Cytokinin response factors regulate auxin-mediated organogenesis

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Running title: CRFs control PIN gene transcription
ABSTRACT

Auxin and cytokinin are key endogenous regulators of plant development. Although cytokinin-mediated modulation of auxin distribution is a developmentally crucial hormonal interaction, its molecular basis is largely unknown. Here we disclose a core mechanistic framework for cytokinin–auxin cross-talk and a direct regulatory link between the cytokinin signaling and the auxin transport machinery. We show that the CYTOKININ RESPONSE FACTORS (CRFs), transcription factors downstream of cytokinin perception, transcriptionally control genes encoding PIN-FORMED (PIN) auxin transporters at a specific PIN CYTOKININ RESPONSE ELEMENT (PCRE) domain. Removal of this cis-regulatory element effectively uncouples PIN transcription from the CRF-mediated cytokinin regulation and attenuates plant cytokinin sensitivity. Hence, CRFs represent the missing cross-talk components which fine-tune auxin transport capacity downstream of cytokinin signaling to control plant development.

Subject terms: auxin, cytokinin, hormonal cross-talk, lateral root organogenesis
**Introduction**

The hormones auxin and cytokinin are essential to control plant growth and development including early embryogenesis\(^1\) and postembryonic organogenic processes, such as root\(^2\)–\(^5\) and shoot\(^6\) branching, phyllotaxis\(^7\), shoot\(^8\),\(^9\) and root apical meristem activity\(^10\),\(^11\), and vasculature development\(^12\),\(^13\). The principal pathways that manage their metabolism, distribution, and perception, and the backbone molecular components have been identified\(^14\)–\(^17\). Importantly, a complex network of interactions and feedback circuits interconnects both pathways and determines the final outcome of the individual hormone actions. Well-established are the mutual regulation of metabolic\(^18\) and signaling pathways\(^2\),\(^8\), as well as the cytokinin-mediated modulation of auxin transport\(^10\)–\(^12\). Cytokinin has been shown to influence cell-to-cell auxin transport by modification of the expression of several auxin transport components and thus to modulate auxin distribution important for root development\(^10\),\(^11\),\(^19\),\(^20\). Through the cytokinin receptor ARABIDOPSIS HISTIDINE KINASE3 (AHK3) and the downstream signaling components ARABIDOPSIS RESPONSE REGULATOR (ARR1) and ARR12, cytokinin has been shown to activate SHY2/IAA3 (SHY2), a repressor of auxin signaling that negatively regulates the PIN auxin transporters\(^10\). However, thus far, the components of the transcriptional complex that directly controls PIN transcription in response to cytokinin are unknown.

Here, we show that the CYTOKININ RESPONSE FACTORS (CRFs)\(^21\) transcriptionally control **PIN-FORMED (PIN)** genes encoding auxin transporters at a specific **PIN CYTOKININ RESPONSE ELEMENT (PCRE)** domain. Removal of this cis-regulatory element effectively uncouples PIN transcription from the CRF-dependent regulation and attenuates plant cytokinin sensitivity. Accordingly, plants with modified CRF activity exhibit alterations in the expression of PIN genes and developmental defects mimicking phenotypes of auxin distribution mutants. We propose that the CRFs act as components of the transcriptional regulatory complex which mediates transcriptional control downstream of cytokinin and fine-tunes PIN expression during plant growth and development.

**Results**

**Loss of a PCRE element results in cytokinin-insensitive PIN7 expression** To explore the upstream pathway mediating cytokinin-dependent PIN transcription, we searched for the
regulatory elements by a promoter deletion analysis in PIN7 and PIN1 promoters (Supplementary Fig. 1a). Initially, we focused on PIN7 of which transcription has previously been shown to be activated by cytokinin\textsuperscript{11,12}. We confirmed that the promoter (1,423 bp upstream of the translational start site) of PIN7 fused to the green fluorescent protein (GFP) reporter gene (Supplementary Fig. 1a) is activated by cytokinin and that this region is sufficient to mediate the hormonal response (Fig. 1a). The abrupt change in the cytokinin response as a consequence of a 200-bp element deletion between 1,423 bp and 1,223 bp upstream of the ATG start codon hinted at the presence of a cis-regulatory element required for the cytokinin-mediated transcriptional control of PIN7 (Fig. 1b). The role of this regulatory element, designated as PIN CYTOKININ RESPONSE ELEMENT7 (PCRE7), in cytokinin-sensitive expression was further tested with a PIN7-GFP translational construct driven by the truncated ΔPIN7 promoter. Quantification of the membrane PIN7-GFP signal demonstrated that the expression, when driven by the truncated promoter, was largely insensitive to cytokinin treatment in the cells of the central root cylinder (Fig. 1c-e), as well as in the initials of lateral root primordia (Supplementary Fig. 2a-e). The ΔPIN7 promoter activity was significantly weakened in the root provasculature and columella cells when compared to the full PIN7 promoter, indicating the importance of this promoter element for the regulation of the PIN7 steady state expression (Fig. 1a and c compared to b, d and e). Hence, the loss of cytokinin sensitivity as a consequence of the promoter truncation implies the presence of a specific cis-regulatory element on which the cytokinin-susceptible transcriptional complex might act to fine-tune PIN7 expression in response to cytokinin. The severely attenuated expression of the ΔPIN7 promoter indicates that cytokinin might, through this regulatory element, participate in the establishment and maintenance of the proper expression level of PIN7.

Cytokinins insensitive PIN7 transcription modulates plant development. A part of cytokinin-regulated plant growth and development has been proposed to be mediated through cytokinin-controlled PIN expression\textsuperscript{10,11}. To dissect the developmental role of cytokinin-regulated PIN expression, plants expressing PIN7-GFP under the control of the truncated cytokinin-insensitive ΔPIN7 promoter or the wild-type PIN7 promoter were crossed with the pin7 mutant background and their seedlings were thoroughly inspected. Root growth analyses revealed that in young seedlings (7 days old), growth of roots expressing either PIN7::PIN7-GFP/pin7 or
ΔPIN7::PIN7-GFP/pin7 was indistinguishable. In contrast, 14-day-old ΔPIN7::PIN7-GFP/pin7 roots were significantly longer than control PIN7::PIN7-GFP/pin7 roots (Fig. 1f and h). Furthermore, attenuation of the cytokinin-mediated PIN7 transcription strongly interfered with the cytokinin sensitivity of root growth (Fig. 1g and h), root meristem size (Fig. 1i), lateral root initiation and development (Fig. 1g and j). Some phenotype features, such as primary root growth and its resistance to cytokinin, were comparable to the pin7 phenotype. However, reduced sensitivity of the root meristem as well as of the lateral root initiation to cytokinin were more pronounced in ΔPIN7::PIN7-GFP/pin7 seedlings.

Altogether these data corroborate the role of the PCRE7 in fine-tuning PIN7 expression and show that cytokinin-controlled transcription of PIN7 through PCRE7 is critical for proper cytokinin-regulated root growth and development.

Cytokinin response factors (CRFs) control PIN7 transcription. To identify the upstream regulatory factors that control PIN7 transcription by direct interaction with PCRE7, we employed a yeast one-hybrid (Y1H) assay. We screened the REGIA open reading frame (ORF) library that contains a set of ~1,300 Arabidopsis transcription factors (TFs) and transcriptional regulators22. The Y1H screen with PCRE7 as bait (Supplementary Fig. 3a), identified CRF2, CRF3, and CRF6 that belong to the cytokinin-inducible subset of the APETALA2/ETHYLENE RESPONSIVE FACTOR family of TFs21 (Fig. 2a). To confirm that the CRFs physically interact with PCRE7, we analyzed the CRF2::CRF2-GFP and 35S::CRF6-GFP transgenic plants through chromatin immunoprecipitation (ChIP) followed by quantitative PCR assays (ChIP-qPCR). Chromatin immunoprecipitated with anti-GFP antibodies was profoundly enriched in the PCRE7 region. No enrichment was detected when other distant sequences in the PIN7 (PIN7 [-553-357]) promoter were tested (Fig. 2c and d; Supplementary Fig. 1c). Hence, CRFs are directly associated with the PCRE7.

To gain insight into the role of the CRFs in the regulation of PIN transcription, we performed a transient expression assay in Arabidopsis protoplasts. The expression of the PIN7::LUCIFERASE (LUC) reporter was strongly activated when co-expressed with CRF2 and CRF6 (Fig. 2e and f), but not with CRF3 driven by constitutive 35S promoter (Supplementary Fig. 3b). Noteworthy, when co-expressed, CRF3 significantly attenuated the positive effect of CRF6 on PIN7::LUC expression (Supplementary Fig. 3c), indicating that individual CRFs might
regulate PIN7 transcription differentially. No increase in the LUC reporter expression was detected when CRF2 was co-expressed with the truncated ΔPIN7::LUC construct (Fig. 2e). Co-expression with CRF6 stimulated ΔPIN7::LUC expression, but less when compared to effect on the PIN7::LUC (Fig. 2f) thus further confirming the importance of the PCRE7 for CRF-dependent transcription. Collectively these data demonstrate that CRFs contribute to the transcriptional control of the PIN7 gene through physical interaction with a specific domain in its promoter.

**CRFs regulate transcription of PIN1 auxin efflux carrier.** Next we examined whether other PIN family members are transcriptionally controlled by CRFs similarly to PIN7. Using promoter deletion analysis we found that removal of the 200 bp between 1417 bp and 1212 bp upstream of the ATG codon resulted in complete cytokinin insensitivity of PIN1 transcription, hinting at the presence of the PCRE1 cis-regulatory element mediating cytokinin-induced transcriptional control in the PIN1 promoter (Supplementary Fig. 1b and 4a-e). A Y1H assay confirmed an interaction of CRF2, CRF3 and CRF6 with this element (Fig. 2b; Supplementary Fig. 3a). Accordingly, chromatin immunoprecipitated with anti-GFP antibodies from CRF2::CRF2-GFP and 35S::CRF6-GFP transgenic plants was profoundly enriched in the PCRE1, while no enrichment was detected for distant sequence in the PIN1 promoter (PIN1 [-512-433]) (Fig. 2c and d). The expression of PIN1::LUC, but not of ΔPIN1::LUC, was strongly activated when transiently co-expressed with CRF2 and CRF6, in protoplasts (Fig.2e and f). Similarly to PIN7, CRF3 did not significantly affect PIN1::LUC expression, but attenuated the CRF6 stimulatory effect when co-expressed (Supplementary Fig. 3d, e). Altogether, these results indicate that PIN1 and PIN7 share common upstream regulatory components controlling their expression in response to cytokinin.

**PCRE1 and PCRE7 contain motifs recognized by CRFs.** Alignment of PCRE1 and PCRE7 elements with the Align program (based on the ClustalW algorithm) displayed a 54% sequence identity, suggesting that the cytokinin-dependent regulation of PIN1 and PIN7 might be governed by transcription factors (TFs) recognizing common types of regulatory motifs (Fig. 2h). Previously, the GCC box (AGCCGCC) has been proposed as a binding motif recognized by ETHYLENE RESPONSE FACTOR1 (ERF1), a TF of the AP2/ERF family, including
CRF2, CRF3 and CRF4\textsuperscript{25}. Scanning of PIN1 and PIN7 promoters revealed that neither PCRE1 nor PCRE7 contains a GCC box, and there is one GCC box located at [-483bp] upstream of ATG in the PIN1 promoter (Supplementary Fig. 1d). However, no significant enrichment for the PIN1 fragment [-512-433] which contains this motif was detected using the ChIP-qPCR assay, indicating that CRF2 and CRF6 do not exhibit an increased affinity to this binding site (Fig. 2c, d). Noteworthy, several studies demonstrated that nucleotides G2, G5, and C7 at conserved positions might be essential for the recognition of the GCC-derived motif by TFs of the AP2/ERF family\textsuperscript{26}. We found that PCRE1 and PCRE7 contain AGCAGAC and AGAAGAC motifs, respectively, with critical nucleotides at conserved positions (Fig. 2h; Supplementary Fig. 1c and d). To examine the relevance of these motifs for CRF binding, we tested whether CRF6 associates with them by ChIP-qPCR. Using specific primer combinations, we inspected enrichment for the short fragments spanning PCRE1\textsuperscript{27}. Significantly increased enrichment detected for fragments either containing or directly neighboring the AGCAGAC site when compared to more distant fragments in PCRE1, strongly supports this motif as a CRFs’ recognition site (Fig. 2 g). Additional thorough scanning for the presence of G2, G5, and C7 motifs revealed one more motif in both PIN1 as well as PIN7 promoters (Supplementary Fig.1c and d).

To further support our conclusion on the CRF-mediated regulation of PIN expression, we tested whether the PIN2 promoter, previously found to be cytokinin insensitive\textsuperscript{28} might be activated by CRF after introducing the AGAAGAC motif. Detailed scanning of the PIN2 promoter sequence revealed that there are no AGCAGAC and AGAAGAC motifs present in the PIN2 promoter sequence within 2500 bp upstream of the translation start and there is one GCCGTC motif located at [-698bp] upstream of the translational start. When the PIN2::LUC reporter was co-expressed with CRF6, a 1.47±0.38 fold increase of LUCIFERASE activity was detected, indicating modest CRF6 activity for the regulation of PIN2 promoter (Fig. 2h). Replacement of the GCCGTC motif in the PIN2 promoter by either one or three copies of the AGAAGAC motif resulted in a 1.75±0.17 and 3.48±1.02 fold increase of the LUCIFERASE activity, respectively (Fig. 2h). Hence, insertion of a AGAAGAC in triplicate into the PIN2 promoter significantly increased sensitivity to CRF6 (Fig. 2h), thus corroborating relevance of the motif identified in the PCRE7 element for CRF6-mediated expression. In line with previous observations, no
dramatic effect on PIN2 as well as on the PIN2 promoter containing PCRE7 motifs could be detected when co-expressed with CRF3 (Fig. 2h).

Previously, type-B ARRs have been proposed to mediate cytokinin regulation of PIN1 and PIN7 expression through direct transcriptional control of the IAA3/SHY2 repressor of auxin signaling\textsuperscript{10}. Three type-B ARRs (ARR10, ARR11 and ARR14) tested in a Y1H assay did not exhibit interaction with either PCRE1 or PCRE7, which indicates that cytokinin transcriptional regulation of PIN1 and PIN7 might not occur through their direct binding to PCREs (Supplementary Fig. 3f).

Altogether, these data suggest that CRF2 and CRF6 might recognize specific motifs within PCRE1 and PCRE7 with G2, G5, and C7 at conserved positions to control PIN1 and PIN7 expression.

**Expression of PIN7 and PIN1 is altered in crf mutants.** The initial expression analysis revealed that the expression patterns of CRFs and PIN1 and PIN7 largely overlap in roots\textsuperscript{29} (Supplementary Fig. 5 compared to Fig. 1a and Supplementary Fig. 4a, c), supporting their role as direct transcriptional regulators. To evaluate the impact of CRFs on PIN7 and PIN1 expression in planta, we examined lines with a modulated activity of CRFs (Supplementary Fig. 6). Analyses of PIN7 expression using qRT-PCR as well as monitoring of PIN7::PIN7-GFP, PIN7::GUS, PIN7::GFP and PIN7::PIN7-GUS reporters revealed a significant increase of PIN7 expression in the root provasculature of RPS::CRF2 and 35S::CRF6, but not of 35S::CRF3 lines (Fig. 3a,b,e and Supplementary Fig. 7a-j). This is largely in agreement with the results of a transient protoplast assay (Fig. 2e and f compared to Fig. 3a-c). Lack of the PCRE7 in the ΔPIN7::PIN7-GFP line interfered with the stimulatory effect of CRF2 on PIN7-GFP expression, regardless of cytokinin levels (Fig. 3c and d). Analysis of PIN7 expression using PIN7::PIN7-GFP, and PIN7::GUS reporters as well as qRT-PCR in mutants lacking either of CRFs revealed attenuated PIN7 expression in crf3 and enhanced PIN7 expression in crf6 roots (Supplementary Fig. 7 k-t).

Similarly to PIN7, PIN1 expression was significantly upregulated in roots overexpressing CRF2 as detected using PIN1::PIN1-GFP and PIN1::GFP reporters as well as by qRT-PCR approach. Deletion of the PCRE1 in the ΔPIN1::PIN1-GFP line interfered with the stimulatory
effect of CRF2 on PIN1 expression in agreement with the proposed role of the PCRE1 in CRF-dependent transcriptional control (Supplementary Fig. 8a-d).

Inspection of crf loss-of-function mutants further confirmed the role of CRFs in the regulation of PIN1 expression in planta. PIN1 expression was significantly reduced in crf2, crf3, crf3crf6 and crf2crf3crf6 loss-of-function mutants (Supplementary Fig. 8d-g), thus resembling the PIN7 expression pattern in these mutant backgrounds.

Altogether, the expression analysis data support a role of the CRFs in balancing the PIN7 and PIN1 transcription. Nevertheless, inconsistency in PIN7 and PIN1 expression patterns observed in lines with modulated CRF expression, such as downregulation of both PIN1 and PIN7 in crf3, and upregulation in crf6, might reflect the presence of intricate in planta regulatory mechanisms, e.g. the existence of a transcriptional complex in which additional components could function as modifiers. This is strongly supported by recent observations that individual CRFs might interact with other family members, as well as with type-B ARRs30. Collectively, our data demonstrate that CRFs contribute to balancing PIN7 and PIN1 expression and that CRF homologs might have specific functions in the control of PIN expression.

CRFs mutants exhibit an auxin transport-defective phenotype. To examine a role of CRFs as direct upstream regulators of PIN transcription, we analyzed in detail the phenotype of plants with modulated CRF expression. Altered expression of PINs in crf loss-of-function mutants might result in an abnormal auxin distribution and, consequently, in developmental and patterning defects as previously demonstrated for auxin distribution mutants1,31. Indeed, auxin measurements in root tips of crf3crf6 mutants revealed an increase in auxin levels (Fig. 4a). Similarly, the auxin accumulation at root tips of a mutant lacking PIN4 auxin efflux carrier activity has been previously observed31.

By closer examination of plants lacking CRF activity, developmental abnormalities were found reminiscent of those caused by impaired auxin transport. A significantly enhanced number of embryonic defects, such as abnormal divisions of upper suspensor cells and in the embryo proper, and occasionally the appearance of double embryos, was observed in embryos of crf2, crf3 and crf3crf6 loss-of-function mutants when compared with control lines (Fig. 4b-i), thus phenocopying the pin1, pin7, and multiple pin embryo defects1. Accordingly, CRF2, CRF3, and CRF6 expression was detected in early embryos (Supplementary Fig. 5). Lack of functional
CRF2, CRF3, both CRF3 and CRF6 or CRF2, CRF3, CRF6 correlated with reductions in root length, root meristem size, and lateral root initiation (Fig. 4j-n), which are developmental aberrations typically associated with defective auxin transport\textsuperscript{3,32,33}. On the contrary, crf6 loss-of-function mutants, in which PIN7 expression was enhanced, exhibited longer roots and a larger root meristem (Fig. 4j-n). Altogether, modulation of CRF activity alters auxin accumulation in the root tips, and leads to developmental defects in many aspects, mimicking phenotypes of auxin distribution mutants\textsuperscript{1,31,32}.

**CRFs fine-tune root system response to cytokinin and auxin.** Typically, an increase in cytokinin activity alters root growth and development: cytokinins restrict root elongation growth, cause shortening of the root meristem size, and compromise the initiation and development of the lateral roots\textsuperscript{4,5,10,11,34,35}. Whereas cytokinin inhibitory effects of root elongation involves ethylene\textsuperscript{11,34}, reduction of the root meristem size, as well as lateral root initiation by cytokinin occurs largely in an ethylene independent manner\textsuperscript{5,11}. To examine the possible role of CRFs in cytokinin-mediated root system establishment, lines with modulated CRF activity were exposed to increased cytokinin concentrations. We found that neither gain nor loss of CRF activity dramatically changed root growth response to cytokinin (Fig. 4l and o). The root meristem cytokinin response was unaffected in crf2 and crf6 mutants and reduced in crf3 and crf3crf6 as well as crf2crf3crf6 mutants (Fig. 4m). Constitutive expression of CRF2, CRF3, and CRF6 reduced root meristem response to cytokinin (Fig. 4p). Noteworthy, most pronounced changes were observed in cytokinin effect on lateral root initiation and development. The significant increase in cytokinin inhibitory effects on both lateral root initiation and development was detected in crf3, crf3crf6 and crf2crf3crf6 mutants (Fig. 4n; Supplementary Fig. 9a-e), whereas CRF2 and CRF6 overexpression attenuated cytokinin effects (Fig. 4r; Supplementary Fig. 9f, g). In contrast to cytokinin, root system response to auxin was reduced in crf3, crf3crf6 and crf3crf3crf6 mutants, which was manifested by an attenuated stimulatory effect of auxin on lateral root initiation (Supplementary Fig. 10a-e). Constitutive expression of CRF2 led to significantly enhanced response to auxin whereas no dramatic changes in lateral root initiation after auxin treatment in either the CRF3 or CRF6 overexposer line when compared to wild type control were detected (Supplementary Fig. 10f-h). Hence, perturbations in CRF expression
affect root response to cytokinin and auxin, and indicate that CRFs through control of auxin transport might fine-tune cytokinin- and auxin-mediated root growth and development.

**Discussion**

Auxin gradients represent universal mechanisms to control plant organogenesis. Modulation of the activity of the transport machinery that regulates auxin distribution directly impacts on organ formation and patterning and, thus, accounts for a developmentally efficient tool to flexibly adapt plant architecture depending on the changing environmental conditions. Recently, evidence accumulates that exogenous factors, such as light or gravity, through their endogenous counterpart, i.e. plant hormones including ethylene, gibberellin, jasmonate and cytokinin, modulate the activity of the polar auxin transport machinery to direct plant growth and development\(^{10,11,19,36–39}\). However, the molecular bases of these regulations are scarcely understood so far.

Here we demonstrate that the expression of auxin efflux transporters can be effectively uncoupled from the cytokinin control through deletion of the \(PCRE\) cis-regulatory element in the promoter of the \(PIN\) auxin efflux carrier gene. The loss of cytokinin-dependent \(PIN7\) transcription impacts on the cytokinin-mediated root growth and branching, thus supporting the developmental significance of the tightly cytokinin-controlled PIN-dependent auxin transport in the establishment of the root system architecture.

Attempts to reveal components of the upstream regulatory pathway acting at the \(PCRE\) led to the identification of CRFs as direct transcriptional regulators. Originally, CRFs have been found as a subgroup of the APETALA 2 (AP2) family of TFs, which are rapidly induced by cytokinin, and they have been proposed to mediate the transcriptional response to cytokinin\(^{21}\). However, the CRFs’ downstream targets and pathways remained unknown so far. Here, we show that through interaction with the cis-regulatory \(PCRE\), presumably through recognition of the \(AGCAGAC\)-like motif, CRFs control expression of \(PIN1\) and \(PIN7\).

Modulation of the CRF activity results in significant changes of the \(PIN1\) and \(PIN7\) expression patterns and in phenotype aberrations reminiscent of mutants with defective auxin transport\(^{1,31,32}\). This, together with a significant overlap in the expression of \(PIN\) and \(CRF\) genes during embryogenesis, in the root meristems and lateral root primordia, strongly supports a role of CRFs in the regulation of \(PIN\) expression. Accordingly, the expression of several \(CRFs\) in
vascular tissue has been correlated with alterations of the vascular patterning in CRF loss-of-function mutants\textsuperscript{38}, similarly to those observed in auxin transport mutants\textsuperscript{39}.

In this light, the identification of the \textit{PIN} genes as direct targets of CRFs reveals a missing direct regulatory link between the cytokinin signaling and the auxin transport machinery. Moreover, \textit{CRF2 (TARGET OF MONOPTEROS [TMO3])} as a direct transcriptional target of the \textit{AUXIN RESPONSE FACTOR5/MONOPTEROS}\textsuperscript{42} might account for an important convergence point to the previously characterized \textit{AHK3-ARR, ARR12-IAA3/SHY2} regulatory chain\textsuperscript{10} and balance both auxin and cytokinin input to control auxin transport. Furthermore, the recent observation that \textit{CRF6} is induced by numerous stresses\textsuperscript{29} hints at a role of CRFs as factors modulating auxin transport in response to environmental signals.

\textbf{METHODS}

\textbf{Plant material and growth conditions.} The transgenic \textit{Arabidopsis thaliana} (L.) Heynh. lines have been described elsewhere: \textit{PIN1::PIN1-GFP\textsuperscript{3}, pin7-2\textsuperscript{3}, PIN7::PIN7-GFP\textsuperscript{32}, PIN7::GUS\textsuperscript{1}, RPS5A::CRF2\textsuperscript{42}, 35S::EGFP- CRF6\textsuperscript{43}}. The previously characterized CRF knockout mutants\textsuperscript{21} were obtained from various T-DNA insertion mutant seed collections: \textit{crf2-1, crf2-2, crf3-1 and crf3-2} from the Salk Institute Genomic Analysis Laboratory (SAIL, former GARLIC) T-DNA insertion lines from the Torrey Mesa Research Institute\textsuperscript{44} and \textit{crf6-S2 and crf6-11.2} from GABI-Kat\textsuperscript{45}. The \textit{crf3crf6} double and \textit{crf2crf3crf6} triple mutants were generated from \textit{crf3-1} and \textit{crf6-S2 and crf2-2, crf3-1 and crf6-S2} respectively. Primers and T-DNA accession numbers are listed in Supplementary Table 2. Seeds of \textit{Arabidopsis} (accession Columbia-0) were plated and grown on square plates with solid half-strength Murashige and Skoog (MS) medium supplemented with 0.5 g/L MES, 10 g/L Suc, and 0.8\% agar. The plates were incubated at 4°C for 48 h to synchronize seed germination and then grown vertically in growth chambers under a 16-h/8-h day/night cycle photoperiod at 18°C.

\textbf{Cloning and generation of transgenic lines.} For promoter analysis of \textit{PIN1} and \textit{PIN7}, particular promoter fragments were amplified by PCR and cloned into the \textit{pGEM-T} vector. The used primers contained unique restriction sites: PstI-sense and BamHI-antisense for \textit{PIN1} and Sall-sense and BamHI-antisense for \textit{PIN7}, allowing digestion and subsequent cloning into the \textit{pGREEN} binary vector. The resulting constructs contained transcriptional fusions between the
PIN1 or PIN7 promoter variants and the enhanced green fluorescent protein (EGFP) with a nuclear localization signal (NLS). Primers used for cloning are listed in Supplementary Table 1. The translational fusion ΔPIN1::PIN1-GFP was obtained by modifying PIN1::PIN1-GFP (in pBINPLUS vector backbone) as follows: the PIN1 promoter sequence from -258 to -2320 relative to the initiating ATG was removed by XbaI digestion and replaced by the PIN1 promoter sequence spanning the -258 to -1212 region. ΔPIN7::PIN7-GFP was derived from PIN7::PIN7-GFP in pBINPLUS by removal of the EcoRI fragment. The truncated promoter construct contained 1141 bp upstream of the translational start site. Expression plasmids were generated by standard molecular biology protocols and Gateway technology (Invitrogen). ORFs were amplified from a cDNA template with Pfu DNA Polymerase (Promega) and fused to the Gateway attB sites by PCR. pDONR221 and p4-p1r were used as ENTRY vectors. The structure and sequence of all destination vectors were as described and are available online at http://www.psb.ugent.be/gateway/ or otherwise referenced. 35S::CRF3 and 35S::CRF6 were obtained by cloning the ORFs of CRF3 and of CRF6 into destination vectors pK7WG2.0 and pK7WG2D, respectively. Overexpression of these lines was confirmed by qPCR, primers are included in Supplementary Table 1. The CRF3 and CRF6 (2-kb) promoters were cloned in pMK7S*NFm14GW, generating the ProCRF3:NLS-GFP-GUS and ProCRF6:NLS-GFP-GUS constructs (transcriptional fusions between the promoters and the gene encoding the EGFP-GUS fusion protein), respectively. The CRF2::CRF2-GFP fusion, used for the ChIP experiments, is a CRF2 promoter fragment (2 kb upstream of the coding sequence of CRF2) cloned into the PgreenIIK vector, resulting in a fusion with the NLS and EGFP. All transgenic plants were generated by the floral dip method. At least two independent transgenic lines were examined for expression pattern.

Quantitative RT-PCR. RNA was extracted with the RNasy kit (Qiagen) from excised root tips of 7-day-old root sample. A DNase treatment with the RNase-free DNase Set (Qiagen) was carried out for 15 minutes at 25°C. Poly(dT) cDNA was prepared from 1 μg of total RNA with the iScript™cDNA Synthesis Kit (Biorad) and analyzed on a LightCycler 480 (Roche Diagnostics) with the SYBR GREEN I Master kit (Roche Diagnostics) according to the manufacturer’s instructions. Targets were quantified with specific primer pairs designed with the Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA). All PCRs were performed
in triplicate. Expression levels were first normalized to ACTIN2 expression levels and then to the respective expression levels in the wild type. The primers used to quantify gene expression levels are listed in Supplementary Table 1.

**Phenotypic analysis.** For the phenotypic analysis of the root parameters (root length and root meristem size) and the lateral root primordia development, 10-20 seedlings of 7- and 10-day-old seedlings were processed as described\(^{49}\). The cytokinin concentrations were adapted to the experimental set up. Typically, to examine root growth response to cytokinin, low 0.25, 0.5 and 0.1 \(\mu\text{M}\) cytokinin concentrations were applied. The cytokinin impact on PIN expression was examined 8 hours after treatment with cytokinin, therefore higher 2 and 5 \(\mu\text{M}\) concentrations were applied. For the analysis of the root growth kinetics, seedlings were recorded every day for 14 days with an EOS035 Canon Rebel Xti camera. Long-term root growth observations for 28 days were performed on Petri dishes of 245 per 245 mm size. All data were analyzed with the ImageJ software (NIH; http://rsb.info.nih.gov/ij) as described\(^{11}\).

**Histochemical and histological analysis.** Seedlings were stained to detect GUS expression and cleared as previously described\(^{49}\). All samples were analyzed by differential interference contrast microscopy (Olympus BX51). Embryos were labelled by immunofluorescence as described\(^{50}\). Primary rabbit anti-GFP and rabbit anti-PIN1 antibodies were diluted 1:600 and 1:400, respectively, and secondary anti-rabbit-Alexa 488 and anti-rabbit Alexa 546 antibodies were diluted 1:600 in 3% bovine serum albumin (BSA) in phosphate buffered saline. Whole-mount root immunolocalization was performed using an automated system (Intavis in situ pro) as previously described\(^{50}\) using anti-PIN1 antibody diluted 1:1000 and CY3-conjugated anti-rabbit antibody (Sigma) diluted 1:600. Live-imaging was done with a confocal laser scanning microscopy (LSM 510, Zeiss). Images were analyzed with the LSM Image Browser (Zeiss).

**Confocal imaging and image analysis.** Confocal microscopy images were obtained with the Zeiss LSM 510, Zeiss LSM 710, or Olympus FV10 ASW confocal scanning microscopes using either 20x or 60x (water immersion) objectives. Fluorescence signals were detected for GFP (excitation 488 nm and emission 507 nm) and propidium iodide (excitation 536 nm and emission 617 nm). Development of lateral root primordia was followed in real time as described\(^{19}\). To
evaluate relative \textit{PIN} expression levels, the GFP signal in root meristems was measured on membranes of pericycle and endodermal cells adjacent to the quiescent center. Fluorescence intensities of the PIN-GFP membrane signals were quantified with ImageJ (NIH; http://rsb.info.nih.gov/ij) as described\textsuperscript{51}. 10-15 seedlings were used for analysis. The statistical significance was evaluated with the Student’s t-test.

\textbf{Auxin accumulation in the root tip of \textit{crf3crf6} double mutants.} Root tips (2-mm segments, about 100 mg) of 6-day-old \textit{Arabidopsis thaliana} (L.) Heynh. seedlings grown on vertical plates were separated and collected in 300 µl Bieleskis solution and homogenized. After overnight extraction at -20°C, the tissues were separated by centrifugation (15,000 g) and extracts were evaporated to dryness. Free IAA determination was done as described\textsuperscript{52}.

\textbf{Yeast one-hybrid screen.} The yeast strain YM4271 and destination vectors \textit{pDEST-MW1} and \textit{pDEST- MW2} have been previously described\textsuperscript{53}. Yeast reporter strains were designed as described\textsuperscript{53}. For the Y1H cDNA library screen, the 200-bp promoter fragments \textit{PCRE1} and \textit{PCRE7} were cloned into the destination vectors \textit{pDEST-MW1} and \textit{pDEST- MW2}, respectively, by Gateway cloning (for primers sequences see Supplementary Table 1). The DNA baits were integrated into yeast using the high efficiency transformation protocol according to the Yeast Protocol Handbook (Clontech) except that 1 µg of linearized plasmid DNA was added to the competent yeast, the heat shock period at 42°C was extended to 20 min and the cells were resuspended in 150 µl TE buffer. The cDNA Y1H library screen was performed with a REGIA and REGULATORS (RR) collection, previously described\textsuperscript{22}. For the transformation of one TF, 20 µl of competent yeast, 2 µl of carrier DNA, 100 ng plasmid (TF) DNA and 100 µl of TE/LiAC/PEG were used. Yeast cells were resuspended in 20 µl TE buffer and spotted on SD-His-Ura-Trp medium. After 3 days of growing, replica plates were made with 0, 15 and 30 mM 3-aminotriazole and positive clones were selected after 6 to 8 days of incubation at 30°C.

\textbf{Transient expression in \textit{Arabidopsis} mesophyll protoplasts.} Mesophyll protoplasts were isolated from rosette leaves of 4-week-old \textit{Arabidopsis} plants grown in soil under controlled environmental conditions in a 16-h/8-h light/dark cycle or under continuous light at 21°C. Protoplasts were isolated and transient expression assays were carried out as described\textsuperscript{54} with
modifications\textsuperscript{55}. Protoplasts were co-transfected with 20 μg of a reporter plasmid that contained \textit{fLUC}, a reporter gene driven by the corresponding promoter, 2 μg of normalization plasmid expressing the \textit{Renilla luciferase (rLUC)} under the control of the 35S promoter, and 20 μg of the effector construct. For the reporter constructs, the \textit{pEN-L4-Pro-R1} vector (with Pro representing \textit{PIN1::LUC}, \textit{ΔPIN1::LUC}, \textit{PIN7::LUC}, and \textit{ΔPIN7::LUC}) was recombined together with \textit{pEN-L1-fLUC-L2} by Multisite Gateway LR cloning with \textit{pm42GW736}. For the effector constructs, the \textit{pEN-L1-ORF-R2} plasmids (with the ORF of \textit{CRF2}, \textit{CRF3}, or \textit{CRF6}) were used to introduce the ORFs by Gateway LR cloning into \textit{p2GW7} for overexpression. The total amount of DNA was equalized in each experiment with the \textit{p2GW7-GUS} mock effector plasmid. After transfection, protoplasts were incubated overnight and then lysed; \textit{fLUC} and \textit{rLUC} activities were determined with the Dual-Luciferase reporter assay system (Promega). Variations in transfection efficiency and technical errors were corrected by normalization of \textit{fLUC} by the \textit{rLUC} activities. The mean value was calculated from three measurements and each experiment was repeated at least three times.

**Transient expression in \textit{Arabidopsis} root suspension culture protoplasts.**

The luciferase assays were performed from 3-days old \textit{Arabidopsis} root suspension culture by PEG mediated transformation. Protoplasts were isolated in enzyme solution (1% cellulose; Yakult, 0.2% Macerozyme; Yakult in B5-0.34M glucose-mannitol solution; 2.2 g MS with vitamins, 15.25 g glucose, 15.25 g mannitol, H2O to 500 mL pH to 5.5 with KOH) with slight shaking for 3-4 hours, centrifuged at 800g for 5 minutes. The pellet was washed with B5-0.34M glucose mannitol solution and resuspended in B5-0.34M glucose mannitol solution to a final concentration of 2 x105 per 50 μL. 2μg of reporter and effector plasmid DNAs were gently mixed together with 50 μL of protoplast suspension and 60 μL of PEG solution (0.1 M Ca(NO3)\textsubscript{2}, 0.45 M Mannitol, 25% PEG 6000) and incubated in the dark for 10 minutes. Then 140 μL of 0.275M Ca(NO3)\textsubscript{2} solution was added to wash off PEG, centrifuged at 800g for 5 minutes and supernatant was removed. The protoplast pellet was resuspended in 200 μL of B5-0.34M glucose mannitol solution and incubated for 16h in the dark at room temperature. The luciferase assays were performed using a Dual-Luciferase\textsuperscript{®} Reporter Assay System (Promega, Madison, WI, USA) as described for mesophyll protoplasts above. For cloning of the \textit{PIN2wt:LUC} construct, 1.5kb fragment of the \textit{PIN2} promoter upstream from the translational
start was PCR amplified from the genomic DNA using SalI-Fw and NcoI-Rv primers and the PCR product was subsequently cloned as SalI+NcoI fragment into the pGreen008-II-Luc vector in frame with the coding sequence of the LUC gene.

For introducing PCRE7 motives mutagenesis by PCR-driven overlap extension technique was used as described previously in. Briefly, intermediate primers containing PCRE7 motives were designed with complementary ends and PCRs were performed using following primer combinations (listed in Supplementary Table1): For introducing PCRE7-1 motif, SalI-Fw primer in combination with PCRE7-1-Rv and NcoI-Rv primer in combination with PCRE7-1-Fw were used and PIN2wt promoter DNA was used as template. The two PCRs products were purified and combined in equal concentrations and were subsequently used as template in the extension PCR round to get a full length PIN2 promoter with PCRE7-1 motif. Similarly for introducing PCRE7-3, SalI-Fw primer in combination with PCRE7-3-Rv and NcoI-Rv primer in combination with PCRE7-3-Fw were used, in this case and PIN2 promoter with PCRE7-1 motif was used as template. The two PCRs products were purified and combined in equal concentrations and were subsequently used as template in the extension PCR round to get a full length PIN2 promoter with PCRE7-3 motif. The PCR products were subsequently cloned into as SalI+NcoI fragment into the pGreen008-II-Luc vector.

**Chromatin immunoprecipitation assay.** ChIP experiments were done as described with minor modifications. One gram of tissue from 8-day-old plants was harvested and immersed in 1% formaldehyde under vacuum for 10 min. Glycine was added to a final concentration of 0.125 M and incubation was continued for 5 min. After washing, the nuclei were isolated and cross-linked DNA/protein complexes were fragmented by sonication with a Bioruptor sonicator (Diagenode), resulting in fragments of approximately 500 bp. After centrifugation (at 500 g), the supernatant was precleared with 80 μl of sheared salmon sperm DNA and protein A agarose (Millipore), of which 10 μl was used as input and the remainder was divided into three samples. To two samples (IP1 and IP2), 25μl GFP-Trap®_A coupled to agarose beads (Chromotec) was added, whereas to the third sample, which served as IgG control, an equal volume of nonspecific control serum was added, consisting of sonicated salmon sperm DNA, BSA, and Protein A (Salmon Sperm DNA/Protein-A agarose-50% slurry; Millipore). The samples were incubated overnight and immunoprecipitates were subsequently eluted from the beads. All centrifugation
steps with bead-containing samples were done at 500 g. Proteins were de-cross-linked and DNA was purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Pellets were resuspended in MiliQ water. The concentration of ChIP DNA was measured with the Quant-iT double-stranded DNA HS assay kit (Invitrogen). The SYBR Green I Master kit (Roche Diagnostics) was used for all quantitative PCRs. ACTIN2 and promoter regions of PIN1 (433-512 bp upstream of the start codon) and PIN7 (357-553 upstream of the ATG) were utilized as negative controls. All primer sequences, including those for PCRE1 and PCRE7, as well as primers used for identification of CRF motif are listed in Supplementary Table 1. To analyze the ChIP enrichment from quantitative PCR data, the Percent Input Method and Fold Enrichment Method were used.

Each ChIP DNA fractions’ Ct value was normalized to the Input DNA fraction Ct value for the same qPCR Assay (ΔCt). ΔCt [normalized ChIP] = (Ct [ChIP] - (Ct [Input] - Log2 (Input Dilution Factor))). In which Input Dilution Factor (Fd) = 1/100 (fraction of the input chromatin saved). The average of normalized ChIP Ct values for replicate samples was calculated. Percent input was then calculated as: % Input = 2 (-ΔCt [normalized ChIP]). The normalized ChIP fraction Ct value was adjusted for the normalized background (IgG) fraction Ct value (first ΔΔCt). ΔΔCt [ChIP/IgG] = ΔCt [normalized ChIP] - ΔCt [normalized IgG]. IP Fold Enrichment above the sample specific background was calculated as linear conversion of the first ΔΔCt: Fold Enrichment = 2(-ΔΔCt [ChIP/IgG]). Standard deviations were calculated for IP1 and IP2 as ln(2)*dSD*FC and for IgG as ln(2)*dSD, with FC the fold change. ChIP data were obtained from single experiments, but similar data were acquired from three independent experiments.
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REFERENCES


**FIGURE LEGENDS**

**Figure 1:** Truncation of *PIN7* promoter results in cytokinin-insensitive *PIN7* transcription. (a–e) Expression of *GFP* (a,b) and *PIN7-GFP* (c,d) is upregulated in response to cytokinin when driven by the full *PIN7* (a,c), but not the truncated Δ*PIN7* (b,d) promoter. Green: nuclear-localized *GFP* (a,b); membrane-localized *PIN7-GFP* (c,d). A semi-quantitative color-coded heat-map of the GFP fluorescence intensity is provided. Quantification of *PIN7-GFP* in the provasculature of primary roots (e). Roots of 7-day-old seedlings (n=15) were treated with control Murashige and Skoog (MS) medium with or without 5 μM of the cytokinin (CK) N6-benzyladenine for 8 h. Student's t-test (**p<0.001, n=15**). (f–j) Seedlings expressing Δ*PIN7::PIN7-GFP* in the *pin7* background exhibit enhanced root growth (f,h) and reduced cytokinin sensitivity of primary root growth (f,g,h), root meristem (RM) size (i) and lateral root initiation (j). Seedlings were grown for 28 (f,g), 14 (h), and 7 days (h,i,j) on MS medium with or without following cytokinins: 0.025 μM N6-benzyladenine (f–h); 0.025, 0.05 and 0.1 μM N6-benzyladenine (i) and 0.025 and 0.05 μM N6-benzyladenine (j). Student's t-test (*p<0.05; **p<0.01; ***p<0.001 (h,j); or *p<0.01; **p<0.001; ***p<0.0001 (i); n=10–15). Δ*PIN7::PIN7-GFP_1* and Δ*PIN7::PIN7-GFP_2* represent two independent transgenic lines
crossed into the pin7 mutant background. Error bars represent standard error. Scale bars, 20 μm (a-d), 2 cm (f-g).

**Figure 2: Cytokinin response factors (CRFs) interact with PCREs.** (a, b) CRF2, CRF3 and CRF6 interaction with PCRE7 (a) and PCRE1 (b) results in HIS3 reporter activation in a Y1H assay in contrast to AP2-79 that does not interact. Yeast cells were grown on SD-H-U-T minimal media without histidin (H), uracil (U) and tryptophan (T), supplemented with 3- amino-1,2,4-triazole (3AT). (c, d) Interaction of CRF2 (c) and CRF6 (d) with PCRE1 and PCRE7 detected by chromatin immunoprecipitation (ChIP). Chromatin immunoprecipitated with anti-GFP antibody is enriched in the PCRE1 and PCRE7 region. No enrichment was detected with PIN7 [-553-357] and PIN1 [-512-433] elements. (e-f) Significantly upregulated expression of PIN1::LUC and PIN7::LUC, by co-expression with CRF2 (e) and CRF6 (f) in contrast to their truncated counterparts (ΔPIN1::LUC, ΔPIN7::LUC) in Arabidopsis mesophyll protoplasts, Student's t-test (*p<0.01, n=3). (g) ChIP of CRF6-GFP reveals a significantly higher enrichment for regions either containing (P1_2) or directly neighboring (P1_3) the AGCAGAC motif when compared to more distant regions in PCRE1 (P1_1 and P1_4) (** p<0.0001, n=3) (h). CRF6 increases expression of PIN2 promoter containing three G2G5C7 motifs in Arabidopsis root cell suspension protoplasts (*p<0.01, n=5) (h). Error bars represent standard error (protoplast assay) and standard deviation (ChIP).

**Figure 3: Expression of PIN genes is altered in CRF-overexpressing lines.** (a,b) PIN7::PIN7-GFP expression is upregulated in the provasculature of CRF2 and CRF6, but downregulated in CRF3-overexpressing lines. (c,d) ΔPIN7::PIN7-GFP is not upregulated in the CRF2-overexpressing line in either absence or presence of cytokinin (5 μM N6- benzyladenine for 5 h). The membrane PIN7-GFP signal was quantified in the pericycle (b,d). Student's t-test (*p<0.05, **p<0.01; ***p<0.001, n =15-20). (e) PIN7 expression in lines overexpressing CRFs monitored by qRT-PCR, no expression detected in pin7 mutant (*p<0.05; ***p<0.0001, n = 3). Error bars represent standard error. Seven-day-old seedlings were analyzed.

**Figure 4: Crf mutants exhibit defective embryogenesis and root development.** (a) Auxin accumulation in the root tip of crf3crf6 mutants. (b-i) Abnormal divisions of upper suspensor
cells, embryo proper, and occasionally double embryos were observed in embryos of *crf2* (c), *crf3* (f) and *crf3crf6* (d,g,h) loss-of-function mutants when compared with controls (b,e,i). (j-n) Lack of *crf2*, *crf3*, *crf6* as well as *crf3crf6* and *crf2crf3crf6* function affects root length (j,l), root meristem size (k,m), and lateral root primordia density (n) and their response to cytokinin. (o-r) Constitutive expression of *CRF2*, *CRF3* and *CRF6* affects root growth (o), root meristem size (p) and lateral root initiation density (r) and their response to cytokinin. Seedlings were grown for 7 days on control MS medium with or without 0.05 μM of the cytokinin (CK) N6-benzyladenine (l-r). Student's t-test (*p<0.05; **p<0.01; ***p<0.001; n=10-15). Arrows indicate the distance between the quiescent center (QC) and the cortex transition zone (k). Error bars represent standard error. Scale bars, (j) 2 cm, (k) 20 μm.