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CK2 promotes jasmonic acid signaling response by phosphorylating MYC2 in *Arabidopsis*

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ABSTRACT

Jasmonic acid (JA) signaling plays a pivotal role in plant development and defense. MYC2 is a master transcription factor in JA signaling, and was found to be phosphorylated and negatively regulated by MAP kinase and receptor-like kinase. However, the kinases that positively regulate MYC2 through phosphorylation and promote MYC2-mediated activation of JA response have not been identified. Here, we identified CK2 as a kinase that phosphorylates MYC2 and thus regulates the JA signaling. CK2 holoenzyme can interact with MYC2 using its regulatory subunits and phosphorylate MYC2 at multiple sites with its catalytic subunits. Inhibition of CK2 activity in a dominant-negative plant line, *CK2mut*, repressed JA response. On the other hand, increasing CK2 activity by overexpression of *CKB4*, a regulatory subunit gene of CK2, enhanced JA response in a MYC2-dependent manner. Substitution of the Ser and Thr residues at phosphorylation sites of MYC2 by CK2 with Ala impaired MYC2 function in activating JA response. Further investigations evidenced that CK2 facilitated the JA-induced increase of MYC2 binding to the promoters of JA-responsive genes *in vivo*. Our study demonstrated that CK2 plays a positive role in JA signaling, and reveals a previously undiscovered mechanism that regulates MYC2 function.

INTRODUCTION

CK2 (casein kinase II) is an evolutionarily well-conserved protein kinase in eukaryotes. The CK2 holoenzyme is a tetramer composed of two catalytic α subunits (CKA) and two regulatory β subunits (CKB), with the two β subunits constituting the structural center of the holoenzyme (1). In plant, CK2 subunits are generally encoded by multiple genes. For instance, the α and β subunits in *Arabidopsis thaliana* are each encoded by four genes with a high degree of functional redundancy (2).

CK2 plays essential roles in various aspects of plant development, and has been proved to phosphorylate many important proteins in different signaling pathways (3). CK2 can phosphorylate the clock regulators CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL) to modulate the output of circadian rhythm and therefore plant flowering time (4–7). In light signaling, CK2-mediated phosphorylation stabilizes two positive regulators of photomorphogenesis, HY5 (ELONGATED HYPOCOTYL 5) and HFR1 (LONG HYPOCOTYL IN FAR-RED LIGHT 1), and induces the degradation of PIF1 (PHY-INTERACTING FACTOR 1), a negative regulator of photomorphogenesis (8–10). During seedling development, CK2 can regulate cotyledon expansion, and root and shoot growth by affecting auxin signaling (11–13). Meanwhile, the roles of CK2 in plant responses to different stresses including salt, cadmium and UV-B, and stress-related abscisic acid (ABA) and salicylic acid (SA) signaling, have also been revealed (14–17). Many ABA signaling proteins have been identified as CK2 substrates in *Zea mays* (maize), among which the SnRK2, a core component of ABA signaling transduction, was proved to be regulated by CK2-mediated phosporylation (18–20). CK2 has also been shown to regulate phosphorus uptake through phosphorylating the phosphate transporter 8 (PT8) in *Oryza sativa* (rice) (21). In *Brassica juncea*, CK2 affects swollen stem formation via controlling the cell cycle (22). Recently, a role of CK2 in root stem cell

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exhaustion under aluminum toxicity and phosphate deficiency was found (23). However, the involvement of CK2 in jasmonic acid (JA) signaling and its related biotic stress response has not yet been established.

JA signaling is indispensable for both plant development and defense response (24,25). In JA signaling transduction, JA-Ile is rapidly synthesized in response to pest attack, wounding, or fungal pathogen infection, and then bound by the co-receptor COI1-JAZ complex, resulting in the degradation of JAZ repressors and the liberation of MYC2 to bind to the G-box *cis*-elements in the promoters of JA-responsive genes and induce their expression (26–29). MYC2 differentially regulates several branches of JA signaling through affecting the expression of numerous JA-responsive genes (30–32), and is a key component mediating the crosstalk between JA signaling and other signaling pathways (32–35). MYC2 protein stability is modulated by the polyubiquitination mediated by PLANT U-BOX PROTEIN10 (PUB10) and BTB/POZ-MATH (BPM) proteins and deubiquitination by ubiquitin proteases UBP12 and UBP13 (36–38).

Protein phosphorylation has been suggested to be involved in JA signaling response (39,40). Treatments with kinase inhibitors affect the expression of JA-responsive genes, implicating kinase activity in JA-mediated transcriptional activation (39). Subsequent studies revealed that MYC2 is phosphorylated *in vivo* (40,41). Specifically, phosphorylation of Thr328 is essential for JA-induced gene expression (40). MPK6 (MAP KINASE 6) phosphorylates MYC2 and inhibits MYC2-mediated JA signaling, as a *mpk6* knockout mutant exhibits enhanced inhibition of root growth after JA treatment, and increased JA-induced expression of the wound-responsive genes (42,43). A receptor-like kinase FERONIA (FER) can inhibit JA signaling by phosphorylating and destabilizing MYC2 (44). However, kinase(s) that promote the MYC2-mediated JA response remain to be identified.

In this study, we identified CK2 as a kinase that positively regulates JA signaling through phosphorylating MYC2. We found that CK2β subunits interact with MYC2, and that recombinant CK2 holoenzyme phosphorylates MYC2 *in vitro*. Overexpression of the CK2β subunit *CKB4* enhances JA-mediated inhibition of root elongation and resistance against *Botrytis cinerea*, and increases the expression of JA-responsive genes. By contrast, the activation of JA-responsive genes is significantly attenuated in the *CK2mut* plants with suppressed CK2 activity, which is consistent with the reduced phosphorylation level of MYC2. We also showed that CK2 promotes the binding of MYC2 to the promoters of its target genes during the activation of JA signaling.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type (WT). The plant materials used in this study, including the *CKB4ox* (45), *CK2mut* (12) and *JAZ1::GUS* (46) lines, have been described previously. The *jin1-9* line (SALK_017005) was obtained from the Arabidopsis Biological Resource Center (ABRC). The mutants and transgenic lines were confirmed by PCR. To generate the *35S::MYC2-GFP* line, the *MYC2* coding sequence was amplified with gene-specific primers by PCR, and then cloned into the pBI-eGFP vector (47) through blunt-end ligation. To generate the *MYC2::MYC2* line, the genomic DNA including *MYC2* coding sequence, and its upstream 1600 bp and downstream 400 bp sequence was amplified with specific primers, and then cloned into pCambia1300 vector. The constructs were confirmed through DNA sequencing. The primers used to generate the constructs are listed in Supplementary Table S1. Transgenic *Arabidopsis* plants harboring these constructs were generated through *Agrobacterium tumefaciens*-mediated transformation. The *Arabidopsis* seedlings were vertically grown on agar medium containing half-strength Murashige and Skoog (1/2 MS) medium supplemented with 1% agar and 1% sucrose, pH 5.8, and at 22°C and 100 μmol m^{-2} s^{-1} illumination under 16 h light/8 h dark conditions.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed using the Matchmaker Yeast Transformation System (Clontech) following the manual. The primers used to generate constructs for the yeast two-hybrid assays are listed in Supplementary Table S1. Yeast AH109 cells were co-transformed with specific bait and prey constructs. All yeast transformants were grown on SD/-Trp/-Leu/-His/-Ade medium for interaction tests.

Bimolecular fluorescence complementation (BiFC)

The coding sequences of *MYC2* and *CKB4* were cloned into the pSPYNE and pSPYCE vectors (48), respectively, to obtain MYC2-nYFP and CKB4-cYFP fusion constructs. Subsequently, these constructs or empty vectors were transformed into *A. tumefaciens* C58C1. *Nicotiana benthamiana* plants were infiltrated with different combinations of the transformed *A. tumefaciens* bacteria as described previously (49). At 3 days after infiltration, YFP fluorescence was visualized using a confocal microscope. Samples were stained with 5 μg/mL of 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) to show the positions of nuclei. BiFC experiments were performed three times with same results observed for each time.

Phosphorylation assay

The 6 × His-tagged proteins were purified using the pET system (Novagen) in *Escherichia coli*. The coding sequences of *MYC2, CKA3*, and *CKB4* were cloned into pET32a vector, and then transformed into the BL21 (DE3) strain of *E. coli*. The proteins were isolated using a Ni-NTA column (Qiagen). A phosphorylation reaction was conducted using the method described previously with CK2 composed of CKA3 and CKB4 (50). The reaction was terminated by adding 4 × SDS loading buffer followed by 5 min of boiling. Proteins were separated using standard gel electrophoresis and the gels were dried and exposed to a phosphorimager screen. The autoradiograph was imaged using a Typhoon 9200 laser scanner.

Identification of phosphorylation site of MYC2

MYC2 proteins were phosphorylated as described above, and then separated by 8% SDS-PAGE electrophoresis. The corresponding gel bands of MYC2 visualized by Commassie Blue (CBB) stain were excised. In-gel digestion with trypsin and phosphopeptide enrichment by immobilized metal affinity chromatography (IMAC) were carried out following standard protocol (51). RPLC-ESI-MS/MS was used to detect the phosphopeptides. LC-MS/MS detection was carried out on a hybrid quadrupole-TOF LC/MS/MS mass spectrometer (TripleTOF 5600+, AB Sciex) equipped with a nanospray source. Raw data from TripleTOF 5600+ were analyzed with ProteinPilot software. Data were searched against the UniProt *A. thaliana* reference proteome database using the following parameters: Sample Type, Identification; Cys Alkylation, Iodoacetamide; Digestion, Trypsin; Special Factors, Phosphorylation emphasis. Search Effort was set to Rapid ID.

Root length measurement

For root growth inhibition assays of *CK2mut*, 5-day-old seedlings that were germinated on medium without treatment were transferred to media with the indicated treatment and allowed to grow for another 5 days. For root growth inhibition assays of *CKB4ox*, seeds were germinated and grown on 1/2 MS medium supplemented or not with 50 μM JA (Sigma-Aldrich) for 10 days. Digital images of 10-day-old seedlings of individual lines were captured. Root length was quantified using ImageJ software. Error bars were obtained based on the measurement of 20 seedlings per treatment from individual lines. At least three replicates were performed for each treatment. Statistical analysis was performed using a Student's *t*-test or ANOVA with Tukey's test.

Gene expression analysis

To evaluate the expression of JA-responsive genes, 10-day-old seedlings of individual lines were submerged in liquid 1/2 MS medium with 100 μM JA for 6 h. The *CK2mut* seedlings were pre-treated with 1 μM Dex for 24 h before JA treatment. For assays using TBB and JA, the wild-type seedlings were pre-treated with 5 μM TBB for 24 h before JA treatment. RNA from the treated seedlings was subsequently isolated, and reverse transcription PCR (RT-PCR) and qRT-PCR were performed as described previously (52). *PROTEIN PHOSPHATASE 2A SUBUNIT A3* (*PDF2*, AT1G13320) was selected as an internal control. The gene-specific primers that were used for qRT-PCR are listed in Supplementary Table S1. The experiments were performed in biological and technical triplicates.

GUS staining

GUS staining and MUG assays were carried out according to the previously described methods (53). The experiments were repeated three times.

Immunoblot and immunoprecipitation analysis

Immunoblot and immunoprecipitation assays were performed as previously described with slight modifications (54). Briefly, 6-day-old seedlings were treated with 100 μM JA for 6 h, and then roots were collected and ground in liquid nitrogen. The *CK2mut* seedlings were pre-treated with 1 μM Dex for 24 h before JA treatment. Proteins were extracted in RIPA buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 5 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin]. Anti-GFP antibody (Santa Cruz) was used to detect MYC2-GFP proteins. For immunoprecipitation assays, protein extracts were pre-incubated for 1 h at 4°C with Protein A agarose (Roche), and then incubated for 4 h at 4°C with Anti-GFP agarose conjugate (Santa Cruz). Immunocomplexes were washed five times with RIPA buffer and eluted by adding 4 × SDS loading buffer followed by 5 min of boiling. The anti-phospho-(Ser/Thr) antibody (Abcam) was used to detect the phosphorylated isoform of MYC2. All the experiments were performed three times and showed similar results.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as previously described (55). Anti-GFP agarose conjugate (Santa Cruz) was used to immunoprecipitate the protein-DNA complex. The isolated chromatin sample before precipitation was used as the input control. The enrichment of DNA fragments was determined by qRT-PCR. The relative enrichment was calculated by normalizing the amount of a target DNA fragment after ChIP against that of a genomic fragment of *ACT2* (AT3G18780) before ChIP (Input). The binding of MYC2 with the G-box motifs in the promoter region of *JAZ1* (–283 bp from ATG), *JAZ3* (–343 and –401 bp from ATG), *LOX3* (–254 bp from ATG), and *CORI3* (–147 bp from ATG) was determined with specific primers listed in Supplementary Table S1. The experiments were performed in biological and technical triplicates.

RESULTS

CK2 interacts with and phosphorylates MYC2

In order to identify potential regulators of MYC2, we performed a yeast two-hybrid (Y2H) screen using the full-length MYC2 from *Arabidopsis* and found that CKB4, a regulatory subunit of CK2, interacted with MYC2. The potential of the other three regulatory subunits of CK2 from *Arabidopsis* to interact with MYC2 was also examined. CKB1 and CKB2 were found to be also able to interact with MYC2 in Y2H assay (Figure 1A). To confirm the interaction between CKB4 and MYC2 *in vivo*, we conducted a bimolecular fluorescence complementation (BiFC) assay. The N-terminus of yellow fluorescent protein (YFP) was fused to MYC2 to create MYC2-nYFP, and the C-terminus of YFP was fused to CKB4 to create CKB4-cYFP, and these expression constructs were introduced into tobacco leaves by *Agrobacterium*-mediated transformation. While no fluorescence was detected in the negative control, the presence of fluorescence in the nuclei of leaf cells that co-expressed MYC2-nYFP and CKB4-cYFP (Figure 1B) indicated that CKB4 interacts with MYC2 *in vivo*.

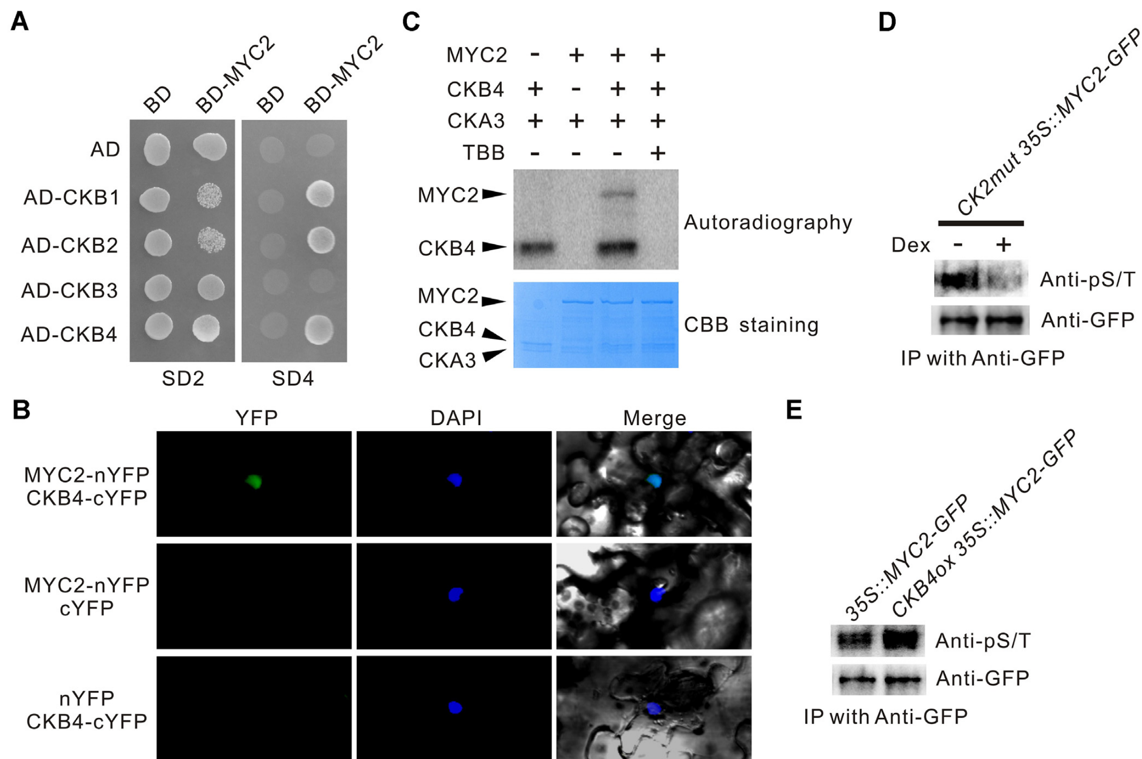

Figure 1. MYC2 is a substrate of CK2. (**A**) Yeast two-hybrid assay to evaluate the interaction between MYC2 and CKBs. Transformants harboring both AD- and BD-derived constructs were grown on SD2 (–Trp/–Leu) medium for growth control and SD4 (–Trp/–Leu/–His/–Ade) medium for the interaction test. (**B**) BiFC assay using nYFP fused with MYC2 and cYFP fused with CKB4. Proteins were transiently co-expressed in *N. benthamiana*. DAPI, 40,6-diamino-2-phenylindol fluorescence. Merge, overlay of YFP, DAPI, and bright field. (**C**) Phosphorylation assay using CKA3/CKB4 as the CK2 holoenzyme and MYC2 as the substrate. TBB, a specific inhibitor of CK2 activity, was used at a concentration of 1 μM. The gel was dried and analyzed by phosphorimaging (upper), and an image of the gel stained with Coomassie Brilliant Blue (CBB, bottom) is shown as the loading control. (**D**) Immunoblot analysis of MYC2 phosphorylation in *CK2mut*. Protein extracts were immunoprecipitated with anti-GFP agarose conjugate and detected for MYC2-GFP (anti-GFP) or phosphorylated MYC2-GFP isoform (anti-pSer/Thr). The *CK2mut MYC2-GFP* seedlings were treated with 1 μM Dex for 24 h before protein extraction. (**E**) Immunoblot analysis of MYC2 phosphorylation in *CKB4ox*. The uncut and repeated immunoblot images can been found in Supplementary Figure S12 and S13.

This interaction between MYC2 and CKB4 led us to test whether CK2 holoenzyme can phosphorylate MYC2. Thus, a CK2 kinase assay was performed using the purified recombinant proteins MYC2, CKB4 and CKA3, a catalytic subunit of CK2. Recombinant CK2 holoenzyme consisting of CKB4 and CKA3 was phosphorylated at CKB4, which is consistent with a previous study (50). When MYC2 protein was added to the reaction, the CK2 holoenzyme phosphorylated MYC2 in a CKB4-dependent manner, and this phosphorylation was inhibited by 4,5,6,7-tetrabromobenzotriazol (TBB), a CK2-specific inhibitor (Figure 1C). These results evidenced that MYC2 is a substrate of CK2 *in vitro*.

We subsequently tested whether CK2 affects the phosphorylation level of MYC2 *in vivo* using the dexamethasone (Dex)-inducible dominant negative plant line *CK2mut*, which has significantly inhibited CK2 kinase activity and shows multiple defects of seedling development in the presence of Dex (12). We crossed *CK2mut* with the transgenic line *35S::MYC2-GFP* and treated the resultant *CK2mut 35S::MYC2-GFP* with Dex to inhibit endogenous CK2 activity. Total protein extracts isolated from the treated plants were immunoprecipitated with an anti-GFP antibody agarose conjugate and assayed by immunoblot with anti-phospho-(Ser/Thr) antibody. The result showed that the phosphorylation level of MYC2 in the Dex-treated *CK2mut 35S::MYC2-GFP* plants was much lower than that in the untreated control (Figure 1D). Meanwhile, we also crossed the *35S::MYC2-GFP* with a transgenic line *CKB4ox*, which contains overexpressed *CKB4* gene and increased CK2 kinase activity, as shown in a previous study (45). Corresponding to the increased CK2 kinase activity, the phosphorylation level of MYC2 in *CKB4ox* plants was markedly higher than that in the WT background (Figure 1E), These results indicate that CK2 contributes to MYC2 phosphorylation *in vivo*.

Inhibition of CK2 activity arrests JA response

Based on the finding that CK2 phosphorylates MYC2, we hypothesized that CK2 plays a role in JA signaling. Thus, JA response in *CK2mut* was examined using a root elongation inhibition assay. We assayed root elongation of *CK2mut* plants induced by different concentrations of Dex (0.1, 0.2 and 0.5 μM) at first, and found that 0.1 μM Dex was sufficient to trigger changes in root growth (Supplementary Figure S1). Then, we measured the root lengths of wild-type (WT) and *CK2mut* plants treated with 0.1 μM Dex in

combination with 0, 10, or 50 μM JA, to evaluate the JA sensitivity of *CK2mut*. It was found that *CK2mut* was less sensitive than WT to both 10 μM and 50 μM JA, based on the root length ratio of plants grown in the presence of both Dex and JA to that of plants grown in the presence of Dex alone (Figure 2A and B). These results suggest that CK2 functions in the JA-mediated inhibition of root elongation.

Next, whether JA-induced transcriptional activation was affected in *CK2mut* was explored by analyzing the expression of the JA-responsive and MYC2-targeted genes *JAZ1*, *JAZ3*, *LOX3,* and *CORI3* using quantitative real-time PCR (qRT-PCR). All tested genes were highly induced by JA in WT plants, as previously reported (56). However, the induction of these genes by JA in *CK2mut* was significantly inhibited (Figure 2C). We crossed *CK2mut* with the marker line *JAZ1::GUS*, in which β-glucuronidase (GUS) expression is driven by the *JAZ1* promoter (46), and examined GUS activity in *CK2mut JAZ1::GUS* in the presence or absence of JA and Dex. GUS staining and a MUG assay of *CK2mut JAZ1::GUS* plants revealed that, while *JAZ1* promoter activity was stimulated by JA, the JA-induced increase in *JAZ1* promoter activity was significantly repressed by the application of Dex and resulted inhibition of CK2 activity (Figure 2D and E). Consistent with these results, JA-induced expression of *JAZ1*, *JAZ3*, *LOX3* and *CORI3* was also suppressed in WT plants that were treated with TBB (Supplementary Figure S2). Together, these results indicate that CK2 activity is required for JA-induced transcriptional activation.

CKB4 overexpression enhances JA response in a MYC-dependent manner

Having shown that inhibition of CK2 activity affected JA-induced gene expression and JA-mediated inhibition of root elongation, we next analyzed whether increased CK2 activity would promote the JA response in the transgenic line *CKB4ox*. While the root length of JA-treated WT plants was 34% of that of the untreated controls, the root length of JA-treated *CKB4ox* plants was 28% of that of untreated *CKB4ox* (Figure 3A–C). These results imply that *CKB4ox* roots are more sensitive to JA than are the roots of WT plants. Because CK2 interacts with and phosphorylates MYC2, MYC2 should be involved in this enhanced inhibition of root growth by JA. We therefore measured the root lengths of *CKB4ox jin1-9* plants*,* which were obtained by crossing *CKB4ox* with *jin1-9*, a MYC2 knock-out mutant. It was found that the JA sensitivity of *CKB4ox jin1-9* resembled that of *jin1-9*, as shown by the root length ratios of the corresponding lines (*CKB4ox*, *CKB4ox jin1-9* and *jin1-9*) grown in the presence and absence of JA (Figure 3A–C).

A qRT-PCR analysis was next conducted to examine whether the JA-induced expression of *JAZ1*, *JAZ3*, *LOX3* and *CORI3* was affected in *CKB4ox* and *CKB4ox jin1-9*. The result showed that the JA-mediated increase in expression of these genes was greater in JA-treated *CKB4ox* than in WT plants (Figure 3D). However, this increased expression of JA-responsive genes in *CKB4ox* was inhibited in *CKB4ox jin1-9* to levels similar to those in *jin1-9* (Figure 3D), suggesting that *CKB4* overexpression enhances JA transcriptional activation in a MYC2-dependent manner. Meanwhile, the direct role of CKB4 on MYC2-mediated transcription activation was checked using transient promoter activation assay. The result showed that transient overexpression of CKB4 can significantly increase the MYC2-induced expression of *JAZ1::LUC* and *LOX3::LUC* reporters (Supplementary Figure S3). In addition, we evaluated the sensitivity of root length of transgenic lines overexpressing *CKB1* or *CKB2* that we constructed previously (15), as CKB1 and CKB2 also interacted with MYC2. Similar to *CKB4ox* line, *CKB1ox* and *CKB2ox* lines showed enhanced inhibition of root growth by JA (Supplementary Figure S4), suggesting that CKB1 and CKB2 also regulate JA response.

In order to further demonstrate the role of CK2 in JA and MYC2-regulated physiological processes such as plant defense against pathogens, the sensitivity of *CKB4ox* to *B. cinerea* was analyzed. Previous studies have evidenced that MYC2 is required for JA-mediated plant resistance to *B. cinerea*, as MYC2 knock-out mutants are more susceptible (57–60). In consistence with the positive role of CK2 on MYC2-induced transcription activation, *CKB4ox* plants displayed smaller lesions after spore infection (Supplementary Figure S5A, B), indicating an increased resistance to *B. cinerea*. The increase of *B. cinerea* resistance of *CKB4ox* was largely dependent on MYC2, as the line *CKB4ox jin1-9* showed resistance lower than the wild type but higher than *jin1-9* (Supplementary Figure S5A, B), supporting the regulatory role of CKB4 on MYC2 function. The different resistance between *CKB4ox jin1-9* and *jin1-9* may be caused by the fact that the defense reaction of plant to *B. cinerea* also employs other signaling pathways besides JA signaling, which may also involve CKB4. All together, these data manifest that CK2 can promote MYC2-mediated JA signaling response.

CK2 promotes the binding of MYC2 to the promoters of target genes in response to JA

To further establish how CK2 modulates MYC2 function, we first examined whether the cellular localization of MYC2 was affected in *CKB4ox* and *CK2mut*. Confocal microscopy analysis of *35S::MYC2-GFP, CK2mut 35S::MYC2-GFP* and *CKB4ox 35S::MYC2-GFP* seedlings showed that MYC2-GFP had a similar nuclear localization in all tested lines (Supplementary Figure S6A and B), suggesting that CK2 does not modulate the subcellular localization of MYC2. Next, the stability of MYC2-GFP was evaluated using cycloheximide (CHX), an inhibitor of *de novo* protein synthesis. The result showed that CHX treatment significantly weakened MYC2-GFP fluorescence in *35S::MYC2-GFP*, consistent with a previous report (40), and also in *CKB4ox 35S::MYC2-GFP* with similar degradation rates of MYC2-GFP (Supplementary Figure S6A and C). Meanwhile, the degradation rate of MYC2-GFP in *CK2mut* in the presence of Dex was not changed compared with that in the absence of Dex (Supplementary Figure S6B and D).

JA treatment was evidenced to induce MYC2 protein accumulation previously (40,61), and we further examined whether this process was regulated by CK2. When the

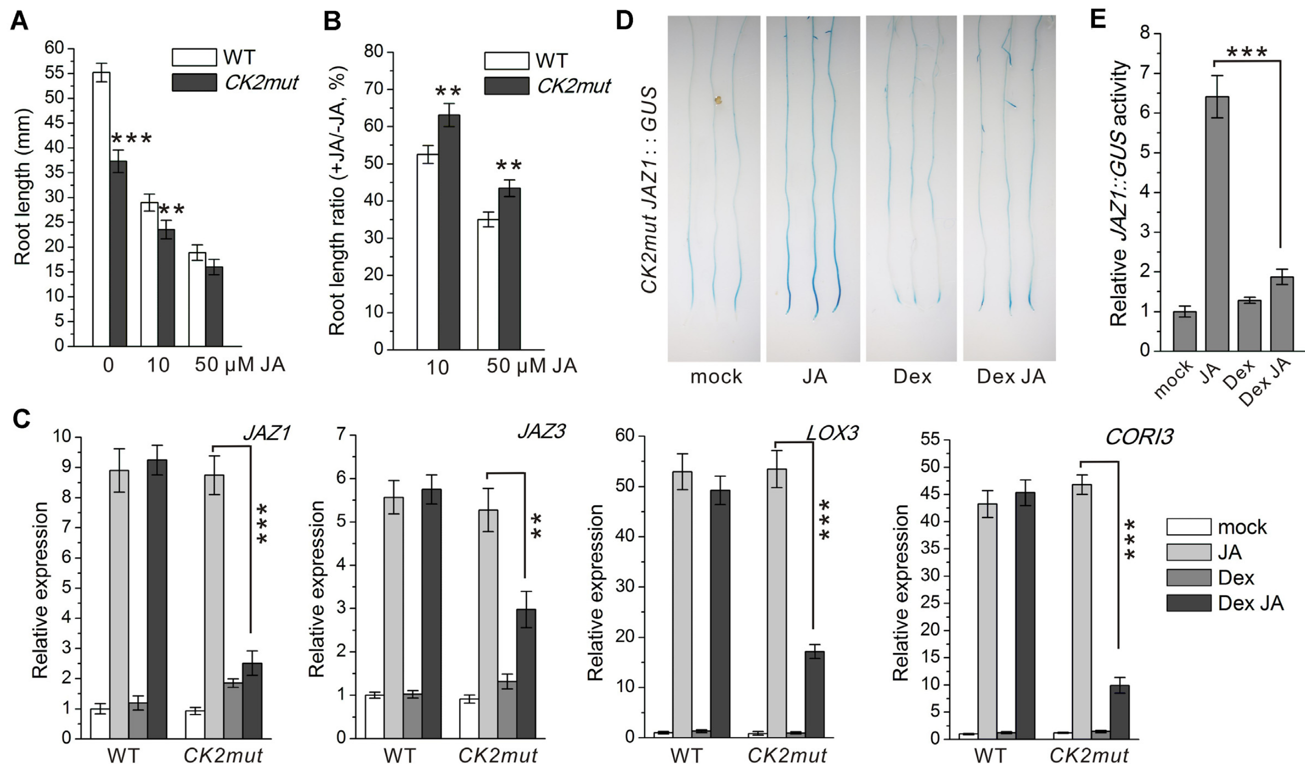

Figure 2. The JA response is inhibited in *CK2mut*. (**A**) Root length of 10-day-old wild-type and *CK2mut* seedlings grown on 1/2 MS medium supplemented with 0.1 μM Dex and the indicated concentration of JA. Error bars represent the SD, and the asterisks indicate that the differences between the wild type and *CK2mut* treated with the indicated concentration of JA are significant (** $P < 0.01$, *** $P < 0.001$, Student's *t*-test, $n = 20$). (**B**) Root length ratio of 10-day-old wild-type and *CK2mut* seedlings. The ratios were obtained by comparing the root length on 1/2 MS medium supplemented with 0.1 μM Dex and 10 or 50 μM JA with the root length on 1/2 MS medium supplemented only with 0.1 μM Dex (designated as 100%). Error bars represent the SD of four biological replicates, and the asterisks indicate that the differences between the wild type and *CK2mut* treated with the indicated concentration of JA are significant (** $P < 0.01$, Student's *t*-test). (**C**) Relative expression of the JA-responsive genes *JAZ1*, *JAZ3*, *LOX3* and *CORI3* in wild-type and *CK2mut* plants treated with JA, Dex or JA and Dex. The expression level of the indicated gene in wild-type plants without treatment was set to 1. Error bars represent the SD of three biological replicates (** $P < 0.01$, *** $P < 0.001$, Student's *t*-test). The wild type was used as a control to show that Dex treatment itself had no effect on the expression of JA-responsive genes. (**D**) GUS staining of 10-day-old *CK2mut JAZ::GUS* seedlings after treatment with JA and Dex. At least 20 seedlings were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) for each treatment, and representative images of roots are shown. The experiment was performed three times with similar results. (**E**) Relative GUS activity of whole *JAZ::GUS* seedlings in (D). The GUS activity of *JAZ::GUS* was determined by a MUG (4-methylumbelliferyl-β-D-glucuronide) assay, and that without treatment was set to 1. Error bars represent the SD of three biological replicates (*** $P < 0.001$, Student's *t*-test).

plants were subjected to JA treatment, the accumulation of MYC2 protein was induced in all assayed lines (Figure 4A and B). However, *CKB4ox* showed similar accumulation of MYC2 protein as WT plants in the presence of JA, revealing that increasing CK2 activity does not affect the level of MYC2 protein (Figure 4A). Moreover, in the presence of JA, *CK2mut* treated with Dex showed similar accumulation of MYC2 protein as that without Dex treatment (Figure 4B), indicating that decreasing CK2 activity does not change MYC2 accumulation either. Thus, the changes of CK2 activity in *CKB4ox* and Dex-treated *CK2mut* plants do not change MYC2 accumulation.

Considering that CK2 has been shown to regulate the promoter-binding ability of several transcription factors (TFs) (3), we examined whether CK2 could regulate the binding of MYC2 to its target promoters. For this purpose, a chromatin immunoprecipitation (ChIP) assay was performed using the *CK2mut 35S::MYC2-GFP* line, to determine the amount of MYC2 binding with the promoters of *JAZ1*, *JAZ3*, *LOX3* and *CORI3*, which are direct targets of MYC2 (56). It was found that under JA treatment, the binding of MYC2 with the G-box motifs in the promoter region of *JAZ1*, *JAZ3*, *LOX3* and *CORI3* was significantly decreased in Dex-treated *CK2mut* compared with untreated plants (Figure 4C), manifesting that inhibition of CK2 activity suppresses JA-induced binding of MYC2 to the promoters of JA-responsive genes. Meanwhile, the ChIP assay using the *CKB4ox 35S::MYC2-GFP* line showed that, the binding of MYC2 to target promoters was markedly increased in *CKB4ox* compared to that in WT background under JA treatment (Figure 4D), indicating that increase of CK2 activity enhances the target promoter binding of MYC2. Taken together, these data suggest that CK2 promotes the JA response by increasing the binding of MYC2 to the promoters of JA-responsive genes.

CK2-mediated phosphorylation is required for MYC2 function in response to JA

To further verify the regulatory role of CK2 on MYC2 through phosphorylation, the phosphorylation sites were

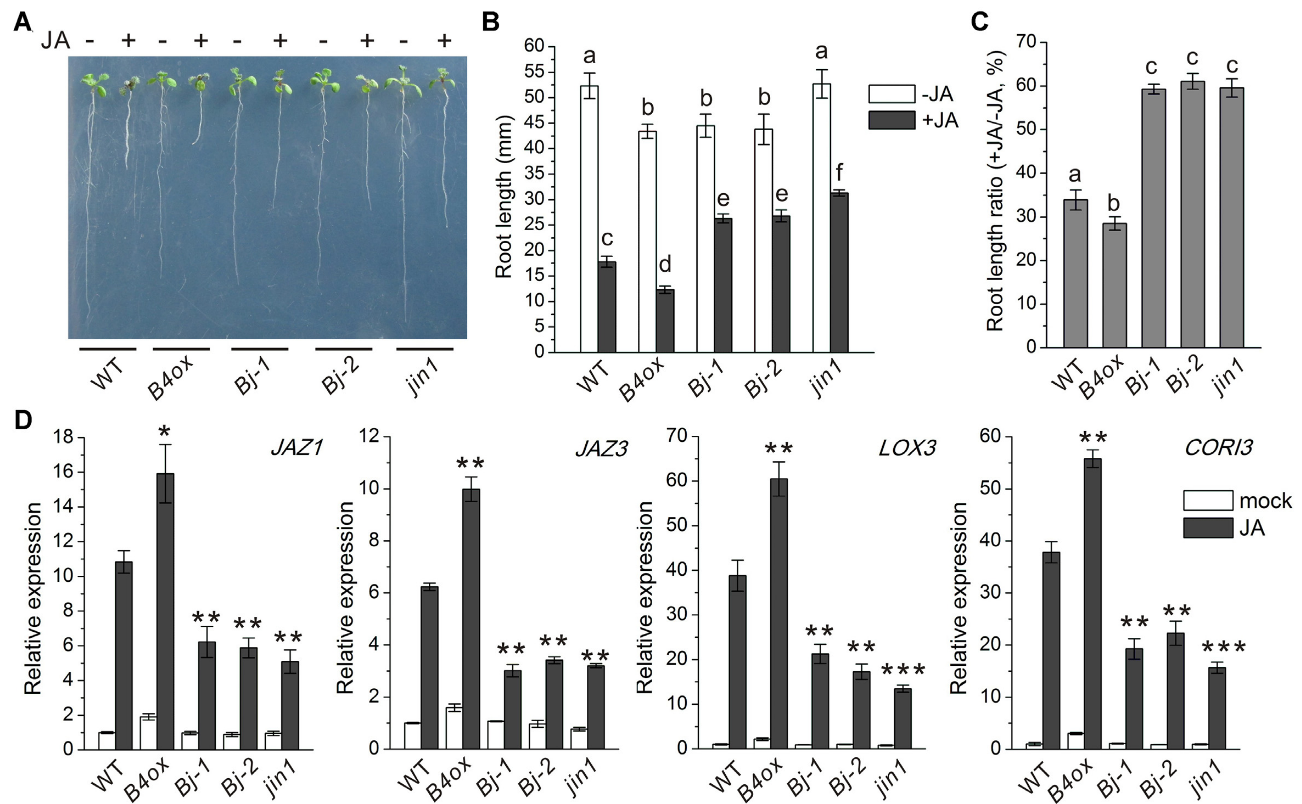

Figure 3. Overexpression of *CKB4* enhances the JA response in a *MYC2*-dependent manner. (**A**) Representative image of 10-day-old wild-type (WT), *CKB4ox (B4ox)*, *CKB4ox jin1-9 (Bj-1* and *Bj-2)* and *jin1-9 (jin1)* plants grown on 1/2 MS medium supplemented or not with 50 μM JA. (**B**) Root length statistics in (A). Error bars represent the SD, and the differences between columns annotated with different letters are significant ($P < 0.05$ by Tukey's test, $n = 20$). (**C**) Root length ratio of 10-day-old wild-type, *CKB4ox*, *CKB4ox jin1-9* and *jin1-9* plants. The ratios were obtained by comparing the root length on 1/2 MS media supplied with 50 μM JA to the root length on media without JA (designated as 100%). Error bars represent the SD for four biological replicates, and the differences between columns annotated with different letters are significant ($P < 0.05$ by Tukey's test). (**D**) Relative expression of JA-responsive *JAZ1*, *JAZ3*, *LOX3* and *CORI3* in wild-type, *CKB4ox*, *CKB4ox jin1-9* and *jin1-9* plants with or without JA treatment. The expression level of the indicated gene in wild-type plants without treatment is set to 1. Error bars represent the SD of three biological replicates, and asterisks represent significant differences compared to WT (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test).

mapped using phosphorylated MYC2 by recombinant CK2 *in vitro* via mass spectrometry. Six sites were identified to be phosphorylated by CK2 (Figure 5A and Supplementary Table S2). All these sites were followed by negatively charged residues (D/E) at +1 to +4 positions, while five sites strictly obeyed the consensus sequence of CK2 substrates, in which the residue at +3 position is negatively charged (S/T-X-X-D/E). T128 was located in the JAZ-interacting domain (JID), and the others were located in the interdomain region between transcription activation domain (TAD) and DNA-binding bHLH domains. When five (S408, T413, S417, S420 and S425) or six (T128, S408, T413, S417, S420 and S425) sites were respectively substituted to Ala to obtain phospho-dead mutants (5A and 6A), the phosphorylation levels showed marked reduction for the mutant MYC2^{5A} and were nearly abolished for MYC2^{6A} (Figure 5B), confirming that these sites are CK2 targets.

Subsequently, the *MYC2*^{6A} mutant gene was introduced into *MYC2* knock-out line *jin1-9* to investigate the role of CK2-mediated phosphorylation *in vivo*. The result showed that the transgenic lines containing *MYC2*^{6A} gene under the control of native *MYC2* promoter and *35S* promoter (with *MYC2* fused with *GFP*) which were constructed independently displayed similar JA insensitivity in term of root growth to the *jin1-9* mutant, while the wild-type *MYC2* gene fully recovered the JA insensitivity (Figure 5C–E and Supplementary Figure S7A–D). Overexpression of *MYC2*^{6A} did not recover the JA sensitivity of *jin1-9* mutant even under the background of *CKB4* overexpression (Supplementary Figure S5C–E). The *B. cinerea* resistance of *CKB4ox jin1-9 35S::MYC2*^{6A}*-GFP* was similar to that of *CKB4ox jin1-9* (Supplementary Figure S5A, B). Moreover, transient overexpression of *MYC2*^{6A} did not significantly increase the expression of *JAZ1::LUC* and *LOX3::LUC* reporters (Supplementary Figure S3). These data indicate that CK2-mediated phosphorylation is essential for MYC2 function in JA response.

Next, the regulatory role of phosphorylation at these sites was further investigated using the *35S::MYC2*^{6A}*-GFP* line. MYC2^{6A}-GFP showed a similar nuclear location and protein expression level with and without JA treatment as the wild-type MYC2-GFP (Figure 5F and Supplementary Figure S7E, F), although the phosphorylation level was remarkably lower (Figure 5G). Consistent with the data obtained with *CK2mut 35S::MYC2-GFP* line, the JA-induced binding of MYC2 to the promoters of *JAZ1*,

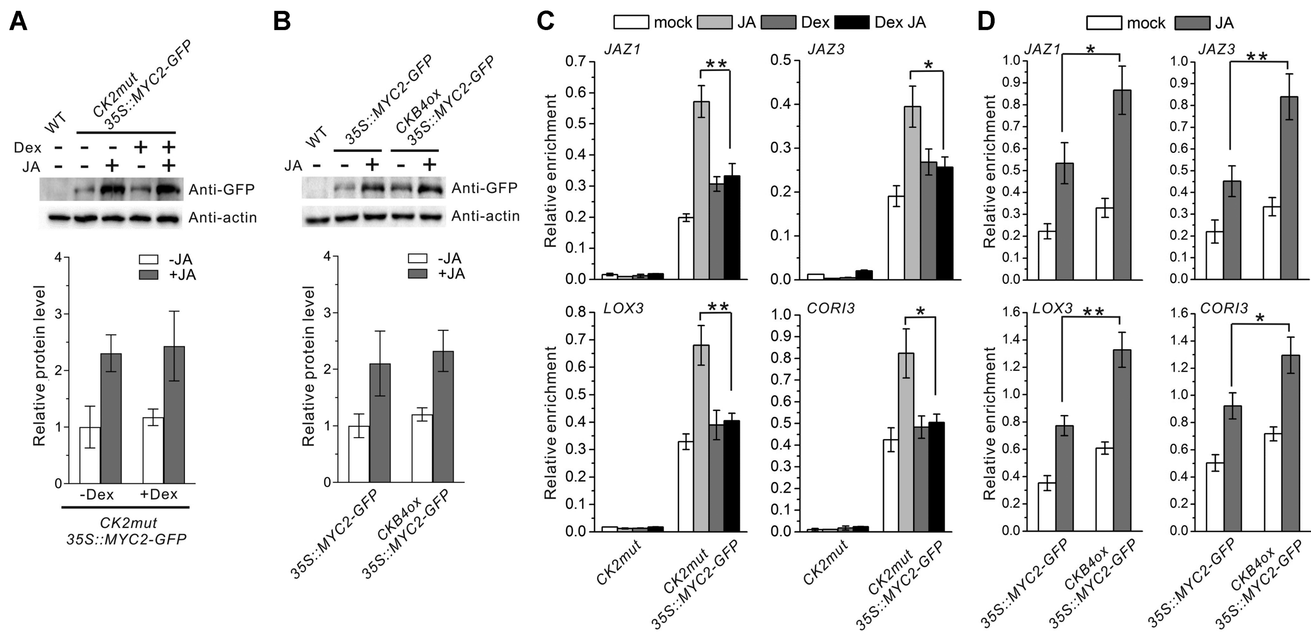

Figure 4. CK2 regulates the binding of MYC2 to the promoters of its target genes. (**A**) Accumulation of MYC2-GFP proteins in *CK2mut 35S::MYC2-GFP* seedlings treated with JA and Dex assayed by immunoblot analysis. Actin was used as loading controls to show equal total protein extract loaded per lane. (**B**) Accumulation of MYC2-GFP in 6-day-old *35S::MYC2-GFP* and *CKB4ox 35S::MYC2-GFP* seedlings treated or not with 100 μM JA for 6 h assayed by immunoblot analysis. All the experiments were performed three times with similar results, and the quantification data of band intensity were shown at bottom in (A) and (B), respectively. Error bars represent the SD of three replicates. The uncut immunoblot images can been found in Supplementary Figure S12. (**C**) ChIP-qRT-PCR analysis of MYC2-GFP binding to the G-box motifs in the promoters of *JAZ1*, *JAZ3*, *LOX3* and *CORI3* in 10-day-old *CK2mut* and *CK2mut 35S::MYC2-GFP* seedlings treated with JA and Dex. The *CK2mut* was used as a control to show that the enrichment of the corresponding DNA fragment is dependent on the *35S::MYC2-GFP* transgene. (**D**) ChIP-qRT-PCR analysis of MYC2-GFP binding to the G-box motifs in the promoters of *JAZ1*, *JAZ3*, *LOX3* and *CORI3* in *35S::MYC2-GFP* and *CKB4ox 35S::MYC2-GFP* seedlings with or without JA treatment. Error bars represent the SD of three biological replicates (* $P < 0.05$, ** $P < 0.01$, Student's *t*-test). The relative enrichment was calculated by normalizing the amount of target DNA fragment after ChIP against that of a genomic fragment of *ACT2* before ChIP.

JAZ3, *LOX3* and *CORI3* was significantly repressed by the MYC2^{6A} mutation as revealed by the ChIP assay (Figure 5H), confirming that CK2-mediated phosphorylation promotes MYC2 binding to target gene promoter. Nevertheless, the specific binding of MYC2 to the consensus DNA *in vitro* was not significantly affected by CK2 phosphorylation or by *MYC2^{6A}* mutation as shown by the band intensities of the MYC2-DNA complex in electrophoretic mobility shift assay (EMSA) (Supplementary Figures S8 and S9A), which is plausible as the identified phosphorylation sites were not located in the bHLH domain. This implies that CK2-mediated phosphorylation regulates the promoter binding of MYC2 not simply through affecting its DNA affinity. Additional Y2H assay showed that the dimerization of MYC2^{6A} was not affected (Supplementary Figure S9B). Considering that T128 is located in the JID domain, we tested the interaction of the MYC2^{6A} mutant and a MYC2^{T128D} (phosphor-mimic) mutant with JAZ3 using Y2H assay. The result showed that both mutants can interact with JAZ3 like wild-type MYC2 (Supplementary Figure S9C), indicating that CK2 phophorylation does not affect the interaction of MYC2 with JAZ repressors. All together, the CK2-mediated phophorylation regulates MYC2 function by affecting its binding to the promoters of JA-responsive genes *in vivo*.

DISCUSSION

The roles of CK2 in regulating plant development and abiotic stress responses have been well investigated (3,52). During plant growth and development, CK2 regulates the circadian clock, light, and auxin signaling through phosphorylating specific proteins (4,10,11). CK2 was suggested to be involved in plant responses to abiotic stress based on the observations that CK2 phosphorylates several proteins involved in ABA signaling, and that mutants of CK2 have altered sensitivity to ABA, salt and cadmium stresses (14,15,18,20). Furthermore, CK2 was also implicated in SA signaling (17,62). Here, we establish a role for CK2 in JA signaling and identify MYC2 as a novel substrate for CK2.

A previous study that used the kinase inhibitor staurosporine implicated protein phosphorylation in the regulation of the JA signaling and wound response (39). Additional studies identified MPK6 as the kinase that negatively regulates JA signaling, and showed that MPK6 can directly phosphorylate MYC2, although the effect of MPK6-mediated phosphorylation is still unclear (42,43). Recently, receptor-like kinase FER was evidenced to inhibit JA signaling by phosphorylating and facilitating the degradation of MYC2 (44). We show here that, through interacting with and phosphorylating MYC2, CK2 primarily promotes JA signaling, because an increase in CK2 activity by overexpression of the CK2β subunit *CKB4* enhances the JA

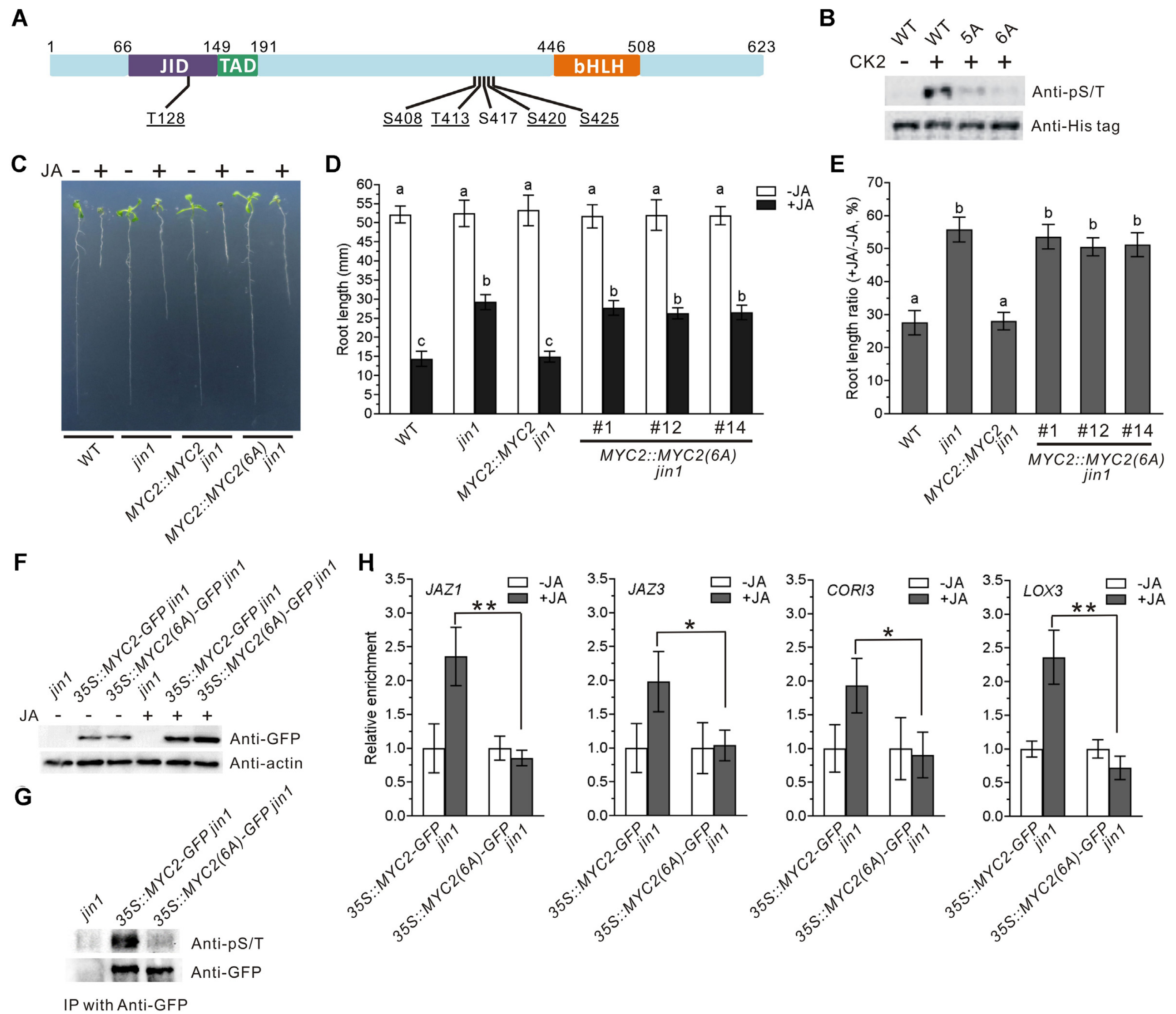

Figure 5. Phosphorylation of MYC2 by CK2 regulates its promoter binding. (**A**) Phosphorylation sites of MYC2 by CK2 identified by mass spectrometry. The underlined residues obey the consensus sequence of CK2 substrate (S/T-X-X-D/E). JID, JAZ-interacting domain; TAD, transcription activation domain; bHLH, basic helix-loop-helix domain. (**B**) Phosphorylation assay of wild-type MYC2 and phosphor-dead mutants MYC2 using CKA3/CKB4 as the CK2 holoenzyme. Phosphorylation was detected with anti-pSer/Thr antibody. Immunoblot with anti-His tag antibody is shown as the loading control. (**C**) Representative image of 10-day-old WT, *jin1-9* (*jin1*), *MYC2::MYC2 jin1-9,* and *MYC2::MYC2^{6A} jin1-9* plants with or without JA treatment. (**D**) Root length statistics in (C). Data of three independent *MYC2::MYC2^{6A} jin1-9* lines are shown. Error bars represent the SD, and the differences between columns annotated with different letters are significant ($P < 0.05$ by Tukey's test, $n = 20$). (**E**) Root length ratio of 10-day-old WT, *jin1-9*, *MYC2::MYC2 jin1-9,* and *MYC2::MYC2^{6A} jin1-9* plants. The ratios were obtained by comparing the root length treated with 50 μM JA to that without treatment (designated as 100%). Error bars represent the SD, and the differences between columns annotated with different letters are significant ($P < 0.05$ by Tukey's test, $n = 4$). (**F**) Accumulation of MYC2-GFP in *35S::MYC2-GFP jin1-9* and *35S::MYC2^{6A}-GFP jin1-9* plants assayed by immunoblot analysis. Actin was used as loading control. (**G**) Phosphorylation of MYC2-GFP in *35S::MYC2-GFP jin1-9* and *35S::MYC2^{6A}-GFP jin1-9* plants assayed by immunoblot analysis. MYC2-GFP was immunoprecipitated with anti-GFP agarose conjugate, and then phosphorylation was detected with anti-pSer/Thr antibody. Immunoblot with anti-GFP antibody is shown as the loading control. The uncut immunoblot images can been found in Supplementary Figure S12. (**H**) ChIP-qRT-PCR analysis of MYC2-GFP binding to the G-box motifs in the promoters of *JAZ1*, *JAZ3*, *LOX3* and *CORI3* in *35S::MYC2-GFP jin1-9* and *35S::MYC2^{6A}-GFP jin1-9* plants with or without JA treatment. Error bars represent the SD of three biological replicates (* $P < 0.05$, ** $P < 0.01$, Student's *t*-test). The relative enrichment in each line was normalized with the value without JA treatment set as 1, respectively.

response in a MYC2-dependent manner, while inhibition of CK2 activity in *CK2mut* or upon treatment with a specific inhibitor of CK2 inhibits the JA response. Moreover, phosphor-dead mutation of MYC2 lost the function of activating JA response. By comparing the binding of MYC2 to the promoters of its target genes in plant lines with changed CK2 activity, we show that CK2 regulates the promoter binding of MYC2 without affecting its protein accumulation at high JA level. This result suggests that CK2 facilitates the promoter binding of MYC2 during JA signaling activation. Combining with previous studies, our data suggest that the protein phosphorylation pathway may modulate JA signaling through multiple mechanisms. How the positive and negative roles of protein phosphorylation in JA signaling are integrated remain to be explored.

It is thought that the CK2β subunits are responsible for the specificity, activity, and stability of the CK2 holoenzyme (1). The CK2-mediated phosphorylation of many proteins was mediated or enhanced by CKBs (4,9,10,21). Similarly, CK2 holoenzyme phosphorylates MYC2 through the physical interaction mediated by three CKBs (CKB1/2/4) with functional redundancy. Thus, protein levels of CKBs may affect the phosphorylation level and therefore the function of MYC2. In consistence, the *CKB4ox* line shows enhanced JA sensitivity and increased promoter binding of MYC2. The accumulation of CKB4 is under the control of the circadian clock, and is more stable during the night (45). By contrast, more JA is synthesized during the day and more MYC2 protein accumulates during the day, specifically at dusk (61,63). This may provide a mechanism to protect the plant against over-activation of the JA response, which can, for example, be observed in the *CKB4ox* plant line that shows enhanced inhibition of growth in the presence of JA.

Due to the central role of MYC2 in JA signaling, many studies have focused on the regulation of its expression and function. Both JA and ABA treatment can up-regulate the mRNA levels of *MYC2* (30), and JA also promotes the accumulation of MYC2 protein via a post-transcriptional mechanism (40). In addition to JA, the circadian clock and light signaling also regulate the accumulation of MYC2 protein (57,61). Moreover, post-translational modification of MYC2 was suggested to be involved in JA response (40). Phosphorylation of Thr328 facilitates the turnover of MYC2 protein, which is essential for the transcriptional activity of MYC2. FER was found to be at least one of the kinases that are responsible for Thr328 phosphorylation (44). But it is reasonable to assume that a proline-directed kinase is also a candidate for this modification, because Thr328 is located in a proline-rich region. Our study suggests that CK2 regulates the promoter binding of MYC2, and reveals a novel aspect of MYC2 regulation. We identified T128, S408, T413, S417, S420 and S425 as the phosphorylation sites of MYC2 by CK2. Ser417 and Ser420 were identified as phosphorylation site of MYC2 *in vivo* in a phosphoproteomics studies previously (41). In addition to MYC2, Ser376 and Ser379 in MYC3, which correspond to S417 and S420 in MYC2, were also found to be phosphorylated (41), inspired by which a sequence alignment among MYC2 and its close homologue proteins MYC3/MYC4/MYC5 was carried out. The result showed that T128 was most conserved as it can be found in all four proteins, while S417, S420 and S425 found in MYC2/MYC3/MYC4, T413 in MYC2/MYC3, and S408 only in MYC2 (Supplementary Figure S10). In consistence with this, CKB1/2/4 were confirmed to interact with MYC3 and MYC4 in Y2H assay (Supplementary Figure S11), suggesting that CK2 may regulate JA signaling at least through MYC2/MYC3/MYC4. MYC5 showed a self-activation in Y2H assay and was not further tested for CKBs interaction.

Phosphorylation regulates the promoter binding of TFs through varied mechanisms. As phosphorylation usually introduces negative charges into TFs and DNA is full of negative charges due to the phosphate backbone, a reduction in binding affinity of TFs to DNA after phosphorylation is reasonable and was found for many TFs (64–66). In the EMSA assay of our study, the binding of MYC2 to the consensus DNA was not significantly affected after CK2 phosphorylation as shown by the band intensity of the MYC2–DNA complex. The other most demonstrated mechanism is that phosphorylation affects the interaction of TFs with their regulatory proteins related to promoter binding. In human, mitogen- and stress-activated kinase 1 (MSK1) phosphorylates retinoic acid receptor RARα allowing its binding with TFIIH and further phosphorylation by cdk7, which promotes the binding of RARα to RA-responsive gene promoters (67). IκB kinase (IKK) phosphorylates RelB in response to TNFα and results in the dissociation of RelB from an inhibitory protein IκBα and then the binding of RelB to the promoters of cell migration-associated genes (68). Our ChIP data obtained using the plants with altered CK2 activity and the transgenic plants carrying $MYC2^{6A}$ mutant which almost abrogated the phosphorylation by CK2, clearly evidenced that CK2 phosphorylation facilitates the binding of MYC2 to the promoters of JA-responsive genes *in vivo*, suggesting that the effect of CK2-mediated phosphorylation is not a simple change of electrostatic interaction with DNA. We speculate that the CK2-mediated phosphorylation may regulate the interaction of MYC2 with currently unidentified proteins and then indirectly affect the promoter binding of MYC2, which waits for further investigation in future.

In brief, our study reveals that CK2 positively regulates JA signaling response and JA-related pathogen resistance, which is achieved through phosphorylating MYC2, the master transcription factor of JA responsive gene expression. The CK2-mediated phophorylation can facilitate the binding of MYC2 to the promoters of JA responsive genes in response of JA. Our study discovers a kinase positively regulating JA signaling by phosphorylating MYC2 for the first time, and can significantly facilitate the understanding of JA signaling regulation through post-translational modification.

DATA AVAILABILITY

The data underlying this article are included in the online Supplementary Data file or available from the corresponding author upon reasonable request.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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