請仔細閱讀提供的文章，並用中文回答下面的問題：

一、 請把文章的摘要(summary)翻譯成中文（25分）。

二、 請列出文章中的主要研究材料和方法，並簡要描述每一種研究方法的用途。(25 分)

三、 請列出文章中的主要研究結果及重要結論（25分）。

四、 請指出此文的優缺點及未來可能的研究方向（25分）。

見背面

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Arabidopsis cyclin-dependent kinase C2 interacts with HDA15 and is involved in far-red light-mediated hypocotyl cell elongation

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SUMMARY

Histone deacetylases (HDAs) regulate many aspects of plant development and responses to environmental changes. Previous studies have demonstrated that the Arabidopsis histone deacetylase HDA15 is a positive regulator in far-red (FR) light-mediated inhibition of hypocotyl elongation. Furthermore, HDA15 can be phosphorylated and its enzymatic activity is negatively regulated by phosphorylation. However, the kinases that can phosphorylate HDA15 are still unknown. Cyclin-dependent kinases (CDKs) are a large family of serine/threonine protein kinases and have been identified as major regulators of the cell cycle and transcription. In this study, we show that the cyclin-dependent kinase CDKC2 interacts with HDA15 both *in vitro* and *in vivo*. *In vitro* kinase assays show that CDKC2 phosphorylates HDA15. Genetic evidence suggests that HDA15 acts downstream of CDKC2 in hypocotyl elongation under FR light. Furthermore, HDA15 and CDKC2 function synergistically in the regulation of FR-mediated cell elongation. The expression of cell wall organization- and auxin signaling-related genes under FR light is increased in *hda15* and *cdkc2/hda15* mutants. Taken together, our study indicates that CDKC2 can phosphorylate HDA15 and plays an important role in FR light-regulated hypocotyl elongation.

Keywords: Arabidopsis, CDKC2, HDA15, far-red light, cell elongation.

INTRODUCTION

Seedlings undergo skotomorphogenesis in the dark, resulting in elongated hypocotyls, closed apical hooks, and undifferentiated chloroplasts. Upon exposure to light, etiolated (dark-grown) seedlings undergo photomorphogenesis, resulting in inhibition of hypocotyl elongation, cotyledon opening, and anthocyanin/chlorophyll accumulation (McNellis & Deng, 1995). Plants have several photoreceptors, such as phytochromes (PHYs), cryptochromes (CRYs), ZEITLUPES, and UVB-RESISTANCE 8 (UVR8) (Wit et al., 2016). In Arabidopsis, five PHYs (PHYA to PHYE) were identified that act as primary light receptors to regulate various light responses (Tepperman et al., 2001). Light changes conformations of PHYs and converts them to the active form (Wit et al., 2016). These active PHYs interact with phytochrome-interacting factors (PIFs) and negatively regulate light signaling in etiolated seedlings (Wit et al., 2016). The active PHYs induce degradation of PIFs through the 26S proteasome and promote photomorphogenesis, resulting in a short hypocotyl phenotype (Adams et al., 2014). Furthermore, PHYs also regulate the light response through repressing the activity of the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1). COP1 interacts with a subset of substrates for degradation in darkness, including the bZIP transcription factors ELONGATED HYPOCOTYL 5 (HY5) and HY5 HOMOLOG (HYH) (Hardtke et al., 2000; Holm et al., 2002; Zhao et al., 2019).

As the main regulators of light responses, PHYA and PHYB are different in light specificity. PHYA regulates the very-low-fluence response, which controls seed germination, and the far-red (FR) light-dependent high-irradiance response, which inhibits hypocotyl elongation and anthocyanin accumulation (Neff et al., 2000). PHYB is involved in the low-fluence response and the red (R) light-dependent high-irradiance response (Lim et al., 2018). The PHYA-mediated signal pathway plays a crucial role in hypocotyl elongation. Moreover, this developmental change is often involved in the expression changes of cell wall organization-related genes such as *XYLOGLUCAN ENDOTRANSGLYCOSYLASE/HYDROLASEs* (*XTHs*) and *PECTIN METHYLESTERASEs* (*PMEs*) and auxin signaling-related genes such as *NDOLE-3-ACETIC ACID INDUCIBLE19*

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(*IAA19*) and *SMALL AUXIN UP RNA36* (*SAUR36*) (Gu et al., 2017).

The lysine residues of histone H3 and H4 can be acetylated (Chen & Tian, 2007; Liu et al., 2016). Hyperacetylation of histones results in a relaxed chromatin structure for activation of gene transcription, whereas hypoacetylation of histones results in a condensed chromatin structure and gene repression (Fisher & Franklin, 2011). The reversible histone acetylation and deacetylation are regulated by histone acetyltransferases (HATs) and histone deacetylases (HDAs or HDACs), respectively (Liu et al., 2014). Arabidopsis HDACs can be categorized into three families: Reduced Potassium Dependence3/Histone Deacetylase 1 (RPD3/HDA1), Silent Information Regulator2 (SIR2), and Histone Deacetylase 2 (HD2) (Alinsug et al., 2009). There are 12 HDACs in the RPD3/HDA1 superfamily, which can be subdivided into three classes: class I (RPD3-like) (HDA6, 7, 9, 10, 17, and 19), class II (HDA5, 8, 14, 15, and 18), and class IV (HDA2) (Alinsug et al., 2009; Chen et al., 2020).

Loss of function of Arabidopsis *HDA15*, which encodes an RPD3/HDA1 class II HDAC, results in a high amount of protochlorophyllide content and increased chlorophyll biosynthesis gene expression in etiolated seedlings (Liu et al., 2013). It has been demonstrated that HDA15 and PHYTOCHROME INTERACTING FACTOR3 (PIF3) interact and repress the expression of genes involved in chlorophyll synthesis and photosynthesis by histone deacetylation (Liu et al., 2013). Furthermore, HDA15 also interacts with HY5 to repress the expression of genes involved in cell wall organization and auxin signaling by histone deacetylation (Zhao et al., 2019).

Studies in mammals have shown that the function of HDACs is affected by phosphorylation (Segré & Chiocca, 2010). In Arabidopsis, phosphomimetics of HDA6 on Ser427 and Ser429 results in increased deacetylase activity, whereas mutation of Ser427 to alanine disrupts the interaction of HDA6 with SUVH5 and SUVH6 (Yu et al., 2017). Phosphorylation of HDA15 negatively regulates its enzymatic activity and results in its relocation from the nucleolus into the nucleoplasm (Chen et al., 2020). Ser448 and Ser452 of HDA15 are also conserved in other Arabidopsis class II HDACs and the human class IIb HDACs HDAC6 and HDAC10 (Chen et al., 2020). Mutation of Ser448 and Ser452 to aspartate in HDA15 abolishes its enzymatic activity, whereas mutation of these two serine residues to alanine retains its enzymatic activity (Chen et al., 2020). However, how HDA15 is phosphorylated remains unknown.

Cyclin-dependent kinases (CDKs) belong to a large family of serine/threonine protein kinases and have been identified as important regulators of the cell cycle and transcription (Mironov et al., 1999; Morgan, 1997). In Arabidopsis, CDKs are classified as CDKA to CDKG. The animal CDK9 plays a role in controlling the transcription machinery by phosphorylating the C-terminal domain (CTD) of RNA polymerase II (RNA Pol II) (Cui et al., 2007; Marshall & Price, 1995). The Arabidopsis CDKCs CDKC1 and CDKC2 are functional homologs of the animal CDK9. CDKC1 and CDKC2 promote RNA Pol II Ser2 phosphorylation to positively regulate transcription elongation and mRNA synthesis with their interacting cyclin T partners CYCT1;3 (Brès et al., 2008; Cui et al., 2007; Wang et al., 2014). CDKC2 is involved in cell division, plant defense, and drought tolerance by modulating the transcription of cell cycle- and stomatal development-related genes (Cui et al., 2007; Zhao et al., 2017). Furthermore, CDKC2 co-localizes with spliceosome components in nuclear bodies and regulates the distribution of spliceosome components (Kitsios et al., 2008).

Our previous studies have shown that HDA15 can be phosphorylated and phosphorylation affects its function and activity (Chen et al., 2020). In this study, we used affinity purification followed by mass spectrometry analysis to identify the protein kinases that can interact with HDA15. We found that HDA15 interacts with the cyclin-dependent kinase CDKC2. Furthermore, CDKC2 can directly phosphorylate HDA15 *in vitro*. CDKC2 acts synergistically with HDA15 in the regulation of cell wall organization- and auxin signaling-related genes in FR light-mediated cell elongation.

RESULTS

HDA15 interacts with protein kinase CDKC2

HDA15 can be phosphorylated at Ser448 and Ser452 in etiolated seedlings (Chen et al., 2020). To investigate which protein kinase could phosphorylate HDA15, we used affinity purification coupled with liquid chromatography–tandem mass spectrometry (LC–MS/MS) to analyze the protein kinases that were co-purified with HDA15 in etiolated seedlings. We found that HDA15 can be co-purified with the cyclin-dependent kinase CDCK2, MKK5, CDPK6, and CPK20 (Figure 1a). Interestingly, CDKC2 was found in all four replicates. The interaction of HDA15 with CDKC2 was, therefore, further examined *in vivo* by bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) assays. For BiFC assays, *HDA15* and *CDKC2* were cloned into the pEarleygate-YC vector and the pEarleygate-YN vector, respectively. The constructs were co-delivered into Arabidopsis protoplasts by polyethylene glycol-mediated transfection (Figure 1b) and into *Nicotiana benthamiana* by *Agrobacterium*-mediated transformation (Figure 1c). Strong YFP signals were observed in the nucleus, supporting that HDA15 interacts with CDKC2 *in vivo*. For Co-IP assays, *N. benthamiana* leaves were infiltrated with *Agrobacterium* cultures carrying *35S:GFP-HDA15* and *35S:CDKC2-3xFLAG*, and leaf extracts were analyzed by Co-IP. As shown in Figure 1(d),

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(a)

Accession	Symbol	Repeat 1 Score	Repeat 1 Num. of significant sequences	Repeat 1 Num. of unique sequences	Repeat 2 Score	Repeat 2 Num. of significant sequences	Repeat 2 Num. of unique sequences	Repeat 3 Score	Repeat 3 Num. of significant sequences	Repeat 3 Num. of unique sequences	Repeat 4 Score	Repeat 4 Num. of significant sequences	Repeat 4 Num. of unique sequences
AT5G64960	CDKC2	54	1	4	48	1	4	87	2	5	49	1	4
AT3G21220	MKK5				69	3	6	84	2	4	42	2	6
AT4G23650	CDPK6	37	1	7	43	2	11				30	1	9
AT2G38910	CPK20				39	1	8	42	1	5	45	1	7

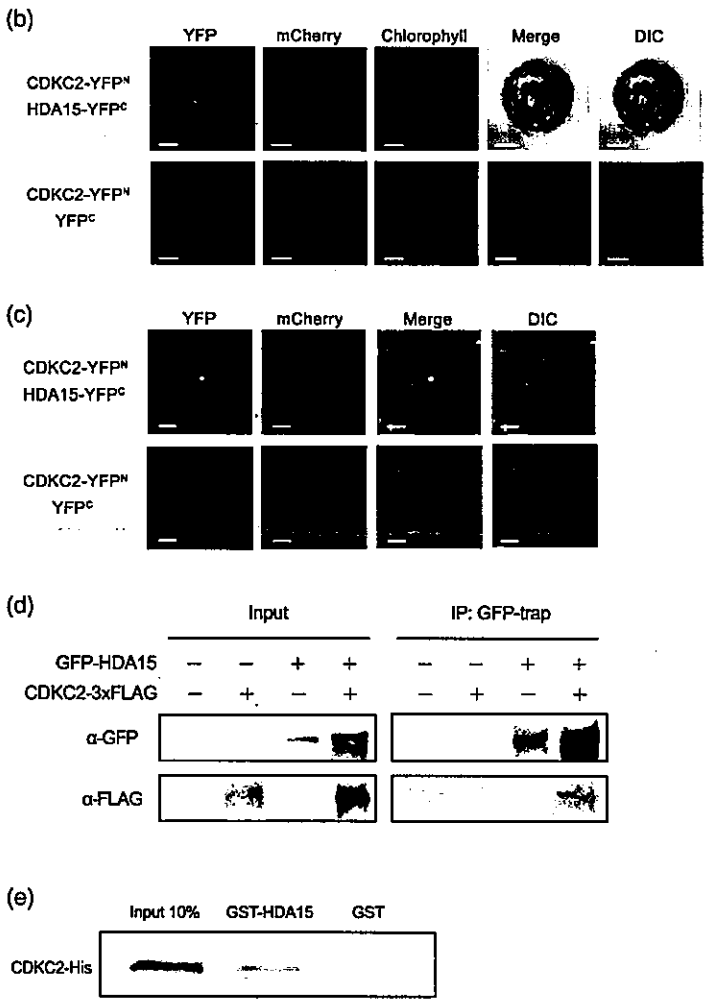

Figure 1. HDA15 interacts with CDKC2.
(a) The protein kinases co-purified with HDA15 in etiolated seedlings.
(b, c) BiFC analysis of the HDA15–CDKC2 interaction *in vivo*. HDA15 and CDKC2 fused with the N- and C-termini of YFP were co-transformed into Arabidopsis protoplasts (b) and *Nicotiana benthamiana* (c). mCherry carrying a nuclear localization signal was used as a nuclear marker. Scale bar, 10 μm. DIC, differential interference contrast.
(d) Co-IP assays of the HDA15–CDKC2 interaction *in vivo*. *35S:CDKC2-3xFLAG* and *35S:GFP-HDA15* were co-transfected into *Nicotiana benthamiana* leaves. Crude extracts (input) were immunoprecipitated (IP) with GFP-Trap and analyzed by Western blot.
(e) *In vitro* pull-down assays of the HDA15–CDKC2 interaction. CDKC2-His was incubated with GST-HDA15 or GST, and the bounded proteins were then detected by Western blot assays using an anti-His antibody.

CDKC2-3xFLAG was coimmunoprecipitated by GFP-HDA15. We further used *in vitro* pull-down assays to identify the direct interaction of HDA15 and CDKC2. The purified recombinant Glutathione S-Transferase (GST) protein GST-HDA15 was incubated with CDKC2-His. We found that CDKC2-His was pulled down by GST-HDA15, but not by GST (Figure 1e). Taken together, these results indicate that HDA15 interacts with CDKC2 both *in vitro* and *in vivo*.

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HDA15 serves as the substrate of CDKC2 *in vitro*

To test if HDA15 could serve as the substrate of CDKC2 *in vitro*, *in vitro* kinase assays were further performed. CYCT1;3 is an essential component of the CDKC2–CYCLIN complex and is required for the kinase activity of CDKC2 (Cui et al., 2007). In the absence of GST-CYCT1;3, CDKC2-His was unable to auto-phosphorylate itself (Figure 2). In the presence of GST-CYCT1;3, CDKC2-His showed auto-phosphorylation, which was enhanced by the addition of more GST-CYCT1;3. Moreover, GST-HDA15 showed clear phosphorylation signals when both CDKC2-His and GST-CYCT1;3 were present (Figure 2), suggesting that CDKC2 directly phosphorylates HDA15 *in vitro*.

HDA15 acts downstream of CDKC2 in FR light-mediated hypocotyl elongation

Previous studies have shown that *hda15* seedlings exhibit longer hypocotyls compared to wild type under R and FR (Liu et al., 2013; Zhao et al., 2019). We also analyzed the hypocotyl phenotype of the *cdkc2* mutants. Two independent T-DNA insertion mutants, *cdkc2-2* (SALK_029546) and *cdkc2-4* (SALK_141647), were obtained from The Arabidopsis Information Resource (https://www.arabidopsis.org/). Semi-quantitative reverse transcription-PCR (RT-PCR) analyses showed that *cdkc2-2* and *cdkc2-4* are two *CDKC2* knockout lines (Figure S1c). There was no significant difference in hypocotyl length between *cdkc2-2* and *cdkc2-4* seedlings and wild type under R light, suggesting that CDCK2 is not involved in R light-mediated hypocotyl elongation. Interestingly, both *cdkc2-2* and *cdkc2-4* seedlings exhibited shorter hypocotyls compared to wild type under FR light, whereas *hda15* exhibited longer hypocotyls (Figure 3). These results indicate that CDKC2 positively regulates the FR light-mediated inhibition of hypocotyl elongation. Moreover, the *cdkc2-2/hda15* double mutants displayed longer hypocotyls similar to *hda15* under FR light, indicating that HDA15 acts downstream of CDKC2 in the regulation of hypocotyl length under FR light conditions.

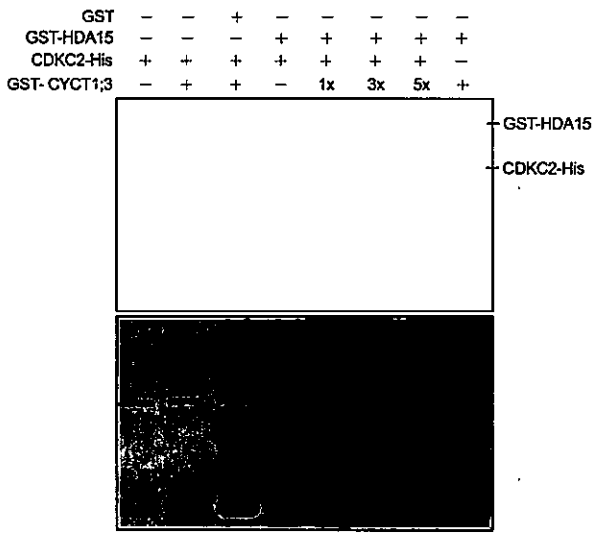

Figure 2. CDKC2 directly phosphorylates HDA15 *in vitro*.
GST-HDA15, CDKC2-His, and GST-CYCT1;3 were incubated for *in vitro* kinase reactions in the presence of [γ-^{32}P] ATP. Autoradiograph (Top) and Coomassie staining (Bottom) show phosphorylation and loading of purified proteins.

HDA15 and CDKC2 act synergistically in the regulation of FR-mediated cell elongation

Plant growth involves both cell proliferation and cell elongation. We further determined the cell length and cell number of 4-day-old *cdkc2-2*, *cdkc2-4*, *hda15*, and *cdkc2-2/hda15* seedlings. Under FR light, the cell number of *cdkc2-2* and *cdkc2-4* was decreased compared to wild type (Figure 4b), suggesting that CDCK2 is involved in cell division. Although the average cell length of *cdkc2-2* and *cdkc2-4* was similar to wild type, the average cell length of *hda15* was increased (Figure 4a,c). Interestingly, the cells of the double mutant *cdkc2-2/hda15* were significantly longer than those of the *hda15* single mutant (Figure 4a,c), indicating that HDA15 and CDKC2 act synergistically in the regulation of FR-mediated cell elongation.

To examine whether the hypocotyl elongation mediated by HDA15 and CDCK2 is related to auxin, the hypocotyl lengths were also measured after treatment with the polar auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA) (Sundberg et al., 1994). The hypocotyl lengths were decreased when treated with NPA under FR conditions (Figure S2), indicating that auxin is involved in the hypocotyl elongation regulated by HDA15 and CDCK2.

HDA15 and CDKC2 synergistically repress the expression of cell wall organization- and auxin signaling-related genes under FR light

Previous studies have shown that HDA15 represses hypocotyl elongation and regulates cell wall organization- and auxin signaling-related genes (Zhao et al., 2019). To investigate how CDKC2 mediates hypocotyl elongation, we further analyzed the expression of cell wall organization- and auxin signaling-related genes under FR light conditions. *XYLOGLUCAN ENDOTRANSGLYCOSYLASE/HYDROLASEs* (*XTH4*, *XTH15*, *XTH17*, and *XTH33*) and *PECTIN METHYLESTERASE16* (*PME16*) are involved in cell wall organization, whereas *INDOLE-3-ACETIC ACID INDUCIBLE19* (*IAA19*) and *SMALL AUXIN UP RNA36* (*SAUR36*) are involved in auxin signaling (Zhao et al., 2019). Quantitative RT-PCR (RT-qPCR) analysis using whole seedlings indicated that the expression of *XTH4*, *XTH15*, *XTH17*, *XTH33*, *PME16*, *IAA19*, and *SAUR36* was upregulated in *hda15* and *cdkc2-2/hda15* but not in *cdkc2-2* and *cdkc2-4* (Figure 5). Moreover, the expression of *XTH4*, *XTH15*, *XTH17*, *XTH33*, *PME16*, and *IAA19* in the *cdkc2-2/hda15* double mutant was significantly higher than

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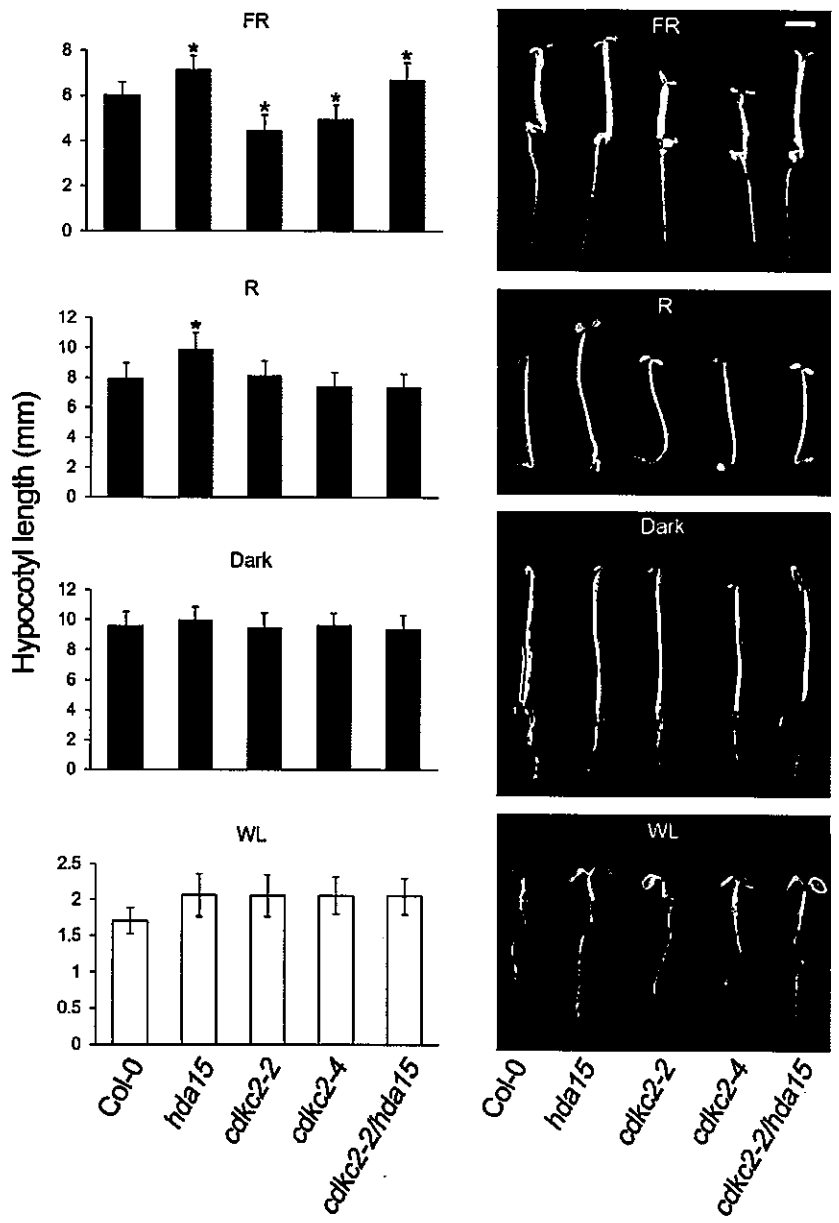

Figure 3. HDA15 acts downstream of CDKC2 in FR light-mediated hypocotyl elongation.
Hypocotyl lengths of 4-day-old Col-0, *hda15*, *cdkc2-2*, *cdkc2-4*, and *cdkc2-2/hda15* seedlings grown under FR light (2.452 μmol m^{-2} sec^{-1}), R light (12.254 μmol m^{-2} sec^{-1}), dark, and white light (WL) conditions. Scare bar, 2 mm. Data are presented as the mean (±SD) of three biological replicates (Student's *t*-test, $*P < 0.05$, $n = 40$).

in the *hda15* single mutant, supporting the synergistically negative role of HDA15 and CDKC2 in FR-mediated cell elongation. Similar results were also obtained when using hypocotyl tissues only (Figure S3).

The expression of cell cycle-related genes such as *CYCLIN D2;1* (*CYCD2;1*) and *CYCT1;3* was also analyzed. The D-type cyclin gene *CYCD2;1* has been shown to act in the G1/S transition (Zhao et al., 2017). The expression of *CYCD2;1* and *CYCT1;3* was decreased in *cdkc2* and *cdkc2/hda15* mutants (Figure 5), indicating that CDKC2 regulates cell division by promoting *CYCD2;1* and *CYCT1;3* expression under FR light conditions. The expression of *CYCD2;1* and *CYCT1;3* was unaffected in the *hda15* mutant (Figure 5), indicating that HDA15 is not involved in cell division.

HDA15 decreases histone H3 and H4 acetylation levels of cell wall organization- and auxin signaling-related genes under FR light

To further investigate how HDA15 and CDKC2 regulate gene expression, we examined the histone H3 and H4

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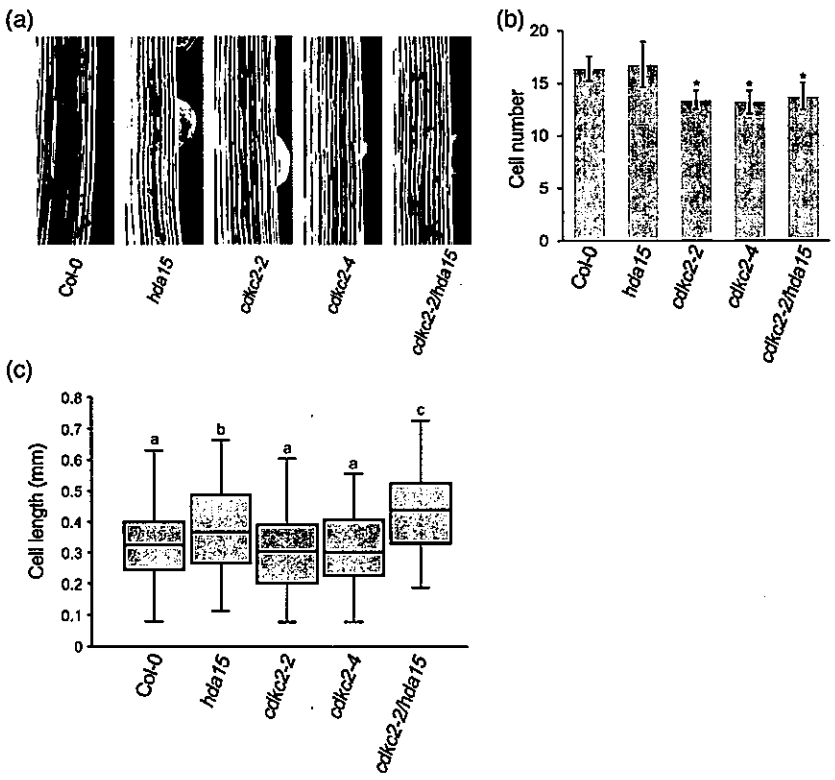

Figure 4. The cell number and cell length of Col-0, *hda15*, *cdkc2*, and *cdkc2/hda15* under FR light.
(a) Scanning electron microscopy analysis of hypocotyl cells of 4-day-old Col-0, *hda15*, *cdkc2-2*, *cdkc2-4*, and *cdkc2-2/hda15* seedlings grown under FR light (2.452 μmol m^{-2} sec^{-1}). Scale bar, 200 μm.
(b) Quantification of the cell number as indicated in (a). Data are presented as the mean (±SD) of three biological replicates (Student's *t*-test, $*P < 0.05$, $n = 20$).
(c) Quantification of the cell length as indicated in (a). Data are presented as the mean (±SD) of three biological replicates. Different letters above the error bars indicate statistically significant differences ($P < 0.05$, post hoc Tukey's HSD test).

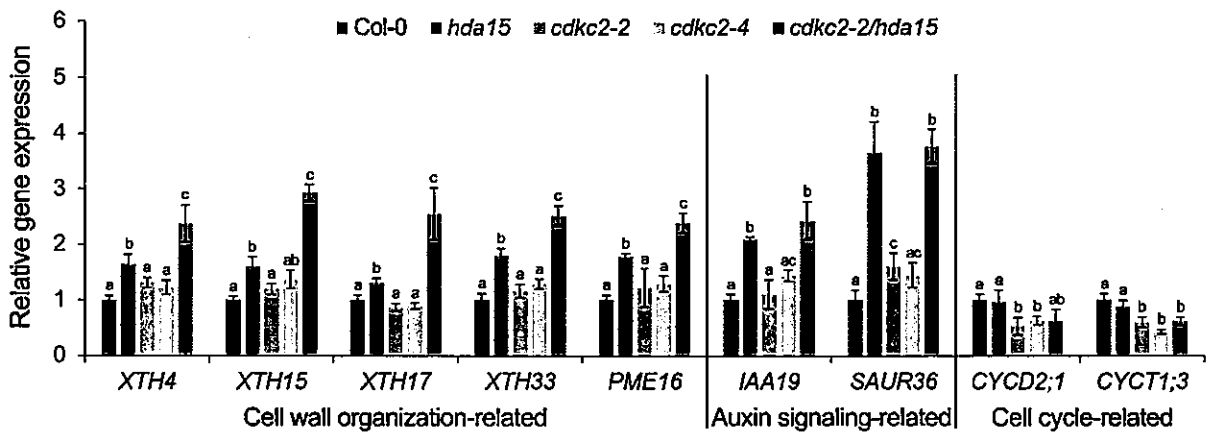

Figure 5. Expression of cell wall organization-, auxin signaling-, and cell cycle-related genes in the whole seedlings of Col-0 and *hda15*, *cdkc2*, and *cdkc2/hda15* mutants.
Expression of *XTH4*, *XTH15*, *XTH17*, *XTH33*, *PME16*, *IAA19*, *SAUR36*, *CYCD2;1*, and *CYCT1;3* was analyzed by RT-qPCR. RNA was extracted from 4-day-old whole seedlings grown under FR light (2.452 μmol m^{-2} sec^{-1}) conditions. *UBQ10* was used as an internal control. Data are presented as the mean (±SD) of three biological replicates. Different letters above the error bars indicate statistically significant differences ($P < 0.05$, post hoc Tukey's HSD test).

acetylation levels of the cell wall organization- and auxin signaling-related genes *XTH4*, *XTH15*, *PME16*, and *SAUR36* in Col-0 and the *hda15*, *cdkc2*, and *cdkc2-2/hda15* mutants by ChIP-qPCR analysis. Increased histone H3 acetylation (H3Ac) levels in the proximal promoters of *XTH4* and *SAUR36* (*XTH4-P* and *SAUR36-P*) and the first exon regions

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of *XTH15* and *PME16* (*XTH15-E* and *PME16-E*) were detected in *hda15* and *cdkc2-2/hda15* mutants compared to wild type (Figure 6a,b). Moreover, increased histone H4 acetylation (H4Ac) levels in *SAUR36-P*, *PME16-P*, and *PME16-E* were also detected in *hda15* and *cdkc2-2/hda15* mutants compared to wild type (Figure 6a,b). The H3Ac and H4Ac levels of the cell cycle-related genes *CYCD2;1* and *CYCT1;3* displayed no obvious changes in *hda15*, *cdkc2*, and *cdkc2-2/hda15* mutants compared to wild type (Figure S4). The H3Ac and H4Ac levels in the *cdkc2-2/hda15* double mutant were similar to those in the *hda15* single mutant, indicating the histone acetylation changes in the mutants were caused by HDA15.

DISCUSSION

CDKC2 interacts with and phosphorylates HDA15

Phosphorylation of HDACs plays a crucial role in the regulation of their functions. The human HDACs HDAC1, HDAC2, HDAC4, HDAC5, and HDAC6 can be phosphorylated

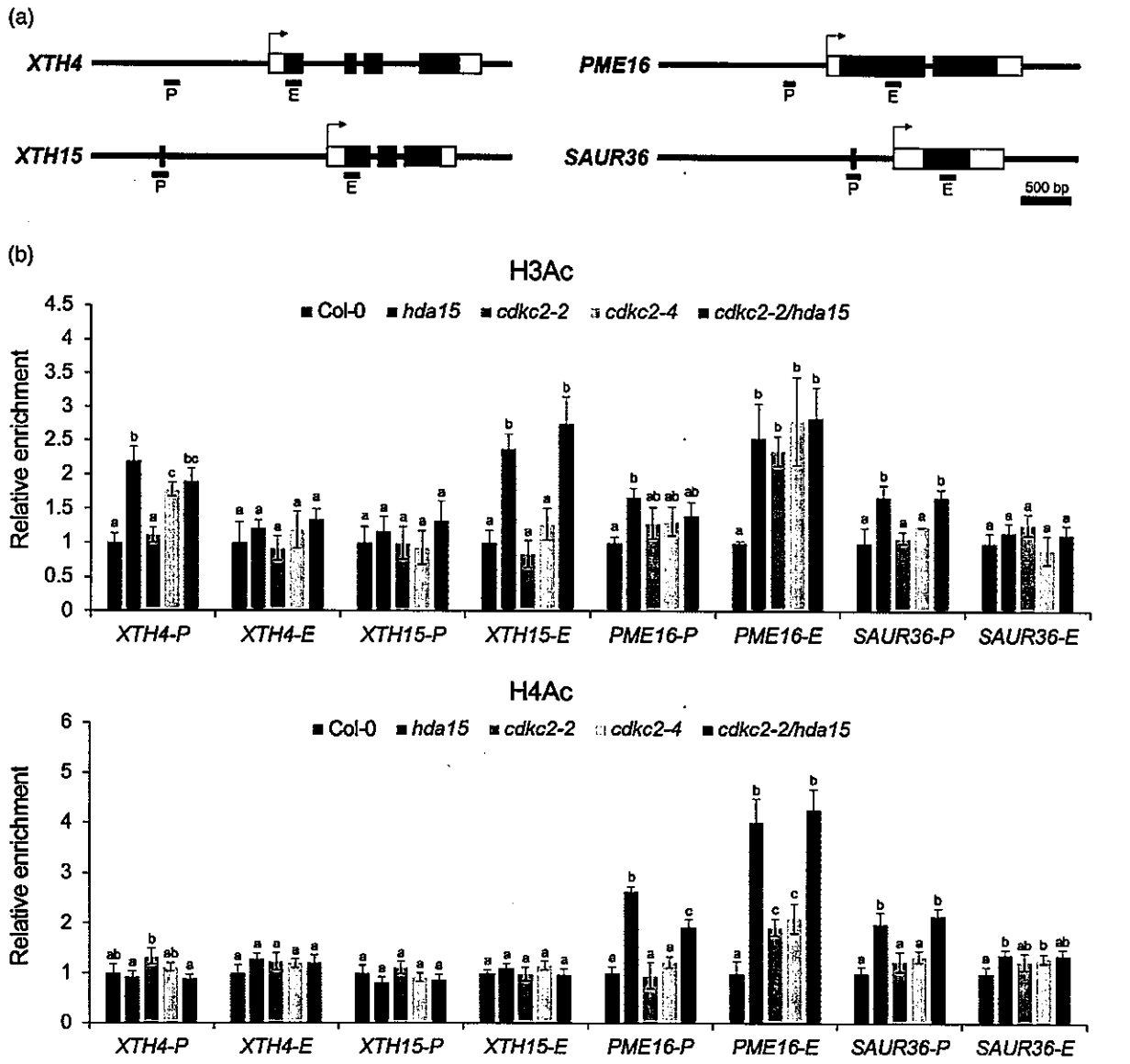

Figure 6. HDA15 decreases the histone H3Ac and H4Ac levels of cell wall organization- and auxin signaling-related genes under FR light conditions.
(a) Schematic diagram of *XTH4*, *XTH15*, *PME16*, and *SAUR36*. The regions used for ChIP-qPCR analysis are shown. P and E indicate proximal promoter and first exon regions, respectively. Red boxes indicate G-box elements (CACGTG).
(b) ChIP-qPCR analysis of histone H3Ac and H4Ac levels of *XTH4*, *XTH15*, *PME16*, and *SAUR36* in Col-0, *hda15*, *cdkc2-2*, *cdkc2-4*, and *cdkc2-2/hda15* grown under FR light (2.452 μmol m^{-2} sec^{-1}) for 4 days. Histone H3 was used as an internal control. Data are presented as the mean (±SD) of three biological replicates. Different letters above the error bars indicate statistically significant difference ($P < 0.05$, post hoc Tukey's HSD test).

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and their functions are regulated by phosphorylation (Lissanu Deribe et al., 2009; McKinsey et al., 2001; Segré & Chiocca, 2010). Human HDAC1 and HDAC2 are the substrates for phosphorylation by the ubiquitous serine/threonine protein kinase casein kinase II, which phosphorylates various substrates that are implicated in cell growth and proliferation and signal transduction (Cai et al., 2001; Gowda et al., 2017; Sun et al., 2007). Phosphorylation on Ser421 and Ser423 of human HDAC1 and Ser394, Ser422, and Ser424 of HDAC2 positively regulates their deacetylase activity, resulting in transcriptional repression of their downstream target genes (Galasinski et al., 2002; Pflum et al., 2001). Furthermore, phosphorylation of HDAC1 also promotes its complex formation (Pflum et al., 2001). Moreover, human HDAC5 is phosphorylated by the serine/threonine kinase Protein Kinase D, which is involved in a variety of cellular functions, including signal transduction and endothelial cell proliferation (Ha et al., 2008). Phosphorylation of HDAC5 promotes its binding to the 14-3-3 chaperone protein, resulting in translocation from the nucleus to the cytoplasm (Grozinger & Schreiber, 2000; McKinsey et al., 2001). Phosphorylation of the Arabidopsis HDACs HDA6 and HDA15 also affects their functions (Chen et al., 2020; Yu et al., 2017). Phosphorylation on Ser427 and Ser429 of HDA6 increases its enzymatic activity, whereas phosphorylation on Ser427 is also important for the interaction of HDA6 with its interacting proteins SUVHs (Yu et al., 2017). By contrast, phosphorylation on Ser448 and Ser452 of HDA15 negatively regulates its enzymatic activity and results in its translocation from the nucleolus into the nucleoplasm (Chen et al., 2020). In the present study, we found that CDKC2, a serine/threonine protein kinase in Arabidopsis, directly interacts with HDA15. Moreover, CDKC2 can phosphorylate HDA15 *in vitro*.

CDKC2 acts upstream of HDA15 in FR light-regulated hypocotyl elongation

Previous studies have shown that CDKC2 influences the phosphorylation status of the CTD of RNA Pol II to regulate transcription (Wang et al., 2014). CDKC2 plays a role in viral infection, the timing of flowering, and stomatal development through affecting the transcriptional activation of viral infection-, cell cycle- and stomatal development-related genes (Cui et al., 2007; Wang et al., 2014; Zhao et al., 2017). On the other hand, HDA15 has been shown to regulate R light- and FR light-mediated inhibition of hypocotyl growth. Furthermore, HDA15 represses the expression of cell wall organization- and auxin signaling-related genes in seedlings under R light to regulate hypocotyl elongation (Zhao et al., 2019). In this study, we found that CDKC2 is also involved in hypocotyl elongation under FR light. The *cdkc2* mutants exhibited shorter hypocotyls compared to wild type under FR light, whereas the *hda15* mutant exhibited longer hypocotyls. Furthermore, the hypocotyl lengths of *cdkc2/hda15* double mutants were similar to those of the *hda15* mutant, indicating that CDKC2 acts upstream of HDA15 in FR light-regulated hypocotyl elongation.

Consistent with these observations, the expression levels of cell wall organization- and auxin signaling-related genes were increased in *hda15* and *cdkc2/hda15* mutants. The Arabidopsis cell wall organization-related genes *XTHs* are expressed in all developmental stages (Becnel et al., 2006). The auxin signaling-related genes *IAA19* and *SAURs* regulate hypocotyl length through promoting cell expansion (Hou et al., 2013; Tatematsu et al., 2004). Moreover, the expression of these genes was significantly higher in the *cdkc2-2/hda15* double mutant than in the *hda15* single mutant, supporting the synergistically negative role of HDA15 and CDKC2 in FR-mediated cell elongation. Taken together, these results suggest that CDKC2 may synergistically modulate the expression of cell wall organization- and auxin signaling-related genes with HDA15 to regulate hypocotyl elongation.

CDKC2 promotes cell division under FR light conditions

Arabidopsis CDKC2, a homolog of human CDK9, is a member of the CDK family (Barrôco et al., 2003). By interacting with cyclin T, CDKC2 plays an important role in phosphorylating the CTD of RNA Pol II (Barrôco et al., 2003; Wang & Fischer, 2008). CDKC2 is also required for the progression of cell division and endoreduplication (Zhao et al., 2017). The cell number of rosette leaves was remarkably increased in the *cdkc2* mutants compared to wild type (Zhao et al., 2017). *hda15* seedlings exhibited longer hypocotyls compared to wild type under R light and FR light conditions, suggesting that HDA15 plays an important role in both R and FR light-mediated hypocotyl elongation (Liu et al., 2013; Zhao et al., 2019). By contrast, there was no significant difference in hypocotyl length between *cdkc2* seedlings and wild type under R light, suggesting that CDCK2 is not involved in R light-mediated hypocotyl elongation. *cdkc2* mutants displayed shorter hypocotyl length and a reduced cell number under FR light conditions. In addition, the expression of the cell cycle-related genes *CYCD2;1* and *CYCT1;3* was also decreased in *cdkc2* mutants. Taken together, these results indicate that CDKC2 might play different roles in different growth stages. CDKC2 acts as a positive regulator in FR-mediated cell elongation, whereas it acts as a negative regulator in the reproductive stage (Zhao et al., 2017).

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis thaliana plants were grown at 23°C under long-day (LD) conditions (16 h light/8 h dark). The T-DNA mutants *hda15-1* (SALK_004027) (Liu et al., 2013), *cdkc2-2* (SALK_029546) (Wang et al., 2014), and *cdkc2-4* (SALK_141647) were obtained from The Arabidopsis Information Resource Center (http://www.arabidopsis.org/).

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For measurement of morphogenetic phenotypes, seeds were sterilized and planted on half-strength Murashige-Skoog medium agar plates containing 0.3% (w/v) sucrose and imbibed for 3 days at 4°C in the dark with or without NPA (1 μM) treatment. After germination was induced under white light for 6 h, the seedlings were placed under various light conditions at 23°C for the indicated times.

LC–MS/MS

The HDA15 protein extracted from 2-day-old etiolated seedlings of Col-0 wild type was immunoprecipitated by an HDA15 antibody (Liu et al., 2013) and isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in four biological replicates (Tu et al., 2022). After trypsin digestion and desalting, peptides were used to perform LC–MS/MS by a Thermo Orbitrap Elite Mass Spectrometer and for Mascot analysis.

BiFC assays and microscopy

The coding sequences of *CDKC2* and *HDA15* were subcloned into the pCR8/GW/TOPO vector and then recombined into the YFP^N (pEarleyGate201-YFP^N) and YPF^C (pEarleyGate202-YFP^C) vectors (Lu et al., 2010), respectively. Constructed vectors were transiently transformed into Arabidopsis protoplasts and fluorescence was observed using a Zeiss LSM 780 confocal microscope. Leaves of 3-week-old *N. benthamiana* were infiltrated with *Agrobacterium tumefaciens* GV3101 containing the YFP^N and YFP^C construct pairs. YFP fluorescence of epidermal cell layers was examined 2 days after infiltration using an Apatome microscope.

Co-IP assays

The coding sequences of *CDKC2* and *HDA15* were cloned into the modified pEarleyGate100 vector containing a 3xFLAG tag (*35S:CDKC2-3xFLAG*) and the pK7WGF2 binary vector (*35S:GFP-HDA15*), respectively. Two days after infiltration with *A. tumefaciens* GV3101, *N. benthamiana* leaves were harvested and ground in liquid nitrogen. Proteins were extracted in extraction buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Igepal CA-630, and 1 mM PMSF) containing protease inhibitor cocktail (Roche, Wilmington, MA, USA). The protein extracts were incubated with GFP-trap beads (Chromotek, Rosemont, IL, USA) overnight at 4°C. The beads were washed with wash buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol, and 1% Igepal CA-630). Immunoblotting was carried out using an anti-FLAG antibody (Sigma; M2 F3165, Burlington, MA, USA) and an anti-GFP antibody (Abcam; ab290, Cambridge, MA, USA).

Pull-down assays

The pull-down assay was performed as previously described (Liu et al., 2013). GST and GST-HDA15 recombinant proteins were incubated with GST resin in binding buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.25% Triton X-100, and 35 mM β-mercaptoethanol) for 2 h at 4°C. The binding reaction was washed three times with binding buffer and then the CDKC2-His recombinant protein was added, followed by incubation for an additional 2 h at 4°C. After washing five times with binding buffer, the pulled down proteins were eluted by boiling, separated by SDS-PAGE, and detected by immunoblotting using an anti-His antibody (Sigma; 70796-M).

In vitro kinase assays

The *in vitro* kinase assay was performed as previously described with some modifications (Cui et al., 2007). The kinase reactions were assembled on ice in kinase buffer (20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, pH 8, and 1.1 mM $CaCl_2$) containing 0.075 μg CDKC2-His or 0.075 μg GST-CYCT1;3 recombinant proteins. The reactions were initiated by adding 0.75 μg GST-HDA15 substrate protein and 2.5 μCi of [γ-^{32}P] ATP and terminated by the addition of SDS loading buffer after 30 min of incubation at 37°C. The reaction mixtures were separated by SDS-PAGE, and phosphorylation was detected by autoradiography.

RNA extraction and RT-qPCR

Total RNA was extracted from 4-day-old plants grown under FR light (2.452 μmol m^{-2} sec^{-1}) conditions with TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. One microgram of total RNA was used to synthesize cDNA. RT-qPCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and a CFX96 real-time PCR system (Bio-Rad). The gene-specific primers used for RT-qPCR are listed in Table S1. Each sample was quantified at least in triplicate and normalized using *Ubiquitin10* (*UBQ10*) as an internal control.

ChIP-qPCR assays

ChIP assays were performed as previously described (Gendrel et al., 2005; Liu et al., 2013). Chromatin was extracted from 4-day-old plants grown under FR light (2.452 μmol m^{-2} sec^{-1}) conditions. After fixation with 1% formaldehyde, the chromatin was sheared to an average length of 500 bp by sonication and then immunoprecipitated with specific antibodies including anti-H3Ac (Millipore; 06-599, Billerica, MA, USA), anti-H4Ac (Millipore; 06-866), and anti-H3 (Abcam; ab1791). The cross-linking was then reversed, and the amount of each precipitated DNA fragment was determined by qPCR using specific primers (Table S1). Three biological replicates were performed, and three technical repeats were carried out for each biological replicate. Representative results from one biological replicate are shown.

ACCESSION NUMBERS

The sequence data of the genes used in this article can be found in The Arabidopsis Information Resource (http://www.arabidopsis.org) under the following accession numbers: *HDA15* (AT3G18520), *CDKC2* (AT5G64960), *CYCT1;3* (AT1G27630), *XTH4* (AT2G06850), *XTH15* (AT4G14130), *XTH17* (AT1G65310), *XTH33* (AT1G10550), *PME16* (AT2G43050), *IAA19* (AT3G15540), *SAUR36* (AT2G45210), and *CYCD2;1* (AT2G22490).

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Identification of *cdkc2* mutant lines.

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Figure S2. Hypocotyl phenotypes of Col-0, *hda15*, *cdkc2-2*, *cdkc2-4*, and *cdkc2-2/hda15* seedlings with NPA treatment.

Figure S3. Expression of cell wall organization-, auxin signaling-, and cell cycle-related genes from hypocotyl tissues in Col-0 and *hda15*, *cdkc2*, and *cdkc2/hda15* mutants.

Figure S4. ChIP-qPCR analysis of the histone H3Ac and H4Ac levels of *CYCD2;1* and *CYCT1;3* under FR light.

Table S1. List of primers used in this study.

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