Fine-tuning of auxin homeostasis governs the transition from floral stem cell maintenance to gynoecium formation

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To ensure successful plant reproduction and crop production, the spatial and temporal control of the termination of the floral meristem must be coordinated. In Arabidopsis, the timing of this termination is determined by AGAMOUS (AG). Following its termination, the floral meristem undergoes gynoecium formation. A direct target of AG, CRABS CLAW (CRC), is involved in both floral meristem determinacy and gynoecium development. However, how floral meristem termination is coordinated with gynoecium formation is not understood. Here, we identify a mechanistic link between floral meristem termination and gynoecium development through fine-tuning of auxin homeostasis by CRC. CRC controls auxin homeostasis in the medial region of the developing gynoecium to generate proper auxin maxima. This regulation partially occurs via direct transcriptional repression of TORNADO2 (TRN2) by CRC. Plasma membrane-localized TRN2 modulates auxin homeostasis. We propose a model describing how regulation of auxin homeostasis mediates the transition from floral meristem termination to gynoecium development.
The inflorescence meristem undergoes two balanced, antagonistic processes: self-renewal of stem cells to maintain their proliferation activity and recruitment of peripheral cells for organogenesis to form flowers\(^1\)–\(^3\). These two mechanisms occur continuously during the reproductive phase. Unlike indeterminate vegetative and inflorescence meristems, floral meristems exhibit a dynamic balance between cell proliferation and organ initiation. Floral meristems sequentially form four types of floral organs without undergoing elongation of the internode between floral organs. Floral meristems are genetically programmed to be terminated at a specific developmental stage after floral organ formation\(^4,5\). Thus, floral meristems are determinate. This precise termination process allows floral meristems to form a female reproductive structure referred to as a gynoecium\(^6,7\).

**Fig. 1** Indeterminate phenotype of the crc knu double mutant is caused by WUS activity. **a–d** The floral meristem activity marker WUS mRNA in longitudinal sections of wild-type (a) crc-1 (b) knu-1 (c), and crc-1 knu-1 (d) floral buds at developmental stage 6. **e–h** Flower phenotypes in wus-1 (e), wus-1 crc-1 (f), wus-1 knu-1 (g), and wus-1 crc-1 knu-1 (h). **i, j** Morphology of crc-1 knu-1 (i) and wus-1 –/+ crc-1 knu-1 (j) fruits. Top: close-up views of fruit tips. Bottom: shapes of whole fruits. Arrowheads indicate stigma structures. Two stigma-like structures were observed in the wus-1 –/+ crc-1 knu-1 mutant due to carpel separation. Images are shown at the same magnification. **k** Quantification of mutant phenotype. \(p\)-values were calculated by \(\chi^2\) test \((n = 30)\). **l, m** Longitudinal sections of crc-1 knu-1 (l) and wus-1 –/+ crc-1 knu-1 (m) fruits. Asterisks indicate ectopic carpels. **n, o** Scanning electron micrographs of fruits with reiterations of floral organs in crc-1 knu-1 (n) and wus-1 –/+ crc-1 knu-1 (o) fruits. Bars = 50 \(\mu\)m in **a–d**; 5 mm in **e–h**; 1 cm in **i, j** bottom; 500 \(\mu\)m in **l, m**; and 1 mm in **n, o**.
The transcriptional regulatory cascade underlying the termination of floral meristems has been characterized in the model plant Arabidopsis thaliana. Floral meristem termination must be completed during stage 6 of flower development when differential gynoecium growth along the medial-lateral and adaxial-abaxial axis has led to central invagination. The C class gene AGAMOUS (AG) plays a central role in this termination process, as well as the establishment of reproductive organ identity. AG turns off the stem cell maintenance program by repressing the expression of the key meristem maintenance gene WUSCHEL (WUS) during stage 6 of flower development in a spatio-temporal-specific manner. WUS expression begins to decline when AG is expressed.
activated\textsuperscript{13,14}. In ag mutants, WUS expression remains active beyond stage 6, which is sufficient to induce floral meristem indeterminacy\textsuperscript{13,16}. Temporal WUS repression by AG is controlled by two different pathways. AG is necessary to recruit Polycomb Group protein (PcG) to methylate histone H3K27 at the WUS locus\textsuperscript{17}. In addition, WUS repression by AG occurs at least partially through the C2H2 zinc-finger protein KNUCKLES (KNU)\textsuperscript{17}. In knu mutants, obvious WUS expression continues after stage 6 in the developing gynoecium\textsuperscript{15}. AG directly activates KNU, a process that requires eviction of the PcG complex, leading to cell cycle-dependent KNU induction\textsuperscript{18,19}. This precise control of KNU activation helps determine the timing of floral meristem termination.

After termination of floral meristems, cells located at the center of stage 6 flowers become gynoecium, which consists of two fused carpels\textsuperscript{20–22}. Previous studies demonstrated that auxin homeostasis contributes to the generation of developmentally important auxin maxima. Not only the well-known PIN-FORMED family of auxin carriers, but also other membrane proteins affect auxin maxima\textsuperscript{20,21}. For example, TORNADO2 (TRN2) encodes a transmembrane protein of the tetraspanin family, the loss-of-function mutant of which shows aberrant auxin maxima in leaves\textsuperscript{22–25}. In flowers, TRN2 is expressed in stigma at flowers\textsuperscript{22,24,25}. Despite the critical importance of the local action of auxin in gynoecium development, its possible function when floral meristem is terminated is not known.

In this study, we obtained insights into a KNU-independent floral meristem termination pathway downstream of AG, which is coordinated by an AG direct target, CRABS CLAW (CRC)\textsuperscript{26–31}. Previous findings reported that crc knu double mutant shows strong indeterminacy in the floral meristem\textsuperscript{31} and crc mutant phenotype is partially rescued by an auxin transport inhibitor\textsuperscript{32}. However, a mechanistic link between floral meristem termination and auxin-mediated gynoecium formation is not known. Here, we found that two direct AG target genes, KNU and CRC, synergistically regulate WUS repression. We further show that CRC represses the expression of TRN2 and controls floral meristem determinacy. Our results highlight the molecular framework underlying the AG-mediated termination of floral meristems through auxin, which is required for subsequent gynoecium development.

**Results**

**Indeterminacy in crc knu is caused by WUS misexpression.** Although CRC is a direct target of AG at stage 6 of flower development\textsuperscript{11,27} (Supplementary Fig. 1), the crc single mutant has only a subtle or no indeterminate floral meristem phenotype\textsuperscript{26}. Double mutants in genetic backgrounds with reduced AG expression or with mutations in the downstream target genes of AG show enhanced indeterminacy\textsuperscript{29–31}. Because the knu single mutant exhibits an indeterminate phenotype\textsuperscript{17}, this mutant provides a sensitized background for studying the role of CRC in floral meristem termination (Supplementary Fig. 2). Before conducting a detailed molecular analysis, we confirmed the previously reported aberrant phenotypes of the crc knu double mutant and parental lines\textsuperscript{34} (Supplementary Fig. 2a–e). Compared to the wild type, style tissue is reduced in the crc-1 mutant, leading to the presence of two unfused carpels at the apical region and two separate stigmas (p = 9.5 × 10\textsuperscript{−13}) (Supplementary Fig. 2a, b, e). Wild-type and crc-1 mutant fruits contain developing seeds enclosed by carpels (Supplementary Fig. 2a, b). By contrast, the knu mutants have bulged gynoecia surrounding additional whorls of floral organs (Supplementary Fig. 2c, f). crc-1 knu-1 double mutant flowers exhibit synergistic indeterminate phenotypes (p = 9.4 × 10\textsuperscript{−14}) (Supplementary Fig. 2d, e, g). Although a few fruits showed a weak phenotype without internode elongation, as shown in Supplementary Fig. 2h ( categorized as weak crc knu), most crc knu fruits showed over-growth of the indeterminate shoot phenotype ( categorized as strong crc knu) (Supplementary Fig. 2d, e, g, i). This ectopic floral internode axis continued to elongate, with approximately ten rounds of carpel production (Supplementary Fig. 3a) and stamens occasionally appearing in a spiral pattern (Supplementary Fig. 2i, inset). Due to these phenotypic variations, we divided the mutant phenotypes into five categories based on their phenotypic severity and quantified the phenotypes in this and subsequent analyses (Supplementary Fig. 2c).

To address the role of CRC in floral meristem activity, we monitored the expression of WUS (Fig. 1a–d and Supplementary Fig. 3b–e). In stage 6 flowers, WUS expression was terminated in wild-type and crc-1 plants (Fig. 1a, b). In the knu-1 single mutant, weak but distinct WUS signals were detected in the centers of stage 6 and later flowers\textsuperscript{18} (Fig. 1a–c and Supplementary Fig. 3b–d). A strong increase in WUS messenger RNA (mRNA) levels was detected in the crc-1 knu-1 (Fig. 1a–d and Supplementary Fig. 3c–e).

To investigate whether the observed phenotype depends on the WUS activity, we next created wus-1 crc-1 knu-1 triple mutants to remove WUS activity from the crc knu double mutant. The triple mutant flowers were indistinguishable from those of the wus-1 single mutant (Fig. 1e–h). These results indicate that wus is epistatic to crc or knu in floral meristems. Furthermore, indeterminacy of the wus-1 →/+ crc-1 knu-1 mutant was significantly rescued compared to the crc-1 knu-1 double mutant (p = 4.3 × 10\textsuperscript{−8}) (Fig. 1i–o), suggesting that the indeterminate phenotype of the crc knu mutant is caused by ectopic WUS expression.

**CRC controls auxin homeostasis and establishes auxin maxima.** Since synergistic effects are observed in crc knu double mutants, disruption of multiple pathways and drastic transcriptional changes are thought to occur in double mutants, but not in...
single mutants. To explore the molecular basis for the CRC downstream pathway that affects WUS expression, we performed expression profiling of knu-1 and crc-1 knu-1. This analysis identified 363 genes whose expression levels were altered more than 2-fold at a 99% confidence level in the crc-1 knu-1 double mutant relative to the knu-1 single mutant (Supplementary Data 1). Among those genes, 210 and 153 genes were up- and downregulated in crc knu, respectively (Supplementary Fig. 4a and Supplementary Data 1).

To investigate the functions of these genes, we performed Gene Ontology (GO) term enrichment analysis using agriGO\textsuperscript{33}. GO terms related to numerous metabolic processes were significantly

\[ \text{Percentage (\%)} = \frac{\text{Number of genes}}{\text{Total number of genes}} \times 100 \]

\[ p = 2.2 \times 10^{-14} \]

\[ p = 6.8 \times 10^{-7} \]

WT-like

crc-like

knu-like

Strong crc knu-like

Weak crc knu-like

\[ \text{Relative WUS} = \frac{\text{WUS expression in treated samples}}{\text{WUS expression in control samples}} \]

\[ p = 2.2 \times 10^{-14} \]

\[ p = 6.8 \times 10^{-7} \]

Mock IAM Mock IAM

Mock IAM Mock IAM

Mock IAM Mock IAM

Mock IAM Mock IAM

Mock IAM Mock IAM

Mock IAM Mock IAM
enriched in crc knu (p < 0.0002, FDR <0.032), and 123 up- or downregulated genes were involved in ‘metabolic processes’ (Fig. 2a, Supplementary Fig. 4b and Supplementary Data 1 and 2). To further determine which of the 125 metabolic genes regulate floral meristem determinacy, we determined which genes contained ‘meristem’ in their GO terms (Fig. 2b, Supplementary Data 2 and Table 1). Among these six genes, two encoded chromatin regulators, CURLY LEAF (CLF) and FASCIA2 (FAS) (Fig. 2b). A ribosomal protein (EMBRYO DEFECTIVE 3105 [EMB3105]), an actin-binding protein (NETWORKED 1A [NET1A]), a HEAT SHOCK PROTEIN 90-like protein (SHEPHERD [SHD]), and a membrane protein (TRN2) were also identified as possible CRC downstream targets (Fig. 2b). Among these six genes, EMB3105 and TRN2 contained ‘auxin polar transport’ in their GO terms (Fig. 2b and Supplementary Table 1). This finding is not unexpected, since metabolic pathways are essential for producing secondary metabolites, including plant hormones. Thus, we hypothesized that a previously hypothesized non-cell-autonomous signal downstream of CRC may be auxin.

The style and stigma phenotypes observed in the crc mutant were reportedly rescued by the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) treatment (p = 3.4 × 10⁻⁶), while wild-type and knu flowers were not significantly affected (Supplementary Figs. 5a–c and 6a–d). Similar effects were observed when we used 1-naphthoxyacetic acid (1-NOA), which inhibits auxin influx (p = 3.1 × 10⁻⁷) (Supplementary Fig. 5d–f). This rescue was not observed when we treated the crc mutant with the auxin signaling inhibitor p-chloro phenoxysubertic acid (PCIB) (Supplementary Fig. 5g–i). To further prove the biological importance of the CRC-mediated role of auxin transport in floral meristem determinacy, crc-1 knu-1 plants were treated by inhibitors. NPA and 1-NOA treatments also brought the crc knu double mutant back to the knu mutant phenotype in terms of floral meristem determinacy (NPA: p = 9.4 × 10⁻¹⁰, 1-NOA: p = 6.1 × 10⁻¹³), while PCIB did not (p = 0.31) (Fig. 2c–i and Supplementary Fig. 7a–f). All of the inner structures in NPA-treated crc-1 knu-1 plants were covered with two carpels, and the number of ectopic carpels was reduced, as observed in the knu-1 mutant (Fig. 2f–i and Supplementary Fig. 6). These results suggest that CRC controls auxin transport, but not signaling required for floral meristem termination.

We next observed the green fluorescent protein (GFP)-tagged PIN1 line (pPIN1::PIN1-GFP) to infer possible changes in auxin distribution. In wild-type floral buds at stage 6, PIN1-GFP signals appeared stronger on the medial domain of the gynoecium, which gives rise to the replum, style, and stigma (Fig. 2j). Basipetal PIN1 polarization caused auxin to move from the top to the bottom of the gynoecium at this stage (Fig. 2k and Supplementary Fig. 8a, b). Consistent with the slight adaxialization of carpels observed in the crc mutant based on ARGONAUTE10 (AGO10) and PHABULOSA (PHB) expression, PIN1 expression domain expanded laterally in the crc mutant, but no difference in its cellular localization was observed (Fig. 2j–m and Supplementary Fig. 8a–d). Furthermore, no obvious PIN1 expression and localization differences between the wild-type and crc knu mutants were observed at stage 5 flowers prior to floral meristem termination (Supplementary Fig. 8e, f). By stage 6, the PIN1 expression domain in crc knu was expanded as seen in the crc mutant (Supplementary Fig. 8g, h). Compared to the wild type, reduced expression of the auxin-response reporter DR5rev::3xVENUS was detected in the crc mutant at the medial domain of the gynoecium (Fig. 2n, o). Since NPA treatment in crc and crc knu led to increased DR5 signals, phenotypic changes observed in crc and crc knu treated with NPA are likely attributable to enhanced auxin maxima (Fig. 2o–r).

**Local auxin production rescues the indeterminacy in crc knu.** To investigate whether local overproduction of auxin is sufficient to bring crc knu back to knu mutant, we expressed the Agrobacterium indole acetamide (IAM) hydrolase (iaaH) gene under the control of the CRC promoter (pCRC::iaaH) in these mutants (Fig. 3a–i). Since iaaH catalyzes the biosynthesis of bioactive auxin (indole-3-acetic acid) from IAM, pCRC::iaaH transgenic plants overproduced auxin in the peripheral region of the floral meristem only when we supplied them with the precursor IAM. In the absence of IAM, the iaaH transgene did not affect mutant phenotypes in either the crc or crc knu mutant background (Fig. 3a, c, e, g, i, k). Overproduction of auxin by IAM application in the peripheral regions of developing carpels triggered significant rescue of crc mutant phenotypes (p = 2.2 × 10⁻¹⁴) (Fig. 3m). IAM-treated pCRC::iaaH fruits exhibited fused carpels (Fig. 3a, b, e, i, j). Likewise, pCRC::iaaH activation also restored the phenotype of the crc knu double mutant to that of knu in terms of floral meristem determinacy (p = 6.8 × 10⁻⁷) (Fig. 3d, h, l, m). Unlike crc knu double mutants, IAM-treated pCRC::iaaH crc knu plants did not show overgrowth of indeterminate shoots (Fig. 3c, d, g, h, k, l). By contrast, we did not detect any phenotypic differences in crc or crc knu treated with the optimal concentration of auxin chemicals (Supplementary Figs. 5j–r and 7g–o), suggesting that auxin maxima may be necessary for proper meristem determinacy. The strong WUS expression observed in crc-1 knu-1 double mutants was attenuated when auxin was locally overproduced (Fig. 3n). Taken together with the NPA treatment result, our findings uncover a possible link between local auxin homeostasis and floral meristem determinacy.

**CRC represses TRN2 expression via a YABBY-binding site.** Based on our expression profiling analysis, we identified two auxin-related targets acting downstream of CRC. Since the role of TRN2 in flower meristem determinacy is largely unknown and the biggest expression changes in crc knu were observed compared knu mutant among six candidate genes (Supplementary Fig. 9a–f), we focused on the TRN2 gene as a putative direct target of CRC. We first investigated whether CRC regulates TRN2 expression. TRN2 was highly expressed throughout crc-1 knu-1 double mutants but was attenuated when auxin was locally overproduced (Fig. 3n). Together with our NPA treatment result, our findings uncover a possible link between local auxin homeostasis and floral meristem determinacy.

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**Fig. 3** Rescue of crc knu indeterminacy by local overproduction of auxin. a–d Morphology of mock-treated crc-1 pCRC::iaaH (a), IAM-treated crc-1 pCRC::iaaH (b), mock-treated crc-1 knu-1 pCRC::iaaH (c), and IAM-treated crc-1 knu-1 pCRC::iaaH (d) fruits. Above: close-up views of fruit tips. Below: shapes of whole fruits. Arrowheads indicate stigma structures. Two stigma-like structures were observed in the mock-treated crc-1 pCRC::iaaH fruits due to carpel separation, while IAM treatment led to carpel fusion. e–h Longitudinal section of mock-treated crc-1 pCRC::iaaH (e), IAM-treated crc-1 pCRC::iaaH (f), mock-treated crc-1 knu-1 pCRC::iaaH (g), and IAM-treated crc-1 knu-1 pCRC::iaaH (h) fruits. Asterisks indicate carpels. i–l Scanning electron micrograph of mock-treated crc-1 pCRC::iaaH (i), IAM-treated crc-1 pCRC::iaaH (j), mock-treated crc-1 knu-1 pCRC::iaaH (k), and IAM-treated crc-1 knu-1 pCRC::iaaH (l) fruits. m Quantification of mutant phenotypes. Although IAM-treated plants had shorter styles than the controls, we categorized the mutants based on the presence or absence of two stigmatic structures. p-values were calculated by χ² test (n = 30). n mRNA abundance of the stem cell marker WUS, in crc-1 pCRC::iaaH and crc-1 knu-1 pCRC::iaaH flowers with or without IAM. Bars = 1 cm in a–d bottom; 500 μm in e–h; and 1 mm in i–l.

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(Fig. 4b, c and Supplementary Fig. 9g), this ectopic expression could be due to the presence of multiple transcriptional activators of TRN2 \(^{25}\), feedback regulation via the auxin pathway, and/or lack of mobile signals from YABBY gene activities \(^{46}\). Moreover, dexamethasone (DEX) activation of biologically functional and glucocorticoid receptor (GR)-tagged CR protein led to a rapid and reproducible decrease in TRN2 expression (Fig. 4a and Supplementary Fig. 10a). These results suggest that CRC represses TRN2 expression in stage 6 floral buds.

We next attempted to identify cis-regulatory elements important for TRN2 expression. By performing phylogenetic shadowing in four different Brassicaceae species, we identified...
three conserved regions in the TRN2 promoter (Fig. 4d). Among these regions, region I and III overlap with DNase hypersensitive sites, where transcription factors can bind. Region III contains a DNase I hypersensitive site only in flowers, where CRC is specifically expressed (Fig. 4d and Supplementary Fig. 10b). Closer inspection of region III revealed the presence of a previously identified potential YABBY-binding site (GA[A/G]AGAAA) (Fig. 4d, e and Supplementary Fig. 10c). Chromatin immunoprecipitation (ChIP) analysis using a Myc epitope-tagged CRC protein and floral tissues from flowers that were synchronized in development showed that CRC was specifically enriched at the region TIII containing a conserved site (Fig. 4f). This result suggests that CRC specifically binds to region III of the TRN2 promoter to repress its transcription in a flower-specific manner, although TRN2 expression in leaves or vasculature is likely regulated by other transcription factor(s) bound by region I of the TRN2 promoter.

To investigate whether the YABBY-binding site is necessary for TRN2 expression, we mutated this site in the TRN2 promoter, yielding pTRN2m (Fig. 4g-i and Supplementary Fig. 10d-g). We did not detect the β-glucuronidase (GUS) activity in developing carpels at stage 6 and the reporter was misexpressed when introduced into crc-1 (Fig. 4g and Supplementary Fig. 10b, i). pTRN2m::GUS was continuously expressed throughout two carpels of stage 6 flowers (Fig. 4g, h). Increases in pTRN2m::GUS signals were also detected in sepalts, most likely due to the activity of other YABBY family proteins (Fig. 4g, h). We analyzed the reporter constructs in an independent population of T1 plants (n ≥ 25) to minimize possible positional effects on transgene expression. Significantly more pTRN2m::GUS lines had stronger signals than pTRN2::GUS lines (p = 0.005) (Fig. 4i). These results suggest that a conserved YABBY-binding site is required for proper repression of TRN2 expression.

**trn2 mutation partially suppresses crc knu.** To examine whether the floral meristem indeterminacy phenotype in crc knu is caused by the ectopic expression of TRN2, we eliminated TRN2 activity in the crc knu double mutant (Fig. 5a–i). The trn2-1 mutant showed reduced TRN2 transcript levels with nonsense mutation and had very small and twisted fruits (Fig. 5a, d, g and Supplementary Fig. 11). Two carpels were properly fused in the trn2-1 mutant (Fig. 5a, d, g). The trn2-1 crc-1 knu-1 mutant exhibited reduced indeterminacy compared to the crc-1 knu-1 parental line (p = 2.2 × 10−6) (Fig. 5b, c, e, f, h, i, j).

We also investigated WUS expression in the triple mutant by both quantitative reverse transcription PCR (qRT-PCR) and in situ hybridization. Consistent with the phenotypic severity, removal of TRN2 activity from crc knu restored WUS mRNA levels to those of trn2-1 (Fig. 5k). While no WUS mRNA was detected in stage 6 trn2-1 flowers, WUS mRNA levels were very high in crc-1 knu-1 flowers at the same stage. In the trn2-1 crc-1 knu-1 triple mutant, compromised WUS levels were detected compared to crc knu (Fig. 5l–n). Taken together, these results indicate that WUS repression by CRC is partially dependent on the TRN2 activity.

**Misexpression of TRN2 in knu mimics crc knu.** To determine whether TRN2 misexpression is sufficient to mimic the crc mutant phenotype, we expressed TRN2 under the control of the CRC promoter (pCRC::TRN2) (Fig. 6a–d). pCRC::TRN2 fruits mimicked the crc mutant and no phenotypic difference was observed between pCRC::TRN2 and the crc-1 mutant (p = 0.15), while the pCRC::TRN2 phenotype differed significantly from the wild type (p = 4.3 × 10−3) (Fig. 6a, b, e). We then examined the contribution of TRN2 to the crc-1 knu-1 phenotype by transforming knu-1 plants with pCRC::TRN2. In contrast to the knu-1 parental line, the pCRC::TRN2 knu-1 plants formed crc knu double mutant-like abnormal fruits bearing extra carpels and stamens due to WUS misexpression (Fig. 6b, c, e, f, h, j–l).

To investigate the link between ectopic TRN2 expression and auxin homeostasis, we treated pCRC::TRN2 lines with NPA. As observed in NPA-treated crc-1 mutants, NPA-treated pCRC::TRN2 plants formed significantly more normal fruits than mock-treated plants (p = 2.3 × 10−7) (Supplementary Fig. 12a–d). Moreover, NPA treatment of pCRC::TRN2 knu-1 plants significantly rescued the iteration phenotype (p = 1.9 × 10−10) (Fig. 6d, e). Unlike pCRC::TRN2 knu-1 plants, NPA-treated pCRC::TRN2 knu-1 plants produced fused carpels (Fig. 6c–i). The number of ectopic carpels that formed inside of fruits was reduced, as observed in the knu-1 mutant (Fig. 6f, g and Supplementary Fig. 6c, d, g, h). In agreement with phenotypic results, qRT-PCR and in situ hybridization showed high levels of WUS expression in pCRC::TRN2 knu-1 plants, but NPA treatment of these plants led to reduced WUS expression (Fig. 6j–m and Supplementary Fig. 12e). These results suggest that ectopic TRN2 expression is sufficient to mimic the crc mutant phenotype.

**Plasma membrane-localized TRN2 modulates auxin homeostasis.** To investigate the function of TRN2, we examined the localization of this protein in reproductive tissues. The gTRN2-GFP transgene we generated rescued the trn2 mutant phenotypes (Supplementary Fig. 13a–f). TRN2-GFP was mainly detected in the shoot apical meristem and vasculature (Fig. 7a). Consistent with the finding that TRN2 encodes a transmembrane protein of the tetraspanin family, TRN2-GFP was localized to the plasma membrane (Fig. 7b–d and Supplementary Fig. 13g, h). We did not detect TRN2-GFP signals in stage 6 carpels, which mimicked the TRN2 promoter expression pattern (Figs. 4b, g and 7e). Compared to the wild type, TRN2-GFP protein levels were elevated in the carpel primordia of the crc floral buds at stage 6 (Fig. 7f).

We next examined PIN1-GFP expression and localization in the trn2 mutant background. In the wild type, PIN1-GFP was highly expressed in the medial domain of the gynoecium (Fig. 7g).
By contrast, PIN1-GFP expression was reduced in \textit{trn2}, while the PIN1-GFP expression domain was expanded in \textit{crc-1}, in which TRN2 is ectopically expressed (Figs. 2j, k and 7g, h). This reduction was not gynoecium-specific, as PIN1-GFP expression in \textit{trn2} inflorescences was lower than in the wild type (Supplementary Fig. 13i, j). We also quantified the transport of radiolabeled IAA in the \textit{trn2} mutant grown on MS plates (Supplementary Fig. 13k–n). This assay showed that \textit{trn2} had reduced IAA transport relative to the wild type (Fig. 7i) ($p = 7.6 \times 10^{-7}$).

Although it was important to ascertain possible roles of TRN2 in auxin transport using \textit{trn2} mutants, changes might be simply due to tissue composition issues. To further examine the role of TRN2 in auxin maxima, we compared the DR5 levels in wild-type and 35S::TRN2 cells, including both \textit{Arabidopsis} T87 cells and tobacco BY-2 cells. We did not observe any differences in terms of cell shape or morphology even if \textit{TRN2} was overexpressed in either cell. Regardless of the cells examined, signals from the DR5::3xVENUS were reduced in 35S::TRN2 compared to the wild type (Fig. 7j, k and Supplementary Fig. 13o, p). We also performed immunostaining with auxin antibody using \textit{Arabidopsis} protoplasts, which allows free auxin to be visualized49–51. Based on immunostaining, we detected fewer auxin foci in 35S::TRN2 protoplasts than in untransformed wild-type cells (Fig. 7l, m). Finally, misexpression of TRN2 under the control of the \textit{CRC} promoter led to reduced DR5 signals in the medial domain of the gynoecium as seen in \textit{crc} mutants (Fig. 7n, o). Taken together, our results suggest that TRN2 modulates auxin homeostasis.

**Discussion**

Here, we show that the direct AG target CRC controls auxin homeostasis in the developing gynoecium through the negative regulation of TRN2, thus preventing the overgrowth of floral meristems when \textit{KNU} is mutated (Fig. 7p). Two direct AG targets, \textit{CRC} and \textit{KNU}, synergistically regulate floral meristem termination, and mutations in these two genes, which act downstream of AG, disrupt the floral meristem determinacy. The confirmation of the striking, synergistic mutant phenotype observed in \textit{crc knu} sheds light on the importance of these key genes. Synergistic effects triggered by two non-homologous genes are thought to be caused by disruption of multiple or different pathways52. Furthermore, strong floral meristem indeterminacy in single \textit{crc} ortholog mutants has been reported in several other angiosperms40. The function of CRC identified in the current study may be conserved during evolution.

The AG-WUS and AG-KNU-WUS pathways play central roles in determining the timing of floral meristem termination13,18,19. Precisely how AG alters the activity of a restricted floral stem cell population was previously unknown. Our findings reveal a molecular framework for the spatial regulation of the floral meristem. CRC controls auxin homeostasis partially through TRN2 repression and generates proper auxin maxima to reduce the activity of the floral meristem for subsequent gynoecium formation. In wild-type floral buds at stage 6, auxin maxima as visualized by DR5 expression were observed in the medial domain of the gynoecium. In this domain, auxin maxima are established via regulation of auxin homeostasis, such as auxin transport and local auxin biosynthesis43. In \textit{crc} or \textit{crc knu} floral buds at stage 6, DR5 expression was greatly reduced. When auxin transport was blocked by auxin transport inhibitors, DR5 expression was recovered in \textit{crc} or \textit{crc knu} flowers. Not only NPA treatment, but also local auxin production by \textit{iaaH} rescued the short style defects in \textit{crc} and restored the phenotype of the \textit{crc knu} double mutant to that of the \textit{knu} mutant in terms of floral meristem determinacy (Supplementary Figs. 5 and 7). Since we could not identify any conditions that rescued one of these defects, we favor a model in which changes in auxin homeostasis in the medial regions (including tip of carpels) of the developing gynoecium cause both
style and floral meristem determinacy defects. CRC-mediated medial domain initiation through auxin homeostasis during stage 6 of flower development may both turn off floral meristem maintenance and trigger medial domain differentiation, resulting in the formation of the style and the normal tip. Although the pathway that functions downstream of auxin to repress \textit{WUS} expression is not yet fully understood, one such transcription factor in this pathway has been identified. \textit{AUXIN RESPONSE FACTOR3 (ARF3)}-mediated auxin perception controls numerous auxin-related events including gynoecium formation\textsuperscript{33–36}.

\textit{ARF3} directly binds to the proximal region of the \textit{WUS} promoter to repress its expression\textsuperscript{37}. Thus, \textit{ARF3} might act downstream of the CRC-TRN2 pathway (Fig. 7p).

For decades, molecular and genetic approaches have been used to identify factors that participate in gynoecium development\textsuperscript{12}. Numerous tissue-specific transcription factors have been identified\textsuperscript{38}. Despite the critical importance of gene regulatory networks regulated by these factors, the downstream target(s) that actually plays a key role in gynoecium formation is currently unknown. Auxin controls gynoecium development\textsuperscript{7}, but the

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**Fig. 6** Ectopic expression of TRN2 mimics the crc mutant phenotype. \textbf{a–d} Morphology of crc-1 (\textbf{a}), pCRC::TRN2 (\textbf{b}), pCRC::TRN2 knu-1 (\textbf{c}), and NPA-treated pCRC::TRN2 knu-1 (\textbf{d}) fruits. Top: close-up views of fruit tips. Bottom: shapes of whole fruits. Arrowheads indicate stigma structures. Two stigma-like structures were observed in the crc-1 mutant as well as pCRC::TRN2 due to carpel separation. Images are shown at the same magnification. \textbf{e} Quantification of mutant phenotypes. \textit{p}-values were calculated by \(\chi^2\) test (\(n = 30\)). \textbf{f–g} Longitudinal sections of mock-treated pCRC::TRN2 knu-1 (\textbf{f}) and NPA-treated pCRC::TRN2 knu-1 (\textbf{g}) fruits. Asterisks indicate carpels. \textbf{h–i} Scanning electron micrograph of pCRC::TRN2 knu-1 fruits with reiterations of floral organs. pCRC::TRN2 knu-1 (\textbf{h}) and NPA-treated pCRC::TRN2 knu-1 (\textbf{i}) fruits. \textbf{j–m} Floral meristem activity marker, \textit{WUS} mRNA, in longitudinal sections of crc-1 (\textbf{j}), pCRC::TRN2 (\textbf{k}), pCRC::TRN2 knu-1 (\textbf{l}), and NPA-treated pCRC::TRN2 knu-1 (\textbf{m}) floral buds at developmental stage 6. Images are shown at the same magnification. Bars = 1 cm in \textbf{a–d}; 500 \(\mu\)m in \textbf{f–g}; 1 mm in \textbf{h–i}; and 50 \(\mu\)m in \textbf{j–m}.
genes involved in auxin homeostasis are often expressed throughout the entire plant. Thus, auxin homeostasis must be fine-tuned in a tissue- or stage-specific manner. This study uncovers a molecular link between tissue-specific transcription factors and a gene controlling auxin maxima. AG-activated CRC represses TRN2, which at least partially controls floral meristem termination. TRN2 expression is correlated with the CRC reporter in the carpel primordia at stage 6 of flower development. Both TRN2 and PIN1 proteins were misexpressed in the crc mutant (Figs. 2j, k and 7e, f). In contrast, trn2-1 showed reduced PIN1 expression with compromised auxin transport activity (Fig. 7g–i). Future investigations are needed to understand how TRN2 controls auxin homeostasis. Although the crc mutant phenotype is only seen in flowers, the trn2 mutant shows a pleiotropic phenotype, such as twisted roots or leaves. Thus, CRC seems to act as a flower-specific repressor rather than a general repressor of TRN2 throughout the plant body. Furthermore, TRN2 was misexpressed throughout the carpel, while CRC is only expressed in the abaxial side of carpels. This misexpression could be due to feedback regulation through auxin homeostasis. Alternatives are lack of mobile signals derived from CRC activity, as non-cell-autonomous activities have been reported for all characterized YABBY genes. Since ETHYLENE RESPONSE FACTOR and PSEUDO-RESPONSE REGULATORY are expressed in carpels, and positively regulate TRN2 expression, those activators and CRC might act antagonistically to control TRN2 expression.

Since introducing the trn2 mutation into the crc knu double mutant led to only partial phenotypic rescue, TRN2 is not clearly the sole downstream target of CRC in the control of floral meristem termination. Such target(s) might be among the 365 misexpressed genes in crc knu compared to knu. In particular, the final six CRC candidate genes, which are most likely involved in floral meristem termination included SHEPHERD (SHD) and CURLY LEAF (CLF). Treatment with the auxin transport inhibitor rescued crc knu mutants more thoroughly than did introduction of the trn2 mutation. The different degree of rescue observed in the triple mutants suggests that an auxin-related...
target might exist that acts directly downstream of CRC and in parallel with TRN2 (Figs. 5 and 7). Because our GO term analysis identified many genes involved in metabolic processes (Fig. 2), it is also possible that CRC has more downstream targets that jointly control auxin transport and metabolism (biosynthesis, conjugation, and degradation) at multiple levels. Further introducing mutations in CRC candidate target genes into the trn2 crc knu triple mutant would provide insight into the importance of other CRC targets and the CRC downstream pathways for floral meristem determinacy.

Methods

Genetic stocks and growth conditions. All Arabidopsis thaliana seed stocks were in the Landsberg erecta (Ler) background or backcrossed into Ler three to four times. The cre-138, knu-117, wus-114, ag-1 35S:AG-GR96, ap1-1 cal-5 35S:API-GR96, pPIN1:PIN1-GFP41, and DR5rev:3xVENUS42 lines were used in this study. For mature plant materials, seeds were sown in pots containing vermiculite and Metro-Mix. For antibiotic selection, plants were grown on Murashige and Skoog (MS) plates. Culture medium consisted of half-strength Murashige and Skoog (MS) salts and 0.8% agar, pH 5.6. All plants were grown at 22 °C under 24-h light conditions.

Phenotypic and statistical analyses. The first five fruits from six individual plants (total of 30 fruits) for the wild type and each mutant were categorized into two to five classes based on phenotypic strength. The first five fruits from twenty individual plants (total of 100 fruits) were characterized for Fig. 5. Seed set was directly compared were grown side-by-side at the same density per pot to minimize potential micro-environmental differences in the growth chamber. Representative images were photographed under an AXIO Zoom.V16 (ZEISS) microscope. Statistical significance was computed using a quadrat test.

Cloning and transformation. To construct gCRC-GFP, gCRC-Myc, and gCRC-GR, a genomic fragment covering the 3492 bp region upstream of the CRC translation start site and the 1204 bp coding region were amplified from Arabidopsis thaliana genomic DNA. The resulting DNA fragments were cloned into the binary vectors pGreen0311 (for mGFP) and pGreen0280 (for Myc and GR) and sequenced (http://www.pgreen.ac.uk). To construct prCRC::TRN2 or pCRC::iaaH, the 3.5 kb CRC promoter and coding sequence of TRN2 or iaaH were sub-cloned into pGreen0311 and sequenced. To amplify the iaaH coding sequence, DNA extracted from transgenic plants containing this gene was used as a template. To construct gTRN2-GFP, a genomic fragment covering the 4.1 kb region upstream of the TRN2 translation start site and its coding region was amplified from genomic DNA and cloned into vector pGreen0311. After cloning of these DNA fragments into the pGreen vector, whole functional fragments were transferred into the pENTR1002 destination vectors for the LR reaction.

For the construction of inferences, seeds were transferred into 5% agar and sliced with a Liner Slicer PRO7 vibratome (Dosiska). The resulting flower sections were placed onto glasses, mounted on a microscope slide with one or two drops of Mountant (Daido Sangyo) and observed under an Axio Scope A1 microscope (Zeiss). More than five flowers were separately observed. For GFP observation, confocal laser scanning microscopy (FV1000: Olympus) with objective lens (UPlanSApo: Olympus) was used. Plants 4–8 cm tall that were directly compared were grown side-by-side at the same density per pot to minimize potential micro-environmental differences in the growth chamber. To interpret the differences in inferences, three independent experiments were performed, and similar results were obtained.

In situ hybridization. For in situ hybridization, inferences were <5 cm long were harvested and fixed in FAA. The resulting tissues were dehydrated in a graded ethanol series, replaced with xylene, and embedded in Paraplast plus (Sigma). Sections (8 μm thick) were cut with an RM2255 microtome (Leica), dried, stained with 0.05% Toluidine Blue (Wako), mounted on a microscope slide with one or two drops of Mountant (Daido Sangyo), and imaged using an Axio Scope A1 microscope (ZEISS).

GFP observation. For GFP observation, confocal laser scanning microscopy (FV1000: Olympus) with objective lens (UPlanSapo: Olympus) was used. Plants 4–8 cm tall that were directly compared were grown side-by-side at the same density per pot to minimize potential micro-environmental differences in the growth chamber. To interpret the differences in inferences, three independent experiments were performed, and similar results were obtained.

Reverse transcription polymerase chain reaction. For RT-PCR, total RNA from floral bud clusters up to stage 10 for mutants9–12 or ap1 cal floral bud clusters at 4 h after DEX treatment was extracted using an RNeasy plant mini kit (Qagen). To renature the DNA, an RNAse-Free Dnase set (Qagen) was used. DNA was synthesized using PrimeScript RT Master Mix (Takara). The cDNA levels were determined with a Light Cycler 480 (Roche) and Light Cycler 480 release 1.5.1.62 SP software (Roche) using FastStart DNA essential DNA Green Master (Roche). The RT-PCR experiments were performed, and similar results were obtained. See Supplementary Table 2 for qRT-PCR primers.

GUS staining. Plant tissue was fixed at room temperature in 90% ice-cold acetone for 15 min, rinsed in GUS staining buffer without 5-bromo-4-chloro-3-indolyl-b-D-glucuronic (X-Gluc), and stained with GUS staining solution (50 mM NaPO4, 2 mM KFe(CN)6, 2 mM KFe(CN)6, 2 mM X-Gluc) at 37 °C overnight. The resulting tissues were incubated in 70% EtOH for more than 1 week. Plants to be directly compared were grown and stained at the same time. Signal strengths were categorized by visual scoring of independent T1 lines. More than 32 were tested for each construct. Statistical significance was calculated using a Student’s t-test. Tissues were mounted on a microscope slide with one or two drops of chloral hydrate-based clearing solution or 0.05% neutral red (Wako), incubated at room temperature for ~1 h, and imaged using an Axio Scope A1 microscope (Zeiss), AxioCam ERC 5 s camera (Zeiss), and Zen black software (Zeiss).

Chromatin immunoprecipitation assay. To investigate the protein binding of stage-specific transcription factors, ap1-1 cal-1 35S:API-GR inferences were treated with 10 μM DEX and harvested 4 days later. Inference tissues were fixed with 1% formaldehyde for 15 min, fixed with liquid nitrogen, and kept ~80 °C until use. Chromatin was extracted and sonicated to produce DNA fragment under 500 bp. The sonicated DNA was pre-cleared with Protein A beads (Thermo Fisher Scientific) for 2 h at 4 °C. After centrifugation, antibody was added. The tubes were incubated overnight at 4 °C on a rotating device. Beads were then added and incubated for more than 6 h at 4 °C on a rotating device. The resulting beads were...
washed twice with low salt buffer, high salt buffer, and TE buffer. DNA was eluted by incubation for 1 h at 65 °C. Protein-DNA crosslink reversal was conducted overnight at 65 °C. DNA was recovered using QIAquick PCR purification kit (Qiagen). The AG antibody⁶⁹ or Myc antibody (9E10; Santa Cruz) was used. The MU gene was used as the negative control locus for ChiP experiments. The ratio of ChiP over input DNA was calculated by comparing the reaction threshold cycle for each ChiP sample to a dilution series of the corresponding input sample and was normalized over mock control loci to obtain fold change. Three independent experiments were performed, and similar results were obtained. The data from one representative experiment are shown. See Supplementary Table 2 for ChiP primers.

Genotype profiling. Microarray analysis with three biological replicates was performed with one NimbleGen 12 × 135 k arrays (Roche). The plants were grown under 12 h light/12 h dark conditions. A total of 10 mg of RNA from three biological replicates of each genotype was hybridized on GC microarrays. The images were analyzed and quantified using ArrayStar (DNAStar) with gene annotations from TAIR7. Genes showing a 2.0-fold change in expression within a 99% confidence interval were considered to be differentially expressed and are presented in Supplementary Data 1. These genes might be involved in various CRC-mediated events, such as floral meristem determination, nectary formation, and gynoecium formation. GO term analysis was performed using agrigo software version 1.2 (http://bioinfo.cau.edu.cn/ agrigo/). AGI codes from Supplementary Data 1 were used as input and hierarchical tree-structured over-represented GO terms in biological process were downloaded from the agrigo website and are shown in Supplementary Data 2 and Supplementary Figure 4b, respectively.

Phylogenetic shadowing and motif analysis. For phylogenetic shadowing, the TRN2 promoter sequences from Arabidopsis thaliana (AHIU000100571), Arabidopsis lyrata (DSF1000128231), and Extrema salutis- nem (AHU10003814.1) were obtained by NCBI blastn using the Arabidopsis thaliana TRN2 promoter sequence as a query (http://www.ncbi.nlm.nih.gov/GenBank/). The promoter sequences were aligned by mVISTA (http://genome.lbl.gov/vista/mvista/submit.shtml). Closer inspection of promoter sequences was performed using CLUSTALW (http://www.genome.ad.jp/tools/clustalw/). The sequence logo of new era of plant developmental research.

Culture and transformation of Arabidopsis and tobacco cell lines. Tobacco BY-2 and Arabidopsis MM2D cells were cultured in MS medium (4.4 g/L MS salt mix, 30 g/L sucrose, 0.2 g/L 2,4-Dichlorophenoxyacetic acid) at 22 °C in the dark. BY-2/ MM2D cells grown on MS agar medium were inoculated into 10 ml of liquid medium and cultured on a rotary shaker at 100 rpm for 1 week. Agrobacterium cells (5 mL of OD600 = 0.8) were washed three times with 5 mL of MS medium (with 250 μg/mL Carbicillin) and resuspended in 5 mL of MS medium. The 10 mL BY-2/MM2D cell cultures were combined with 100 μL of Agrobacteria in MS and cultured for ~36 h. After incubation, the cells were harvested. Gene expression was analyzed by Arraystar (DNAStar) with gene annotations from NCBI blastn using the Arabidopsis thaliana promoter sequences from Arabidopsis halleri (AHIU01003814.1) were obtained by NCBI blastn using the Arabidopsis thaliana promoter sequence as a query (http://www.ncbi.nlm.nih.gov/genbank/). The sequence logo of the promoter sequences was aligned by mVISTA (http://genome.lbl.gov/vista/mvista/submit.shtml). The W6 buffer was carefully removed, and the cells were fixed with 4% formaldehyde in W6 solution and incubated for 1 h at room temperature. The cells were washed three times with 300 μL of TSW buffer for 10 min per wash for permeabilization, followed by incubation with primary antibody⁴⁹–⁵⁵ (anti-auxin) (Sigma-Aldrich) diluted in 300 μL of TSW buffer at 4 °C for 16 h in a moist chamber. The samples were washed three times with 300 μL of TSW buffer for 10 min per wash to remove the unbound secondary antibody, the samples were mounted with 70 μL of DAPI, covered with a coverslip, and sealed with clear nail polish for longer storage. The VENUS signals were observed under a Zeiss LSM510 microscope. The VENUS signals were observed under a Zeiss LSM510 microscope. More than 10 cells were observed, and representative images are shown.

Data availability. The data that support the findings of this study are available from corresponding author on request. The microarray data have been deposited in GEO under accession number GSE89869.

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Author contributions
N.Y., J.H. and T.I. conceived this study. N.Y. and J.H. performed all experiments except
for microarray analysis and auxin transport assay. The microarray experiment was
conducted by J.H. and analyzed by J.H., N.Y. and Y.X. The auxin transport assay was
conducted by N.Y. and K.T.; N.Y. and T.I. wrote the paper with input from J.H., Y.X. and
K.T. All authors read and approved the final version of the manuscript.

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