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Regulation of Leaf Maturation by Chromatin-Mediated Modulation of Cytokinin Responses

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SUMMARY

Plant shoots display indeterminate growth, while their evolutionary decedents, the leaves, are determinate. Determinate leaf growth is conditioned by the CIN-TCP transcription factors, which promote leaf maturation and are negatively regulated by *miR319* in leaf primordia. Here we show that *CIN-TCPs* reduce leaf sensitivity to cytokinin (CK), a phytohormone implicated in inhibition of differentiation in the shoot. We identify the SWI/SNF chromatin remodeling ATPase *BRAHMA* (*BRM*) as a genetic mediator of CIN-TCP activities and CK responses. An interactome screen further revealed that SWI/SNF complex components including BRM preferentially interacted with basic-helix-loop-helix (bHLH) transcription factors and the bHLH-related CIN-TCPs. Indeed, TCP4 and BRM interacted in planta. Both TCP4 and BRM bound the promoter of an inhibitor of CK responses, *ARR16*, and induced its expression. Reconstituting *ARR16* levels in leaves with reduced CIN-TCP activity restored normal growth. Thus, CIN-TCP and BRM together promote determinate leaf growth by stage-specific modification of CK responses.

INTRODUCTION

Plant shoots are characterized by indeterminate growth, while leaves undergo a gradual differentiation to reach a finite size and shape. Leaves are initiated at the flanks of the shoot apical meristem (SAM), and following the establishment of dorsiventrality they begin to expand laterally to generate a flat lamina (reviewed in Efroni et al., 2010). Lamina expansion is associated with progressive loss of morphogenetic potential in regions at leaf margins termed "marginal blastozones," which generate the lamina and its lateral elaborations (e.g., serrations and leaflets; Hagemann and Gleissberg, 1996). These aspects of leaf maturation are correlated with protracted changes in gene expression (Efroni et al., 2008). The relationship between the gradual gene expression changes and the progressive loss of morphogenetic potential is not understood.

The earliest known markers of lamina formation are the CIN-TCP transcription factors (TFs), whose induction requires the establishment of leaf dorsiventrality (Sarojam et al., 2010). The CIN-TCPs form a subclade of the class II TCPs, a family of non-canonical basic-helix-loop-helix (bHLH) TFs. Five of the eight *Arabidopsis CIN-TCPs* are regulated by the *miR319* microRNA (miRNA; Palatnik et al., 2003). The CIN-TCPs jointly promote leaf maturation and progression of the cell-division arrest front (Nath et al., 2003; Ori et al., 2007; Efroni et al., 2008). In addition, CIN-TCPs promote leaf senescence by direct induction of *LOX2*, the product of which catalyzes the first dedicated step in jasmonic acid biosynthesis (Schommer et al., 2008). In *Arabidopsis*, simultaneous downregulation of five or all eight CIN-TCPs results in large, crinkly leaves with extended leaf margin proliferation and a delay in the sequential progression of gene expression profiles that characterize the normal leaf maturation schedule (Efroni et al., 2008).

The plant hormone cytokinin (CK) plays a role in leaf maturation that is opposite to that of the CIN-TCPs. CK promotes mitotic cell divisions, the formation of marginal leaf serrations, and marginal blastozone activity; moreover, CK inhibits leaf senescence and delays leaf differentiation (Miller et al., 1955; Gan and Amasino, 1995; Werner et al., 2003). CK sensing by the AHK receptor leads to the activation of B-class *Arabidopsis* response regulators (ARRs) (Müller and Sheen, 2007). B-class ARRs promote the transcription of various CK downstream genes, including A-class ARRs (D'Agostino et al., 2000). A-class ARRs in turn inhibit B-class ARRs, forming a negative feedback loop. Attempts to unravel the developmental roles of A-class ARRs have been hindered by their extensive redundancy (To et al., 2004). For example, downregulation of multiple A-class ARRs is required for indeterminate growth of the SAM (Leibfried et al., 2005).

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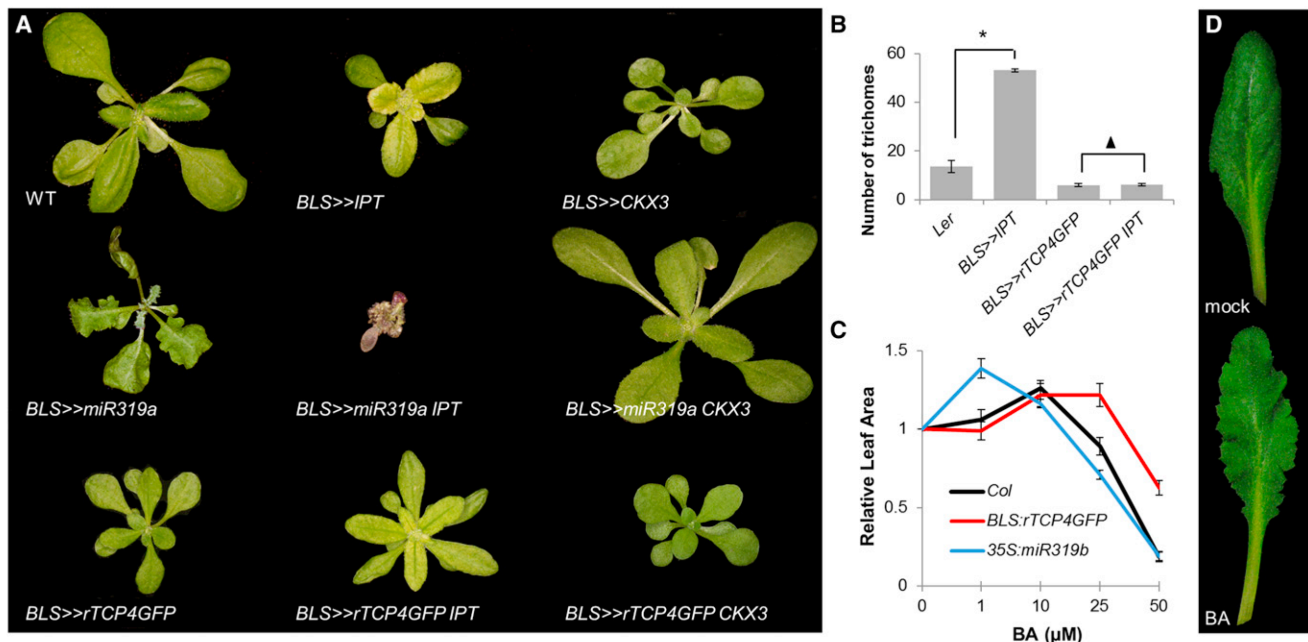


Figure 1. CIN-TCPs Regulate *Arabidopsis* Leaf Response to CK

(A) Images of 21-day-old plants expressing the CK biosynthesis enzyme IPT or CK deactivating enzyme CKX3 from a promoter active in young leaves (*BLS*). Plants with reduced or increased CIN-TCP levels overexpress *miR319* or a miRNA-insensitive form of TCP4, respectively.

(B) Prevalence of trichomes, a marker for CK activity, on leaf 3. *$p < 0.01$, Student's t test. The triangle indicates insignificant difference ($p > 0.3$, Student's t test). Error bars are SE, n = 15.

(C) Effects of biweekly exogenous application of BA on the relative growth of leaf 4 (mean leaf size at 0 μM BA is 137.9, 94.3, and 114.7 mm^2 for Col, *BLS:rTCP4GFP*, and *35S:miR319b*, respectively). Error bars are SE, n = 7–10.

(D) Expanded leaf 10 from short-day-grown Col plants after 12 weeks of 40 μM BA application.

See also Figure S1.

In metazoans, as well as in plants, the proper execution of many developmental programs depends on the accessible genome in the context of chromatin. Recent studies have highlighted the role of SWI/SNF complexes in this process (Ho and Crabtree, 2010, Kwon and Wagner, 2007). These chromatin remodeling complexes use the energy derived from ATP hydrolysis to direct nucleosome disassembly or to alter the position or conformation of the nucleosome (Clapier and Cairns, 2009). SWI/SNF complexes do not have DNA-binding specificity on their own, but are frequently recruited to their target loci by interaction with DNA-binding TFs. SWI/SNF ATPases alter the nucleosome position or conformation to allow access of sequence-specific binding proteins to the genomic DNA (Ho and Crabtree, 2010). In *Arabidopsis*, mutants in the SWI/SNF ATPase *BRAHMA* (*BRM*) fail to repress the embryonic/seed-specific programs and display other developmental defects in leaves (Tang et al., 2008; Hurtado et al., 2006). To date, few direct leaf targets of BRM are known, and little information is available about the sequence-specific DNA-binding proteins with which BRM acts in concert. Here we show that BRM and the CIN-TCPs modulate leaf responses to CK and hence promote determinate leaf growth.

RESULTS AND DISCUSSION

CIN-TCPs Regulate Leaf Sensitivity to CK

Leaves with reduced *CIN-TCP* activity display delayed maturation and, as a consequence, extended proliferation and maintenance of morphogenetic potential (Ori et al., 2007; Efroni et al., 2008), in similarity to plants with elevated CK levels. To examine whether enhanced CK responses account for the delayed leaf maturation in *cin-tcp* mutants, we modulated CK levels in the developing leaf by expressing the enzyme isopentenyl transferase (IPT), which catalyzes CK production (Kakimoto, 2001), or CKX3, which catalyzes irreversible CK inactivation (Werner et al., 2003). To restrict manipulations to the relevant leaf tissue, we used the *BLS* promoter, which drives expression in young leaves (Lifschitz et al., 2006). Increased CK levels in leaves of *BLS≫IPT* plants resulted in small yellow leaves with excessive serrations, dense trichomes, and anthocyanin accumulation typical of external CK application (Figures 1A and 1B; Figures S1A and S1C available online; Greenboim-Wainberg et al., 2005). By contrast, reduction of CK levels by *BLS≫CKX3* resulted in plants with smaller, rounder leaves, as previously reported (Figure 1A; Werner et al., 2003).

The leaves of plants that overexpressed *miR319* from the *BLS* or *35S* promoters were large and curly (Figure 1A; Efroni et al., 2008). *IPT* overexpression in *BLS ≫miR319a* plants resulted in severely dwarfed purple plants that failed to reach maturity (Figure 1A), suggesting that these plants are hypersensitive to CK. Reducing CK levels in the *BLS≫miR319a* background by overexpression of *CKX3* resulted in strong suppression of the leaf buckling phenotype and elimination of the excessive serrations (Figure 1A), suggesting that some phenotypes caused by a reduction of CIN-TCP function are due to elevated CK responses.

Constitutive overexpression of a *miR319*-insensitive version of the CIN-TCP gene *TCP4* (*rTCP4* hereafter) results in precocious arrest of shoot and leaf growth (Palatnik et al., 2003; Ori et al., 2007). By contrast, *BLS≫rTCP4GFP* plants are fertile and are characterized by small, smooth-edged, dark green leaves with very few trichomes (Figure 1A; Figure S1B; Efroni et al., 2008). When *IPT* and *rTCP4* were coexpressed, no additional serrations or trichomes were formed on the leaves of *BLS≫IPT rTCP4GFP* relative to *BLS≫rTCP4GFP* plants (Figures 1A and 1B; Figures S1B and S1D). Likewise, expressing *CKX3* in *BLS≫rTCP4GFP* plants had little effect on plant morphology (Figure 1A).

We next assayed the effects of CIN-TCP levels on leaf growth in response to CK application by repeatedly spraying Col seedlings with varying concentrations of the CK 6-benzylaminopurine (BA) followed by measuring the area of leaf 4. A bell-shaped response curve was obtained: low CK concentrations promoted the development of larger leaves, whereas higher CK concentrations inhibited leaf growth (Figure 1C). Moreover, the dose-response curve of leaf 4 to exogenous CK application was dependent on CIN-TCP activity. We found that 25 μM BA increased leaf growth in *BLS:rTCP4GFP* (identical in phenotype to *BLS≫rTCP4GFP*) but inhibited it in wild-type (WT) and *35S:miR319b* (Figure 1C; $p < 0.05$; Student's t test). In contrast, leaf growth of *35S:miR319b* was inhibited by 10 μM BA, a concentration that still promoted leaf growth in the WT (Figure 1C; $p < 0.05$; Student's t test). The fact that the bell-shaped dose-response curve to CK was maintained in all genotypes tested suggests that plants with altered CIN-TCP activity display altered leaf sensitivity to CK rather than altered steady-state CK levels. As an independent test of leaf CK responses, we performed a callus induction assay. Here too, CK responses were enhanced in *35S:miR319b* and reduced in *BLS:rTCP4GFP* plants (see Figure S1E and its legend for details). Finally, we repeatedly sprayed the leaves of WT plants grown in short days (a condition that delays leaf maturation) with 40 μM BA. This caused marginal elaborations, generating buckling leaves similar to those of the *35S:miR319* plants (Figure 1D; Palatnik et al., 2003). Taken together, our studies suggest that the CIN-TCPs, including TCP4, dampen leaf responses to CK.

BRAHMA Activity Is Required for Promotion of Leaf Maturation by *TCP4*

To understand how the CIN-TCPs modulate leaf CK responses, we identified factors required for TCP4 activity. Toward this end, we mutagenized seeds of plants that displayed precocious leaf maturation due to *rTCP4GFP* overexpression from the *BLS* promoter. Leaves of *BLS:rTCP4GFP* formed few adaxial trichomes and had smooth margins (Figures 1A, 2A, and 2B; Figures S1A and S1B). Three extragenic suppressors were identified from the progeny pools of 1,000 M1 plants; their leaves were wider and larger, lighter green in color, and had many more trichomes and serrations than the parental *BLS:rTCP4GFP* plants (Figures 2C–2E). The three mutants also had a short stature with compact inflorescences and short pedicels. Complementation tests revealed them to be allelic to a previously described mutant, *ffo3* (Levin et al., 1998). Map-based cloning (see Supplemental Experimental Procedures) identified all suppressors as new *brahma* (*brm*) alleles. We named these *brm-104* to *brm-107* to match common terminology for *BRM* mutants (Figure 2F; Bezhani et al., 2007).

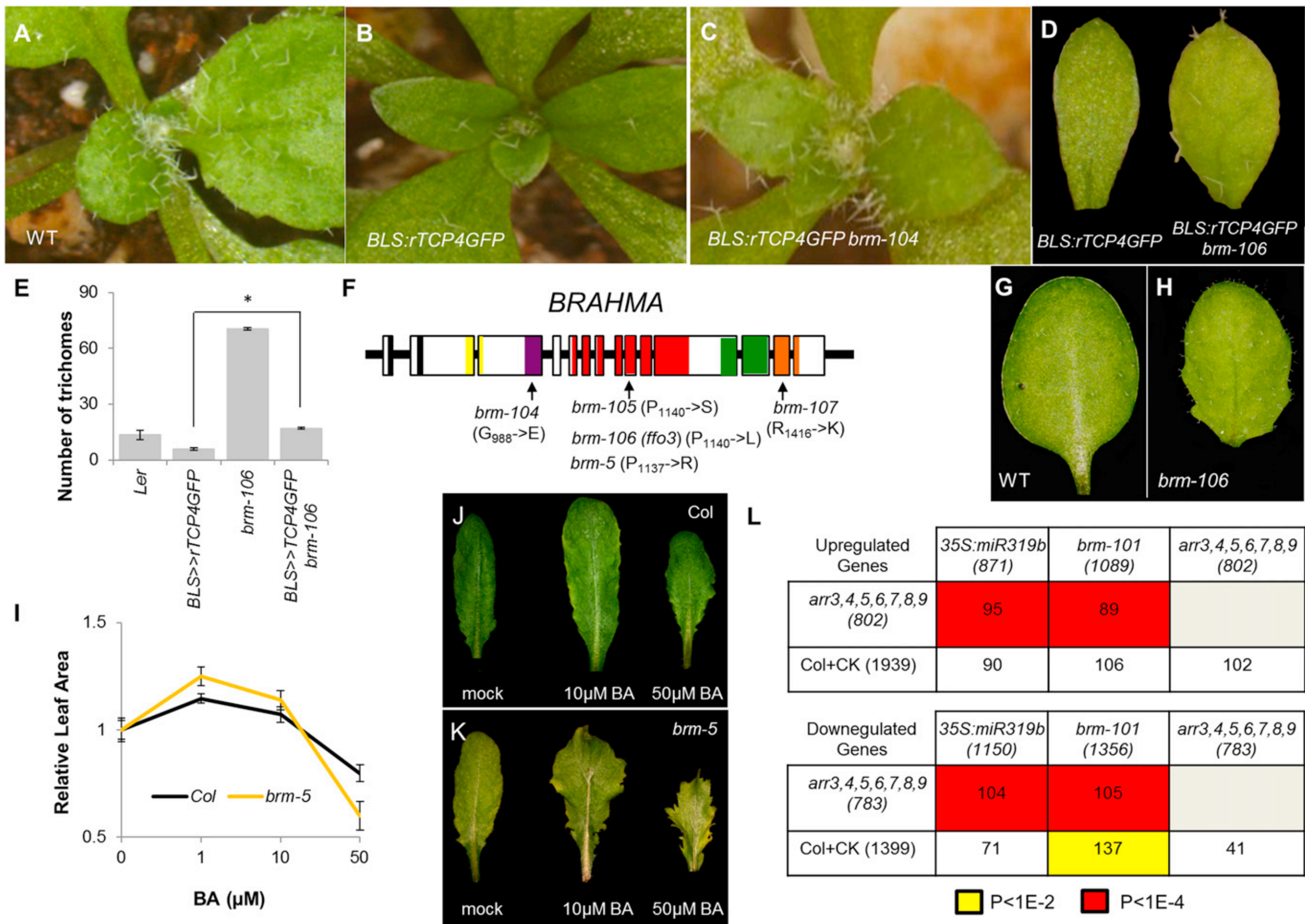
Null mutations in *BRM* cause severe phenotypes and are sterile (Hurtado et al., 2006; Kwon et al., 2006), suggesting that the new alleles are hypomorphs. In agreement with this, all four mutants carried missense mutations in important BRM domains (Figure 2F; Clapier and Cairns, 2009). The *brm* hypomorph alleles were small, fertile, and early flowering, and had curled leaves when grown in long days (this study; Farrona et al., 2004). However, when grown in short days, *brm-106* leaves exhibited excessive intervein leaf growth resulting in an uneven lamina, as well as pronounced serrations of the leaf margins and light green color (Figures 2G and 2H). These phenotypes resemble partial loss of CIN-TCP activities (Schommer et al., 2008) and are consistent with a delay in leaf maturation.

The phenotypes of the *brm* hypomorphs suggested that CK sensitivity might be altered in these mutants. To test this, we treated WT Ler, *brm-106*, *BLS:rTCP4GFP*, and *brm-106 BLS:rTCP4GFP* seedlings with BA as described above (Figure S2A). Seedlings of the Ler cultivar plants proved more resistant than those of the Col cultivar to CK application, and showed clear leaf growth inhibition only at 100 μM BA ($p < 1E-4$; Student's t test; compare Figure S2A with Figure 1C). By contrast, growth of *brm-106* leaves was already significantly inhibited at a CK dose of 25 μM BA ($p < 0.01$; Student's t test). Whereas the leaves of *BLS:rTCP4GFP* did not show a significant response to any of the BA treatments used ($p > 0.15$, ANOVA), CK responsiveness was restored to *BLS:rTCP4GFP* leaves with reduced BRM activity (*BLS:rTCP4GFP brm-106*; $p < 1E-7$, ANOVA), with a characteristic bell- shaped dose-response curve (Figure S2A).

Given the stronger responses shown by seedlings of the Col cultivar to BA treatment, we next compared the BA dose response of *brm-5*, a hypomorph allele in the Col background (Tang et al., 2008), with that of Col. Similarly to the *35S:miR319b* leaves, the *brm*-5 leaves showed increased growth relative to the WT at low CK concentration (1 μM BA; $p < 0.05$, Student's t test; Figure 2I) and reduced growth compared with the WT at high concentrations (50 μM BA; $p < 0.05$, Student's t test). The *brm* mutants also had more pronounced leaf serrations and increased leaf width compared with the WT when similar CK concentrations were applied to both (Figures 2J and 2K). Additional support for altered CK response of *brm* mutants and of plants with altered TCP levels comes from the significant overlap of genes differentially expressed in *brm* and CK response mutants (Figure 2L).

TCP4 Interacts with BRM and its Complex Member, SWI3C

A reduction in BRM activity suppressed TCP4 overexpression and both *brm* and *cin-tcp* mutants were more sensitive to CK than the WT, suggesting that both factors may act together to coordinately regulate downstream targets. We conducted a yeast-two-hybrid interactome study aimed at identifying TFs that can recruit SWI/SNF complexes to the genomic loci they regulate, using a library of 1,400 *Arabidopsis* TFs as prey (Song et al., 2008). This identified a total of 400 pairwise interactions involving 210 unique TFs from 25 different families (Table S1). TFs from seven families were significantly enriched as SWI/SNF interacting (Figure 3A). The highest enrichment was



Upregulated Genes	35S:miR319b (871)	brm-101 (1089)	arr3,4,5,6,7,8,9 (802)
arr3,4,5,6,7,8,9 (802)	95	89	
Col+CK (1939)	90	106	102

Downegulated Genes	35S:miR319b (1150)	brm-101 (1356)	arr3,4,5,6,7,8,9 (783)
arr3,4,5,6,7,8,9 (783)	104	105	
Col+CK (1399)	71	137	41

Figure 2. TCP4 and BRM Jointly Promote Leaf Maturation

(A–D) The effect of TCP4 on leaf trichome production (A–C) or leaf growth (D) was suppressed by weak alleles of the SWI/SNF ATPase *BRM* (B and D).

(E) *brm-106* restores the number of trichomes of *BLS:rTCP4GFP* to WT levels, whereas CK application does not (*p < 1E-10, Student's t test). Error bars are SE, n = 15.

(F) Hypomorph *brm* alleles identified. Domains: yellow, QLQ; purple, HSA; red, ATPase; green, AT-hook; orange, bromodomain.

(G and H) Weak serrations and uneven lamina in short-day-grown WT (G) and *brm-106* (H) leaf 3.

(I–K) Effects of biweekly exogenous BA application on the relative growth of leaf 4 (I), and on serrations and marginal growth of leaf 6 (J and K) for long-day-grown Col and *brm-5* plants. Mean leaf size at 0 μM BA: 127.6 mm^2 (Col) and 127.4 mm^2 (*brm-5*). Error bars are SE, n = 7–10.

(L) Overlap of genes differentially expressed in plants with altered TCP or BRM activity, and in plants with genetic (*arr* mutants) or chemical (+CK) alteration of CK responses (p values, hypergeometric test).

observed for the bHLH and TCP TF families (p < 1E-4, Fischer's exact one-tailed). bHLH and bHLH-related DNA-binding proteins such as TCPs may thus play an important role in SWI/SNF complex recruitment in *Arabidopsis*.

The SWI/SNF core complex in yeast and metazoans consists of four proteins: one catalytic subunit (a SWI/SNF ATPase), two SWI3 proteins, and one SNF5 subunit (Clapier and Cairns, 2009; Kwon and Wagner, 2007, Jerzmanowski, 2007). The interactome screen included as baits the SWI/SNF ATPases BRM and SYD, three SWI3 proteins (A, B, and C), and the SNF5 subunit BUSHY. Intriguingly, BRM and the proposed BRM complex subunit SWI3C (Archacki et al., 2009; Hurtado et al., 2006) interacted frequently with TCP TFs (Table S1). We repeated the interaction tests for seven of the eight CIN-TCPs, and found that TCP3, TCP4, and TCP5 interacted with BRM and SWI3C (Figure S2B). We further verified the physical interaction between BRM or SWI3C and TCP4 in planta using bifluorescence molecular complementation. We observed strong binding of TCP4 to BRM and weaker binding of TCP4 to SWI3C (Figures 3B–3E). Our combined data suggest that TCP4 and other CIN-TCPs may act together with the BRM chromatin remodeling complex to regulate downstream target genes. Genetic support for this hypothesis comes from the finding that *brm-106* did not dramatically enhance the leaf phenotypes of *35S:miR319a* plants. An enhancement would be expected if both were modifying CK responses independently (Figure S2C).

Genes Altered in *cin-tcp* and *brm* Mutants Overlap Extensively

If the CIN-TCPs and BRM act in concert, we would expect a significant overlap between the genes with altered expression in each mutant. Indeed, there was a significant overlap in genes

A

TF category	TFs in library	Total Interactions	Interacting TFs	P-Value
HLH	113	71	38	1.65E-07
TCP	22	36	13	1.96E-06
E2F	5	6	4	0.0021
bZIP	66	36	18	0.0060
TriHelix	23	15	8	0.0144
B3	15	15	6	0.0163
CO-like	32	17	9	0.0394

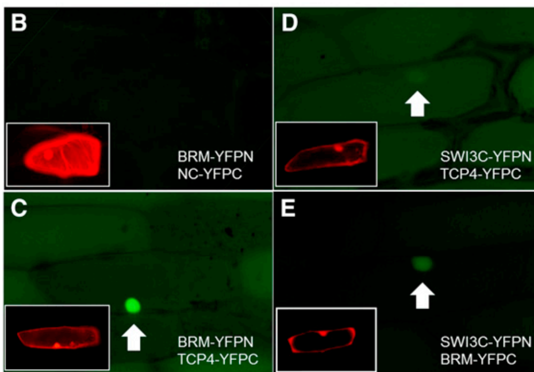


Figure 3. CIN-TCPs and BRM Interact to Regulate a Common Set of Genes

(A) TF families with significantly enriched SWI/SNF interacting TFs. TFs from 32 different families were tested (Table S1). Seven families were significantly enriched (Fisher's exact one-tailed test, $p < 0.05$).

(B–E) Transfected epidermal onion cells with NC (B) or interaction tests (C–E). Red fluorescent protein marks transformed cells and nuclei (insets). Arrows point to nuclei.

(F) Overlap of genes differentially expressed in apices with altered TCP4, *miR319*, and BRM levels.

See also Figure S2 and Tables S1 and S2.

coordinately down- or upregulated in young *35S:miR319a* and *brm-101* seedlings (Efroni et al., 2008, Bezhani et al., 2007): 200 genes were downregulated in both conditions, and 89 genes were upregulated in both conditions ($p < 0.001$, hypergeometric test; Figure 3F). In contrast, the overlap between genes upregulated in one genotype and downregulated in the other was as expected by chance alone (data not shown). When we probed for genes coordinately regulated in *brm-101*, *35S:miR319a* plants and in *BLS≫rTCP4* plants, we identified 102 genes as positively regulated ($p < 1E-39$, hypergeometric test) and 17 as negatively regulated ($p < 1E-5$, hypergeometric test; Figure 3F; Table S2). Interestingly, of these 119 putative TCP4 and BRM target genes, 52 (44%) were also differentially expressed in the expected manner in leaves of *TCP5* overexpressors (Table S2), consistent with the observed physical interaction between TCP5 and BRM (Figure S2B).

Given the physical interaction between TCP4 and BRM, and the similarity in the transcriptional responses of the two mutants, we hypothesized that TCP4 and BRM might reside together on the promoter of common target genes. Among the common putative BRM and TCP4 targets (Table S2), we identified *ARR16*, an A-class ARR and inhibitor of CK responses (To et al., 2004; Ren et al., 2009). An independent quantitative RT-PCR (qRT-PCR) experiment verified the microarray result (Figure 4A). In addition, we found that *brm-106* abolished the increased *ARR16* RNA accumulation in *BLS:rTCP4GFP* leaves (Figure 4A). Analysis of A-class *ARR* expression across leaf development based on a published data set (Schmid et al., 2005; Efroni et al., 2008) showed that *ARR16* and several *CIN-TCPs* were most highly expressed in young expanding leaves (Figures S3A and S3B). Moreover, the expression of five *CIN-TCPs*, including *TCP4*, was correlated with that of *ARR16* across leaf development ($R = 0.61$, $p < 0.05$; Figure S3C).

TCP4 and BRM Bind the Promoters of Genes that Direct Hormonal Responses

The 5′ promoter sequence of *ARR16* has two repeats of the TCP4 binding motif GTGGTCCA and a repeat of the core TCP motif TGGTCC (Figure 4B; Schommer et al., 2008), providing potential sites for TCP4 recruitment to the *ARR16* promoter. To test whether *ARR16* is a direct TCP4 target gene, we performed chromatin immunoprecipitation (ChIP) with GFP-tagged TCP4 (*BLS:rTCP4GFP*). Indeed, TCP4 associated with the 5′ intergenic region of the *ARR16* gene (Figure 4C). As a positive control, we confirmed association of TCP4 with the *LOX2* promoter, as previously demonstrated in vitro (Schommer et al., 2008). Next, we employed an epitope-tagged version of BRM, pBRM:BRM-hemagglutinin (pBRM:BRM-HA), which fully rescues the morphological defects of *brm-1* null mutants (Han et al., 2012) for ChIP. BRM-HA strongly bound to the 5′ intergenic region of both *ARR16* and *LOX2* (Figure 4D). Thus, TCP4 and BRM associated with common regulatory regions in *Arabidopsis* leaves, among which was the promoter of *ARR16*, a gene that is downregulated in plants overexpressing *miR319* and in *brm* mutants.

Given the significant role of BRM and TCP4 in leaf CK responsiveness, they may coordinately regulate other *ARRs* in addition to *ARR16*. Consistent with this hypothesis, we detected TCP4 and BRM association with the promoter of *ARR6*, a gene whose expression was high in young expanding leaves (Figure S3A) and responsive to the level of BRM or TCP4 activity (Figures S3D–S3F).

Leaf Expression of *ARR16* Can Partially Substitute for CIN-TCPs

Since CK delays differentiation in leaf cells (Shani et al., 2010), the delayed maturation of *35S:miR319a* leaves may be due to a compromised negative CK signaling feedback. We therefore wished to examine the effect of altered *ARR16* activity on plants with different *CIN-TCP* levels. *arr16-1* plants did not show morphological defects (Figures S3G–S3I). However, when tested for leaf CK responses, *arr16-1* leaves were more sensitive to CK, displaying enhanced growth at 2 μM BA and reduced growth at 40 μM BA relative to the WT ($p < 1E-4$ and $p < 0.01$, respectively; Student's t test; Figure 4E). Notably, *tcp4-2* single

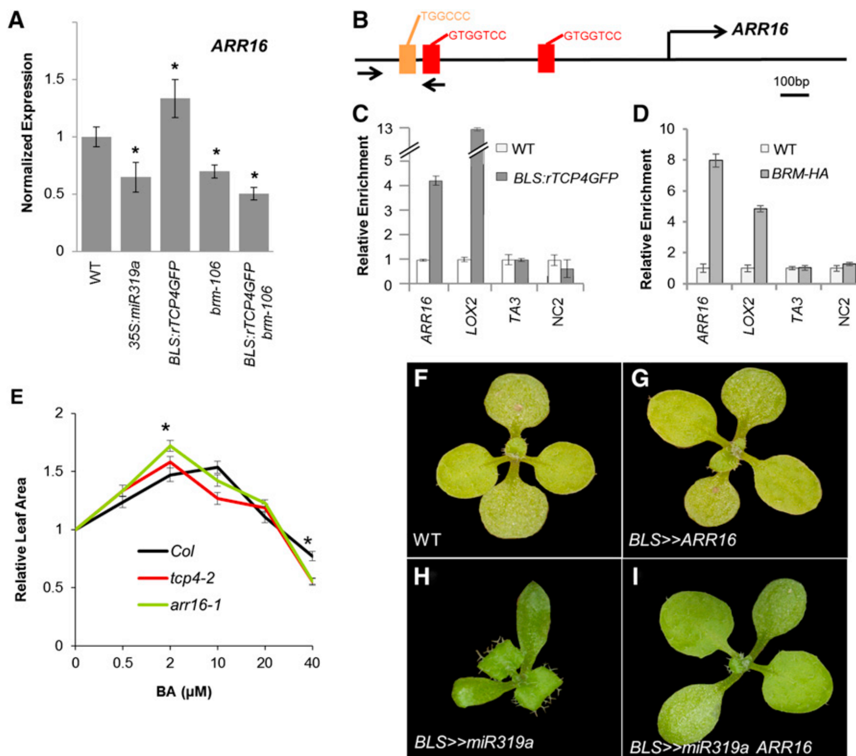


Figure 4. CIN-TCPs and BRM Modulate the Expression of a CK Response Gene

(A) qRT-PCR measurement of *ARR16* expression; *significant difference from WT ($p < 0.05$, Student's t test). Error bars are SE, n = 3.

(B) TCP4 binding motifs in the promoter of *ARR16*. Red, full motif; orange, core motif.

(C and D) ChIP from *pBLS:TCP4GFP* (C) or *pBRM:BRM-HA* (D) followed by qPCR of the *ARR16* promoter (arrows in B mark the primers used), the *LOX2* promoter, and two NCs (TA3 and NC2). ChIP was repeated at least three times, and a representative result is shown. Error bars are SE for three technical repeats.

(E) Effect of biweekly exogenous BA application on the size of leaf 4 (mean leaf size at 0 μM BA is 98.2, 106.9, and 105.8 mm^2 for Col, *tcp4-2*, and *arr16-1*, respectively). *$p < 0.01$, Student's t test. Error bars are SE, n = 10.

(F–I) Overexpression of *ARR16* rescues the growth defects of leaves expressing *miR319*.

See also Figure S3.

mutants exhibited similar hypersensitivity to CK ($p < 0.01$ and $p < 0.01$, respectively; Student's t test; Figure 4E).

We next tested whether *ARR16* expression driven from a heterologous promoter could revert the leaf maturation defects of plants with compromised CIN-TCP activity. Expression of *ARR16* from the *BLS* promoter resulted in plants with essentially unchanged leaves (Figures 4F and 4G). In contrast, expression of *BLS≫ARR16* in *BLS≫miR319a* leaves resulted in a significant rescue of the excessive growth phenotype, flattening of the leaf lamina, and a reduction in the buckling that is typical of *miR319*-overexpressing plants (Figures 4H and 4I). The same result was obtained when both *ARR16* and *miR319* were expressed from the *35S* promoter (Figure S3J). Moreover, expression of *BLS≫ARR16* could largely rescue the growth inhibition of the CK-overproducing *BLS≫IPT* plants (Figures S3K and S3L). To test whether *ARR16*-mediated rescue of CIN-TCP loss of function resulted from a general inhibition of the CK response, we crossed *35S:miR319* to previously described plants overexpressing another A-class ARR, *ARR5* (Salomé et al., 2006; Ren et al., 2009). In contrast to *ARR16* overexpressors (Figures 4G and 4I; Figure S3J), *ARR5* overexpressors did not alter the leaf phenotypes of plants with elevated *miR319* levels (Figures S3M–S3P), supporting the previous finding (Ren et al., 2009) of functional specificity among A-class ARRs.

CK, Chromatin, Differentiation, and Organ Size

CK responses are critical for the balance between indeterminate growth and differentiation in multiple plant tissues. In the *Arabidopsis* shoot, indeterminacy is maintained by the homeodomain TF WUSCHEL, which represses the expression of several A-class *ARR*s (Leibfried et al., 2005). Similarly, maintenance of the tomato leaf marginal blastozone is aided by CK activity (Shani et al., 2010), and partial loss of CK degradation results in larger *Arabidopsis* organs (Bartrina et al., 2011). We propose here that the TCP4 CIN-TCP, which is expressed at the onset of lamina formation, regulates leaf maturation through interaction with a BRM chromatin-remodeling complex and modification of the chromatin state of promoters of common targets such as *ARR16* and *ARR6*. This provides a temporal cue to dampen CK responses, thus restricting morphogenetic programs that initially are active throughout leaf primordia and are later restricted to leaf margins (blastozones). Consistent with this hypothesis, minor changes in *TCP4* levels, like those in CK levels, can have dramatic effects on overall leaf growth (Efroni et al., 2008).

The class I TCPs TCP14 and TCP15 were recently shown to sensitize *Arabidopsis* leaf responses to CK (Steiner et al., 2012), a function opposite to the one we describe for a class II CIN-TCP in leaf development. Consistent with our findings, the *BRANCHED* class II CIN-TCP gene, which is specifically expressed in axillary meristems, dampens apical dominance release—a classical CK response (Braun et al., 2012). Together, these findings implicate the TCP family, as a whole, in regulating developmental responses to CK. It was previously suggested that a balance of the antagonistic activities of class I and class II TCPs may regulate the cell cycle and plant growth, possibly via opposite effects on common target genes (Li et al., 2005). We propose here that one of the interaction points of these opposing classes of TCP TFs is the CK response pathway.

The leaf maturation rate and hence the duration of leaf growth are highly variable even within the same plant, depending on the growth conditions. This plasticity requires that input signals, such as CK, can be modulated in response to the environment. Such modulation may rely, at least in part, on the chromatin status of target genes. In agreement with this idea, mutations in chromatin remodeling complexes, such as PICKLE (Furuta

et al., 2011) and BRM (this study), modulate CK responses, potentially enabling environmental regulation of the leaf maturation schedule.

EXPERIMENTAL PROCEDURES

Plant Material

Plants were grown on soil under fluorescent light at 20°C in long day (16 hr light), unless short day (10 hr light) is indicated. *Op:CKX3* seeds were provided by Eilon Shani. *Op:IPT* seeds were previously described (Greenboim-Wainberg et al., 2005). The plants were of the Ler ecotype, except for *brm-5*, *35S:miR319b*, and *BLS:rTCP4GFP* that were used for CK response and callus induction experiments, and the two *35S:ARR5* lines that were crossed with *35S:miR319b* (Salomé et al., 2006; Ren et al., 2009). Plasmid construction is detailed in Supplemental Experimental Procedures. Transgenic lines were generated as described previously (Pekker et al., 2005). A representative single T-DNA insertion line was selected for further analysis. For the suppressor screen, *BLS:rTCP4GFP* seeds (0.2 g) were incubated in 0.3% ethyl methanesulfonate. M2 seeds were collected in pools of five M1 plants. To ensure the absence of transgene silencing in plants carrying multiple transgenes, we monitored for the presence of morphological defects caused by each transgene in all genetic backgrounds.

Yeast Two-Hybrid Screens

Six different yeast hosts, each carrying an SWI/SNF chromatin remodeling complex component as bait, were transformed with one of 1,400 TFs in the prey vector (Song et al., 2008). See Supplemental Experimental Procedures for further details on the interactome screen. Interactions between CIN-TCPs and BRM/SWI3C were confirmed by cotransforming the bait and prey plasmids into yeast.

Tissue Collection, RNA Preparation, and qRT-PCR

To measure *ARR16* or *ARR6* levels, RNA was extracted using the RNeasy Kit (QIAGEN) from 7 days after stratification (DAS) long-day-grown or 21 DAS short-day-grown plants, with qualitatively similar results. qRT-PCR was performed on 1 μg of total RNA according to Steiner et al. (2012) on an Applied Biosystems 7300 RT-PCR system. For 21 DAS short-day-grown plants, 2 μg of purified RNA was used, and qRT-PCR was performed as in Han et al. (2012). UBI21 (AT5G25760) or EIF4A1 (AT3G13920) served as internal controls. The primer sequences can be found in Supplemental Experimental Procedures.

Hormone Treatment and Callus Induction

For CK treatment, seven to eight plants of each genotype were sprayed twice a week with different concentrations of BA (Sigma) or with water after the first two leaves appeared. The area of the fully expanded leaf 4 was measured. For callus induction, plants were germinated on 1/2 Murashige and Skoog basal salt mixture (MS) plus 1% sucrose agar plates. Leaf 3 was removed from 14-day-old seedlings and transferred to 1/2 MS sucrose plates containing varying amounts of 2-4D (Sigma) and kinetin (Sigma). The plates were sealed and kept at constant light at 23°C for 4 weeks.

ChIP

ChIP and ChIP-qPCR were performed as previously described (Han et al., 2012) on 21-day-old short-day-grown plants, and 500 mg of *BLS:rTCP4GFP* seedlings were used for GFP ChIP using 5 μl of anti-GFP antibody (A6455; Invitrogen). For anti-HA ChIP, 1,000 mg *brm-1 BRM:BRM-HA* plants and 20 μl of anti-HA antibody (12CA5; Roche) were used. The retrotransposon TA3 (Han et al., 2012) and the genomic region 3′ of *ARR16* (AT2G40660; NC2) served as negative controls (NCs). The primer sequences can be found in Supplemental Experimental Methods.

Bimolecular Fluorescence Complementation

Bimolecular fluorescent complementation plasmids were introduced into onion epidermal cells using particle bombardment with BioRad PDS-1000/He. *35S:2xmCherry* was used as a transformation control and to mark the nuclei. Images were taken with an Olympus MVX100 epifluorescence microscope. The NC construct *pCL113 Tdy1-NLS* was previously described (Ma et al., 2009).

Bioinformatics Analysis

Raw microarray data were analyzed in R (2.12.0), and bioconductor (2.5). MAS5 expression values were normalized to a median of 50 (except for data from Buechel et al., 2010, which were processed with GeneChip robust multi-array averaging). Genes with normalized expression of <30 were considered absent. An arbitrarily log2 value cutoff of >|0.8| (1.74-fold change) was selected to identify genes that were significantly differentially expressed.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.01.019.

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