Casein Kinase 1 Regulates Ethylene Synthesis by Phosphorylating and Promoting the Turnover of ACS5

Graphical Abstract

Highlights
Arabidopsis plants lacking Casein Kinase 1.8 show constitutive ethylene responses

CK1.8 deficiency leads to ethylene overproduction and ACS5 accumulation

CK1.8 phosphorylates ACS5 at threonine 463 and promotes its interaction with ETO1

Overexpression of CK1.8 results in delayed fruit ripening in tomatoes.

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In Brief
The role of casein kinase1 (CK1) in plants is largely unknown. Tan and Xue report that a CK1 isoform, CK1.8, acts as a negative regulator of ethylene biosynthesis in Arabidopsis by promoting, via phosphorylation, the degradation of an ethylene biosynthesis enzyme, ACS5. In Arabidopsis, loss of CK1.8 leads to phenotypes consistent with overproduction of ethylene, whereas in tomatoes overexpression delays fruit ripening, a result with potential agricultural implications.
Casein Kinase 1 Regulates Ethylene Synthesis by Phosphorylating and Promoting the Turnover of ACS5

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SUMMARY

The casein kinase 1 (CK1) family participates in various cellular processes in eukaryotes, but CK1 function in higher plants remains largely unknown. Here, we characterize the function of Arabidopsis CK1 in the regulation of ethylene biosynthesis. Etiolated seedlings of a CK1.8-deficient mutant, ck1.8-1, showed characteristic ethylene-specific constitutive responses due to overaccumulation of ethylene. Biochemical and physiological studies showed that CK1.8 phosphorylates ACS5, a key enzyme of ethylene biosynthesis, at threonine 463 to promote its interaction with the E3 ubiquitin ligase Ethylene Overproduction 1 (ETO1). Deficiency of CK1.8 leads to the accumulation of ACS5, and transgenic plants harboring a dephosphorylation-mimic ACS5T463A showed constitutive ethylene responses, confirming the role of CK1.8 in regulating ACS5 stability by phosphorylation and demonstrating that CK1.8 is an important regulator of ethylene biosynthesis. CK1.8 expression is feedback regulated by ethylene. Our studies provide insight into the regulation of ACS5 and ethylene biosynthesis.

INTRODUCTION

Ethylene is an important and unique gaseous phytohormone that regulates various physiological processes including seed germination, seedling growth, leaf and flower senescence and abscission, photomorphogenesis, fruit ripening, etc. (Ecker, 1995; Schaller and Kieber, 2002; Li et al., 2012). Exogenous ethylene treatment induces triple responses of etiolated seedlings in Arabidopsis, including the inhibited elongation of hypocotyls and roots, radial swelling of hypocotyls, and exaggerated curvature of apical hook (Ecker, 1995; Guzmán and Ecker, 1990). Endogenous ethylene promotes the ripening process of many vegetable and fruit crops, such as tomato (Solanum lycopersicum L.) and apple (Malus domestica), which is important for seed release during evolution but shortens the shelf life of fruits, and disruption of either synthesis or signaling pathway of ethylene could prevent ripening in these species (Klee and Giovannoni, 2011).

Synthesis of ethylene includes two key steps. S-adenosylmethionine (SAM) is first converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), and then ACC is oxidized by ACC oxidase (ACO) to produce ethylene. Studies showed that ACS is a rate-limiting step in ethylene biosynthesis and is responsible for ethylene dosage control in most tissues (Schaller and Kieber, 2002). ACS is encoded by a multigene family in many plant species, and analysis revealed that there are eight active ACS isoforms (ACS2, ACS4–9, and ACS11) and an inactive ACS1 in Arabidopsis (Yamagami et al., 2003). The tight and dynamic control of quantity or activity of ACSs is vital for plant morphogenesis and resistance, and studies have shown that ACSs are regulated by many biotic and abiotic cues both transcriptionally and posttranslationally, including ubiquitin-26S proteasome degradation (Chae et al., 2003; Wang et al., 2004; Nomoto et al., 2004; Lyzenga et al., 2012), proteolysis, and reversible phosphorylation (Tatsuki and Mori, 2001; Liu and Zhang, 2004; Han et al., 2010; Skottke et al., 2011).

In Botrytis cinerea-induced ethylene biosynthesis, the pathogen-responsive MAPK3 and MAPK6 phosphorylate and stabilize type I ACSs, ACS2, and ACS6, to stimulate ethylene biosynthesis and confer pathogen resistance (Liu and Zhang, 2004; Han et al., 2010). Reversible phosphorylation of ACS is regulated by the PP2A protein phosphatase, and studies showed that the stability of ACS2 and ACS6 is negatively, whereas that of ACS5 is positively, regulated by PP2A (Skottke et al., 2011). In addition, recent studies showed that a phospho-binding protein, 14-3-3, binds to and stabilizes ACS5 (Yoon and Kieber, 2013), suggesting that phosphorylation may involve in the regulation of ACS5 stability. Of the eight characterized ACSs in S. lycopersicum, LeACS2 and LeACS4 are the major enzymes functioning during ripening (Klee and Giovannoni, 2011), and knockdown of them prevented fruit ripening (Oeller et al., 1991), and interestingly LeACS2 could be phosphorylated by a calcium-dependent protein kinase (CDPK) (Tatsuki and Mori, 2001). Type II ACSs, especially ACS5 and ACS9, are degraded by the ubiquitin-26S proteasome pathway, which is mediated by broad complex/tramtrack/bric-a-brac (BTB) type E3 ligases ETO1 (Ethylene Overproduction 1)/EOLs (Chae et al., 2003; Wang et al., 2004; Christians et al., 2009). Mutation of the carboxy-terminal sequences of ACS5 (a dominant mutant, eto2) and ACS9 (a dominant mutant, eto3) eliminates the recognition by ETO1/EOLs, leading to ethylene overproduction (Chae et al., 2003; Wang et al., 2004).
Phytohormones cytokinin and brassinosteroids regulate ethylene biosynthesis by stabilizing ACS5 (Chae et al., 2003; Hansen et al., 2009). Two receptor like kinases FEl1 and FEI2 interact with ACSs, linking the cell-wall biosynthesis with ethylene production during root growth (Xu et al., 2008). In addition, ACS4 (type II) and ACS7 (type III) are regulated by XBAT32 E3 ligases (Nodzon et al., 2004; Prasad and Stone, 2010; Prasad et al., 2010; Lyzenga et al., 2012), and ACS4 and ACS5 are the targets of E3 ligases, for degradation through ubiquitin-26S proteasome (Christians et al., 2009; Lyzenga et al., 2012). Although ACS proteins are modified at various levels to involve in different developmental process, the underlying regulatory mechanisms remain poorly understood and need further investigation.

Casein kinase 1 (CK1) is a conserved Ser/Thr kinase family in eukaryotes and regulates different physiological processes by phosphorylating various substrates (Gross and Anderson, 1998; Knippschild et al., 2005). Most of the knowledge about CK1 is accrued from yeast and animal studies, whereas only sketchy reports of the CK1 function in higher plants exist. Arabidopsis casein kinase 1 like 6 (CKL6) is a paralog of the tobacco plasmodesmal-associated protein kinase and phosphorolylates a subset of non-cell-autonomous proteins, such as tobacco mosaic virus movement proteins (Lee et al., 2005). In addition, CKL6 regulates the organization of cortical microtubules (Ben-Nissan et al., 2008). Recently, Arabidopsis CK1.3 (CKL3) and CK1.4 (CKL4) were identified as key regulators in blue light signaling via phosphorylating the receptor cryptochrome 2 (Tan et al., 2013). In rice, EARLY FLOWERING1 is involved in gibberellin signaling by regulating the activity and stability of SLENDER RICE1 (Dai and Xue, 2010), and OsCK1 is involved in brassinosteroid signaling (Liu et al., 2003). Analysis showed that there are 17 CK1 isoforms in Arabidopsis and 15 in rice; however, most of them have not been functionally characterized. Considering the important role of CK1 in other organisms, studies of that in plants will provide insights into the CK1 functions.

We have carried out systemic biochemical and genetic studies to characterize the physiological roles of Arabidopsis CK1s. Our studies showed that CK1.8 regulates ethylene synthesis by phosphorylating ACSs. Expression of CK1.8 in fruits results in the delayed ripening of tomatoes. These findings reveal the existence of an explicit biochemical mechanism that regulates ethylene biosynthesis and provide a strategy for prolonging the fruit preservation.

**RESULTS**

**Arabidopsis CK1.8 Encodes an Active Casein Kinase 1**

CK1 plays crucial roles during plant growth and development, and there are 17 isoforms of CK1 in the Arabidopsis genome (Tan et al., 2013), which exhibit differential subcellular localizations (Lee et al., 2005). We obtained all the T-DNA mutants of Arabidopsis CK1 members to systematically characterize the physiological functions of them by genetic studies. Of these CK1 members, CKL8 presents high similarity to OsCK1 (Li et al., 2003), and an in vitro kinase assay showed that CKL8 can phosphorylate casein (Figure 1A), confirming CKL8 is an active CK1. We thus rename CKL8 as CK1.8 to avoid confusion. Expression pattern analysis by quantitative RT-PCR (qRT-PCR) and promoter-reporter gene (35S-glucuronidase, GUS) fusion revealed that CK1.8 is expressed in roots, leaves, stem, flowers, mature stamens, and pistil tips (Figure S1A). Interestingly, the expression of CK1.8 in shoots presented a dynamic pattern under dark, i.e., low at early stages (before 96 hr), while increased to maximal at 6 days (Figure 1B). At early stages, CK1.8 is expressed in the most parts of seedlings and is restricted to the upper region of hypocotyls (below the apical hook) during hypocotyl elongation and apical hook formation (Figures S1A and S1B). Considering the crucial role of ethylene in young seedling development, analysis of GUS staining under ACC (ethylene precursor) treatment showed that CK1.8 transcription is not altered under white light but significantly reduced under dark (Figure 1C), which is further confirmed by qRT-PCR analysis showing the CK1.8 expression in shoots is reduced by ACC treatment under dark (Figure 1D), suggesting that CK1.8 might involve in the ethylene-regulated skotomorphogenesis.

**Deficiency of CK1.8 Caused Overaccumulated Ethylene**

To investigate the physiological role of CK1.8, a T-DNA insertion mutant, ck1.8-1, was identified from the SALK T-DNA insertion population (Alonso et al., 2003). The T-DNA insertion was located in the second intron of CK1.8 (Figures S1C and S1D) and completely abolished the CK1.8 expression as revealed by qRT-PCR analysis (Figure 1E), indicating ck1.8-1 a knockout mutant.

Phenotypic observations showed that ck1.8-1 adult plants grew similarly to wild-type (WT) under normal conditions, but ck1.8-1 seedlings exhibited shortened hypocotyls and exaggerated apical hooks under dark (Figures 1F and 1G) that are typically observed in ethylene-overproducing or signal-enhancing mutants as constitutive triple responses (CTR). Interestingly, unlike the typical CTR mutants, such as ctr1, eto1, eto2, or eto3, ck1.8-1 showed no difference in root length (Figure 1F; Figure S2A), indicating a specific role for CK1.8 in regulating ethylene responses of the seedling aerial parts.

Analysis of the genotype-phenotype linkage of the F2 generation by backcrossing ck1.8-1 with WT showed that the homozygous ck1.8-1/ck1.8-1 resulted in constitutive ethylene response, whereas the heterozygous CK1.8/ck1.8-1 plants exhibited a normal phenotype (Figure S2B). In addition, the segregation ratio of constitutive ethylene response:normal in F2 generation was 1:3 (Table S1), indicating ck1.8-1 a single recessive mutation. Further complementation studies showed that rescued CK1.8 expression driven by either native or Cauliflower mosaic virus (CaMV) 35S promoter (Figure 1E) resulted in the normal growth (Figure 1G), confirming that CK1.8 is responsible for the phenotype of ck1.8-1. However, transgenic plants overexpressing CK1.8 (p35S::CK1.8, Figure S2C) showed no obvious phenotype.

Gas chromatography analysis showed that ck1.8-1 produced more ethylene than WT (approximately one-third of ethylene produced in eto1; Figure 2A). Furthermore, a key ethylene-responsive gene, ERF1, was upregulated in ck1.8-1 both under light and dark conditions (Figure 2B), confirming that CK1.8 deficiency resulted in the enhanced ethylene response in planta, which is mainly by overproduced ethylene.
Some chemical compounds have been previously identified as antagonists of ethylene synthesis or signaling, which were then utilized to test whether the phenotype of ck1.8-1 was resulted from the altered ethylene biosynthesis or signaling. Both Ag⁺ (ethylene receptor inhibitor) and AVG (ACS inhibitor) treatment completely recovered the ck1.8-1 seedling phenotype (Figure 2C), which is consistent with the increased ethylene amount and enhanced ethylene signaling in ck1.8-1 and suggests CK1.8 a negative regulator in ethylene biosynthesis.

Alignment with known CK1s from human and Arabidopsis (Ben-Nissan et al., 2008) identified the key amino acids of CK1.8 required for the kinase activity: lysine 34 (Lys38) and aspartic acid 128 (Asp128) (Figure S2D). Indeed, analysis of the two point mutants CK1.8 K38R (lysine to arginine) and CK1.8D128N (aspartic acid to asparagine) by in vitro kinase assays indicated that both CK1.8K38R and CK1.8D128N lose the kinase activity toward casein (Figure S2E). Furthermore, after transforming CK1.8K38R, CK1.8D128N, or CK1.8, driven by CaMV35S promoter, into ck1.8-1 (Figure S2F), observations showed that the wild-type version of CK1.8 could recover the ethylene phenotype of ck1.8-1, but CK1.8K38R and CK1.8D128N could not (Figure 2D). These results indicate that the kinase activity is required for CK1.8 function in ethylene response.

CK1.8 Phosphorylates ACS5 In Vitro and Negatively Regulates ACS5 Stability In Planta

The fact that AVG treatment rescued the phenotype and the increased ethylene accumulation of ck1.8-1 suggested that altered ACS is the possible cause. Among the eight ACSs of Arabidopsis, ACS5 is an important isoform responsible for early seedling development. Analysis of ck1.8-1 acs5-1 double mutant showed that ACS5 deficiency completely suppressed the phenotype of ck1.8-1 (Figure 3A), revealing that acs5-1 is epistatic to ck1.8-1. It was thus hypothesized that CK1.8 might directly regulate ACS5.

Figure 1. Arabidopsis CK1.8 Encodes an Active CK1 and Deficiency of CK1.8 Results in the Constitutive Ethylene Response
(A) In vitro kinase assay by 32P-γ-ATP autoradiograph confirmed CK1.8 a casein kinase (upper) by using recombinant CK1.8 (1 μg) from E. coli. Dephosphorylated casein and BSA (as control) were used as substrates (10 μg). Coomassie brilliant blue (CBB) staining indicated the loading of proteins (10 μg, bottom).
(B) qRT-PCR analysis revealed the dynamic expression of CK1.8 during seedling development. Shoots from etiolated seedlings at different stages (60, 72, 84, 96, 108, 120, 132, 144, 156, and 168 hr after germination) were analyzed. Experiments were repeated three times, and error bars represent SE. (C) Promoter-reporter gene analysis revealed the reduced expression of CK1.8 under ACC treatment in dark. Four-day-old etiolated seedlings under white light (upper) or dark (bottom) were treated with 10 μM ACC (MS medium as control). Scale bars, 1 mm.
(D) qRT-PCR analysis confirmed the reduced expression of CK1.8 in shoots under ACC treatment in dark. Four-day-old etiolated seedlings were treated with 10 μM ACC (MS as control). ACTIN7 was used as an internal reference. Error bars represent SE. Statistical analysis was performed using a two-tailed Student’s t test (**p < 0.01; compared with “-ACC”).
(E) qRT-PCR analysis confirmed the deficient expression of CK1.8 in ck1.8-1 and rescued expression of CK1.8 in ck1.8-1 transformed with CK1.8 cDNA driven by its own native promoter or CaMV35S promoter. Relative expression levels of CK1.8 were normalized with ACTIN7. Experiments were repeated three times, and error bars represent SE.
(F) ck1.8-1 exhibited constitutive ethylene response under dark (grown for 90 hr). Scale bar, 1 mm.
(G) Hypocotyl length of etiolated seedlings (grown in dark for 90 hr) of WT, ck1.8-1, and individual complemented lines. Experiments were repeated three times, and error bars represent SD (n > 20). Statistical analysis was performed by using a two-tailed Student’s t test.

Some chemical compounds have been previously identified as antagonists of ethylene synthesis or signaling, which were then utilized to test whether the phenotype of ck1.8-1 was resulted from the altered ethylene biosynthesis or signaling. Both Ag⁺ (ethylene receptor inhibitor) and AVG (ACS inhibitor) treatment completely recovered the ck1.8-1 seedling phenotype (Figure 2C), which is consistent with the increased ethylene amount and enhanced ethylene signaling in ck1.8-1 and suggests CK1.8 a negative regulator in ethylene biosynthesis.

Alignment with known CK1s from human and Arabidopsis (Ben-Nissan et al., 2008) identified the key amino acids of CK1.8 required for the kinase activity: lysine 34 (Lys38) and aspartic acid 128 (Asp128) (Figure S2D). Indeed, analysis of the two point mutants CK1.8 K38R (lysine to arginine) and CK1.8D128N (aspartic acid to asparagine) by in vitro kinase assays indicated that both CK1.8K38R and CK1.8D128N lose the kinase activity toward casein (Figure S2E). Furthermore, after transforming CK1.8K38R, CK1.8D128N, or CK1.8, driven by CaMV35S promoter, into ck1.8-1 (Figure S2F), observations showed that the wild-type version of CK1.8 could recover the ethylene phenotype of ck1.8-1, but CK1.8K38R and CK1.8D128N could not (Figure 2D). These results indicate that the kinase activity is required for CK1.8 function in ethylene response.

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CK1 phosphorylation sites exist in ACS5 protein (Figure S2G). To test whether CK1.8 may directly regulate ACS5, a yeast two-hybrid analysis was performed, and results showed that CK1.8 directly interacts with ACS5 (but not with ACS4, Figure 3B). Further in vitro kinase activity assay by using recombinant ACS5 and CK1.8 proteins showed that CK1.8 directly phosphorylates ACS5 (Figure 3C), suggesting that CK1.8 may regulate ethylene synthesis through directly phosphorylating ACS5.

ACS5 fused with Myc (driven by CaMV35S promoter) was then transformed into WT and ck1.8-1 to study the effects of CK1.8 on ACS5 in vivo. Three lines expressing comparable Myc-ACS5 fusion proteins were selected to examine the phosphorylation status of ACS5 (Figure S3A). Further, a quick cross was performed to obtain the isogenic Myc-ACS5 plants and clarify the role of CK1.8 in ACS5 regulation. Pollens from Myc-ACS5 (in ck1.8-1) homozygous lines were spread on the stigma of WT and ck1.8-1, respectively, and analysis of the F1 plants with comparable ACS5 expression levels (Figure S3B) by western blot showed that more Myc-ACS5 is accumulated in ck1.8-1 than in WT and ck1.8-1 (Figure 3D), suggesting that CK1.8 and partially rescued that of ck1.8-1. After pretreatment with a proteasome inhibitor, MG132, to block the degradation of Myc-ACS5, western blot showed that more Myc-ACS5 is accumulated in ck1.8-1 than that in WT and ck1.8-1 (Figure 3D), suggesting the crucial role of CK1.8 in ACS5 stability. After pretreatment with a proteasome inhibitor, MG132, to block the Myc-ACS5 degradation, western blot assay showed a shifted band near to the Myc-ACS5 in ck1.8-1 (Figure 3D), which is observed in many phosphorylated proteins and could not be observed after treatment with shrimp alkaline phosphatase (SAP) (Figure 3E), confirming the phosphorylation of ACS5. Being consistent, the phosphorylated Myc-ACS5 was barely detected in ck1.8-1, indicating that CK1.8 stimulates ACS5 phosphorylation in vivo (Figure 3E).

Further analysis of transgenic WT or CK1.8 overproduced ethylene in ck1.8-1 Background (Figure 2A). Gas chromatography analysis revealed an overproduction of ethylene in ck1.8-1 and ck1.8-1 mutants. Geminated seeds (20 per vial) were incubated for 48 hr before analysis. Experiments were repeated three times, and error bars represent SD. (B) qRT-PCR analysis revealed the enhanced expression of ERF1 in ck1.8-1. Relative expression level of ERF1 was normalized by ACTIN7. Experiments were repeated three times, and error bars represent SD. Statistical analysis was performed using a two-tailed Student’s t test (**p < 0.01; compared with WT).

CK1.8 Phosphorylation Promotes the ACS5-ETO1 Interaction

Previous studies showed that ETO1 directly interacts with and ubiquitinates type II ACSs, including ACS4, ACS5, and ACS9 (Wang et al., 2004; Christians et al., 2009; Yoshida et al., 2005). ck1.8-1 displayed increased ethylene and shortened hypocotyl similar to eto1, eto2, and eto3, whereas overexpression of CK1.8 (p35S::CK1.8) showed no significant difference from WT, similar to the ethylene deficient mutants acs5-1, acs9-1, or acs5-1 acs9-1 (Figure 4A). In addition, AVG treatment (10 μM) rescued the constitutive ethylene response of eto1 and ck1.8-1 and partially rescued that of eto2 (Figure 4B).

Considering the ETO1 effects in ubiquitinating ACS5, it is thus speculated that CK1.8 might regulate the ETO1-ACS5 interaction and hence the accumulation of ACS5. Yeast three-hybrid
Threonine 463 Is an Important CK1 Phosphorylation Site Responsible for ACS5 Stability

In silico analysis identified threonine (Thr) 209, 236, 258, 416, 426, and 463 and serine (Ser) 67, 122, 261, 440, and 441 of ACS5 as the candidate phosphorylation sites (Figure S2G). Previous studies showed a dominant mutation of ACS5 locus, eto2, harbors a 1 bp insertion destructing the 12 amino acid at C terminus (459–470 aa) of ACS5, and the ACS5eto2 protein is stable in planta because it cannot interact with ETO1 (Chae et al., 2003). In addition, the TOE motif at C terminus is required and efficient for ETO1 targeting ACS5 (Christians et al., 2009; Yoshida et al., 2005). Because only Thr463 exists in the TOE motif, we thus chose this site for further analysis. In vitro kinase assay showed that Thr463 in planta.

Figure 3. CK1.8 Interacts with and Phosphorylates ACS5 In Vitro and Is Required for ACS5 Turnover In Planta

(A) Constitutive ethylene response of ck1.8-1 depends on the ACS5 function. Analysis of hypocotyl length of 90-hr-old etiolated seedlings of WT, ck1.8-1, acs5-1, and ck1.8-1 acs5-1 double mutant revealed that ACS5 is epistatic to CK1.8. Experiments were repeated three times, and error bars represent SD (n > 15). Statistical analysis was performed by using a two-tailed Student’s t test (“p < 0.01; compared with WT).

(B) Yeast two-hybrid assays revealed a direct interaction between CK1.8 and ACS5. ACS4 was used as a negative control. Transformed yeast cells were grown on SD with dropout (-Leu-Trp, left) or SD with dropout (-Leu-Trp-His, right).

(C) In vitro kinase assays by 32P-y-ATP autoradiograph revealed that CK1.8 phosphorylates ACS5 (upper). Recombinant CK1.8 (0.1 μM) and ACS5 (10 μM) from E. coli was used. The protein loading was shown by CBB staining (bottom).

(D) Western blot analysis showed that deficiency of CK1.8 led to more accumulated Myc-ACS5. Protein extracts of 7-day-old transgenic CK1.8/ck1.8 or ck1.8-1 seedlings expressing Myc-ACS5 were analyzed with a Myc antibody (upper). The protein loading was shown by CBB staining (bottom).

(E) Western blot analysis revealed the reduced phosphorylation of ACS5 in ck1.8-1 in planta. Proteins were extracted from 7-day-old transgenic seedlings expressing Myc-ACS5 (in CK1.8/ck1.8 [L1] or ck1.8-1 [L1]) pretreated with MG132 (50 μM) and then incubated in MS medium supplemented with CHX (100 μM, to block the synthesis of new protein) for different times (0, 15, 30, 60, 120, or 240 min) and then analyzed using a Myc antibody (upper). The protein loading was shown by CBB staining (bottom).
Myc-ACS5 showed that (Wang et al., 2004; Christians et al., 2009; Yoshida et al., 2005), ETO1 acts as an E3 ligase to ubiquitinate ACS5 for degradation interaction. Combined with the previous studies showing that Trp-Met+Asp, left) and SD with dropout (-Leu-Trp-His-Met+Asp+), right). Yeast three-hybrid analysis by growth on auxotroph plate indicated the crucial roles of CK1.8 in ETO1-ACS5 interaction. Empty pBridge vector (BD), BD-ETO1, BD-ETO1-CK1.8, BD-ETO1-CK1.8K38R, and BD-ETO1-CK1.8D128N were analyzed. Transformed yeast cells were grown on SD with dropout (-Leu-Trp-His-Met+Asp+X-Gal, right).

Figure 4. CK1.8 Promotes the ETO1-ACS5 Interaction
(A) Hypocotyl length of 90-hr-old etiolated seedlings of WT, ck1.8-1, eto1, eto2, eto3, acs5-1, acs9-1, and p3SS::CK1.8 lines. Experiments were repeated three times, and error bars represent SD (n > 20). Statistical analysis was performed using a two-tailed Student’s t test (**p < 0.01; compared with WT).
(B) Measurements and statistical analysis of hypocotyl length of 90-hr-old etiolated seedlings of WT, ck1.8-1, eto1, eto2, acs5-1, and p3SS::CK1.8 lines under different dose of AVG. Experiments were repeated three times, and error bars represent SD (n > 20). Statistical analysis was performed using a two-tailed Student’s t test (**p < 0.01; compared with WT).
(C) Yeast three-hybrid analysis by growth on auxotroph plate indicated the crucial roles of CK1.8 in ETO1-ACS5 interaction. Empty pBridge vector (BD), BD-ETO1, BD-ETO1-CK1.8, BD-ETO1-CK1.8K38R, and BD-ETO1-CK1.8D128N were analyzed. Transformed yeast cells were grown on SD with dropout (-Leu-Trp-Met+Asp, left) and SD with dropout (-Leu-Trp-His-Met+Asp+X-Gal, right).

interaction. Combined with the previous studies showing that ETO1 acts as an E3 ligase to ubiquitinate ACS5 for degradation (Wang et al., 2004; Christians et al., 2009; Yoshida et al., 2005), we hypothesize that the phosphorylation of ACS5 by CK1.8 might promote its recognition by ETO1 for ubiquitin-26S proteasome-mediated degradation. Further analysis by transforming Myc-ACS5T463A and Myc-ACS5T463E into WT plants (transgenic lines harbored comparable protein levels, Figure S5B) showed that Myc-ACS5 in either WT or ck1.8-1 background, and Myc-ACS5T463A in WT resulted in strong effect in constitutive ethylene response phenotype, whereas Myc-ACS5T463E in WT did not (Figure 5C), revealing the important role of Thr463 site in regulating ACS5 stability. Further in vivo degradation assays indicated that the Myc-ACS5T463A was more stable than Myc-ACS5T463E, which was quickly degraded after CHX treatment (Figure 5D; Figure S5C). Being consistent, cell-free degradation assays indicated that His-ACS5T463A was more stable than His-ACS5T463E (Figure S5D). These results concluded that phosphorylation by CK1.8 at Thr463 is important for ACS5 degradation, and possibly through promoting ACS5-ETO1 interaction (Figure 5E).

Fruit-Specific Expression of CK1.8 Delays Fruit Ripening in Tomatoes
Ethylene is essential for various developmental processes, especially in fruit ripening. Modulation of ethylene dosage or signaling will result in the altered fruit maturation, which is crucial for quality and preservation of fruits. During fruit ripening, LeACS2 (type I) and LeACS4 (type III) of tomato are highly expressed and play dominant roles in ethylene synthesis (Yoshida et al., 2005). Interestingly, CK1 phosphorylation sites are also presented in type I ACSs including ACS6 and LeACS2, although they are not targeted by ETO1/EOLs (Yoshida et al., 2005). To test the potential application of CK1.8, we transformed CK1.8 and CK1.8D128N, driven by a fruit-specific promoter pE8 (Kneissl and Deikman, 1996), into tomatoes (Figure 6A). Observation of the fruit ripening showed that when control (LA1781) and transgenic plants expressing CK1.8D128N grew to the breaker stage (turning orange) at 30 days postanthesis (dpa), plants expressing CK1.8 remained green and kept orange for over 10 days (Figure 6B). Detailed statistical analysis showed that fruits of control and plants expressing CK1.8D128N completely turned red at ~37 dpa, whereas that was more than 45 dpa for plants expressing CK1.8 (Figure 6C), indicating a much delayed ripening under CK1.8 expression, which is possibly due to the reduced ethylene amount. These results are consistent with the fact that LeACS2 harbors a CK1 phosphorylation site (Figures S5A and S6). However, the detailed regulatory mechanism for CK1.8 in tomato ethylene synthesis might be different and needs further studies, because LeACS2 is not targeted by ETO1/EOLs. It is speculated that, in addition to the ETO1-dependent regulation, CK1.8 might also function in ETO1-independent regulation through different mechanisms and other factors (for example, E3 ligases) that may be involved in the process.

DISCUSSION
Our studies characterized the role of CK1 in ethylene synthesis and expanded the knowledge on the distinct function of CK1 in plants. The gaseous phytohormone ethylene is crucial for crop growth, especially the ripening process, and ACSs are key enzymes controlling ethylene synthesis. Although a tight regulation of ethylene biosynthesis is important for plant growth and development, the relevant mechanisms are still unclear. ETO1/EOLs are important regulators of ethylene synthesis through acting as BTB family E3 ligases to target type II ACSs (including ACS5 and ACS9) for ubiquitination-26S
proteasome-mediated degradation (Wang et al., 2004; Christians et al., 2009). Our studies showed that deficiency of CK1.8 results in the accumulation of ethylene and revealed CK1.8 a key negative regulator of ethylene synthesis through promoting the interaction between ETO1 and ACS5. Previous studies showed that TOE motif is responsible for ACS5 stability (Christians et al., 2009; Yoshida et al., 2005), and, considering Thr463 is a unique phosphorylation site in TOE motif in many species, identification of Thr463 as an important site for ACS5 phosphorylation and stability will help to illustrate the detailed regulatory mechanism of ACS5.

Expression of CK1.8 exhibits a dynamic complementary pattern with the ethylene dosage change in young shoots (Figure S5E), providing an accurate regulation of ethylene synthesis and seedling growth: at the early stage (1–4 days after germination), CK1.8 is expressed at low levels, and ACS5 accumulates to produce high levels of ethylene that contributes to restrict the morphogenesis by forming apical hooks; later on (5 days after germination), CK1.8 is expressed at high levels, and ACS5 accumulates to produce high levels of ethylene that contributes to restrict the morphogenesis by forming apical hooks.

![Figure 5. Thr463 Is the CK1 Phosphorylation Site and Is Crucial for ETO1 Interaction and ACS5 Stability](image)

A) In vitro kinase assay revealed that T463A substitution of ACS5 results in significantly reduced phosphorylation by CK1.8 (left upper). Recombinant ACS5, ACS5T236A (short as T236A), and ACS5T463A (short as T463A) from E. coli were used as substrates (10 μg). Protein loading was shown by western blot using His antibody (left bottom). Relative phosphorylation level of different His-ACS5 versions versus the western blot bands were calculated by Image J program (right). The quantity of “His-ACS5” was set as 1.0.

B) GST pull-down assay confirmed that the T463E substitution of ACS5 resulted in the enhanced binding ability to ETO1 than T463A substitution and native ACS5. Recombinantly GST, GST-ETO1, His-ACS5, His-ACS5T463A, and His-ACS5T463E were expressed in E. coli and purified for analysis. GST and GST-ETO1 were used as bait to detect the binding ability to different ACS5 versions. A mouse His antibody (upper) and a mouse GST antibody (bottom) were used to detect the proteins.

C) Measurement of hypocotyl length of 90-hr-old etiolated seedlings of WT, ck1.8-1, and transgenic WT lines harboring different versions of Myc-ACS5 (Myc-ACS5, Myc-ACS5T463A, or Myc-ACS5T463E) revealed the suppressed constitutive ethylene response of Myc-ACS5T463E lines compared to Myc-ACS5 and Myc-ACS5T463A lines. Three independent lines were analyzed, and error bars represent SD (n > 20). Statistical analysis was performed by using a two-tailed Student’s t test (*p < 0.01; Myc-ACS5T463E compared with Myc-ACS5 [L1, red] or Myc-ACS5T463A [L2, black], respectively).

D) In vivo degradation assays revealed that dephosphorylated Myc-ACS5T463A version was more stable than phosphorylation-mimic Myc-ACS5T463E. Transgenic WT plants expressing Myc-ACS5T463A (L2) or Myc-ACS5T463E (L9) were analyzed. Seven-day-old seedlings were incubated in liquid MS medium supplemented with CHX (100 μM) for 0, 15, 30, 60, or 120 min and then analyzed by western blot with a Myc antibody (upper). CBB staining showed the equal protein loading (bottom).

E) A hypothetic model of how CK1.8 functions. CK1.8, the expression of which is feedback regulated by ethylene (dashed line), phosphorylates ACS5 and promotes the interaction of which with ETO1, resulting in the enhanced ubiquitination and degradation of ACS5. Ub, ubiquitin.
Studies by fruit-specific expression of CK1.8 indicate the crucial role of CK1.8 in the ripening of tomatoes; however, whether CK1.8 functions through a similar mechanism to regulate ethylene synthesis in tomatoes and other plant species needs further investigations. CK1 recognizes and phosphorylates S/T in the “D/EX1-2S/T” or “ps/TX1-2S/T” sequences. Although Thr463 is conserved in several ACSs (Arabidopsis ACS5, ACS9, ACS6, and LeACS2), analysis of ACS5 homologs from different species revealed the presence of a highly conserved putative CDPK phosphorylation site (Ser461 in Arabidopsis ACS5, Ser460 in LeACS2; Tatsuki and Mori, 2001; Hernández-Sebastiá et al., 2004) and whose phosphorylation might prime the phosphorylation of ACS5 at Thr463. Interestingly, there is also a potential CK1 site (Ser462) at pS+2 position following Ser460 in the TOE motif of LeACS2 as well as other orthologs, with some diversity and an instead Asp in many species (Figure S6). Because Asp has negative charge as phospho-Thr or Ser, the relatively conserved “S-W/F-T/S/D” motif suggests a potential regulatory mechanism of CK1-CDPK crosstalk during plant evolution. However, orthologs with a basic amino acid at same position may not be regulated by CK1; whether the “S-W/F-T/S/D” or “S-W/F-D” motif represents two different kinds of regulation of ACS proteins would be an interesting question for further studies.

In addition, ETO1 could not interact with LeACS2 (Yoshida et al., 2005), suggesting that other E3 ligases might involve in its stability regulation. Recently, the RING finger E3 ligase XBAT32 was shown to target ACS4 and ACS7 for ubiquitin-26S proteasome degradation during Arabidopsis lateral root development (Nodzon et al., 2004; Prasad et al., 2010), indicating that other factors may involve in the regulation of ethylene synthesis to regulate distinct developmental processes. Tomato is one of the most important fleshy fruits worldwide for nutrient source, like vitamin A and C (Klee and Giovannoni, 2011). During ripening, the respiration rate and ethylene level exhibit a sharp increase with flesh softening, which is a serious problem for storage. Previous studies identified several nonripening tomato mutants due to the defective ethylene pathway, such as Never-ripe (Nr, an ethylene receptor), suggesting the possibility to delay fruit ripening and prolong the shelf life through modifying the native target genes (Klee and Giovannoni, 2011). Our studies characterized a negative regulator of ethylene synthesis from model plant Arabidopsis and demonstrated its role in tomatoes, providing a knockin strategy for endogenous ethylene dosage control. Given the importance of ethylene in various kinds of plants, CK1.8 is a potential gene for further genetic modification in crops.

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Growth Conditions**

All Arabidopsis thaliana plants were with Columbia-0 (Col-0) background. The T-DNA insertion mutants, ck1.8-1 (SALK_025753C), acs5-1 (CS16567), acs9-1 (SALK_129805C), and acs5-1 acs9-1 (CS16593) were obtained from the Arabidopsis Biological Resource Center (ABRC). The tomato (Solanum lycopersicum L) plants for transformation were in LA1781 background.

Seeds were sterilized and plated on Murashige and Skoog (MS) medium (Duchefa Biochemie). After stratification at 4°C for 4 days, seedlings were grown in phytofon with a 16-hr-light/8-hr-dark cycle (22°C). Tomatoes were grown in phytofon with a 12-hr-light/12-hr-dark (22°C) cycle.

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For seedlings grown in dark, after stratification, plates containing seeds of WT, the mutants used in this study, or different transgenic lines were exposed to light for 3 hr (22°C) and then transferred to dark for 90 hr (22°C). For pharmacological treatments, unless otherwise stated, following final concentrations were added to the plates, 10 μM ACC (Sigma, A9036), 10 μM AVG (Sigma, A1284), or 100 μM AgNO₃. Seedlings were photographed, and measurements were performed by using ImageJ (NIH).

**Constructs and Plant Transformation**

For expressing CK1.8 in ck1.8-1, the coding sequence of CK1.8 was amplified with primers (CK1.8-3 and CK1.8-4) and subcloned into the pCambia1302 vector; the CaMV35S promoter was replaced with the native promoter of CK1.8, which was amplified using primers (CK1.8p-1 and CK1.8p-2), resulting in the construct pCK1.8:CK1.8. For expressing CK1.8, CK1.8Δ128N, and CK1.8Δ128N in WT or ck1.8-1, the coding sequence of CK1.8, CK1.8Δ128N, and CK1.8Δ128N was amplified with primers (CK1.8-1 and CK1.8-4, CK1.8-5 and CK1.8-6, and CK1.8-7 and CK1.8-8, respectively) and subcloned into a pHB vector (driven by 2 x CaMV35S promoter) and confirmed by sequencing.

For expression of Myc-ACS5, Myc-ACS5ΔED, and Myc-ACS5ΔEDE in WT or ck1.8-1, the coding sequences of ACS5, ACS5ΔED, and ACS5ΔEDE were amplified into pEGAD-4XMyc vectors with N-terminal fusion (the fused amino acids were MRRYRFIMMEKLIASEEDNLNEQKLEIEDNLMEQKLEILEDNLEEDDLT). For expressing Myc-ETO1 in WT and ck1.8-1, the coding sequence of ETO1 was amplified (primers ETO1-1 and ETO1-5) and subcloned into pEGAD-4XmY vectors with N-terminal fusion. For tomato transformation, the E8 promoter was amplified using genomic DNA (from LA1781) as template and subcloned into a pHB vector. CK1.8 or CK1.8Δ128N sequences were cloned into the pHB-E8 vector.

*Arabidopsis* transformation was performed by floral dip methods (Clough and Bent, 1998). For tomato transformation, constructs pE8::CK1.8 and pE8::CK1.8Δ128N were first introduced into Agrobacterium GV3101 and then transformed into the callus from tomato cotyledon explants. The positive transformants were selected by hygromycin resistance (50 mg/l) and confirmed by PCR analysis. Primers used are listed in Table S2.

**Quantitative Real-Time RT-PCR Analysis**

qRT-PCR analysis was performed to examine the transcription of CK1.8 in various tissues, ERF1 under dark or light, and expression levels of CK1.8, CK1.8Δ128N, and CK1.8Δ128N in transgenic plants. Total RNAs were extracted and used to synthesize the cDNAs by reverse transcription. Primers used are listed in Table S2. The ACTIN7 (AT5G09810) gene was amplified and used as an internal reference.

**Ethylene Measurement**

Seeds of WT, ck1.8-1, and eto1 were sterilized and plated on sterilized filter paper. After stratification at 4°C for 4 days, plates were exposed to light for 3 hr and then transferred to dark for 2 days (22°C). After germination, 20 seedlings per sample (n = 5) were transferred to a gas chromatography vial (2 ml) with 0.5 ml MS medium and incubated for 2 days under dark. Accumulated ethylene was measured by gas chromatography with a flame ionization detector (Zhang and Wen, 2010).

**Promoter-Reporter Gene Fusion Studies**

The promoter of CK1.8 was amplified using genomic DNA as template (with primers CK1.8p-1 and CK1.8p-2; Table S2) and subcloned into a modified pCambia1300 vector (Liu et al., 2003). The histochemical GUS assay was performed as previously described (Wu and Xue, 2010). Stained samples were observed using DIC microscopy (Nikon SMZ1500), and representative images were shown.

**Recombinant Expression of CK1.8, ETO1, and Various Versions of ACS5 and In Vitro Kinase Assay**

The coding region of CK1.8 was amplified (primers CK1.8-1 and CK1.8-2) and subcloned into the pET32a vector (Novagen). The resulting construct was used to generate the kinase inactive forms His-CK1.8Δ128N and His-CK1.8Δ128N (primers CK1.8-5 to CK1.8-8) using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Stratagene).

The coding region of ETO1 was amplified (primers ETO1-1 and ETO1-5) and subcloned into the pGEX-4T-1 vector (GE Healthcare).

The coding region of ACS5 (primers ACS5-2 and ACS5-3) was amplified and subcloned into vector pET28a. The resulting construct was used to generate the point-mutated versions of ACS5 (ACS5Δ128N, ACS5ΔED, and ACS5ΔEDE, primers ACS5-7 to ACS5-12) using the QuickChange II Site-Directed Mutagenesis Kit. Primer sequences are listed in Table S2, and all constructs were confirmed by sequencing.

Proteins were expressed in *E. coli* (strain BL21) supplemented with 1 mM IPTG (28°C, 3 hr) and then purified using Ni-NTA His binding resin (Novagen) or Glutathione Sepharose (Novagen) according to the manufacturer protocols. The assay of kinase activity was performed according to previous description (Klimczak and Cashmore, 1993; Tan et al., 2013) with a few modifications.

**In Vivo Degradation Assays and Immunoblot Analysis**

Isogenic Myc-ACS5 plants were first prepared by crossing the Myc-ACS5 (in ck1.8-1) homozygous lines (as the male parent) with WT and ck1.8-1, respectively. Because Myc-ACS5 transgene harbors a Basta gene as a selectable marker, only introgressed transgenic F1 plants (Myc-ACS5 [CK1.8/ck1.8] or Myc-ACS5 [ck1.8-1]) could survive under supplemented herbicide (120 mg/l glufosinate ammonium, Sigma). Due to the low level of Myc-ACS5 in CK1.8/ck1.8 background, 4-week-old plants were first pretreated with 50 µM MG132 for 1 hr and then transferred to a new Petri dish filled with liquid MS medium containing 100 µM CHX (C7698, Sigma-Aldrich) to block the synthesis of new proteins. Approximately 200 mg of leaves was collected at different time points (0, 15, 30, 60, or 120 min) and frozen in liquid nitrogen. Proteins extracts were resuspended in 100 µl extraction buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Tween-20, 1 mM EDTA, 1 mM DTT) containing a protease inhibitor cocktail (Roche, “Complete,” 0493161001). After addition of an equal volume of 2x SDS buffer, samples were boiled for 5 min, fractionated by 10% SDS-PAGE, and transferred to a PVDF membrane by semidy blotting. The blots were incubated with a mouse anti-Myc antibody (Millipore) and then with a goat anti-mouse immunoglobulin G AP-conjugated secondary antibody (Santa Cruz Biotechnology). AP activity was detected by the BCIP/NBT kit (Invitrogen).

**Yeast Two-Hybrid and Three-Hybrid Assays**

Coding sequence of CK1.8 was amplified (primers CK1.8-1 and CK1.8-2) and subcloned into pGBK7 vector, and coding sequences of ACS5 (primers ACS5-1 and ACS5-2) and ACS5 (primers ACS5-2 and ACS5-3) were amplified and subcloned into pGADT7 vectors. The candidate interaction pairs were cotransformed into yeast strain AH109 (Clontech), and the transformed yeast cells were selected on synthetic dropout (-Leu/-Trp/-His) medium. For auxotroph assays, four individual clones were streaked on synthetic dropout (-Leu/-Trp/-His) medium and grown at 30°C for 4 days. Colonies showing continuous growth represented interactions.

Coding sequence of ETO1 was amplified (primers ETO1-1 and ETO1-2) and subcloned into pBridge vector, and CK1.8, CK1.8Δ128N, and CK1.8Δ128N were then amplified (primers CK1.8-15 and CK1.8-16) and subcloned into pBridge-E1O1 construct, respectively. Together with pGAD7-ACS5 (used for two-hybrid analysis), the candidate interaction pairs were cotransformed into yeast strain AH109, and the transformed yeast clones were selected on synthetic dropout (-Leu/-Trp/-His) medium and grown at 30°C for 4 days. Colonies showing continuous growth represented interactions.

Coding sequence of ETO1 was amplified (primers ETO1-1 and ETO1-2) and subcloned into pBridge vector, and CK1.8, CK1.8Δ128N, and CK1.8Δ128N were then amplified (primers CK1.8-15 and CK1.8-16) and subcloned into pBridge-E1O1 construct, respectively. Together with pGAD7-ACS5 (used for two-hybrid analysis), the candidate interaction pairs were cotransformed into yeast strain AH109, and the transformed yeast clones were selected on synthetic dropout (-Leu/-Trp/-His) medium and grown at 30°C for 4 days. Colonies showing continuous growth represented interactions.

**In Vitro GST Pull-Down Assay**

GST pull-down assays between GST-ETO1 and different His-ACS5 versions (ACS5, ACS5Δ128N, and ACS5ΔEDE) were performed according to previous description (Tan et al., 2013).

**Detailed experimental procedures are included in the Supplemental Experimental Procedures.**
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.047.

AUTHOR CONTRIBUTIONS

S.-T.T. performed acquisition of data as well as analysis and interpretation of data and drafted the article. H.-W.X. was responsible for conception and design as well as analysis and interpretation of data and drafted and revised the article.

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