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

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http://dx.doi.org/10.1016/j.devcel.2013.01.019

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 noncanonical 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).
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::TCP4 plants are fertile and are characterized by small, smooth-edged, dark green leaves with very few trichomes (Figure 2A; 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::TCP4 plants (Figures 2A and 1B; Figures S1B and S1D). Likewise, expressing CKX3 in BLS::TCP4 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 dose-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::TCP4 plants (identical in phenotype to BLS::TCP4) but inhibited it in wild-type (WT) and 3SS::miR319b (Figure 1C; p < 0.05; Student’s t test). In contrast, leaf growth of 3SS::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 steadystate CK levels. As an independent test of leaf CK responses, we performed a callus induction assay. Here too, CK responses were enhanced in 3SS::miR319b and reduced in BLS::TCP4 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) (Tang et al., 2008), with that of Col. Similarly to the WT Col cultivar to BA treatment, we next compared the BA dose response of brm-106 leaves with reduced BRM activity (BLS::TCP4; p < 1E-7, ANOVA), with a characteristic bell-shaped dose-response curve (Figure S2A).

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 mutatedized seeds of plants that displayed precocious leaf maturation due to rTCP4 overexpression from the BLS promoter. Leaves of BLS::TCP4 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::TCP4 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; Bezhanis 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 interven 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::TCP4, and brm-106 BLS::TCP4 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::TCP4 did not show a significant response to any of the BA treatments used (p > 0.15, ANOVA), CK responsiveness was restored to BLS::TCP4 leaves with reduced BRM activity (BLS::TCP4; 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 3SS::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
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).

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 mm² (Col) and 127.4 mm² (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).

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
CIN-TCPs were most highly expressed in young expanding leaves (Figures S3A and S3B). Moreover, the expression of five (Figure 4A). Analysis of A-class ARR16 increased (Figure 4A). In addition, we found that RT-PCR (qRT-PCR) experiment verified the microarray result (To et al., 2004; Ren et al., 2009). An independent quantitative ARR16 increased (Figure 4A). Consistent with this hypothesis, we detected TCP4 and BRM association with the promoter of common target genes. Among the common putative TCP4 and BRM target genes, 52 (44%) were also differentially expressed in the expected manner in leaves of TCP4 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 TCP4 and BRM 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:TCP4GFP 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 more 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).

chromatin immunoprecipitation (ChIP) with GFP-tagged TCP4 (BLS:TCP4GFP). 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 coordinate regulation of 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
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-CK 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 ARRs (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:IPF seeds were previously described (Greenboim-Wainberg et al., 2005). The plants were of the Ler ecotype, except for bm-5, 3SS:mil319b, and BLS:TCP4GFP that were used for CK response and callus induction experiments, and the two 3SS:ARR5 lines that were crossed with 3SS:mil319b (Salomé et al., 2006; Ren et al., 2009). Plasmid construction is detailed in Supplemental Experimental Procedures. Transgenic lines were generated as described previously (Pelkiro et al., 2003). A representative single T-DNA insertion line was selected for further analysis. For the suppressor screen, BLS:TCP4GFP 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/SWIIC 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). UB21 (AT5G25760) or EFA4A1 (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 CK response, 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: TCP4GFP seedlings were used for GFP ChiP using 5 μl of anti-GFP antibody (A6455, Invitrogen). For anti-HA ChiP, 1,000 mg bm-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.

**Biennial Fluorescence Complementation**

Bimolecular fluorescent complementation plasmids were introduced into onion epidermal cells using particle bombardment with BioRad PDS-1000/He. 3SS:2×mCherry 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). MA55 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.

**ACKNOWLEDGMENTS**

We thank D. Weiss, J. Bowman, and N. Ori for comments; J. Reyes for pDBLeu-SYD; J. Pfluger for help with the interaction assays; C. Winter for statistical analyses; N. Ohad for technical advice; B. Ren and E. Shani for seeds; and J. Lohman for sharing expression datasets. This work was supported in part by a grant from the Next-Generation BioGreen 21 Program (SSAC No. 2011); the Rural Development Administration, Republic of Korea; the Basic Science Research Program through the National Research Foundation of Korea (MEST 2010-0012801 to J.C.H.); MINERVA (ISF 1294-10 to Y.E.); NIH (R01 GM64650-01 to D.W.); NSF (IOS 0849298 to D.W.); and EMBO (LT185-2010 to I.E.).

Received: May 1, 2011

Revised: November 7, 2012

Accepted: January 23, 2013

Published: February 25, 2013

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