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CDK8 is associated with RAP2.6 and SnRK2.6 and positively modulates abscisic acid signaling and drought response in *Arabidopsis*

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Summary

CDK8 is a key subunit of Mediator complex, a large multi-protein complex that is a fundamental part of the conserved eukaryotic transcriptional machinery. However, the biological functions of *CDK8* in plant abiotic stress responses remain largely unexplored.

Here, we demonstrated *CDK8* as a critical regulator in the ABA signaling and drought response pathways in *Arabidopsis*. Compared to wild type, *cdk8* mutants showed reduced

sensitivity to ABA, impaired stomatal apertures, and hypersensitivity to drought stress. Transcriptomic and chromatin immunoprecipitation analysis revealed that *CDK8* positively regulates the transcription of several ABA-responsive genes probably through promoting the recruitment of RNA polymerase II to their promoters.

We discovered that both CDK8 and SnRK2.6 physically interact with an ERF/AP2 transcription factor RAP2.6, which can directly bind to the promoters of *RD29A* and *COR15A* with GCC or DRE elements, thereby promoting their expression. Importantly, we also showed that *CDK8* is essential for the ABA induced expression of *RAP2.6* and *RAP2.6*-mediated up-regulation of ABA-responsive genes, indicating that CDK8 could link the SnRK2.6-mediated ABA signaling to RNA polymerase II to promote immediate transcriptional response to ABA and drought signals.

Overall, our data provide new insights into the roles of CDK8 in modulating ABA signaling and drought responses.

Key words: Mediator complex, CDK8, abiotic stress responses, RAP2.6, SnRK2.6, ABA responsive genes, Arabidopsis

Introduction

The phytohormone abscisic acid (Papdi *et al.*, 2008) plays a prominent role in plant development and abiotic stress tolerance (Finkelstein *et al.*, 2002; Cutler *et al.*, 2010; Klingler *et al.*, 2010; Finkelstein, 2013; Zhu, 2016). ABA signaling involves three core components: the ABA receptors (the PYRABACTIN RESISTANCE 1 [PYR1])/PYR1-Like [PYL] /REGULATORY

COMPONENT OF ABA RECEPTOR [RCAR] family of proteins), the negative regulators (PP2Cs), and the kinases that function as positive regulators (SnRK2s). In the absence of ABA, SnRK2s are inactivated by PP2Cs, which prevent SnRK2s from phosphorylating their downstream targets. Upon ABA perception, the PYL ABA receptors change their conformation, which enables them to associate with and inhibit PP2Cs, leading to activation of the SnRK2s (Fujii *et al.*, 2009; Ma *et al.*, 2009; Park *et al.*, 2009; Hou *et al.*, 2016). Several SnRK2 substrates have been identified, including the AREB/ABF family of transcription factors (TFs), RAV1 (Related to ABI3/VP1) and ion channels such as SLAC1 (Slow Anion Channel-Associated 1), which are required for the induction of ABA-responsive genes and stomatal movement (Sirichandra *et al.*, 2010; Fujii & Zhu, 2012; Wang *et al.*, 2013; Feng *et al.*, 2014), respectively. In addition to the core components, several new components that involve in the ABA signaling have recently been identified as well. Enhancer of ABA co-receptor1 (EAR1), for instance, negatively regulates ABA signaling by enhancing PP2C activities (Wang *et al.*, 2018). However, more downstream components of ABA signaling pathway remain to be identified.

Mediator is a large multi-protein complex that is evolutionarily conserved across eukaryotic species to serve as a co-regulator of gene transcription by connecting TFs to RNA polymerase II (RNAP II) (Kelleher *et al.*, 1990; Myers & Kornberg, 2000; Carlsten *et al.*, 2013). Mediator complex, which was first described in yeast, comprises of 25-30 subunits (Flanagan *et al.*, 1991; Kim *et al.*, 1994; Tsai *et al.*, 2014). Based on previous reports , the structure of the complex is divided into the head module, the middle module, the tail module, and the CDK8 kinase module which consists of the cyclin-dependent kinase 8 (CDK8), C-type cyclin, MED12, and MED13 (Myers & Kornberg, 2000; Tsai *et al.*, 2014). Studies in the human system revealed that CDK8 module could associate with core Mediator via MED13 in a reversible manner to affect transcription (Davis *et al.*, 2013; Allen & Taatjes, 2015). Thus far, more than 20 Mediator subunits in plants were able to be co-purified from *Arabidopsis* cell suspension cultures, but the kinase module was not purified from such cultures even though it was known to exist in plants (Backstrom *et al.*, 2007). Over the last decade, emerging evidence has indicated that several Mediator subunits are crucial for various developmental processes, hormone signaling, plant defense, and abiotic stress tolerance (Samanta & Thakur, 2015; Ito *et al.*, 2016; Malik *et al.*, 2017). For instance, MED14 and MED16, which are subunits of the tail module,

are critical for cold acclimation and plant defense, since *med14* and *med16* mutants are more susceptible to cold stress and plant pathogens than the wild type (WT) (Zhang *et al.*, 2012; Zhang *et al.*, 2013; Hemsley *et al.*, 2014; Wang, C *et al.*, 2015). Interestingly, MED16 is also involved in cellulose formation and iron homeostasis (Yang *et al.*, 2014; Sorek *et al.*, 2015). MED25, also termed as PHYTOCHROME AND FLOWERING TIME 1 (PFT1), is another multi-functional Mediator subunit that contributes to jasmonic acid (JA)-mediated plant defense, ABA signaling, salt tolerance, and flowering control by directly interacting with multiple TFs including MYC2, ABI5, and DREB2A (Elfving *et al.*, 2011; Cevik *et al.*, 2012; Chen *et al.*, 2012; Inigo *et al.*, 2012). MED18, which is located in the head module, was recently found to be important for flowering regulation, ABA response, and plant immunity (Zheng *et al.*, 2013; Lai *et al.*, 2014; Fallath *et al.*, 2017; Zhu *et al.*, 2017). CDK8 is the unique kinase in the Mediator complex with other names of HUA ENHANCER3 (HEN3) and CDKE1 that functions in floral organ determination; as indicated by its partial activities in the AGAMOUS (AG) pathway (Wang & Chen, 2004). CDK8 has also been recognized as a positive regulator in mitochondrial retrograde signaling (Ng *et al.*, 2013), the JA pathway, and plant immunity (Zhu *et al.*, 2014; Chen *et al.*, 2019). However, the biological roles of *CDK8* remain largely unknown in plants.

In the present work, we provide genetic, transcriptomic and biochemical evidence to demonstrate CDK8 as a key regulator in the ABA signaling and drought response. We showed that both CDK8 and SnRK2.6 interact with the key ETHYLENE RESPONSIVE FACTOR /APETALA2 (ERF/AP2) type TF RAP2.6 to promote the transcription of several ABA-responsive genes. More importantly, we revealed that *CDK8* is required for *RAP2.6* mediated upregulation of several ABA-responsive genes, probably through affecting the RNA polymerase II recruitment to those promoters.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana plants used in this study were in the Columbia-0 ecotype (Col-0) background. T-DNA insertion mutants *cdk8-1* (SALK_138675), and *cdk8-2* (SALK_016169) were previously described (Zhu *et al.*, 2014). The *snrk2.6* (*ost1*) mutant and the *snrk2.2/2.3/2.6* triple mutant were obtained as described by (Fujii *et al.*, 2007). *rap2.6* mutant (SAIL_1225_G09) was ordered from The

Nottingham Arabidopsis Stock Centre-BBSRC.

Plasmid construction and generation of transgenic plants

35S:CDK8-HA transgenic lines were generated by transforming Col-0 plants with *35S:CDK8-HA* (pCAMBIA99-1). *35S:CDK8-MYC* and *35S:CDK8*$_{D176A}$*-MYC* transgenic plants were generated by transforming *cdk8-1* mutants, which were described in (Zhu *et al.*, 2014). *CDK8* complementation lines were created by transforming *cdk8-1* mutants with *CDK8pro:CDK8-GFP*. *RAP2.6* over-expression lines were created by transforming WT (Col-0) plants with *35S:RAP2.6-GFP*. Independent transgenic lines were identified by immunoblotting analysis and RT-qPCR. Homozygous transgenic plants were used in this study.

Affinity purification/mass spectrometry (AP-MS)

Proteins were extracted from 14-day-old transgenic lines and WT (Col-0) seedlings with protein lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM DTT, 0.1 (v/v) Triton X-100, and Protease Inhibitor Cocktail (Sigma-Aldrich). After centrifugation at 15800 g (rcf) and 4°C for 15 min, the supernatant was incubated with monoclonal anti-HA agarose (Sigma-Aldrich), or GFP-Trap agarose (Chromotek) at 4°C for about 4 h with gentle rotation before washing four times with lysis buffer. After digestion, the protein complex was identified using Q-TOF LC/MS/MS (Zhu *et al.*, 2017).

ABA treatment, drought stress treatment and assessment of stomatal aperture

For seed germination and cotyledon green expansion experiments, *Arabidopsis* seeds were surface sterilized with 10% bleach and grown on ½ strength Murashige and Skoog (MS) medium containing 1% (w/v) sucrose and 0.8% (w/v) agar in a growth chamber at 23°C and with a 16 h light/8 h dark photoperiod. At least 100 seeds per genotype were grown on plates containing ½ MS or ABA for green cotyledon calculation. For post-germination root growth assays, 3-day-old seedlings were first germinated on vertical plates containing ½ strength MS medium and were then transferred to ABA or mannitol-supplemented medium; primary root growth was measured 7 days after transfer.

Wild type, mutants and indicated transgenic plants were grown in pots containing of soil. After reaching two weeks old, thirty-two plants of each genotype were subjected to drought stress

under a 12 h light/12 h dark photoperiod in a 22°C growth room. The location of the pots was randomized in the growth room to avoid location effects. After water was withheld for a period (ranging from 10-18 days), drought phenotypes were recorded. After re-watering for 2 days, the recovery and survival rate of each genotype were documented. The drought stress experiment was repeated at least three times. Stomatal apertures were measured as previously described (Wang, P *et al.*, 2015; Dong *et al.*, 2018). In brief, mature rosette leaves from 5-week-old plants were detached and incubated in "stomata open solution" (5 mM KCl, 1 mM $CaCl_2$, and 10 mM MES pH 6.1) in a growth chamber for 2 h before ABA treatment. Stomatal apertures were measured 2 h after treatment with 10 µM ABA. The apertures of at least 40 stomata were measured in three independent experiments.

Water loss measurement and Toluidine-Blue staining

The whole aboveground tissues of the indicated genotypes were detached, kept at room temperature, and weighed at the indicated time points. The rate of water loss was expressed as the percentage of the initial fresh weight. The experiment was repeated three independent times. At least 10 fully expanded leaves from each genotype were excised and submerged in a 0.05% toluidine blue solution for about 30 min at room temperature before taking images.

Yeast two-hybrid (Y2H) assay

The full-length coding sequences of the indicated genes were amplified with high-fidelity Taq polymerase (Takara PrimeSTAR MAX) and were separately cloned into the pGBKT7 and pGBAD7 vectors (Clontech). Pairs of GAL4-BD and GAL4-AD vectors were co-transformed into *Saccharomyces cerevisiae* AH109 cells as previously described (Gietz & Schiestl, 2007). After transformation, yeast colonies were suspended in distilled water, and drops of the suspensions were then added to the indicated selection medium (Hou *et al.*, 2016). The colonies were photographed after incubation at 30°C for 3 days.

Protoplast isolation and transient expression

Arabidopsis Col-0 plants were grown under short-day condition (8 h light/16 h dark), and 5-week-old plants were used for protoplast isolation and transient expression as previously described (Yoo *et al.*,

2007; Zhu, Y *et al.*, 2010). All of the plasmids were purified using the QIAGEN Plasmid Maxi or Midi Kit.

BiFC and co-Immunoprecipitation assay

The full-length or truncated coding sequences of the indicated genes were amplified by PCR using primers listed in Table S1. The BiFC plasmids were introduced into GV3101 agrobacterial cells for transient transformation in tobacco leaves (Kudla & Bock, 2016). After co-infiltration for 48 h, the YFP signal was assessed by confocal microcopy (Zeiss LSM-710).

The CDK8-HA and SnRK2.6-MYC were described as (Zhu *et al.*, 2014; Hou *et al.*, 2016). Indicated plasmids were transiently expressed in protoplasts. After overnight transformation, the protoplasts were collected by centrifugation at 100 *g* for 2 min and were then suspended in 1 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM DTT, 0.1 [v/v] Triton X-100, and Protease Inhibitor Cocktail [Sigma-Aldrich]) on ice for 30 min. The preparation was then centrifuged at 13000 rpm and 4°C for 15 min before one-tenth of the supernatant was kept for input and the rest of the supernatant was incubated with pre-equilibrant GFP/MYC Trap (Chromotek) or anti-HA agarose (Sigma) for at least 4 h with gentle rotation. The agarose was washed at least four times with lysis buffer at 4°C before boiling in 4× SDS loading buffer for 10 min. Protein samples were separated by SDS-PAGE and transferred to nitrocellulose blotting membranes. The proteins were further detected with polyclonal anti-HA (Abcam ab9110), anti-MYC (Abcam ab9106) or anti-GFP antibody (Roche).

Transactivation assay

Arabidopsis protoplasts were co-transformed with indicated reporter construct and internal control ZmUBQ:GUS, with or without indicated effectors. The transactivation assays were performed as described in (Zhu *et al.*, 2014; Hou *et al.*, 2016).

Expression and purification of recombinant proteins from *E. coli*

The coding sequences of indicated genes were amplified by PCR and were cloned into pGEX4T1 or pMAL-c5X vectors. Recombinant proteins were purified using glutathione–agarose beads (Sigma-Aldrich) or maltose binding protein (MBP) agarose (GE Healthcare Life Science) according to the

manufacturer's instructions.

In vitro kinase assay

The recombinant proteins were purified and incubated at room temperature for 30 min in kinase reaction buffer (25 mM Tris-HCl pH 7.4, 12 mM $MnCl_2$, 1 mM DTT) with 1 μCi [ϒ-^{32}P] ATP. After incubation, 4× SDS buffer was added to the reaction mixture and boiled for 5 min. The samples were then separated by SDS-PAGE. Radioactivity was detected with a phosphor-imager (BIO-RAD).

RNA-seq and data analysis

Three biological replicates of 10-day-old seedlings of Col-0 WT and *cdk8-1* mutants were grown on ½ MS plates and were treated with either mock or 50 μM ABA for 3 hours at room temperature. Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instruction. RNA-seq of Col-0 and *cdk8-1* samples was conducted at the Shanghai Center for Plant Stress Biology (Shanghai, China). ABA-responsive genes were identified using cuffdiff in Cufflinks (P-value < 0.05 and at least 4-fold change after ABA treatment). Gene Ontology enrichments were analyzed at http://geneontology.org/. The RNA-seq data has been deposited to NCBI (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135607).

RNA isolation and quantitative real-time PCR

RNAs were isolated with Qiagen RNeasy mini kits according to the manufacturer's instructions. Reverse transcription and real-time PCR were performed (Zhu *et al.*, 2014; Hou *et al.*, 2016). Primers are listed in Table S4 (see later).

ChIP-qPCR

ChIP experiments were conducted according to (Saleh *et al.*, 2008) with minor modifications. Ten-day-old seedlings were treated with mock or 50 μM ABA for 1 h, and then at least 2 gram of seedling tissue was cross-linked. The chromatin complex was isolated and precipitated with anti-Pol II antibody (Abcam ab817), anti-MYC (Abcam, ab32) or anti-GFP (Abcam, ab290) at 4°C overnight. The protein-DNA complex was captured with salmon sperm DNA/Protein A agarose (Millipore). The immunoprecipitated DNA was detected with qPCR. ChIP enrichment was normalized to its input,

fold enrichment was converted as the ratio of control WT plants which was set as 1. Primers are listed in Table S4 (see later)**.**

EMSA experiment

EMSA experiments were performed as described previously with minor modifications (Wang *et al.*, 2019). Purified recombinant proteins GST-RAP2.6 or MBP-CDK8 was incubated with indicated FAMA-labeled oligonucleotides in the binding buffer (20 mM Tris-HCl, pH 8.0, 1 mM DTT, 100 mM NaCl, 2 mM $MgCl_2$, and 10% glycerol) at room temperature for 20 min. The reaction mixtures were separated in 8% native PAGE. Fluorescent signal was detected with excitation light of 470 nm and emission light of 543 nm.

Results

CDK8 regulates ABA response

In a screen for altered response to abiotic stress, we tested several mediator mutants in response to exogenous ABA. From the screen, we found that *cdk8* mutants exhibited altered ABA response. Relative to the WT, the *cdk8* mutants exhibited reduced sensitivity to exogenous ABA at the seed germination stage as indicated by a significantly higher ratio of seedlings with green cotyledons (Fig. 1a and b). *cdk8* mutants also showed enhanced ABA insensitivity at the post-germination stage in that their primary root lengths were longer than that of the WT after transferring to and growing on MS plates supplemented with ABA, though the *cdk8* mutants had shorter roots than WT under normal condition (Fig. 1c). The quantitative root length and root length (%) change of WT and two lines of *cdk8* mutants were present in Fig. S1 and Fig. 1d, respectively. Interestingly, the expression of *CDK8* was also increased with the ABA treatment (Fig. S2), which implied the involvement of *CDK8* in the ABA signaling.

We consequently generated *35S:CDK8* transgenic plants by transforming WT plants. The *CDK8* expression in these transgenic lines was significantly higher than that in WT plants (Fig. S3a) and the CDK8-HA fusion protein expression was also confirmed by western blot (Fig. S3b). Accordingly, *35S:CDK8-HA* transgenic lines exhibited hypersensitivity to ABA. As shown in Fig. 1e

and f, all the WT, *35S:CDK8* #1 and *35S:CDK8* #2 transgenic lines grew similarly on the control MS plates. In contrast, the two *35S:CDK8-HA* transgenic lines displayed a significantly reduced green cotyledon expansion rate and the seedling size compared to WT when germinated on MS medium containing 0.5 μM ABA (Fig. 1e and f). In addition, we also examined the post-germination seedling growth and found that the *35S:CDK8-HA* transgenic lines evidently exhibited shorter primary roots than WT after transferring to ABA containing MS plates (Fig. 1g and h), further suggesting that CDK8 may be a positive regulator in the ABA signaling.

We further investigated if CDK8 kinase activity is required for the ABA response, we examined the post-germination root growth of WT, *35S:CDK8-MYC #1*, *35S:CDK8-MYC #2*, and two *CDK8* kinase-dead transgenic lines *35S:CDK8D176A-MYC#1* and *35S:CDK8D176A-MYC#2*. Those transgenic materials were described in (Zhu *et al.*, 2014; Mao *et al.*, 2019). As shown in Fig. S4, the root length of *35S:CDK8D176A-MYC#1* and *35S:CDK8D176A-MYC#2* was similar to WT. In contrast, the root length of *35S:CDK8-MYC* lines was obviously shorter than WT, consistent with the ABA hypersensitivity of *35S:CDK8-HA* transgenic plants. Those results imply that CDK8 kinase activity is required for the ABA response.

CDK8 is critical for drought resistance

In addition to examining their ABA response, we also determined whether *cdk8* mutants would show altered responses to drought stress. Relative to the WT, the two mutant alleles of *cdk8* displayed hypersensitivity to drought stress (Fig. 2a). After water withheld, survival rate (as indicated by recovery after re-watering) was significantly lower for the *cdk8* mutant plants compared to the WT plants (Fig. 2a). The rate of water loss from detached leaves was much higher for the *cdk8* mutants, which was comparable to that of *ost1* (Fig. 2b). We then determined whether the drought hypersensitivity of the *cdk8* mutants was due to altered stomatal function and we found that stomatal closure induced by exogenous ABA was impaired in the *cdk8* mutants (Fig. 2c and d). Recent studies have shown that *cdk8* mutants and several ABA-insensitive mutants such as *aba3*, *pyl* sextuple mutants, and *snrk2.2/3/6* triple mutants exhibit increased cuticle permeability (Zhu *et al.*, 2014; Cui *et al.*, 2016). As indicated by staining with toluidine blue (Zinsmeister *et al.* 2016). cuticle permeability was greater for the leaves of the *cdk8-1* mutant, the *ost1* mutant, and the *snrk2.2/3/6* triple mutant than

for leaves of the WT (Fig. 2e), consistent with previous study that showed the enhanced cuticle permeability of *cdk8* mutants (Zhu *et al*., 2014). Interestingly, the cuticle permeability of *35S:CDK8-MYC* lines was slightly reduced compared to WT, but the cuticle permeability of *35S:CDK8D176A-MYC* was still similar to *cdk8* mutants (Fig. S4c), suggesting the CDK8 kinase activity is also essential for its role in cuticle permeability regulation. Moreover, we also examined the mannitol response of WT and *cdk8* mutants and revealed that *cdk8* mutants were more sensitive to mannitol than WT as the root length of *cdk8* mutants are obviously shorter than that of WT after transferred to mannitol-containing medium (Fig. 2f and g).

Since we had *CDK8* over-expression and complementation transgenic lines, we also examined their drought responses. As illustrated in Fig. 3a, two independent lines of *35S:CDK8-HA* transgenic plants exhibited enhanced drought tolerance compared to WT with higher survival rate and reduced water loss rate (Fig. 3b). In addition, the *CDK8pro:CDK8-GFP* complementation rescued the drought hypersensitivity of *cdk8* mutants (lower survival rate and faster water loss rate) to wild type levels (Fig. 3c and d), further suggesting the essential roles of *CDK8* in drought response.

The expression pattern of *CDK8* in plants

To understand the expression pattern of *CDK8* in plants, we generated transgenic lines that express a β-glucuronidase (Carlsten *et al.*) reporter driven by the *CDK8* native promoter. GUS staining of young seedlings of *CDK8pro:GUS* transgenic lines showed CDK8pro:GUS expression throughout the seedlings, with enrichment in the hypocotyl, meristem, and root tips, as well as in vascular tissues (Fig. 4a, b and c). In the leaves, GUS expression was primarily found in trichomes, vascular bundles, and guard cells (Fig. 4d, e and f). GUS was also highly expressed in the stamen and stigma (Fig. 4g and h), indicating a function in floral development. In contrast, there was obviously less GUS expression in siliques and developing seeds (Fig. 4i). To examine the subcellular localization of CDK8, we transiently co-expressed the 35S:CDK8-YFP fusion protein and nuclear indicator H2B-mCherry in tobacco leaves (Gao *et al.,* 2020), finding it to be localized in the nuclei of tobacco epidermal cells (Fig. 4j-m). Additionally, we investigated the subcellular localization of *CDK8* in transgenic lines that express native promoter driven *CDK8* with GFP fusion reporter in *cdk8-1* mutant background. As revealed by confocal microcopy (Fig. 4n and 4o), fluorescence is

widely observed in the nuclei of root tissues in the *CDK8pro:CDK8-GFP* transgenic lines.

Affinity purification by CDK8 in plants

To identify the proteins that are in complex with CDK8, we performed affinity purification-mass spectrometry (AP-MS) using *35S:CDK8-HA* and *CDK8pro*:*CDK8-GFP* transgenic plants. Components of the kinase module, including MED12, MED13, and one C-type cyclin, were successfully co-purified by CDK8 from both transgenic lines (Fig. 5a); suggesting that the configuration of the kinase module is conserved throughout eukaryotic cells including those of plants. Several Mediator subunits such as MED21 and MED32 were co-purified by CDK8 in two independent AP-MS assays. In addition, two histone superfamily proteins were found in the CDK8 immunocomplex, which is consistent with previous human studies indicating that CDK8 is a histone kinase (Knuesel *et al.*, 2009). Interestingly, SnRK2.6 was also immunopurified, supporting the involvement of CDK8 in the ABA signaling pathway. Nevertheless, CDK8 did not directly interact with SnRK2.6 in Y2H assays (Fig. 5b), which raises the possibility that CDK8 may associate with SnRK2.6 through other proteins.

CDK8 physically interacts with RAP2.6

To identify more putative CDK8 interacting proteins, we performed Y2H screening and we successfully detected several putative CDK8 interactors including C-type Cyclin CycCa, which has been reported previously (Zhu *et al.*, 2014). One of new CDK8 interactors was identified as RAP2.6, which encodes an ERF- type TF that participates in various biotic and abiotic stress response (Okamuro *et al.*, 1997; Papdi *et al.*, 2008; Zhu, Q *et al.*, 2010; Gasch *et al.*, 2016). We further validated the physical interaction between RAP2.6 and CDK8 by multiple approaches. It was demonstrated that the yeasts co-expressing CDK8-AD and RAP2.6-BD could grow on yeast selection medium in Y2H assays (Fig. 5c); and that co-expression of CDK8-CYFP and RAP2.6-NYFP could lead to a strong YFP signal in the nuclei of tobacco leaves by BiFC assays (Fig. 5d). In contrast, the co-expression of truncated RAP2.6-C-NYFP (121-192 amino acid, C-half part) and CDK8-CYFP did not show any detectable YFP signals (Fig. S5a), suggesting the specific interaction between CDK8 and RAP2.6. Moreover, CDK8-HA were immunoprecipitated by RAP2.6-GFP in Co-IP assays (Fig. 5e), confirming the physical interaction between CDK8 and RAP2.6.

SnRK2.6 directly interacts with RAP2.6

The key stress-relative protein kinase SnRK2.6 was detected in the CDK8 immuno-complex from our AP-MS experiments. Given the physical interaction between CDK8 and RAP2.6, we also examined if SnRK2.6 could interact with RAP2.6. Using Y2H and BiFC assays, SnRK2.6 was demonstrated to directly interact with RAP2.6 (Fig. 6a and b). In contrast, the co-expression of RAP2.6-C-CYFP (121-192 amino acid, C-half part) and SnRK2.6-NYFP did not lead to YFP signaling (Fig. S5b), implying the specific interaction between SnRK2.6 and RAP2.6 in BiFC assays. Moreover, we also validated their interaction in Co-IP assays. The results revealed that SnRK2.6-MYC was present in RAP2.6-GFP immune-complex and that RAP2.6-GFP was also pulled down by SnRK2.6-MYC (Fig. 6c and d), implying the physical interaction between SnRK2.6 and RAP2.6.

CDK8 may associate with SnRK2.6 through RAP2.6

Although we have demonstrated the interactions between CDK8-RAP2.6 and RAP2.6-SnRK2.6, it remains unclear whether those three proteins could form a complex. Thus, we performed Co-IP assays by co-expressing CDK8-HA, RAP2.6-GFP and SnRK2.6-MYC in *Arabidopsis* protoplasts. As revealed in Fig 7a, SnRK2.6-MYC was not immunoprecipitated by CDK8-HA alone, consistent with the Y2H results (Fig. 5b). But SnRK2.6-MYC was immunoprecipitated by CDK8-HA when RAP2.6-GFP was co-expressed (using anti-HA agarose). Moreover, we also verified the ternary complex formation with anti-GFP agarose (Fig. 7b). Both CDK8-HA and SnRK2.6-MYC were present in the RAP2.6-GFP precipitates when those three proteins were co-expressed. Similarly, CDK8-HA was also immunoprecipitated by RAP2.6-GFP. However, ABA treatment did not obviously affect the interactions between those proteins probably due to transient expression (Fig. 7b).

SnRK2.6, but not CDK8, could phosphorylate RAP2.6 *in vitro*

To determine the potential phosphorylation relations within those three proteins (two kinases and one TF), we purified the recombinant proteins MBP-CDK8, GST-SnRK2.6 and GST-RAP2.6 and performed *in vitro* kinase assays. It was revealed that CDK8 did not obviously phosphorylate RAP2.6 *in vitro* as no detectable phosphorylation signal was observed when MBP-CDK8 and GST-RAP2.6 were incubated, while CDK8 could clearly phosphorylate CTD (C-terminal domain of RNA

polymerase II) (Fig. 7c). Interestingly, SnRK2.6 could phosphorylate RAP2.6 and the positive control RAV1 *in vitro* when GST-SnRK2.6 was incubated with GST-RAP2.6 or GST-RAV1 (Fig. 7d), suggesting that RAP2.6 is a putative substrate of SnRK2.6. We are also curious about whether CDK8 could phosphorylate SnRK2.6 or vice versa. When recombinant protein MBP-CDK8 was incubated with a kinase-dead version of GST-SnRK2.6 G33R (Belin *et al.*, 2006), no obvious phosphorylation signal was detected at the GST-SnRK2.6 (G33R) protein band (Fig. S6a). Similarly, incubation of GST-SnRK2.6 and GST-CDK8-KD (kinase-dead) recombinant proteins did not lead to an obvious phosphorylation signal at the GST-CDK8-KD band (Fig. S6b), suggesting that CDK8 may not directly phosphorylate SnRK2.6, or vice versa, *in vitro*.

Transcriptomic analysis reveals a positive role of *CDK8* in ABA response

To explore the genome-wide effect of mutation in *CDK8* in the ABA-triggered transcriptome changes, we performed RNA-sequencing experiments. Under mock conditions, at least 951 genes displayed >2-fold changes in expression in *cdk8* mutant plants relative to WT plants (Table S2). The heat map generated with those 951 DE genes was shown in Fig. S7a. Consistent with a previous report (Zhu *et al.*, 2014), expression levels of several *PLANT DEFENSIN* genes (including *PDF1.2*, *PDF1.2b*, and *PDF1.3*) were significantly suppressed in *cdk8* mutant plants. Gene Ontology (GO) analysis revealed that the DE genes regulated by *CDK8* under mock condition were primarily enriched in these categories: response to chemical stimulus, response to stimulus, response to stress, and DNA-dependent transcription; suggesting that *CDK8* is involved in plant responses to environmental and transcription regulation (Fig. S7b).

After ABA treatments, there were about 2,000 ABA-responsive genes identified in the WT (Table S3). Among those ABA-responsive genes, at least 692 genes were differentially expressed in *cdk8* compared to the WT (Table S4), indicating that these ABA-responsive genes are likely affected by *CDK8*. For instance, the ABA-responsive genes *RESPONSIVE TO DESSICATION 29B* (*RD29B*), *COR27*, *DREB2A*, and *RAP2.6* were induced to a lesser degree in the *cdk8* mutant than in the WT. A heat map generated with CDK8-affected ABA-responsive genes shows that the *CDK8* mutation partially suppresses many ABA-responsive genes (Fig. 8a). We further classified *CDK8* affected ABA-responsive genes into *CDK8* positively regulated genes and *CDK8* negatively regulated genes

which contain 395 and 297 genes, respectively. The heat map generated with *CDK8* negatively- or positively- regulated genes were illustrated in Fig 8b and c. GO analysis further revealed that *CDK8* negatively regulated genes were enriched in categories such as oxidoreductase activity, tetrapyrrole binding, and monooxygenase activity (Fig. 8d upper part). While *CDK8* positively regulated genes were enriched in categories such as response to stimulus, response to chemical stimulus and response to stress (Fig. 8d lower part). As we focused on the role of *CDK8* in abiotic stress response, therefore we mainly pursued *CDK8* positively regulated genes.

Recruitment of RNA polymerase II to the promoters of several ABA-responsive genes requires CDK8

To validate our RNA-seq data, we measured the transcript levels of several ABA-responsive genes *RD29B*, *COR15A*, and TF *DREB2A* in the WT, *cdk8-1* mutants and the *snrk2.2/3/6* triple mutant following mock and ABA treatments. The ABA induced expression of those genes was significantly reduced in the *cdk8* mutants and in the *snrk2.2/2.3/2.6* triple mutants compared to the WT (Fig. 8e), indicating that *CDK8* positively regulate the ABA induced expression of those ABA-responsive genes. To test whether the attenuated expression of those ABA-responsive genes was due to the altered recruitment of RNA Pol II, we performed ChIP assays with an antibody against Pol II C-terminal domain repeats in order to determine Pol II occupancy after ABA treatment. Several primers were designed to amplify genomic sequences at specific sites along the genes *DREB2A* and *RD29B*. The results revealed a significant reduction of Pol II occupancy at the indicated genomic sites in *cdk8* mutants when compared to the WT after ABA treatments (Fig. 8f), indicating that the reduction in gene expression in *cdk8* mutants is probably due to failures in Pol II recruitment.

CDK8 is essential for the ABA induced expression of *RAP2.6* and *RAP2.6*-dependent activation of ABA-responsive genes

From our RNA-seq data, we noticed that ABA induced transcription of *RAP2.6* was significantly lower in *cdk8* mutants than that in WT. Therefore, we performed RT-qPCR for further validation. As illustrated in Fig. 9a, *RAP2.6* expression was greatly induced by ABA treatment in WT, but such induction was significantly suppressed in *cdk8-1* mutants. Consistently, CDK8 was enriched to the promoter region (500 bp upstream) other than the coding region of *RAP2.6* after ABA

treatments by ChIP assays (Fig. 9b). However, CDK8 was not significantly enriched at the *RAP2.6* promoter and coding region under mock conditions (Fig. S8), suggesting the requirement of CDK8 in the ABA induced transcriptional activation of *RAP2.6*. To further understand the roles of RAP2.6 in the regulation of ABA-responsive genes, we performed Electrophoretic mobility shift assay (EMSA) assays. The results revealed that recombinant GST-RAP2.6 could directly bind to DRE, GCC-motif and the promoter of an ABA-responsive gene of *COR15A* (Fig. 9c). However, CDK8 did not directly associate with those elements or promoter (Fig. S9), implying that CDK8 may regulate the transcription of ABA-responsive genes through RAP2.6. Since the promoters of ABA-responsive genes *RD29A* and *COR15A* contain GCC-motif, we amplified the *RD29A* promoter sequence and cloned it into reporter construct to test if RAP2.6 could activate the expression of *RD29A*. When co-transforming RD29Apro:LUC and internal control GUS with or without RAP2.6 in *Arabidopsis* protoplasts, it was shown that protoplasts expressing RAP2.6 significantly induced higher *RD29Apro:LUC* activities under both control and ABA treatments than those only expressing *RD29Apro:LUC* (Fig. 9d). To investigate if CDK8 affects RAP2.6 mediated activation of *RD29Apro:LUC* in response to ABA, we co-expressed RAP2.6-YFP, RD29Apro:LUC and internal control GUS in protoplasts isolated from WT, *cdk8-1* and *ost1* mutants with mock and ABA treatments. As indicated in Fig. 9e, the RD29Apro:LUC activities were similar between WT and mutants under mock conditions. After ABA treatments, RAP2.6 activated RD29Apro:LUC was significantly higher in WT and *ost1* mutant protoplasts than that in *cdk8-1* protoplasts. Consistently, ABA-induced expression of *RD29A* was also obviously lower in *cdk8-1* mutants than that in the WT (Fig. 9f), indicating that CDK8 is required for RAP2.6 mediated activation of *RD29A*.

RAP2.6 over-expression confers ABA and mannitol hypersensitivity

We next examined if *rap2.6* mutants showed altered ABA response. T-DNA PCR confirmed the *rap2.6* mutants carry homozygous T-DNA insertions in *RAP2.6* gene (Fig. S10a), and RT-qPCR confirmed that the expression of *RAP2.6* is remarkedly reduced in the mutants (Fig. S10b). When transferred to ABA-containing MS medium, *rap2.6* mutants did not display altered ABA phenotype compared to WT as the root length of *rap2.6* was similar to WT (Fig. S10c and d). Therefore, we generated two independent *RAP2.6-GFP* over-expression (OE) lines. The transcription of *RAP2.6* was expressed at significantly higher levels than in those two transgenic lines by RT-qPCR assays (Fig.

S11a); and RAP2.6-GFP fusion protein was also detected by Western blot (Fig. S11b). Confocal imaging showed that RAP2.6-GFP was located in the nuclei with enrichment in the root tip (Fig. S11c). We next examined the ABA and mannitol response of WT and *RAP2.6* OE lines. It was revealed that both *RAP* OE#1 and #2 lines displayed hypersensitivity to ABA and mannitol as the root length of those transgenic lines is shorter than the WT after transferred to ABA or mannitol-containing medium (Fig. 10a and b). Additionally, we found that the cuticle permeability of *RAP2.6* OE lines is enhanced compared to WT (Fig. 10c), suggesting the involvement of *RAP2.6* in cuticle development. Importantly, the ABA-induced expression of *RD29A* and *COR15A* was evidently higher in two *RAP2.6* OE lines than those in WT (Fig. 10d and e), suggesting the positive role of *RAP2.6* in activating the transcription of ABA-responsive genes. We further performed ChIP assays to examine if RAP2.6 could be enriched at the promoters of ABA-responsive genes. As indicated in Fig. 10f. RAP2.6 was enriched at the promoters and TATA box regions of *COR15A* and *RD29A* compared to WT control. Interestingly, this enrichment was significantly increased by ABA treatment, indicating that RAP2.6 is responsible for the ABA induced expression of several ABA-responsive genes.

Discussion

Although the key components of ABA signaling pathway have been characterized, more essential components in the downstream signaling pathway remain to be identified. The Mediator complex, a multi-protein complex, is an important co-regulator of transcriptional machinery and its roles in the ABA signaling transduction remains elusive. In the present work, we provided genetic, transcriptomic and biochemical evidence to demonstrate that *CDK8* is a critical regulator in the ABA signaling pathway and drought response. Notably, CDK8 associates with RAP2.6 and SnRK2.6, and positively regulate the transcription of ABA-responsive genes.

Previous studies reported that *MED25* negatively regulates ABA and drought tolerance as *med25* mutants displays hypersensitivity to ABA but enhanced tolerance to drought (Elfving *et al.*, 2011; Chen *et al.*, 2012). The stress-responsive genes were also up-regulated in *med25* mutants compared to WT plants, indicating the negative role of *MED25* in the ABA signaling and drought response. Although CDK8 and its kinase Module components are typically reported as negative regulators of gene expression in yeast, metazoan cells and plants (Nonet & Young, 1989; Nemet *et al*.,

2014; Ito *et al*., 2016), increasing evidence suggested that *CDK8* could also play a positive role in transcriptional regulation, especially in plants. It has been demonstrated that *CDK8* positively regulates the expression of defense-responsive (*PDF1.2*, *AACT1* and *NPR1*), SA-biosynthesis genes (*ICS1* and *EDS5*) and ABA-responsive genes (such as *RAP2.6*, *RD29A*, *RD29B* and *COR15A* in our present study) (Zhu *et al*., 2014; Huang *et al*., 2019; Chen *et al*., 2019). It is interesting that *CDK8* and its kinase module components have been mainly reported as a transcriptional activator in plants, which is different from their homologue genes in yeast and metazoan cells. In our study, we showed that *CDK8* positively regulates the ABA signaling and drought response. Moreover, transcriptomic analysis indicated that about 30% of the ABA-responsive genes are affected by *CDK8*, most of which are down-regulated in *cdk8* mutants relative to WT (*CDK8* positively regulated genes). The expression of several important TFs (*DREB2A* and *RAP2.6*) and ABA-responsive genes (*RD29A*, *RD29B* and *COR15A*) was significantly lower in *cdk8* mutant plants, indicating the positive role of *CDK8* in modulating ABA-induced transcription. Our ChIP analysis further illustrated that *CDK8* is essential for the ABA induced RNA Polymerase II recruitment to the promoters of ABA-responsive genes. However, the TFs which collaborate with CDK8 in this process remains unknown. Therefore, we performed a Y2H screen and successfully identified a new CDK8 interactor, RAP2.6, an ERF/AP2 type TF that involves in biotic and abiotic stress responses. After confirming the physical interaction between CDK8 and RAP2.6, we subsequently showed that CDK8 is enriched at the promoter region of *RAP2.6* in response to ABA, indicating the essential role of CDK8 in regulation of *RAP2.6* transcription. Interestingly, RAP2.6, but not CDK8, could directly associate with the DRE or GCC motif and *RD29A* or *COR15A* promoters. In addition, RAP2.6 could be enriched at the *RD29A* and *COR15A* promoters in response to ABA. Those evidence raise the possibility that that CDK8 may regulate the expression of ABA-responsive genes through RAP2.6. However, we could not exclude the possibility that other TFs may also interact with CDK8 to regulate the expression of ABA-responsive genes. Furthermore, we showed that RAP2.6-mediated activation of *RD29A* is attenuated in *cdk8* mutants, indicating that *CDK8* is essential for recruiting RNA polymerase II to the promoters of RAP2.6 target genes. Consistent with the biochemical results, over-expression of *RAP2.6* resulted in hypersensitivity to ABA and mannitol at seedling stage as well as higher expressions of several

ABA-responsive genes. Our findings revealed that RAP2.6 and CDK8 could fine-tune the transcription of ABA-responsive genes, especially those genes containing DRE/GCC-motifs.

Although no direct interaction and phosphorylation between CDK8 and SnRK2.6 were detected in our study, both CDK8 and SnRK2.6 could physically interact with RAP2.6. Interestingly, we revealed that CDK8 may associate with SnRK2.6 through RAP2.6. It is worth noting that our data are not sufficient to demonstrate that those three proteins function or exist within a relevant complex because they are artificially overexpressed within the experimental system. We further analyzed the potential phosphorylation within the complex and found that SnRK2.6, not CDK8, could phosphorylate RAP2.6 *in vitro*. However, the biological significance of this phosphorylation remains unknown. It is still not clear if RAP2.6 could be phosphorylated *in vivo* in plants thus far. Whether the phosphorylation of RAP2.6 could affect its protein stability or transcriptional activity need to study in future. In yeast cells, the SnRK homologue Snf1 is a conserved protein kinase that regulates transcription and metabolism under glucose deprivation and other stresses (Carlson, 1999; Hardie, 1999). In response to glucose deprivation, Snf1 controls the expression of various genes by directly interacting with RNA Pol II holoenzymes including the Mediator component Srb10, which is the plant CDK8 orthologue in yeast (Kuchin *et al.*, 2000). Several Mediator mutants were identified as suppressors of *Snf1* mutation, indicating genetic interactions between Snf1 and the Mediator complex (Balciunas & Ronne, 1995; Kuchin *et al.*, 1995; Song *et al.*, 1996). Nevertheless, no direct phosphorylation between Snf1 and CDK8 has been observed in yeast cells to date. Interestingly, our findings indicated that the interactions between SnRK2.6, RAP2.6 and CDK8 may provide a regulatory “shortcut” mechanism for transcriptional control in response to ABA and drought signals in plants, which connects core signaling pathway components directly to RNA polymerase II transcriptional machinery.

Based on previous and our present results, we have proposed a working model for the roles of *CDK8* in the ABA and drought responses. ABA or drought signals were transmitted to the key protein kinase SnRK2.6, which directly interacts with and phosphorylates the TF RAP2.6 to activate the expression of several ABA-responsive genes. CDK8 could be recruited by RAP2.6 and is essential for the RNA polymerase II recruitment to the promoters of RAP2.6 target genes, which links the core ABA signaling pathway to RNA Polymerase II to immediately initiate downstream transcriptional

changes (Fig. 11). In summary, we have solidified the vital roles of the *CDK8* in ABA signaling and drought responses and provided new insights into the underlying mechanism of the Mediator complex in regulating abiotic stress responses.

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Author contributions

Y.Z. design of the research; Y.Z., P.H., P.G., L.C., G.Y., X.S., T.H., Y.L., CC.H., W.G. performance of the research; K.T., Y.Z., C.Z., W.A.T., T.M. performed data analysis, collection, or interpretation; and Y.Z. and JK.Z. wrote the manuscript.

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Figure legend

Fig. 1 *CDK8* regulates ABA response in *Arabidopsis*. (a) Images showing the ABA insensitivity of *cdk8* mutants at the germination stage. (b) Green cotyledon percentage of the WT and *cdk8* mutants in (a). Values are means ± SD of three replicates. Within each treatment, means with asterisks are significantly different from the WT (Student's *t*-test, P< 0.05). At least 100 seeds per genotype were germinated on MS or ABA containing MS medium for green cotyledon calculation. The experiments were repeated three times with similar results. (c) The phenotype of root growth of the WT and *cdk8* mutants under normal or ABA treatment at the post-germination stage. (d) Changes in primary root length (%) of the WT and *cdk8* mutants on ½ MS plates without and with ABA. The average root length of each genotype under normal conditions (untreated with ABA) was set as 100. Values are means ± SD (n=20). (Student's *t*-test, *P< 0.05). (e) The ABA hypersensitivity of *35S:CDK8* transgenic lines at the germination stage. (f) Green cotyledon percentage of the WT and *35S:CDK8* transgenic lines in (e). At least 100 seeds per genotype were germinated on MS or ABA containing MS medium for green cotyledon calculation. The experiments were repeated three times with similar results. (g) The root growth of the WT and *35S:CDK8* transgenic lines transferred to ABA containing MS medium at the post-germination stage. (h) Average primary root length of WT and *35S:CDK8* transgenic lines on plates without and with ABA. Values are means ± SD (n=20). (Student's *t*-test, *P< 0.05).

Fig. 2 Drought hypersensitivity of *cdk8* mutants in *Arabidopsis*. (a) Representative phenotype of the WT and *cdk8* mutants under well-watered (control) conditions (top), after (10-14 days) drought stress (middle), and 2 days after re-watering (bottom) and their survival rate after re-watering. At least 24 plants per genotype were subjected to drought stress. The drought experiment was repeated three independent times with similar results. Asterisks indicate significantly different from the WT (Student's *t*-test, **P< 0.01). (b) Water loss percentage from detached leaves of the WT, *cdk8-1* and *ost1* mutants at indicated time points. Values are means ± SD (n = 9) from three biological replicates. Asterisks indicate significantly different from the WT at the same time point (Student's *t*-test, **P< 0.01). (c) Stomata status of WT and *cdk8* mutants by SEM after ABA treatments. Scale bar indicates 200 μM. (d) Stomatal responses to ABA in the WT and *cdk8* mutants. Stomatal aperture was measured in epidermal strips peeled from rosette leaves of 5-week-old plants, which were incubated for 2 h in "stomata open solution" without or with 10 μM ABA. Data represent means ± SD from

three biological replicates (n=40). Different letters indicate statistically significant differences by two-way ANOVA with Tukey's post-hoc test ($P < 0.05$). (e) Cuticle permeability of the WT and of *cdk8*, *ost1*, and *snrk2.2/2.3/2.6* mutants as indicated by Toluidine-Blue staining. (f) Images showing the hypersensitivity of *cdk8* mutants in response to mannitol. (g) Primary root length of WT and *cdk8* mutants under mock or mannitol treatments. Values are means ± SD (n=20) of three biological replicates. Different letters indicate statistically significant differences by two-way ANOVA with Tukey's post-hoc test ($P < 0.05$).

Fig. 3 The drought responses of *CDK8* over-expression and complementation lines in *Arabidopsis*. (a) Representative phenotypes of WT and two independent *CDK8* over-expression lines before drought treatment (top panel), after (14-18 days) drought stress (middle panel) and 2-days after re-watering (bottom panel) and their survival rate. The drought experiments were repeated three times with similar results. Asterisks indicate significantly different from the WT (Student's *t*-test, ** $P< 0.01$). (b) Percentage of water loss from detached leaves of indicated genotypes at different time points. Data represent mean value ± SD from three biological replicates (n = 9). Asterisks indicate significantly different from the WT at the same time point (Student's *t*-test, *$P<0.05$, **$P< 0.01$). (c) Representative phenotypes of WT, *cdk8-1* mutants and *CDK8* complementation lines under well-watered condition (top panel), 14-day after drought stress (middle panel) and 2-days after re-watering (bottom panel) and their survival rate. The drought experiments were repeated three times with similar results. (d) Percentage of water loss from detached leaves of indicated genotypes at different time points. Value is mean ± SD (n = 9) from three biological replicates. Asterisks indicate significantly different from the WT at the same time point (Student's *t*-test, **$P< 0.01$).

Fig. 4 Expression of *CDK8pro*:GUS and subcellular localization of CDK8-GFP in *Arabidopsis*. GUS expression in *CDK8pro:GUS* transgenic seedlings such as cotyledon (a), root tip (b), root elongation area (c), mature leaves (d), guard cells (e and f) and flower (g and h) as well as silique (i). The nuclei subcellular localization of CDK8 (j to o). Transient co-expression of CDK8-GFP and H2B-mCherry in tobacco leaves. The images observed from light field (j), GFP channel (k), RFP (l) and merged channel (m). Scale bar =50μM. GFP signal in the root of *CDK8pro-CDK8-GFP* transgenic plants from GFP channel (n) and higher magnification of Fig. n (o). Scale bar =100 μM in (n) and 10 μM in (o).

Fig. 5 CDK8 physically interacts with RAP2.6 in *Arabidopsis*. (a) List of CDK8 co-purified proteins identified by AP-MS analysis. The results were from two independent AP-MS experiments. "Coverage" indicates the percentage of amino acid residues covered by the identified peptides. "# Peptides" refers to the total number of identified unique peptides matching the protein. (b) Y2H assays showing no interaction between CDK8 and SnRK2.6. CDK8-BD was co-transformed with empty AD or SnRK2.2/2.3/2.6-AD in yeast cells. The interactions were determined by yeast growth on selection medium. (c) Y2H assays showing physical interaction of CDK8 with RAP2.6. (d) Bimolecular fluorescence (BiFC) analysis of interaction between CDK8 and RAP2.6 in tobacco cells. Reconstituted YFP signals were detected in the nuclei of tobacco leaves when RAP2.6-nYFP and CDK8-cYFP were transiently co-expressed. Scale bars = 40 μm. (e) Co-immunoprecipitation (Co-IP) assays showing the interaction between CDK8 and RAP2.6 in Arabidopsis protoplasts. 35S:CDK8-HA and 35S:RAP2.6-GFP, or 35S:CDK8-HA only, or 35S:RAP2.6-GFP only were transformed in Arabidopsis protoplasts. RAP2.6-GFP was then immunoprecipitated with GFP-Trap agarose. CDK8-HA was detected with anti-HA antibody.

Fig. 6 The interaction between SnRK2.6 and RAP2.6 in *Arabidopsis*. (a) Y2H assays showing interaction of SnRK2.6 with RAP2.6. (b) BiFC assays showing the interaction between CDK8 and RAP2.6 in tobacco cells. Scale bars = 40 μm. (c) Co-IP assays showing the interaction between SnRK2.6 and RAP2.6 with GFP Trap Agarose in Arabidopsis protoplasts. RAP2.6-GFP was then immunoprecipitated with GFP-Trap agarose. SnRK2.6-MYC was detected with anti-MYC antibody. (d) CO-IP assays showing the SnRK2.6-RAP2.6 interaction with MYC Trap Agarose. 35S:SnRK2.6-MYC and 35S:RAP2.6-GFP, or 35S:SnRK2.6-MYC only, or 35S:RAP2.6-GFP only were transformed in Arabidopsis protoplasts. SnRK2.6-MYC was immunoprecipitated with MYC-Trap agarose, RAP2.6-GFP was detected with anti-GFP antibody.

Fig. 7 SnRK2.6 could phosphorylate RAP2.6 and may form a ternary complex with CDK8 in *Arabidopsis*. (a) CDK8, RAP2.6 and SnRK2.6 may form a ternary complex in Co-IP assays with anti-HA agarose. CDK8-HA/RAP2.6-GFP/SnRK2.6-MYC or CDK8-HA/SnRK2.6-MYC were co-expressed in *Arabidopsis* protoplasts. Anti-HA agarose was used to immunoprecipitated CDK8-HA and its interacting proteins. NS indicates non-specific bands. The experiments were repeated three times with similar results. (b) CDK8, RAP2.6 and SnRK2.6 could form a protein complex

independent of ABA. GFP-Trap agarose was used to immunoprecipitated RAP2.6-GFP and its interacting proteins. Mock or ABA treatment was applied to protoplasts for 1h before total protein extraction. The experiments were repeated twice with similar results (c) CDK8 does not phosphorylate RAP2.6 *in vitro*. (d) SnRK2.6 could phosphorylate RAP2.6 *in vitro*. Autoradiograph (right) and Coomassie staining (CBB, left) results were present in both c and d.

Fig. 8 RNA-seq analysis shows the global impacts of *CDK8* in response to ABA in *Arabidopsis*. (a) Heat map depiction of the expression of ABA-responsive genes in the WT and *cdk8* mutants under mock and ABA treatments. (b) Heat map of *CDK8* negative-regulated ABA-responsive genes. (c) Heat map of *CDK8* positive-regulated ABA-responsive genes. (d) Gene ontology enrichment analysis of *CDK8* negative-regulated (upper part) and positive-regulated ABA-responsive genes (lower part). The statistically enriched GO categories were identified based on the number and percentage of DE genes (P-value < 0.05). (e) Relative expression of *RD29B*, *COR15A*, and *DREB2A* in WT, *cdk8-1* and *snrk2.2/2.3/2.6* triple mutants under mock and ABA treatment. Relative gene expression was normalized against the reference gene *ACTIN2*. The expression of indicated genes in the WT under mock treatment was set as 1. The error bars indicate SD (n=3). Different letters indicate statistically significant differences by two-way ANOVA with Tukey's post-hoc test ($P < 0.05$). (f) *CDK8* is required for ABA-induced recruitment of RNA Pol II to *DREB2A* and *RD29B* promoter regions as determined by ChIP-qPCR (ten-day-old seedlings were treated with 50 μM ABA for 1 h before cross-linking). Actin was used as a negative control. The association of RNA Pol II with the indicated promoter regions in WT was set at 1. Values are means ± SD (n=3). Asterisks indicate significant difference (Student's *t*-test, *P< 0.05).

Fig. 9 CDK8 positively regulates the transcription of *RAP2.6* and its targeted genes in *Arabidopsis*. (a) The relative expression of *RAP2.6* in WT and *cdk8-1* mutants under mock and ABA treatments. Relative gene expression was normalized against the reference gene *ACTIN2*. Error bars indicate SD (n=3). Different letters indicate statistically significant differences by two-way ANOVA with Tukey's post-hoc test ($P < 0.05$). (b) CDK8 associates with the *RAP2.6* promoter regions with ABA treatment by ChIP assays. The association of CDK8 with the indicated promoter regions in WT was set at 1. Values are means ± SD (n=3). Asterisks indicate significant difference (Student's *t*-test, *P< 0.05). (c) EMSA assays showing the binding of RAP2.6 to the indicated DNA sequences. (d) RAP2.6 is

required for the activation of *RD29A*. The activity of reporter gene *RD29Apro:LUC* was normalized to the internal control GUS activity. Relative LUC activities represent mean values from three independent replicates (n=3). Different letters indicate statistically significant differences by two-way ANOVA with Tukey's post-hoc test ($P < 0.05$). (e) *CDK8* is required for RAP2.6 mediated activation of *RD29Apro:LUC*. Relative LUC activities represent mean values from three independent replicates (n=3). Different letters indicate statistically significant differences by two-way ANOVA with Tukey's post-hoc test ($P < 0.05$). (f) The relative expression of *RD29A* in WT and *cdk8-1* mutants under mock and ABA treatments. Relative gene expression was normalized against the reference gene *ACTIN2*. Error bars indicate SD (n=3). Different letters indicate statistically significant differences by two-way ANOVA with Tukey's post-hoc test ($P < 0.05$).

Fig. 10 The hypersensitivity of *RAP2.6* over-expression lines in response to ABA and mannitol in *Arabidopsis*. (a) The root growth phenotype of WT and *RAP2.6* OE lines after transferred to MS, MS+ABA and MS+ mannitol medium for 5 days. Scale bars indicate 1cm. (b) Root lengths of indicated genotype describe in a. Values are means ± SD (n=20). (Student's *t*-test, *$P< 0.05$). (c) The enhanced cuticle permeability of *RAP2.6* OE lines by Toluidine-Blue staining. (d) The relative expression of *RD29A* in WT and two *RAP2.6* OE lines under mock and ABA treatments. (e) The relative expression of *COR15A* in WT and *RAP2.6* OE lines under mock and ABA treatments. Relative gene expression was normalized against the reference gene *ACTIN2* in (d) and (e). The expression of the indicated genes in the WT under mock treatment was set as 1. The error bars indicate SD (n=3). Different letters indicate statistically significant differences by two-way ANOVA with Tukey's post-hoc test ($P < 0.05$). (f) ChIP assays showing the enrichment of RAP2.6 at the promoters of *COR15A* and *RD29A* under mock and ABA treatment. 10-day-old WT and *RAP2.6* OE seedlings were used for chromatin isolation. The enrichment with the indicated promoter regions in WT was set at 1. Error bars indicate SD (n=3). Different letters indicate statistically significant differences by two-way ANOVA with Tukey's post-hoc test ($P < 0.05$).

Fig. 11 A proposed working model of CDK8 in the ABA signaling and drought response in *Arabidopsis*. ABA and drought signals are transmitted from SnRK2.6 to RAP2.6 through direct interaction and phosphorylation. RAP2.6 can directly bind to the promoters of ABA-responsive genes containing GCC/DRE-motif, such as *RD29A* and *COR15A*, and promote their expressions. The

Mediator CDK8 physically interacts with RAP2.6 and facilitates the RNA Polymerase II recruitment to the promoters of ABA-responsive genes regulated by RAP2.6.

Supporting Information

Fig. S1 Root lengths of WT and two lines of *cdk8* mutants transferred to MS and MS+ABA (50 μM) medium at post-germination stage.

Fig. S2 Increased expression of *CDK8* by ABA treatments.

Fig. S3 The expression of *CDK8* in *35S*:CDK8 transgenic lines.

Fig. S4 The requirement of CDK8 kinase activity for the ABA response.

Fig. S5 BiFC assays showing no interactions between SnRK2.6/CDK8 and RAP2.6 C-half (121-190 amino acid).

Fig. S6 No obvious phosphorylation was detected between CDK8 and SnRK2.6 *in vitro.*

Fig. S7 Analysis of *CDK8* -regulated genes under mock conditions.

Fig. S8 The enrichment of CDK8 at the promoter region of *RAP2.6* under mock condition.

Fig. S9 EMSA assays showing no direct binding between CDK8 and DRE, GCC-motif and *COR15A* promoter sequences.

Fig. S10 *rap2.6* mutants did not show altered response to ABA.

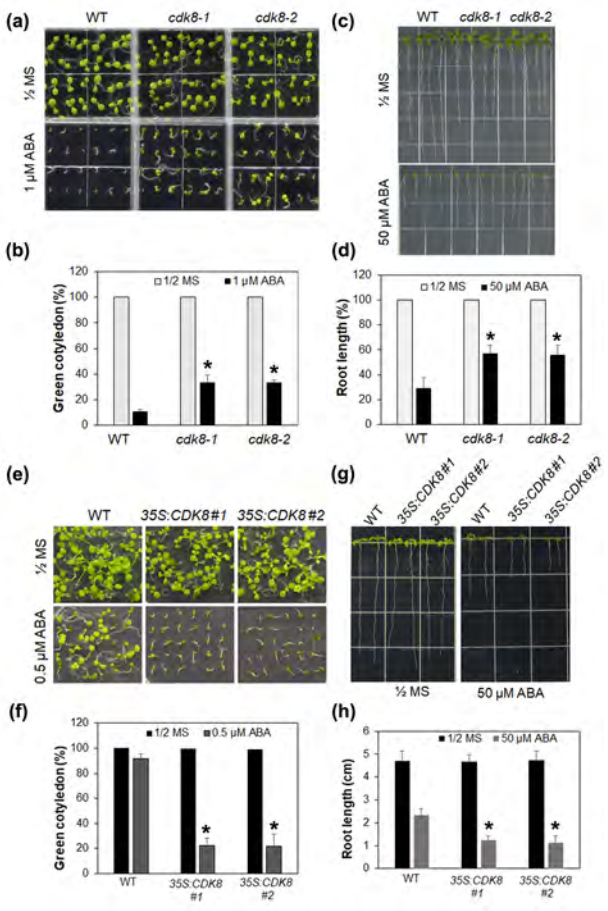
Fig. S11 Transcription, protein level and subcellular localization of RAP2.6 in *RAP2.6* over-expression lines.

Table S1 Primers used in this study.

Table S2 List of *CDK8*-regulated DE genes identified under mock conditions.

Table S3 List of ABA-responsive genes identified in the wild type.

Table S4 List of ABA-responsive genes regulated by *CDK8.*



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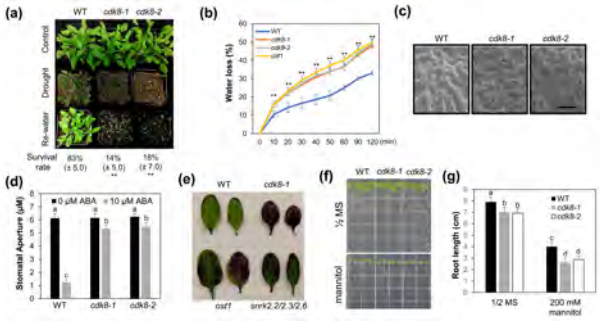

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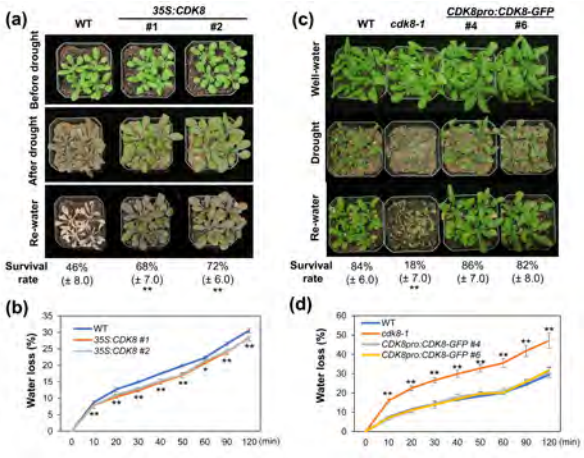

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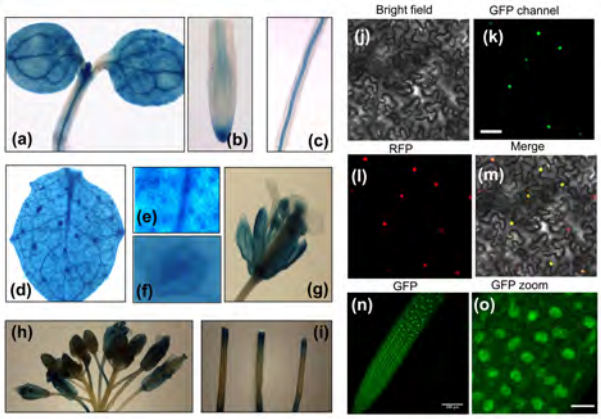

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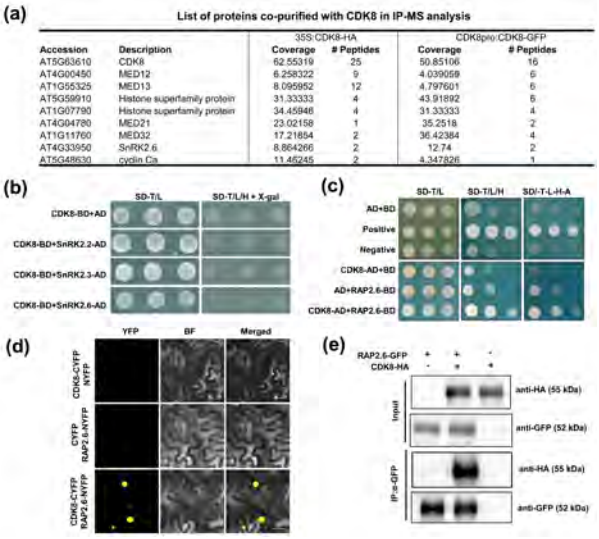

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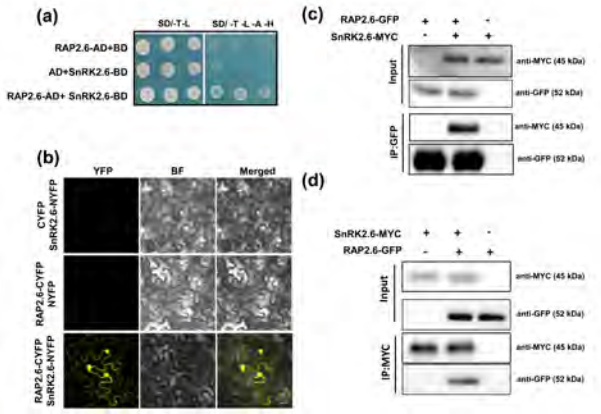

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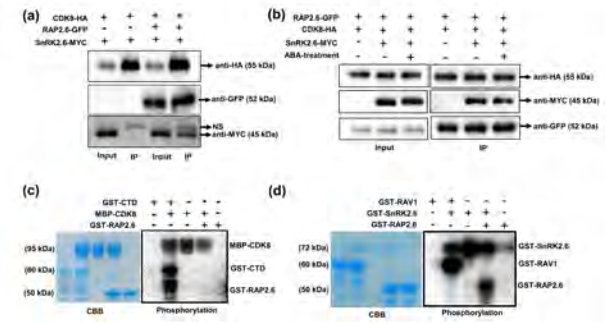

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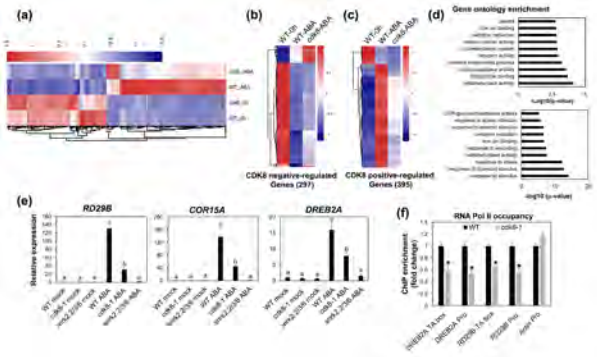

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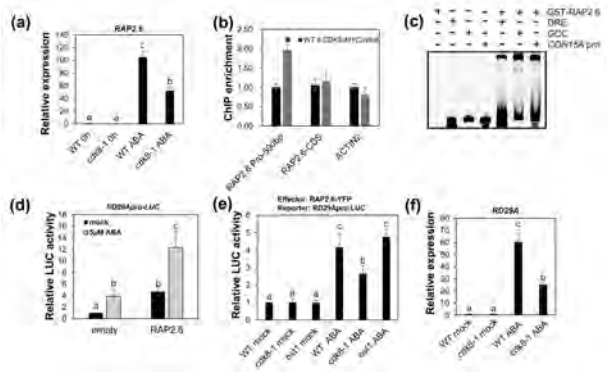

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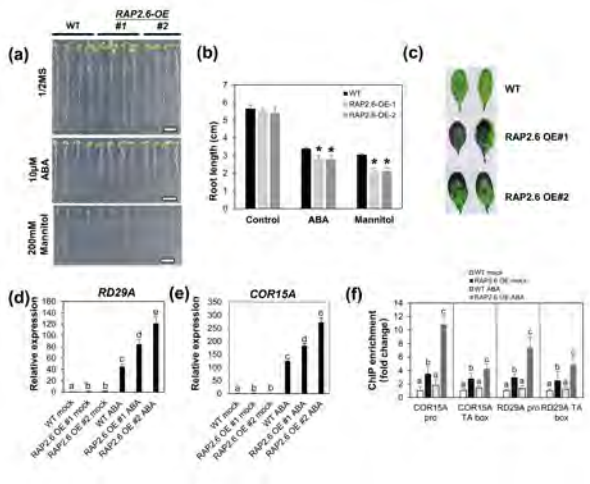

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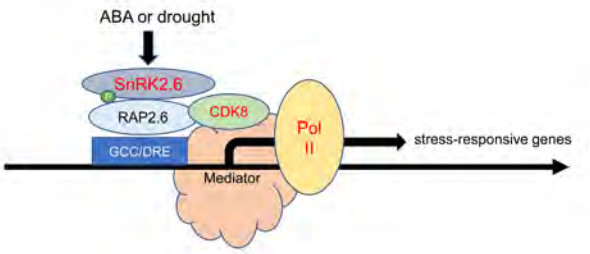

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