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The B'ζ subunit of protein phosphatase 2A negatively regulates ethylene signaling in Arabidopsis

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ABSTRACT

Ethylene is a major plant hormone that regulates plant growth, development, and defense responses to biotic and abiotic stresses. The major pieces of the ethylene signaling pathway have been put together, although several details still need to be elucidated. For instance, the phosphorylation and dephosphorylation processes controlling the ethylene responses are poorly understood and need to be further explored. The type 2A protein phosphatase (PP2A) was suggested to play an important role in the regulation of ethylene biosynthesis, where the A1 subunit of PP2A was shown to be involved in the regulation of the rate-limiting enzyme of the ethylene biosynthetic pathway. However, whether other subunits of PP2A play roles in the ethylene signal transduction pathway is yet to be answered. In this study, we demonstrate that a B subunit, PP2A-B'ζ, positively regulates plant germination and seedling development, as a *pp2a-b'ζ* mutant is very sensitive to ethylene treatment. Furthermore, PP2A-B'ζ interacts with and stabilizes the kinase CTR1 (Constitutive Triple Response 1), a key enzyme in the ethylene signal transduction pathway, and like CTR1, PP2A-B'ζ negatively regulates ethylene signaling in plants.

1. Introduction

Ethylene, a gaseous hormone, is produced in most plant tissues, where it regulates plant growth and development including seed germination, seedling growth, leaf, stem, shoot, root and flower development, fruit ripening, organ senescence, leaf and fruit abscission, and responses to environmental and biotic stresses (Binder, 2020). Ethylene is synthesized from S-adenosyl methionine (SAM) in a simple 2-step biochemical pathway by 1-aminocyclo propane-1-carboxylic acid (ACC) synthase and ACC oxidase (ACS and ACO, respectively) (Pattyn et al., 2021). The regulation of ACS and ACO fine tunes ethylene production. Several studies revealed the transcriptional and post-translational regulation of ACS and ACO (reviewed in Pattyn et al., 2021). In addition to ethylene biosynthesis, how plants transduce ethylene signal has also been well studied. A relatively complete ethylene transduction pathway was elucidated in recent years (Ju et al., 2012; Qiao et al., 2012; Binder, 2020). In the absence of ethylene, the ethylene receptors, e.g. ETHYLENE RESPONSE 1 (ETR1), ETHYLENE RESPONSE 2 (ETR2), ETHYLENE RESPONSE SENSOR 1 (ERS1), ETHYLENE RESPONSE SENSOR 2 (ERS2), and ETHYLENE INSENSITIVE 4 (EIN4), activate the downstream component of the signaling pathway, a protein kinase called CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), which phosphorylates the C-terminus of another key component of the pathway, ETHYLENE INSENSITIVE 2 (EIN2), thereby preventing the cleavage of EIN2 and its nuclear localization (Alonso et al., 1999; Ju et al., 2012). EIN2 is then degraded by the 26S proteasome with the help of the F-box protein EIN2 TARGETING PROTEIN 1 and 2 (ETP1/2) (Qiao et al., 2009). When ethylene is present, the ethylene receptors become inactive, leading to the inactivation of CTR1, which allows the C-terminus of EIN2 (i.e. EIN2 C-end) to be cleaved and translocated into nucleus where EIN2 C-end activates downstream events such as activation of transcription factors and ethylene regulated gene expression. The transcription factors such as ETHYLENE INSENSITIVE 3 (EIN3)/EIN3-LIKE 1 (EIL1) and ETHYLENE-RESPONSE FACTOR 1 (ERF1) are the ones up-regulated by ethylene binding to its receptors. Studies have shown that CTR1 plays a key negative role in ethylene signaling pathway (Huang et al., 2003), as *ctr1* mutant displays a constitutive triple response phenotype that is the typical response

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regulated by ethylene (Kieber et al., 1993). Clark et al. (1998) demonstrated the interaction between ethylene receptors and CTR1, where the N-terminal region of CTR1 was expressed with the kinase domain of ETR1 and ERS proteins in the yeast two-hybrid system. The interaction between these two regions was detected, suggesting that CTR1 acts in the ethylene transduction pathway and that ethylene signaling pathway involves the interaction of CTR1 and ethylene receptors. More recent studies indicated that CTR1 and ETR1 function as a complex in ethylene signaling (Gao et al., 2003; Shakeel et al., 2015). Co-purification of ETR1 from the affinity purification of TAP (tandem affinity purification)-tagged CTR1 revealed that ETR1 and CTR1 could indeed be found in the same protein complex. However, whether CTR1 is regulated by phosphorylation or dephosphorylation is yet to be revealed.

The type 2A Ser/Thr protein phosphatase (PP2A) is one of the most common protein phosphatases in plants, and the holoenzyme of PP2A is made of three subunits: the scaffolding subunit A, the regulatory subunit B, and the catalytic subunit C. In Arabidopsis, with various combinations of the 3 A subunits, 17 regulatory B subunits, and 5 catalytic C subunits, there could be 255 different PP2A enzyme activities formed, giving PP2A enormous diversity in its roles in plant growth and development, stress response, and cellular metabolism (Farkas et al., 2007). PP2A was reported to play critical roles in auxin distribution in embryo and root development (Michniewicz et al., 2007; Ballesteros et al., 2013), brassinosteroid signal transduction (Tang et al., 2011), abscisic acid (ABA) signaling (Kwak et al., 2002; Pernas et al., 2007; Hu et al., 2014; Waadt et al., 2015), light stress response (Konert et al., 2015), and salt stress tolerance (Leivar et al., 2011; Hu et al., 2017). However, the knowledge about PP2A's function in plant hormone signaling was still limited, and those existing studies mainly involved the A1 subunit of PP2A (i.e. PP2A-A1), which is also called RCN1 as it was first named after its mutant *rcn1* (*roots* curl *in NPA*, Garbers et al., 1996), while almost no studies involved the B subunits of PP2A despite that far more B subunits are found in plants.

Larsen and Chang (2001) showed that a *PP2A-A1* mutant, *eer1* (*enhanced ethylene response 1*), displays enhanced ethylene response, and this mutant contains an increased ethylene content. Later Skottke et al. (2011) demonstrated that RCN1 was involved in the biosynthesis of ethylene by regulating ACS6 phosphorylation status, as *rcn1* mutant displayed shorter hypocotyl with or without ACC present due to higher ACS6 activity and high ACC content. Other research showed that the *PP2A-B″* mutant *ton2* (*TONNEAU2/FASS*) contains an increased ethylene content in seedlings and the genetic study with crossing *fass/ton2* to *etr1* indicated that *fass/ton2* is epistatic to *etr1* (Fisher et al., 1996). CTR1 was shown to physically interact with the ethylene receptors (Clark et al., 1998), while the interaction between a C subunit of PP2A and CTR1's kinase domain was also reported (Larsen and Cancel, 2003). A recent study indicated that the A1 and C4 subunits of PP2A modulate ethylene signaling to alleviate Cd-induced growth inhibition (Chen et al., 2020). Nevertheless, direct evidence for the role of PP2A's other subunits in regulating the ethylene signal transduction pathway is still lacking. In this study, we report that a B subunit of PP2A, PP2A-B'ζ, interacts with CTR1, a key component of the ethylene signaling pathway, and this interaction is important for the stability of CTR1 in plant cells, therefore PP2A-B'ζ is an important negative regulator in the ethylene signaling pathway.

2. Results

2.1. Loss of function in PP2A-B'ζ leads to higher sensitivity to ethylene

To understand the function of *PP2A-B'ζ* (At3g21650) in Arabidopsis, we analyzed a T-DNA insertion mutant of *PP2A-B'ζ*. The genotyping PCR experiment confirmed that the T-DNA is inserted into the first exon of *PP2A-B'ζ* (Fig. 1A and B) and this insertion leads to no *PP2A-B'ζ* transcript in the *pp2a-b'ζ* mutant according to the result of reverse-transcription PCR analysis (Fig. 1C). Because ethylene is one of the most important hormones that regulates plant growth, development, and response to environmental and biotic stresses, we analyzed how the *pp2a-b'ζ* mutant would behave in the presence of the ethylene precursor ACC in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). We sowed Arabidopsis seeds on MS plates with or without 10 μM ACC and grew these seeds vertically for 5 days in darkness, then we found that the *pp2a-b'ζ* mutant was very sensitive to ACC (Fig. 2A and B). The hypocotyl length of the *pp2a-b'ζ* mutant is about 30% shorter than that of the *ctr1-8* mutant, and approximately 42% shorter than wild-type plants (Fig. 2A and B). However, under normal conditions, the *pp2a-b'ζ* mutant did not show significant difference to wild-type plants. To find whether this phenotype is due to the involvement of PP2A-B'ζ in the ethylene signaling pathway, we added silver nitrate ($AgNO_3$), an ethylene receptor inhibitor, to the MS media to block ethylene signaling. The results showed that in the presence of $AgNO_3$, the *pp2a-b'ζ* mutant became completely insensitive to ACC (Fig. 2C and D), indicating that the shortening of hypocotyl in *pp2a-b'ζ* mutant was ethylene signaling related.

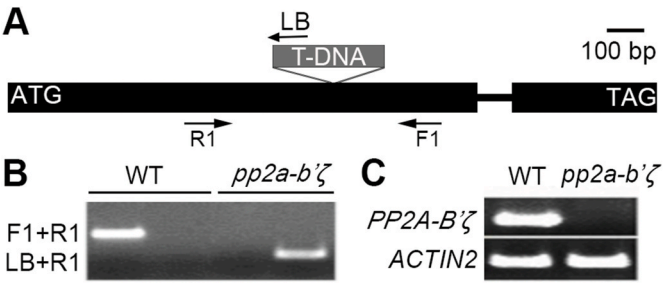

Fig. 1. Molecular analysis of the *pp2a-b'ζ* mutant. **A.** The position of the T-DNA insertion in the *PP2A-B'ζ* gene and the primers used for genotyping PCR experiments to confirm the *pp2a-b'ζ* mutant. Black boxes indicate exons and the line indicates the intron. LB, primer designed from the left border sequence of T-DNA; F1 and R1, two primers designed from the *PP2A-B'ζ* gene. **B.** Genotyping PCR experiments to confirm the *pp2a-b'ζ* mutant. Genomic DNAs extracted from wild-type and the *pp2a-b'ζ* mutant were used for PCR experiments and primer pairs used are marked on the left. **C.** Using the reverse transcription PCR experiment to analyze the PP2A-B'ζ transcript level in wild-type and the pp2a-b'ζ mutant plants. The gene *ACTIN2* was used as the internal control.

2.2. PP2A-B'ζ regulates plant growth at early stages of seedling development

To gain a better understanding of the function of *PP2A-B'ζ* in the ethylene signaling pathway, we analyzed the germination rate and seedling development of the *pp2a-b'ζ* mutant and *PP2A-B'ζ*-overexpressing plants. Based on RNA blot analysis (Supp. Fig. 1), two independent *PP2A-B'ζ*-overexpressing lines, OE1 and OE2, that expressed the *PP2A-B'ζ* transcript at high levels, were selected for further analysis. Because both ethylene and ABA are involved in regulating seed germination and seedling development and they inhibit each other's biosynthesis (Arc et al., 2013), we analyzed the germination rate and seedling development of these plants in the presence of ABA. Arabidopsis seeds of wild-type, *pp2a-b'ζ* mutant, two *PP2A-B'ζ*-overexpressing lines (OE1 and OE2), and the ethylene signaling mutant *ctr1-8* were sown on MS plates with or without 0.5 μM of ABA (Fig. 3A). Our results showed that on MS plates without ABA, the *pp2a-b'ζ* mutant displayed lower germination rate than other lines in the first 3 days, but reached the same level as others on day 4 (Fig. 3B). However, on MS plates supplemented with ABA, these plants showed different rates of germination at early stage, with *ctr1-8* being the lowest on day 1 and *pp2a-b'ζ* mutant the lowest on day 2, and they both reached the same level as others on day 3 (Fig. 3C). The two *PP2A-B'ζ*-overexpressing lines OE1 and OE2

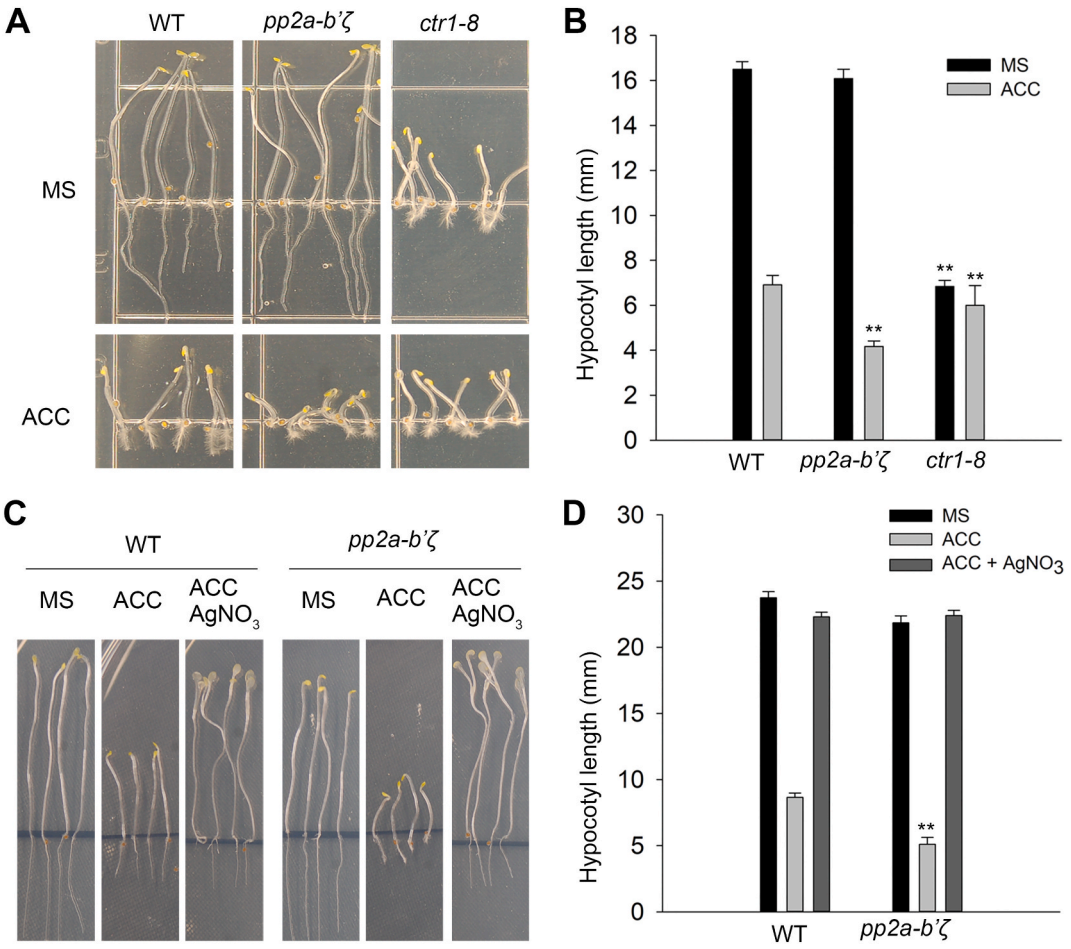

Fig. 2. The pp2a-b'ζ mutant is sensitive to ethylene precursor ACC treatment. **A**. Phenotypes of wild-type, *pp2a-b'ζ*, and *ctr1-8* plants on MS plates and MS plates supplemented with ACC. Plants were kept in darkness for 5 days in the absence or presence of 10 μM of ACC. WT, wild-type plant. **B**. Hypocotyl lengths of seedlings shown in A. **C**. Phenotypes of wild-type and *pp2a-b'ζ* plants on MS plates and MS plates supplemented with ACC or ACC plus AgNO3. Plants were kept in darkness for 7 days in the absence or presence of 10 μM of ACC or 10 μM of ACC plus 10 μM of AgNO3. **D**. Hypocotyl lengths of seedlings shown in C. Values represent means ± SD (n = 20 individual replicate plants). * and ** indicated significant difference from that of WT at $p < 0.05$ and $p < 0.01$, respectively, by one-way ANOVA analysis with a post-hoc Tukey honestly significant difference (HSD) test.

showed higher germination rates in the first 3 days after stratification (Fig. 3A and C). Interestingly, both *ctr1-8* and *pp2a-b'ζ* mutants developed green cotyledon leaves at much low rates than other lines at later stages of seedling development on ABA plates (Fig. 3D).

2.3. *PP2A-B'ζ is highly expressed in dry seeds*

To study the expression pattern of *PP2A-B'ζ*, we conducted real-time quantitative PCR (RT-qPCR) experiments to analyze the transcript levels of *PP2A-B'ζ* at different developmental stages and in different organs. We found that the *PP2A-B'ζ* transcript was highly expressed in dry seeds, moderately expressed in flowers and siliques, and expressed at low level in leaves of different developmental stages (Fig. 3E), indicating that *PP2A-B'ζ* is likely expressed in most tissues throughout plant growth and development. Like CTR1, *PP2A-B'ζ* is clearly needed for early seedling development, and overexpression of *PP2A-B'ζ* appears to promote germination and seedling development, as well as chloroplast development (greening process during seedling development).

2.4. *PP2A-B'ζ negatively regulates gene expression in ethylene signaling*

Since PP2A-B'ζ is negatively involved in ethylene signaling, we speculated that PP2A would affect ethylene regulated gene expression in Arabidopsis. To test this hypothesis, we added cantharidin, a PP2A inhibitor, to MS media to reduce PP2A activity for plants grown on MS plate and then compared how *pp2a-b'ζ* mutant and other plants would respond. Arabidopsis seeds were sown on MS plate or MS plates supplemented with 10 μM ACC, 10 μM cantharidin, or both 10 μM ACC and 10 μM cantharidin, and then plants were allowed to grow vertically in darkness for 7 days. In the presence of cantharidin, except *rcn1-6*, the hypocotyl length of other lines was reduced to roughly half of their length when grown without cantharidin in the media in darkness (Fig. 4A and B). Interestingly, the hypocotyl length of *pp2a-b'ζ* mutant was almost the same as wild-type in the presence of cantharidin (Fig. 4A and B). The *rcn1-6* mutant, which already has reduced PP2A activity, showed even shorter hypocotyl length in the presence of cantharidin (about 30% of the length that *rcn1-6* would show when grown in the absence of cantharidin in darkness). These results indicated that PP2A-B'ζ plays a vital role in regulating hypocotyl elongation in darkness. Among plants treated with both ACC and cantharidin, the hypocotyl length of all lines was severely reduced (Fig. 4A and B). The hypocotyl lengths of wild-type, *PP2A-B'ζ*-overexpressing plants, *ctr1-8,* and *rcn1-6* were reduced to about 50% of their lengths under ACC treatment, but the hypocotyl length of *pp2a-b'ζ* was reduced more, about 70% to its length under ACC treatment. This result indicates that PP2A is involved in the regulation of hypocotyl elongation through the ethylene pathway, and the B'ζ subunit is a key factor in this regulation.

To test whether ethylene regulated gene expression is affected in the

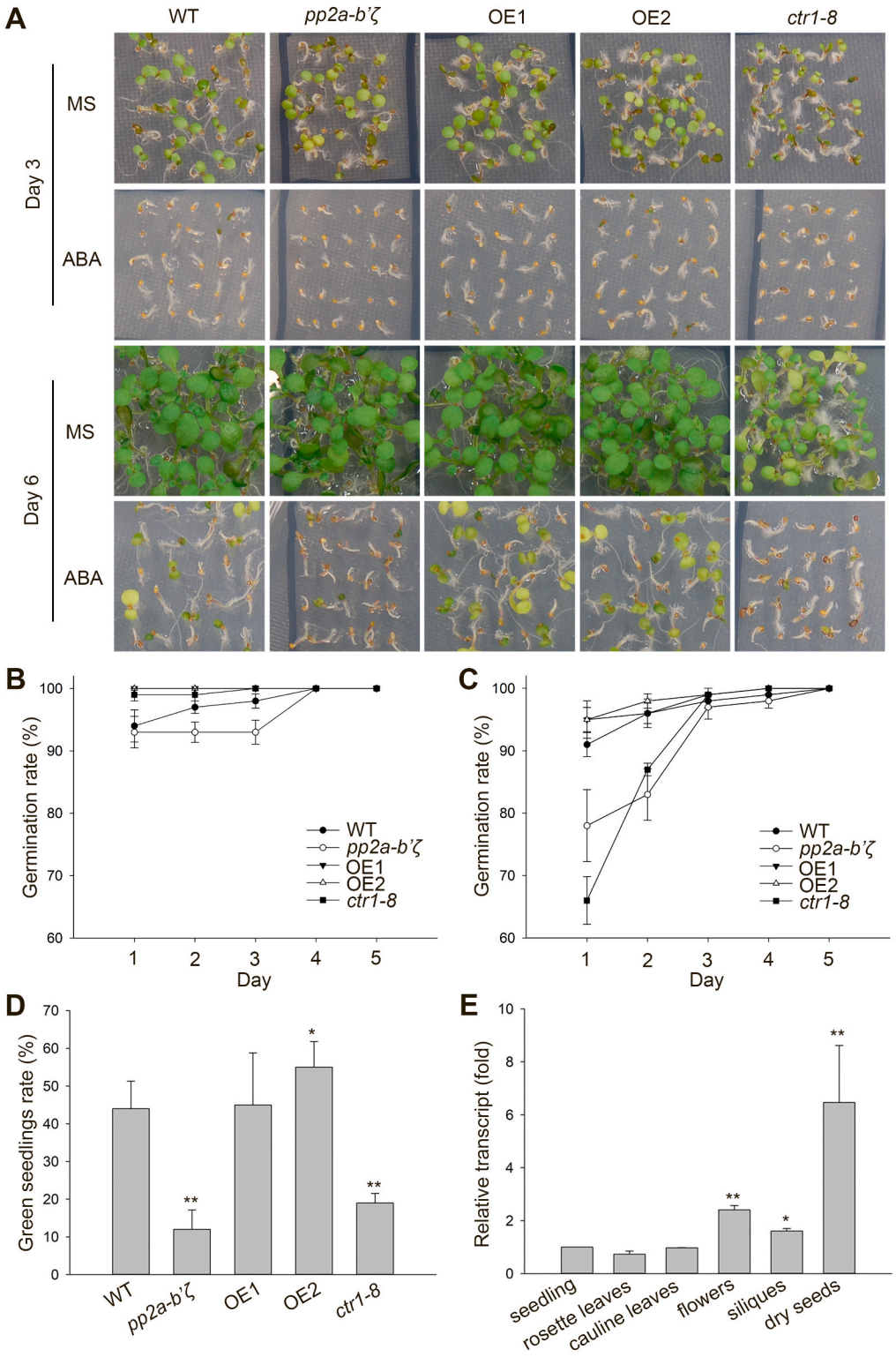

Fig. 3. Germination and seedling development of *pp2a-b'ζ* mutant and *PP2A-B'ζ*-overexpressing plants in the absence and presence of ABA and *PP2A-B'ζ* transcript analysis in different tissues. **A**. Phenotypes of wild-type, *pp2a-b'ζ* mutant, *PP2A-B'ζ*-overexpressing, and *ctr1-8* mutant plants on MS plates and MS plates supplemented with ABA. MS, MS plates; ABA, MS plates supplemented with 0.5 μM ABA; WT, Wild-type. **B**. Germination rates of wild-type, *pp2a-b'ζ* mutant, *PP2A-B'ζ*-overexpressing, and *ctr1-8* mutant plants on MS plate. **C**. Germination rates of wild-type, *pp2a-b'ζ* mutant, *PP2A-B'ζ*-overexpressing, and *ctr1-8* mutant plants on MS plate supplemented with ABA. **D**. Percentage of green seedlings to germinated seeds of wild-type, *pp2a-b'ζ* mutant, *PP2A-B'ζ*-overexpressing, and *ctr1-8* mutant plants in the presence of ABA on day 6. **E**, *PP2A-B'ζ* transcript level at different developmental stages and in different tissues by using RT-qPCR technique. Values represent means ± SD (n = 3 individual replicate experiments). * and ** indicated significant difference from that of WT or seedling at $p < 0.05$ and $p < 0.01$, respectively, by one-way ANOVA analysis with a post-hoc Tukey honestly significant difference (HSD) test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

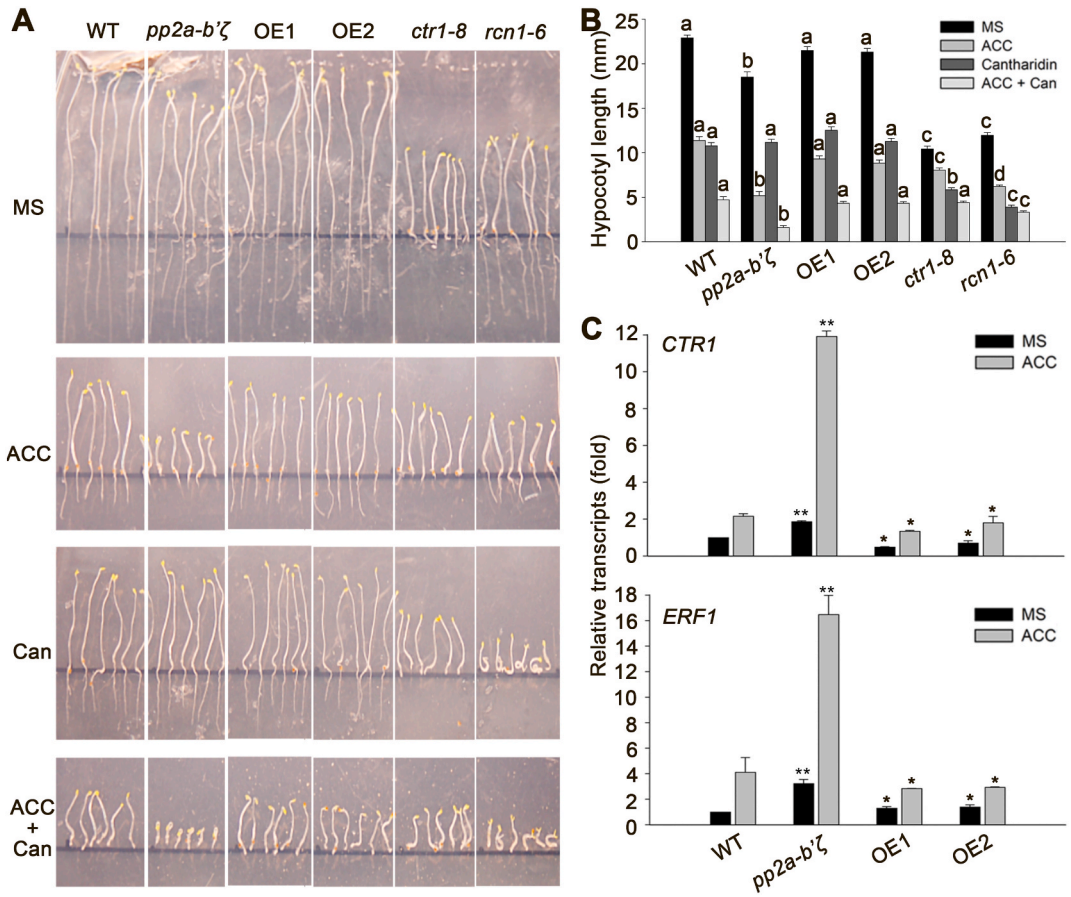

Fig. 4. Analysis of the hypocotyl length of wild-type, *pp2a-b'ζ* mutant, *PP2A-B'ζ*-overexpressing, *ctr1-8* mutant, and *rcn1-6* mutant plants on MS plates and MS plates supplemented with ACC, cantharidin, or ACC plus cantharidin and transcript analysis of two ethylene regulated genes in wild-type, *pp2a-b'ζ* mutant, and *PP2A-B'ζ*-overexpressing plants. **A** and **B**. Hypocotyl length of different lines on MS, ACC, cantharidin and ACC plus cantharidin plates. WT, wild-type; OE1 and OE2, two *PP2A-B'ζ*-overexpressing lines; MS, MS plates; ACC, MS plates supplemented with 10 μM ACC; Can, MS plates supplemented with 10 μM cantharidin; ACC + Can, MS plates supplemented with 10 μM ACC and 10 μM cantharidin. Values represent means ± SD (n = 18 plants). Samples denoted by different letters are significantly different ($P < 0.05$, Tukey correction). Samples are compared to wild-type plants in same treatment group. **C**. Transcript level of *CTR1* and *ERF1* from wild-type, *pp2a-b'ζ* mutant, and two *PP2A-B'ζ*-overexpressing lines with or without 10 μM ACC treatment. Values represent means ± SD (n = 3 experiments). * and ** indicated significant difference from that of WT at $p < 0.05$ and $p < 0.01$, respectively, by one-way ANOVA analysis with a post-hoc Tukey honestly significant difference (HSD) test.

pp2a-b'ζ mutant, we analyzed the transcript levels of two important genes in the ethylene signaling pathway, *CTR1* and *ERF1*, by using RT-qPCR technique (Fig. 4C). Since CTR1 interacts with the ethylene receptor ETR1 on the endoplasmic reticulum (ER) membrane (Gao et al., 2003), it functions at the early step in the ethylene signaling pathway. The transcription factor ERF1 (ethylene response factor 1) regulates expression of ethylene responsive genes, consequently it functions at the last step in the pathway. In the absence of ACC, the transcript level of *CTR1* was the highest in the *pp2a-b'ζ* mutant and the lowest in *PP2A-B'ζ*-overexpressing plants (Fig. 4C), roughly 2-folds higher in the *pp2a-b'ζ* mutant and 50% lower in *PP2A-B'ζ*-overexpressing plants when compared to wild-type plants. In the presence of ACC, although the *CTR1* transcript level was increased in all lines, the increase was substantially higher in the *pp2a-b'ζ* mutant (Fig. 4C). The *CTR1* transcripts were increased by 2, 20, 1.5 and 1.8 folds in wild-type, *pp2a-b'ζ*, and *PP2A-B'ζ*-overexpressing plants, respectively (Fig. 4C). Like the *CTR1* gene, in the absence of ACC, the *ERF1* transcript was higher in the *pp2a-b'ζ* mutant than in wild-type and *PP2A-B'ζ*-overexpressing plants. However, in the presence of ACC, the *ERF1* transcript level was dramatically increased as well, with about 4-fold increase in wild-type and *pp2a-b'ζ* mutant plants and about 2-fold of increase in *PP2A-B'ζ*-overexpressing plants (Fig. 4C). Based on these results, it appears that PP2A-B'ζ is a negative regulator of CTR1 or a negative regulator in the ethylene regulated gene expression, as overexpression of *PP2A-B'ζ* reduces the transcripts of both *CTR1* and *ERF1* under ACC treatment (Fig. 4C). Without functional PP2A-B'ζ, plants become more sensitive to ACC treatment, and plants produce more *CTR1* and *ERF1* transcripts.

2.5. *PP2A-B'ζ functions upstream of EIN2*

To explore the relationship between PP2A-B'ζ and components of the ethylene signaling pathway, we studied the phenotype of the double mutant of *pp2a-b'ζ* and *ein2* since EIN2 is the central component of the ethylene transduction pathway (Alonso et al., 1999). We crossed *pp2a-b'ζ* mutant with *ein2* mutant, and from the F_2 progenies we identified the *pp2a-b'ζ ein2* double mutant, which was then confirmed by genotyping PCR experiments (Supp. Fig. 2A). We next analyzed the hypocotyl length of the double mutant on MS plate in the absence or presence of ACC and we found that the etiolated hypocotyl length of the double mutant was similar to that of the single mutant *ein2*, whether or not ACC was in the MS media (Fig. 5A and B). Zhang et al. (2014) showed that the *ctr1-1 ein2* double mutant displayed the phenotype of *ein2*, not the phenotype of *ctr1*, indicating that *CTR1* functions upstream of *EIN2*. Similar to the study of the *ctr1-1 ein2* double mutant, we conclude that *PP2AB'ζ* functions upstream of *EIN2*. Because the

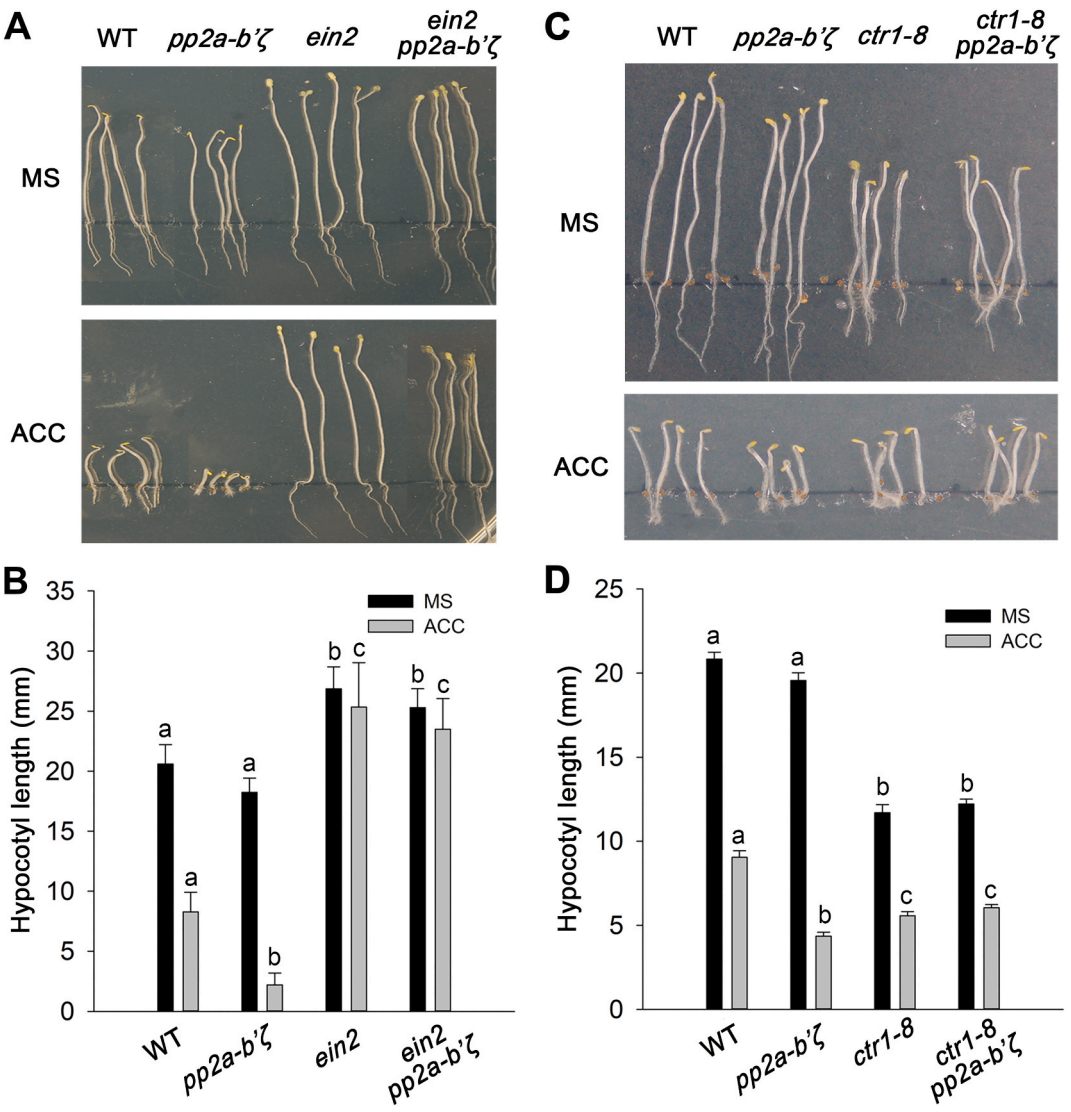

Fig. 5. Analysis of double mutants *pp2a-b'ζ ein2* and *pp2a-b'ζ ctr1-8* places *PP2A-B'ζ* functions upstream of *CTR1* and *EIN2* in the ethylene signaling pathway. **A** and **B**. The *pp2a-b'ζ ein2* double mutant shows the *ein2* mutant phenotype. **C** and **D**. The *pp2a-b'ζ ctr1-8* double mutant shows the *ctr1-8* mutant phenotype. MS, MS plates; ACC, MS plates supplemented with 10 μM ACC. Values represent means ± SD (n = 20 individual replicate plants). Samples denoted by different letters are significantly different ($P < 0.05$, Tukey correction). Samples are compared to wild-type plants in same treatment group.

immediate upstream component of *EIN2* is *CTR1*, we then analyzed the relationship between *PP2A-B′ ζ* and *CTR1* by creating and analyzing the *ctr1-8 pp2a-b'ζ* double mutant. From the F_2 progenies of the cross between dihybrid F_1 parents (i.e. *CTR1 ctr1-8 PP2A-B′ ζ pp2a-b'ζ*), we found 19 out of 94 seedlings showing *ctr1-8* phenotype (i.e. smaller size; Supp. Fig. 2B). The genomic DNAs of these 19 plants were extracted and examined by PCR in order to identify the *pp2a-b'ζ* mutant (Supp. Fig. 2C). Three homozygous double mutants were identified. Then the hypocotyl length of the *ctr1-8 pp2a-b'ζ* double mutant was analyzed along with wild-type, *pp2a-b'ζ*, and *ctr1-8* in the absence or presence of ACC. After 5 days of growth in darkness, we found that the hypocotyl length of the *ctr1-8 pp2a-b'ζ* double mutant mimics that of the *ctr1-8* mutant, whether or not ACC was in the MS media (Fig. 5C and D). Our result showed that *PP2A-B'ζ* is also a negative factor in the ethylene transduction pathway, and it could work upstream of *CTR1* or at the same site of *CTR1*. Because the upstream component of CTR1 is the ethylene receptor ETR1 (Ecker, 1995; Chang, 2016) and CTR1 and ETR1 physically interact with each other (Clark et al., 1998), we hypothesized that PP2A-B'ζ would function at the site of CTR1 as another upstream regulator of CTR1.

2.6. PP2A-B'ζ physically interacts with CTR1

To test our hypothesis, we analyzed if PP2AB'ζ physically interacts with CTR1 by conducting the pull-down experiments. To demonstrate the protein-protein interaction between one protein derived from plant cells and another derived from bacterial cells, two transgenic lines overexpressing *CTR1* with Human influenza hemagglutinin (HA) tagged CTR1 protein were generated, one in wild-type background (COW) and the other in the *pp2a-b'ζ* mutant background (Cob). A transgenic line from each genetic background with similar transgene transcript level (around 3 folds of their individual background) was selected for the pull-down experiments (i.e. COW2 and COb4, respectively, in Supp. Fig. 3). The HIS-tagged PP2A-B'ζ protein (i.e. 6× histidine residues fused to PP2A-B'ζ) made in bacterial BL21 cells were incubated with cellular extracts from wild-type, COW and COb plants in phosphate buffer and then recovered from resin beads that retained His-tagged protein. The pull-down experiments showed that HA-CTR1 interacted with His-PP2A-B'ζ either in the presence or absence of ACC, but without functional PP2A-B'ζ in host plant cells, the HA-tagged CTR1 was less stable in the presence of ACC (Fig. 6A). To further confirm this interaction,

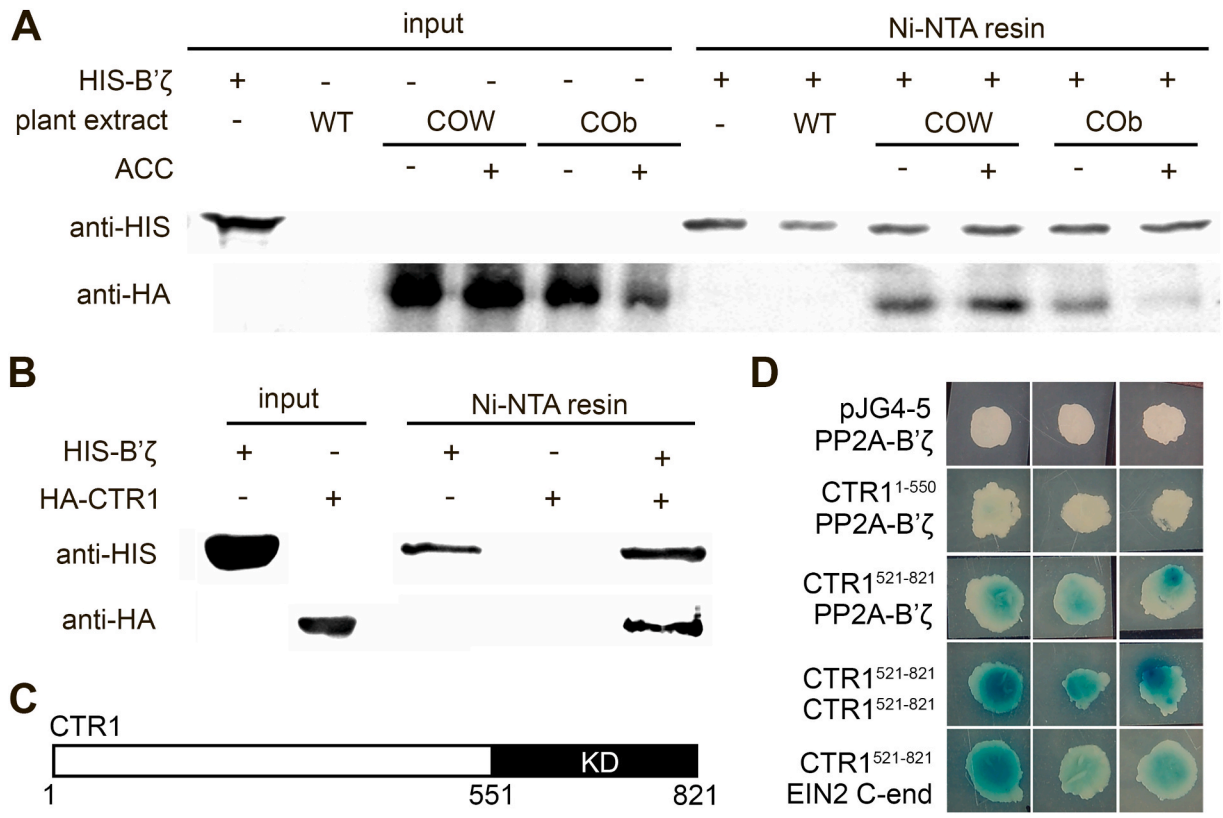

Fig. 6. Protein-protein interaction between PP2A-B'ζ and CTR1. **A**. The pull down experiment shows the interaction between *E coli.* expressed PP2A-B'ζ and plant expressed CTR1. + and -, presence or absence of added protein, ACC, 10 μM of ACC treatment; anti-HIS and anti-HA, antibodies against HIS tag and HA tag, respectively. **B**. The pull down experiment shows interaction between *E coli.* expressed PP2A-B'ζ and CTR1 proteins. **C**. The diagram of the functional domains of CTR1. The N-terminal binding domain (1–550) and the C-terminal kinase domain (KD, 551 to 821) are shown. **D**. The yeast two-hybrid experiments show protein-protein interactions between two CTR1 KDs, between CTR1 KD and PP2A-B'ζ, and between CTR1 KD and EIN2 C-end. Three repeats on X-gal plates were shown for each interaction. The combination of pEG202-EIN2 C-end and pJG4-5-CTR1$^{521-821}$ was used as positive control, combination of pEG202-PP2A-B'ζ and pJG4-5 empty plasmid was used as negative control.

both proteins were expressed in bacterial BL21 cells, then we incubated HA-tagged CTR1 with His-tagged PP2A-B'ζ in phosphate buffer in test tubes and conducted the pull-down experiment again. If PP2A-B'ζ interacts with CTR1, it should pull down CTR1 from the incubation solution. Indeed, we found that the Ni-NTA resins that retained PP2A-B'ζ also retained CTR1 (Fig. 6B).

In addition to the protein-protein interaction demonstrated *in vitro*, we also used the yeast two-hybrid system to analyze the protein-protein interaction between PP2A-B'ζ and CTR1 *in vivo*. The CTR1 is made of two domains: N-terminal domain (residues 1 to 550) that interacts with ethylene receptor ETR1, and C-terminal domain (residues 551 to 821) that contains a kinase domain (Fig. 6C) (Clark et al., 1998). The kinase domain can interact with the kinase domain of another CTR1 in the absence of ethylene to form an active enzyme that phosphorylates EIN2 (Mayerhofer et al., 2012; Ju and Chang, 2012; Ju et al., 2012). We first tested if the C-terminal part of one CTR1 (521–821) could interact with the C-terminal part of another CTR1 by fusing the C-terminal part of CTR1 (residues 521–821) to the bait vector pEG202 and another C-terminal part of CTR1 (residues 521–821) to the prey vector pJG4-5 of the LexA based yeast two-hybrid system (Golemis et al., 1996). Furthermore, we tested if this part also interacts with PP2A-B'ζ and the C-terminal portion of EIN2 (i.e. EIN2 C-end). Our results not only confirmed that the C-terminal part (521–821) of CTR1 could indeed interact with another CTR1 in this region, but also this region could interact with PP2AB'ζ and EIN2 C-end (Fig. 6D). Based on the intensity of the blue color, it appears that the CTR1-CTR1 interaction is stronger than the PP2AB'ζ-CTR1 interaction. It is clear that this part of CTR1 is the site critical for CTR1's function and regulation.

2.7. *PP2A-B'ζ stabilizes CTR1 in plant cells*

To further explore the relationship between PP2A-B'ζ and CTR1, we expressed HA-tagged CTR1 in wild-type and *pp2a-b'ζ* mutant plants, and then studied the steady-state level of the expressed HA-CTR1 in plant cells. To keep the native status of protein samples, no reducing agents were used during sample preparing. Based on the Western blot analysis, we found that CTR1 exists in two forms in wild-type plants, the monomer form and the dimer form (90 KDa and 180 KDa, respectively) (Fig. 7). We could not detect the monomer form of CTR1 when we expressed the HA-CTR1 in the *pp2a-b'ζ* mutant background and the amount of the CTR1 dimer is also reduced in the *pp2a-b'ζ* mutant background (Fig. 7). This result indicates that the CTR1 dimer is more stable and the CTR1 monomer is not stable in the *pp2a-b'ζ* mutant,

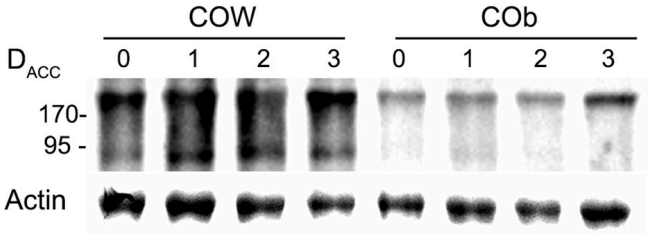

Fig. 7. The CTR1 stability in plant cells depends on functional PP2A-B'ζ. COW, HA-tagged CTR1 protein overexpressed in wild-type plants; Cob, HA-tagged CTR1 protein in pp2a-b'ζ mutant plants. D_{ACC}, days under ACC treatment; Actin, protein loading control.

suggesting that CTR1's stability in plant cells depends on functional PP2A-B'ζ protein.

3. Discussion

PP2A has been implicated in playing important roles in plant hormone signaling for two decades. The A1/RCN1 subunit of PP2A (or PP2A-A1) was first shown to be involved in auxin transport in Arabidopsis (Garbers et al., 1996), then it was found that this subunit plays important roles in all stages of development, especially in seeding development (Deruere et al., 1999; Zhou et al., 2004; Blakeslee et al., 2007). Furthermore, the PP2A-A1 was shown to play critical roles in guard cell closure by regulating ABA signaling (Kwak et al., 2002) and in hypocotyl gravitropism by regulating EIN2 in ethylene signaling (Muday et al., 2006). In an effort to study ethylene signaling, Larsen and Chang (2001) isolated the enhanced ethylene response mutant *eer1* and later they found that this mutant was a different allele of *PP2A-A1* (Larsen and Cancel, 2003). All these studies involved the PP2A-A1 subunit, clearly indicating that this subunit plays a critical role in PP2A's function in plants (Zhou et al., 2004). The roles of B subunits in plant hormone signaling were largely unknown other than that a B subunit mutant, *ton2,* was shown to contain higher amount of ethylene (Fisher et al., 1996). The research reported here provides a clear evidence that the B subunit of PP2A, PP2A-B'ζ, plays an important role in ethylene signaling pathway.

We showed that the loss of function in *PP2A-B'ζ* leads to higher sensitivity to ethylene. Although in the absence of ethylene, the hypocotyl length of *pp2a-b'ζ* mutant was similar to that of wild-type plants, in the presence of ethylene the hypocotyl length of *pp2a-b'ζ* mutant was very short (Fig. 2), indicating that the function of *PP2A-B'ζ* is to repress ethylene signaling, which is similar to the function of CTR1. We showed that PP2A-B'ζ is involved in the early seedling development, as the *pp2a-b'ζ* mutant displayed similar phenotypes of *ctr1-8*, e.g. delayed germination rate and seedling greening (Fig. 3). Early studies of CTR1 showed that CTR1 is a key factor linking ethylene receptors to nuclear events through its N- and C-terminal regions (Clark et al., 1998; Ju et al., 2012). Unlike other *ctr1* mutants, the *ctr1-8* has a point mutation at its N-terminal side (i.e. G354E) with a functional C-terminal region (Huang et al., 2003). This mutation leads to a weak phenotype, but it serves as a good material for studying its C-terminal region that interacts with other proteins. Under normal growth condition, *pp2a-b'ζ* mutant displayed similar germination rate (i.e. >90% germination from day 1 to day 3) and chloroplast development to wild-type plants, however, the application of ABA delayed germination and chloroplast development in both *pp2a-b'ζ* and *ctr1-8* mutants (Fig. 3A and D). Hu et al. (2014) showed that ABA inhibits PP2A activity in Arabidopsis seedling indicating that PP2A plays important role in ABA-regulated seedling development. In this study, ABA increased the differences of germination rate and chloroplast development between wild-type plants and *pp2a-b'ζ* mutant, and this difference is due to the involvement of ethylene signaling component, as *pp2a-b'ζ* mutant is very sensitive to ACC treatment and more sensitive to the combined treatment of ACC and PP2A inhibitor cantharidin (Fig. 4A and B).

Having made a connection between *PP2A-B'ζ's* function and ethylene signaling, we tested ethylene regulated gene expression in the *pp2a-b'ζ* mutant. We found that the transcript levels of *CTR1* and *ERF1* were highly up-regulated in the presence of ACC in the *pp2a-b'ζ* mutant (Fig. 4C), again supporting that *PP2A-B'ζ'* plays a negatively role in ethylene regulated gene expression. To place where *PP2A-B'ζ'* might function in the ethylene signaling pathway, we conducted genetic analysis with the *pp2a-b'ζ* mutant and two ethylene signaling mutants *ein2* and *ctr1-8*. From analyzing the phenotype of the *pp2a-b'ζ ein2* double mutant, we conclude that *PP2A-B'ζ* functions upstream of *EIN2* as the double mutant displayed the same phenotype as *ein2* in the presence of ACC (Fig. 5A). Interestingly, the phenotype of the *pp2a-b'ζ ctr1-8* double mutant is similar to the *ctr1-8* mutant in the presence or absence of ACC (Fig. 5C), suggesting that *PP2A-B'ζ'* should also function upstream of *CTR1*. The literature indicates that the ethylene receptor ETR1 interacts with CTR1 on ER membranes (Clark et al., 1998), then PP2A-B'ζ might function as the other upstream partner of CTR1. To test this possibility, we conducted protein-protein interaction experiments and indeed we found that PP2A-B'ζ physically interacts with CTR1 in pull-down experiments and in yeast two-hybrid experiments (Fig. 6). The interaction between PP2A-B'ζ and CTR1 is clearly important for the stability of CTR1 monomers in plant cells as we could not detect CTR1 monomers in the *pp2a-b'ζ* mutant.

Based on the data presented here, we propose a model to explain the function of PP2A-B'ζ in the ethylene signaling pathway. As a Raf-like protein kinase, CTR1 is active when formed as a dimer, not as a monomer (Mayerhofer et al., 2012). When a CTR1 polypeptide is newly made, it could form a dimer with another CTR1 monomer, thereby becoming stable. We hypothesize that the binding of ethylene to its receptor ETR1 triggers the phosphorylation and separation of CTR1 dimer from ETR1 dimer and from each other, resulting in the release of phosphorylated CTR1 monomer from ETR1 on ER membranes, which becomes the substrate for PP2A-B'ζ. If PP2A-B'ζ binds to the phosphorylated CTR1 monomer, which converts it to dephosphorylated CTR1 monomer that can bind with another dephosphorylated CTR1 monomer to form CTR1 dimer. The function of PP2A with the B subunit PP2A-B'ζ in removing the phosphate from CTR1 monomer is to allow CTR1 dimer to be formed, thereby recycling the phosphorylated CTR1 monomer in the presence of ethylene (Fig. 8). In this model, the function of PP2A-B'ζ is to keep CTR1 to its maximal level in dimer form so that the ethylene signaling is kept silent in the absence of ethylene. When ethylene binds to its receptors, CTR1 dimer becomes phosphorylated by a kinase or by ETR1, then dissociates from ETR1 and released as the phosphorylated CTR1 monomer that can no longer phosphorylate EIN2, thereby allowing EIN2 to be cut by an unknown enzyme to produce EIN2 C-end that enters nucleus to trigger ethylene regulated gene expression. In the *pp2a-b'ζ* mutant, because the steady-state level of CTR1 dimer is further reduced due to the lack of protection for phosphorylated CTR1 monomer from PP2A-B'ζ, the mutant becomes hyper-sensitive to ethylene treatment.

In this report, we provide strong evidence that PP2A-B'ζ negatively regulates ethylene transduction pathway through controlling CTR1 activity by dephosphorylating CTR1 to stabilize CTR1. For this model to work, two assumptions must be made: 1, ethylene binding to its receptor ETR1 leads to the release of phosphorylated CTR1 monomers to cytosol; 2, there is a specific E3 ligase that recognizes the phosphorylated CTR1 monomer and targets it to the 26S proteasomes for degradation. At this time, it is not known what happens to CTR1 dimer on ER membranes when ethylene binds to its receptor ETR1. It is urgently needed to study the function of the kinase domain of ETR1 in ethylene signaling, especially its relationship with CTR1 upon ethylene binding. Equally important is to understand why dephosphorylated CTR1 monomer is not stable in the *pp2a-b'ζ* mutant. For a polypeptide to disappear quickly in plant cells, normal proteinases are not likely very effective. We assume it is the action of 26S proteasomes that can remove phosphorylated CTR1 monomer effectively. The next challenge is to find an E3 ligase that recognizes the phosphorylated CTR1 monomer and targets it to the 26S proteasome for degradation, and then it would provide a solid support to our model.

4. Material and methods

4.1. Plant materials

The *pp2a-b'ζ* mutant (Salk_107944C) and the *ein2* mutant (Salk_143,201) were obtained from Arabidopsis Biological Resources Center (ABRC, Ohio State University, USA). The *ctr1-8* mutant was kindly provided by Dr. Eric Schaller of Dartmouth College, and the *rcn1-6* mutant was obtained from Dr. Alison DeLong of Brown University. The

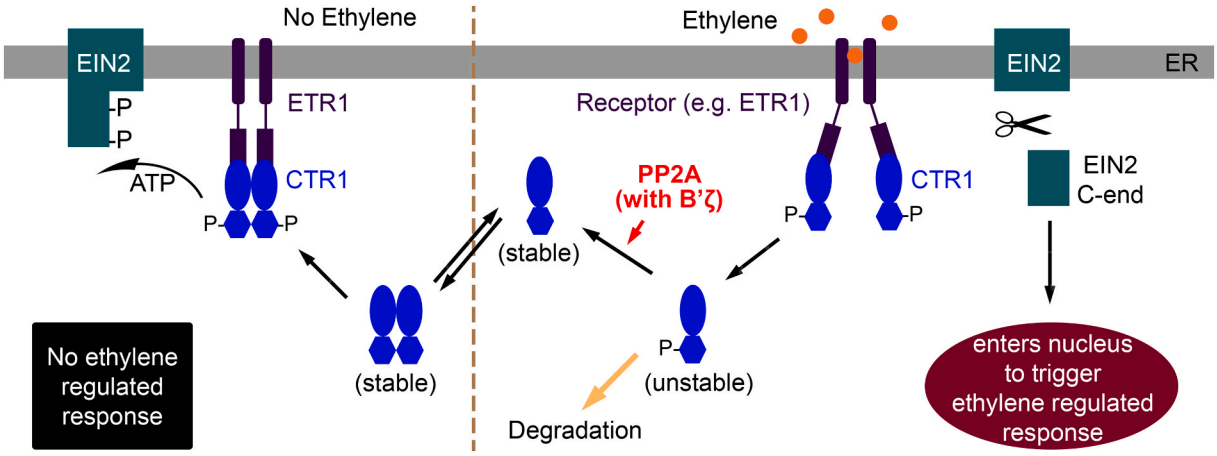

Fig. 8. Proposed model on PP2A-B'ζ's function in stabilizing CTR1 protein in plant cells. In the absence of ethylene, CTR1 binds to ethylene receptor as phosphorylated dimer form that can phosphorylates the C-terminal end of EIN2, therefore repressing ethylene regulated response. In the presence of ethylene, due to the conformation change in ethylene receptor, CTR1 is no longer associated with the ethylene receptor and is released as phosphorylated monomer, leading to the cleavage of EIN2 C-end, which enters nucleus to trigger ethylene regulated gene expression. The unstable and phosphorylated CTR1 monomer is also the substrate of PP2A-B'ζ that can convert the phosphorylated CTR1 monomer to regular CTR1 monomer that is stable in wild-type cells. The interaction between PP2A-B'ζ and CTR1 monomer stabilizes CTR1 and facilitates the formation of CTR1 dimer and dimer-receptor complex in the absence of ethylene, thereby together with CTR1 forming the negative regulator in the ethylene signaling pathway.

pp2a-b'ζ ein2 double mutant was created by crossing *pp2a-b'ζ* mutant with *ein2* mutant and from the F_2 progenies we screened for the double mutant by using genotyping PCR with T-DNA specific primer LBa1 and gene specific primers b'zeta-F1/b'zeta-R1, and ein2-F1/ein2-R1. Similarly, the *pp2a-b'ζ ctr1-8* double mutant was created by crossing the *pp2a-b'ζ* with the *ctr1-8* mutant and confirmed through genotyping PCR by using T-DNA primer LBa1 and gene specific primers b'zeta-F1/b'zeta-R1. The F_2 progenies that show the *ctr1* phenotype were checked by using primers b'zeta-F1/b'zeta-R1. Plants that showed the *ctr1* phenotype and contained T-DNA yet with no *pp2a-b'ζ* transcript were considered as *pp2a-b'ζ ctr1-8* double mutants. The *PP2A-B'ζ*-overexpressing lines were generated with a full-length cDNA of *PP2A-B'ζ* constructed in the pBI121 based vector under the control of CaMV 35S promoter. There were 11 homozygous lines obtained and their transcript levels were analyzed by using RT-qPCR. Two *PP2A-B'ζ*-overexpressing lines were selected for further experiments, which were designated as OE1 and OE2, respectively (Supp. Fig. 1). HA-tagged *CTR1*-overexpressing lines were generated in the pFGC5941 vector under the control of CaMV 35S promoter. The HA tag was fused to the N-terminus of the full-length CTR1. Homozygous transgenic plants were identified based on the segregation analysis of the Basta resistance (no more Basta sensitive plants were found among the T_3 transgenic plants), and the transgenic plants were verified by PCR analysis using primers LBa1 and b'zeta-R1. The transcript levels were analyzed by using RT-qPCR. Two lines with moderate levels of transgene (i.e. COW2 and COb4, around 3 folds of their own background plants, Supp. Fig. 3) were selected for further experiments, and they were labelled as COW and COb in text and in Figs. 6 and 7.

4.2. Plant growth conditions

For ABA and ACC treatments, Arabidopsis seeds were grown on plates in MS medium with or without ABA or ACC. Arabidopsis seeds were sterilized in 15% Clorox for 15 min (with gentle shaking), then washed extensively with water three times. Sterilized seeds were then kept at 4 °C in darkness for 72 h. For germination assay, sterilized seeds were grown on MS plates with or without 0.5 μM ABA at 22 °C under continuous light. Germination rates were recorded every 24 h until more than 99% of seeds germinated on MS plates. For ACC treatment experiment, seeds were grown on MS plates with or without 10 μM ACC at 22 °C in darkness. Hypocotyl lengths were then measured after one week's growth under ACC treatment.

4.3. RT-qPCR analysis

To analyze the transcript levels of *PP2A-B'ζ*-overexpressing plants in different tissues, total RNAs were extracted from *PP2A-B'ζ*-overexpressing plants and *HA-CTR1*-overexpressing plants by using the PureLink Plant RNA Reagent (Thermo Fisher Scientific, Waltham, MA) according to the product manual. The concentrations of total RNAs were determined by using the Nanodrop machine (Thermo Fisher Scientific, Waltham, MA), and one μg of total RNAs was used for reverse transcription. First strand cDNA was synthesized using the PrimeScript RT reagent Kit with gDNA eraser (Takara, Kyoto, Japan) according to manufacturer's instruction. Then cDNAs were diluted to 200 μL for RT-qPCR analysis. Gene specific primers were used in the PCR, while the *Actin 2* gene was used as the internal control. The RT-qPCR was performed using PowerUp SYBR Green Master Mix (Life technologies, Hercules, CA) on the Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). The changes in relative transcript level were calculated by using the ΔΔCt method.

4.4. Yeast two-hybrid analysis

The full-length coding sequence of *PP2A-B'ζ* was amplified from a cDNA library that was made of mRNAs of one-week-old seedling by using the primers B'zeta-YF and B'zeta-YR. The amplified cDNA fragment was digested with *Eco* RI and *Xho* I, then cloned into the bait vector pEG202 (Golemis et al., 1996). The N-terminal sequence (1–550 AA) and the C-terminal sequence (521–821 AA) of CTR1 were separately cloned into the prey vector pJG4-5 (Golemis et al., 1996). The EIN2 C-end fragment and the CTR1 fragment (521–821 AA) were used as positive controls in the yeast two-hybrid system. The yeast strain EGY48 was used for Y2H assay. The respective combination of bait plasmid and prey plasmid were co-transformed into EGY48 cells. Transformants were grown on -Ura-His-Trp dropout medium plates as describe in Golemis et al. (1996).

4.5. Pull-down experiment

Both HIS-tagged PP2A-B'ζ and HA-tagged CTR1 were expressed in the bacterial strain BL21 with the pET30b vector (EMD Biosciences,

Watertown, MA). The full-length cDNA of *PP2A-B'ζ* was cloned into pET30b, and then the recombinant vector was introduced into the bacterial strain BL21. The full-length of *CTR1* coding sequence with a HA tag was cloned into the pET30b vector in which the HIS sequence was deleted. Next, this recombinant construct was expressed in BL21 strain. Bacterial cells were collected in falcon tubes after centrifugation, re-suspended in phosphate buffer (PBS pH 7.4, 0.5% Triton X-100, 0.5 mM PMSF), and bacterial cells were sonicated (30 s interval). HIS-PP2A-B'ζ and HA-CTR1 were mixed and incubated at room temperature for 2 h and purified by using the HIS bind resin (EMD Biosciences, Watertown, MA) according to the manufacturer's instructions. Afterwards, the resin was washed, and the proteins bound to resin were eluted and collected. Protein samples were separated in the SDS-PAGE gel, and later transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The His-PP2A-B'ζ and HA-CTR1 were detected by using anti-HIS and anti-HA antibodies, respectively. HIS bands were detected using AP Conjugated Substrate Kit (Bio-Rad, Hercules, CA) and HA bands were detected using Amersham ECL Western Blotting Analysis System (GE Healthcare, Chicago, IL). Plant protein samples were extracted from HA-CTR1 transgenic plants using phosphate buffer, HA-CTR1 protein from plant tissues and His-PP2A-B'ζ protein from BL21 cells incubated in the test tubes, then pull-down experiment were conducted as above, then analyzed by using Western blot experiments.

4.6. Western blot analysis

The 10-day-old seedlings of COW and COb plants were grown in MS liquid media with or without 10 μM ACC, then protein samples were extracted with extraction buffer (50 mM MOPS, pH 7.8, 0.1 mM DTT, 2% Triton X-100, 1 mM PMSF and protease inhibitor cocktail) at days 0, 1, 2 and 3 after ACC was added to the media. Protein concentrations were determined by using the Bio-Rad Protein Assay System (Bio-Rad, Hercules, CA). A total of 10 μg of each sample was separated in an 8% SDS-PAGE gel. Proteins were then transferred onto a nitrocellulose membrane with Towbin buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3]. The nitrocellulose membrane was incubated with non-fat milk in TBST (5% milk in TBS buffer with 0.1% Tween-20) at room temperature for 1 h to block non-specific sites. Then the membrane was washed 3 times with TBST before it was incubated with the primary antibody (Anti-HA-Peroxidase, Roche, Switzerland) for another hour at room temperature. After incubation with antibody, the membrane was washed with TBST 3 more times, and then the signal was analyzed using Amersham ECL Western Blotting Analysis System (GE Healthcare, Chicago, IL). Image was taken with the ChemiDoc MP Imaging software (Bio-Rad, Hercules, CA).

4.7. Statistical analysis

The data presented as the mean ± SD were analyzed by a one-way ANOVA analysis with post-hoc Tukey honestly significant difference (HSD) test. p-values of <0.05 or <0.01 were considered to be statistically significant (Figs. 2, 3 and 4C, S1 and S3). Tukey's method was used for pairwise comparison among more than two groups of plants at the significant level of $\alpha = 0.05$ (Figs. 4B and 5).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2021.10.037.

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