As the number of peaks detected is smaller than 1,000 and negative binomial distributions-based statistics used by DESeq or edgeR could lose powers, pseudo-peaks were be generated from the 200bp promoter regions of annotated protein-coding genes that do not overlap with any detected peak for quality control and normalization. Raw read counts per peak (or pseudo-peaks if present) per sample were quantified with HTSeq v0.5.4p1 (https://www-huber.embl.de/users/anders/HTSeq/). Peaks showing differential binding between case and control were determined using DESeq [51] with the method = "blind," sharingMode = "fit-only" and P value cutoff of 0.05 [50]. (Also see Data S1A)

Calculating the probability of observing zero high-affinity binding sites at the RCO locus under a neutral evolutionary model

To test whether the absence of high-affinity binding sites for RCO at the RCO locus can be explained by chance alone, we simulated DNA under a neutral evolutionary model and subjected the simulated sequences to the same PWM-scoring method that has been used on the empirical data. For each simulated dataset, we calculated the number of high-affinity binding sites predicted by the PWM scoring algorithm and used this distribution to calculate the probability of observing no high-affinity binding sites. Neutral DNA simulations were done with seq-gen v1.3.4 using the following options "-mHKY -L 5000 -k 1." The input tree was a simple bifurcation representing the duplication of RCO and LMI1 with branch length Ks/2, where Ks is the synonymous divergence between RCO and LMI1. For each simulation, the ancestral DNA sequence was initialized by a random DNA sequence with the same nucleotide composition as observed within a 200kb region centered around RCO and LMI1. For every simulated DNA sequence, we ran the PWM scoring method implemented in fimo v5.0.5 with the following option "--thres 0.000619 --max-strand." The threshold was set such as to identify sites with a PWM score larger than the highest PWM score observed in the real RCO regulatory region. The annotated script used for this analysis can be found here: https://gitlab.mpcdf.mpg.de/slaurent/statistical_analyses_for_hajheidari_et_al.

Cytokinin analysis by LC-MS/MS

Cytokinins and their related metabolites were extracted from seedlings with modified Bieleski buffer and consequently purified by using MCX separation cartridges (Waters) [52]. Samples were analyzed in 4 biological replicates. Stable isotope-labeled standards (Olchemim) for all measured compounds were added to samples before extraction. Mass spectrometry analysis and quantification was performed by LC-MS/MS, which consisted of the 1290 Infinity Binary LC System coupled to 6490 Triple Quad LC/MS System with Jet Stream and Dual Ion Funnel technologies (Agilent Technologies) [53]. Concentrations were then calculated using a standard isotope dilution method. All solvents used were of analytical or higher grade (Sigma Aldrich GmbH).

Statistical Analysis

As indicated in the text, statistical analyses were performed using a two-tailed unpaired Student’s t tests, assuming equal variances (with NS, not significant; *p < 0.05; **p < 0.01) or, for Figures 3B, 3D, S2C–S2F, and S3E, using a one-way ANOVA followed by a Tukey’s HSD test for multiple-pairwise comparisons (as implemented in the R base package).

GO analysis

Gene ontology (GO) analysis was done using agriGO with default settings (http://bioinfo.cau.edu.cn/agriGO/). (Also see Data S1E and S1F)

DATA AND CODE AVAILABILITY

The raw ChIP-Seq data have been deposited at the European Nucleotide Archive under project name PRJEB29759 (ENA: PRJEB29759) (ENA, https://www.ebi.ac.uk/ena/data/view/PRJEB29759). The raw RNA-Seq data are available at the European Nucleotide Archive under project name PRJEB29757 (ENA: PRJEB29757) (ENA, https://www.ebi.ac.uk/ena/data/view/PRJEB29757). The codes for RNA-Seq and ChIP-Seq data analysis are available upon request. The R scripts implementing all the statistical analyses and the visualization can be found at https://gitlab.mpcdf.mpg.de/slaurent/statistical_analyses_for_hajheidari_et_al. Raw data for cellular-morphology analysis in Figure 6 is available at Edmond repository (https://edmond.mpdl.mpg.de/imeji/collection/EBFSUTS1h4UmUfPq) [https://doi.org/10.17617/3.32].