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Phosphorylation of 1-Aminocyclopropane-1-Carboxylic Acid Synthase by MPK6, a Stress-Responsive Mitogen-Activated Protein Kinase, Induces Ethylene Biosynthesis in Arabidopsis [W]

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Mitogen-activated protein kinases (MAPKs) are implicated in regulating plant growth, development, and response to the environment. However, the underlying mechanisms are unknown because of the lack of information about their substrates. Using a conditional gain-of-function transgenic system, we demonstrated that the activation of SIPK, a tobacco (*Nicotiana tabacum*) stress-responsive MAPK, induces the biosynthesis of ethylene. Here, we report that MPK6, the *Arabidopsis thaliana* ortholog of tobacco SIPK, is required for ethylene induction in this transgenic system. Furthermore, we found that selected isoforms of 1-aminocyclopropane-1-carboxylic acid synthase (ACS), the rate-limiting enzyme of ethylene biosynthesis, are substrates of MPK6. Phosphorylation of ACS2 and ACS6 by MPK6 leads to the accumulation of ACS protein and, thus, elevated levels of cellular ACS activity and ethylene production. Expression of ACS6^{DDD}, a gain-of-function ACS6 mutant that mimics the phosphorylated form of ACS6, confers constitutive ethylene production and ethylene-induced phenotypes. Increasing numbers of stress stimuli have been shown to activate Arabidopsis MPK6 or its orthologs in other plant species. The identification of the first plant MAPK substrate in this report reveals one mechanism by which MPK6/SIPK regulates plant stress responses. Equally important, this study uncovers a signaling pathway that modulates the biosynthesis of ethylene, an important plant hormone, in plants under stress.

INTRODUCTION

Plants have sophisticated surveillance systems to sense the ever-changing environment, including various stress conditions. After the sensing step, signals generated at the receptors/sensors are converted to cellular responses through various signal transduction pathways. The very early signaling events, such as calcium influx and protein phosphorylation/dephosphorylation, occur within minutes, which are followed by the generation of several signaling compounds/phytohormones, including reactive oxygen species, nitric oxide, ethylene, jasmonic acid, and salicylic acid (Lamb and Dixon, 1997; Shinozaki and Yamaguchi-shinozaki, 1997; Dong, 1998; Scheel, 1998; Chang and Shockey, 1999; Bowler and Fluhr, 2000; McDowell and Dangl, 2000; Knight and Knight, 2001; Romeis, 2001; Zhu, 2002; Lamattina et al., 2003). Change of global gene expression in response to these primary and secondary signals eventually alters the metabolism/physiology of plants and leads to their adaptation to the new environment.

Mitogen-activated protein kinase (MAPK) cascades are major pathways downstream of sensors/receptors that transduce extracellular stimuli into intracellular responses in eukaryotes (Mizoguchi et al., 1997; Widmann et al., 1999; Davis, 2000; Chang and Karin, 2001; Innes, 2001; Tena et al., 2001; Zhang and Klessig, 2001; Jonak et al., 2002). Recent studies from several laboratories demonstrated that SIPK and WIPK, two tobacco (*Nicotiana tabacum*) MAPKs, as well as their functional orthologs in other plant species, including MPK6 and MPK3 in *Arabidopsis thaliana*, SIMK and SAMK (also named MMK1 and MMK4, respectively) in alfalfa (*Medicago sativa*), LeSIPK (also named LeMPK1/LeMPK2) and LeWIPK (also named LeMPK3) in tomato (*Lycopersicon esculentum*), and PcMPK6 and PcMPK3 (also named ERMK) in parsley (*Petroselinum crispum*), are activated in plants under various stresses, such as wounding, osmotic shock, high salinity, drought, UV irradiation, ozone, extreme temperature, oxidative stress, and pathogen infection (Mizoguchi et al., 1997; Tena et al., 2001; Zhang and Klessig, 2001; Asai et al., 2002; Jonak et al., 2002; MAPK Group, 2002; Ekengren et al., 2003; Holley et al., 2003; Kroj et al., 2003; del Pozo et al., 2004; Lee et al., 2004). Stress-induced activation of SIPK/MPK6/SIMK/LeMPKs/PcMPK6 occurs within one to several minutes, representing one of the earliest responses in plants under stress, which potentially allows these MAPKs to influence a variety of other early, intermediate, and late stress responses.

Ethylene is involved in regulating plant responses to both biotic and abiotic stresses, in addition to its functions in plant growth and development (Zarembinski and Theologis, 1994; Johnson and Ecker, 1998; Chang and Shockey, 1999; Bleecker and Kende, 2000; Schaller and Kieber, 2002; Wang et al., 2002; Hall and Bleecker, 2003). Increase in ethylene biosynthesis occurs in plants under a wide variety of stresses. The two key

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steps in ethylene biosynthesis are the conversion of *S*-adenosyl-L-Met to 1-aminocyclopropane-1-carboxylic acid (ACC) and the oxidative cleavage of ACC to form ethylene (Yang and Hoffman, 1984; Kende, 1993; Zarembinski and Theologis, 1994). The enzymes catalyzing these two reactions are ACC synthase (ACS) and ACC oxidase (ACO), respectively. Both enzymes are encoded by small gene families. In general, the basal level activity of ACS is very low in tissues that do not produce a significant amount of ethylene. Stress-induced ethylene production is associated with a rapid increase in cellular ACS activity. By contrast, ACO activity is constitutively present in most vegetative tissues. Therefore, ACS is the rate-limiting enzyme and the major regulatory step in stress-induced ethylene production (Yang and Hoffman, 1984; Kende, 1993; McKeon et al., 1995; Bleecker and Kende, 2000; Wang et al., 2002; Chae et al., 2003).

An increasing variety of stress stimuli that induce ethylene production were shown to rapidly activate SIPK in tobacco or its orthologs in other plant species (Mizoguchi et al., 1997; Tena et al., 2001; Zhang and Klessig, 2001; Asai et al., 2002; Jonak et al., 2002; MAPK Group, 2002; Holley et al., 2003). Protein phosphorylation has been implicated in regulating ethylene biosynthesis (Spanu et al., 1994; Tatsuki and Mori, 2001; Wang et al., 2002). However, the kinase(s) involved remain to be elucidated. Recently, using a dexamethasone (DEX)-inducible gain-of-function transgenic system, we demonstrated that the activation of SIPK by NtMEK2^{DD}, the active mutant of NtMEK2, resulted in a dramatic increase in ethylene production (Kim et al., 2003). The increase in ethylene biosynthesis after the activation of SIPK coincides with a dramatic increase in ACS activity. Strong activation of ethylene-response genes was observed after the ethylene production in *NtMEK2^{DD}* plants. After functionally connecting these two stress-inducible events, we set out to understand the molecular mechanism underlying this process using the Arabidopsis system because of the available mutants. Here, we report that selected isoforms of the ACS enzyme are direct targets of this plant stress-responsive MAPK pathway. Phosphorylation of ACS2/ACS6 by MPK6 stabilizes the ACS proteins in vivo, which leads to elevated levels of cellular ACS activity, ethylene production, and ethylene-induced phenotypes.

RESULTS

MPK6 Is Required for NtMEK2^{DD}-Induced Ethylene Production in Arabidopsis

We recently reported that the activation of SIPK by NtMEK2^{DD}, a constitutively active mutant of NtMEK2, induces ethylene production (Kim et al., 2003). Similar conditional gain-of-function transgenic Arabidopsis plants were generated using *NtMEK2^{DD}*, *MKK4^{DD}*, and *MKK5^{DD}* under the control of the same steroid-inducible promoter (Aoyama and Chua, 1997; Ren et al., 2002). Arabidopsis MKK4 and MKK5 are two functional orthologs of tobacco NtMEK2. We found that tobacco NtMEK2^{DD} can activate the endogenous MPK6/MPK3 in Arabidopsis, and Arabidopsis MKK4^{DD} and MKK5^{DD} can activate the endogenous SIPK/WIPK in tobacco (Ren et al., 2002) (Figure 1B). These results suggest that, over the course of evolution, tobacco NtMEK2 and Arabidopsis MKK4/MKK5 remain conserved enough to be functionally interchangeable.

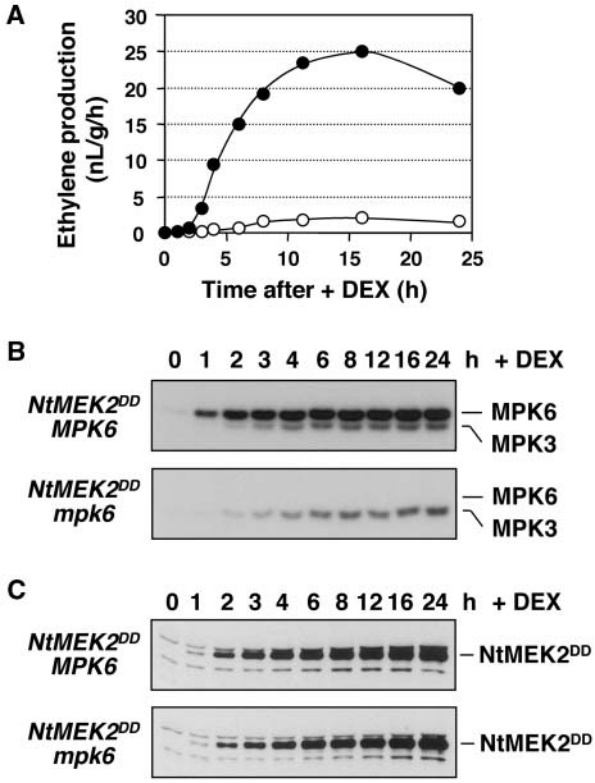

Figure 1. Endogenous MPK6 Is Required for NtMEK2^{DD}-Induced Ethylene Production in Arabidopsis.

(A) MPK6 is required for NtMEK2^{DD}-induced ethylene production. Twelve-day-old *NtMEK2^{DD}* (closed circles) and *NtMEK2^{DD}/mpk6* (open circles) seedlings grown in 50-mL gas chromatography (GC) vials were treated with DEX (2 μM). Ethylene levels in the headspace were determined at various times. The seedlings were harvested and used for analyses in **(B)** and **(C)**.

(B) Activation of endogenous MAPKs by NtMEK2^{DD} in wild-type and *mpk6* mutant Arabidopsis. Proteins were extracted from *NtMEK2^{DD}* and *NtMEK2^{DD}/mpk6* seedlings treated with DEX for various times. In-gel kinase assays were performed using myelin basic protein (MBP) as a substrate.

(C) NtMEK2^{DD} expression after DEX treatment is similar in wild-type and *mpk6* mutant backgrounds. Flag-tagged NtMEK2^{DD} in the protein extracts was detected by immunoblot analysis using anti-Flag antibody.

Induction of *NtMEK2^{DD}*, *MKK4^{DD}*, or *MKK5^{DD}* expression by the application of DEX elevated the rates of ethylene biosynthesis in Arabidopsis (Figure 1A; data not shown). This event was preceded by the activation of MPK6 and MPK3 (Figure 1B, top panel). To determine if the endogenous MAPK is required for the ethylene induction, we crossed the steroid-inducible promoter:*NtMEK2^{DD}* transgene into *mpk6* T-DNA insertion mutants. We used *NtMEK2^{DD}* transgenic Arabidopsis in this study because *MKK4^{DD}* and *MKK5^{DD}* transgenic lines that we generated are not as stable because of gene silencing. Some *MKK4^{DD}* and *MKK5^{DD}* plants lost their transgene inducibility as they were propagated through the generations. This prohibited the use of

these lines in the genetic analysis. By contrast, the *NtMEK2^{DD}* line is very stable.

Three *mpk6* mutant alleles were used for crosses. *mpk6-1* was identified from the BASTA population at the Wisconsin Arabidopsis Knockout Facility and is in Wassilewskija-0 (Ws-0) background (Sussman et al., 2000). *mpk6-2* and *mpk6-3* are Salk lines in Col-0 background (Alonso et al., 2003). In *NtMEK2^{DD}/mpk6-1* plants, the production of ethylene after DEX application was greatly reduced (Figure 1A). Similar results were obtained in *NtMEK2^{DD}/mpk6-2* and *NtMEK2^{DD}/mpk6-3* plants (data not shown). In these plants, no MPK6 activity was detectable, although MPK3 was activated normally (Figure 1B, bottom panel). The minor amount of ethylene production seen in the *NtMEK2^{DD}/mpk6* plants is likely a result of MPK3 activation. Nonetheless, the major reduction (>90%) in the ethylene induction in *NtMEK2^{DD}/mpk6* plants suggests that MPK3 plays a less important role in the process. Immunoblot analysis showed comparable Flag-tagged NtMEK2^{DD} induction in both plants (Figure 1C). These results demonstrate that MPK6 is required for the NtMEK2^{DD}-induced ethylene production in Arabidopsis.

ACS6 Is Involved in MPK6-Induced Ethylene Production

The addition of aminooxyacetic acid (AOA), an ACS inhibitor, or Co^{2+}, an ACO inhibitor (Abeles et al., 1992), blocked ethylene production in *NtMEK2^{DD}* transgenic Arabidopsis after DEX treatment (data not shown), suggesting that both ACS and ACO activities are required for the MPK6-induced ethylene production. The very rapid induction of ethylene after MPK6 activation is associated with the increase in ACS activity (Figure 2A), similar to that observed in *NtMEK2^{DD}* transgenic tobacco (Kim et al., 2003). In the control *NtMEK2^{KR}* transgenic Arabidopsis plants, no increase in ACS activity was observed (Figure 2A). NtMEK2^{KR} is an inactive mutant of NtMEK2 with the catalytically essential Lys in the kinase domain mutated to Arg. Previously we demonstrated that the induction of NtMEK2^{KR} expression does not activate MPK6 and MPK3 in Arabidopsis (Ren et al., 2002). In contrast with the induction of ACS activity, ACO activity stayed high before and after DEX treatment as determined by an in vivo ACO activity assay (Figure 2B). These results suggest that ACS is the rate-limiting enzyme in the process, and the activity of one or more ACS isozymes are induced after MPK6 activation.

ACS6 was implicated in stress-induced ethylene production based on its transcriptional activation by stresses (Vahala et al., 1998; Wang et al., 2002) (data not shown). To find out if ACS6 is involved, we used an immune complex ACS assay in which the ACS6 protein was first immunoprecipitated from the total protein extract using an ACS6-specific antibody, and the ACS activity in the immune complex was then determined. Among the three recombinant ACS proteins (ACS2, ACS5, and ACS6) that we tested, this antibody only recognized ACS6 as determined by immunoblot analysis (Figure 3A). As shown in Figure 3B, high ACS6 activity was detected in *NtMEK2^{DD}* plants after DEX treatment but not before DEX treatment. Addition of the peptide to which the antibody was raised blocked the immunoprecipitation of ACS6 activity, demonstrating the specificity of the assay. Wild-type Arabidopsis (Col-0) seedlings treated with Flg22,

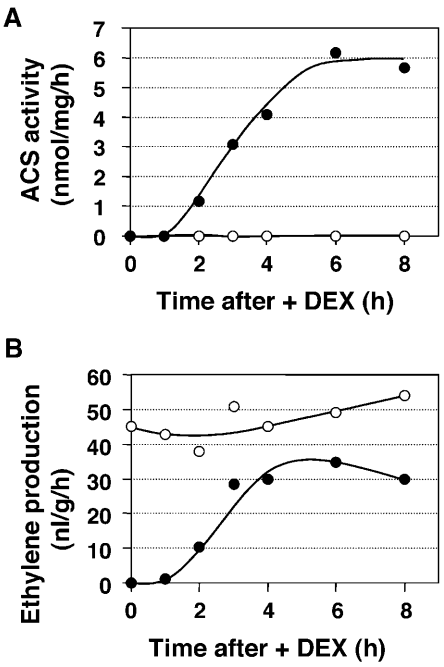

Figure 2. MPK6-Induced Ethylene Production in *NtMEK2^{DD}* Transgenic Arabidopsis Is Associated with the Increase in ACS Activity.

(A) Induction of ACS activity in *NtMEK2^{DD}* transgenic plants after DEX treatment. ACS activity in total protein extracts from *NtMEK2^{DD}* (closed circles) and *NtMEK2^{KR}* negative control (open circles) plants was determined at various times after DEX treatment. NtMEK2^{KR} is an inactive mutant of NtMEK2 with the catalytically essential Lys in the kinase domain mutated to Arg. The induction of NtMEK2^{KR} expression does not activate downstream MPK6 and MPK3 (Ren et al., 2002).

(B) ACO activity is not the limiting step in the MAPK-induced ethylene biosynthesis as determined by an in vivo ACO activity assay. Two sets of *NtMEK2^{DD}* seedlings grown in 50-mL GC vials were treated with DEX (2 μM). At various times, ACC (1 mM final concentration) was added to one set of the vials (open circles), and the other set received no ACC and was used as controls (closed circles). The GC vials were flushed and then capped. Ethylene levels in the GC vial were determined 1.5 h later. Similar levels of ethylene production in the presence of ACC before (0 time point) and after DEX treatment indicate constitutively high ACO activity. No ethylene generation was detected in vials with only medium plus ACC. In addition, ethylene production stopped in ACC-treated vials after the seedlings were removed, demonstrating that the ethylene production is a result of ACO activity in the seedlings.

a peptide elicitor derived from bacterial flagellin that activates MPK6 (Gómez-Gómez et al., 1999; Nühse et al., 2000), also showed elevated levels of ACS6 activity (Figure 3D). In the *mpk6* mutant background, both NtMEK2^{DD}- and Flg22-induced elevation of ACS6 activity was abolished (Figures 3C and 3D), providing genetic evidence that MPK6 is required for the induction of ACS6 activity.

Phosphorylation of ACS2 and ACS6 by MPK6

To determine if the increase in ACS6 activity is a result of *ACS6* gene activation, we generated transgenic plants that

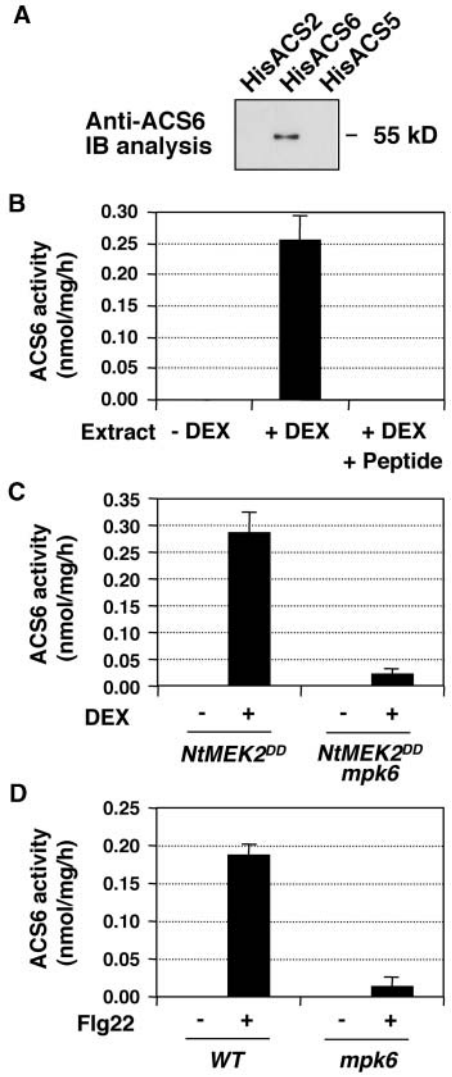

Figure 3. Elevated ACS6 Activity in Col-0 Seedlings after Flg22 Treatment or *NtMEK2^{DD}* Seedlings after DEX Treatment Requires MPK6.

(A) Anti-ACS6 raised against the C-terminal peptide of ACS6 specifically recognized the ACS6 protein. Five nanograms each of recombinant HisACS2, HisACS6, and HisACS5 were subjected to immunoblot (IB) analysis with anti-ACS6. After incubation with a horseradish peroxidase–conjugated secondary antibody, the complex was visualized.

(B) Increase in ACS6 activity after MPK6 activation in *NtMEK2^{DD}* plants. ACS6 activity in *NtMEK2^{DD}* transgenic plants before and after DEX treatment (final concentration of 2 μM for 6 h) was determined by the immune complex ACS assay using an ACS6-specific antibody. The specificity of the assay was assessed by the addition of peptide (6 μg/mL), to which the antibody was raised. Error bars indicate standard deviation ($n = 3$).

(C) *MPK6* is required for the induction of ACS6 activity in *NtMEK2^{DD}* plants after DEX treatment. Total protein extracts were prepared from *NtMEK2^{DD}* and *NtMEK2^{DD}/mpk6* seedlings without DEX treatment (−) or treated with DEX for 6 h (+). ACS6 activity was determined by the immune complex ACS assay using an ACS6-specific antibody.

(D) *MPK6* is required for the induction of ACS6 activity in wild-type seedlings treated with Flg22. Total protein extracts were prepared from wild-type and *mpk6* mutant seedlings without Flg22 treatment (−) or treated with 0.2 μM of Flg22 for 1 h (+). ACS6 activity was determined by the immune complex ACS assay using ACS6-specific antibody.

overexpress *ACS6*. To our surprise, these plants did not have higher levels of ACS activity and did not overproduce ethylene (data not shown; see Figure 5), suggesting that an additional level of regulation, possibly at the posttranslational level, is involved. A closer examination of all Arabidopsis ACS sequences revealed potential MAPK phosphorylation sites in ACS1, ACS2, and ACS6, which form a unique clade in the ACS phylogenetic tree (Figure 4A) (Chae et al., 2003; Yamagami et al., 2003). These phosphorylation sites are conserved in ACSs from other plant species, inferring a functional importance (see Supplemental Figure 1 online).

Recombinant His-tagged ACS2 and ACS6 were prepared along with ACS5, which was used as a negative control. We did not examine ACS1 because it is a nonfunctional ACS (Liang et al., 1995; Yamagami et al., 2003). Activated recombinant MPK6 phosphorylated both ACS2 and ACS6 but not ACS5 (Figure 4B). To determine if the native MPK6 from protein extracts can phosphorylate ACS6, we performed the in-gel kinase assay using the recombinant ACS6 as an embedded substrate. As shown in Figure 4C, activated endogenous MPK6 from *NtMEK2^{DD}* plants after DEX treatment or nontransgenic plants after Flg22 or wounding treatment could phosphorylate ACS6. In the *mpk6* knockout background, no phosphorylation was detected, demonstrating that MPK6 is the kinase responsible for the ACS6 phosphorylation. Recombinant ACS2 is much more difficult to purify because it is poorly expressed in *Escherichia coli*. As a result, we did not test ACS2 using the in-gel kinase assay.

MAPKs are Pro-directed protein kinases (Cohen, 1997). Four potential phosphorylation sites (S400, S480, S483, and S488) were identified in ACS6 where Ser residues are followed by Pro (Figure 4A). We generated recombinant ACS6 proteins with single, double, and triple Ser (S) to Ala (A) or Asp (D) mutations. The ability of these proteins to serve as a MPK6 substrate was determined by phosphorylation assays. As shown in Figure 4D, MPK6 could not phosphorylate the S480A/S483A/S488A triple mutant, whereas various single and double mutants could still be phosphorylated. The levels of phosphorylation were proportional to the number of phosphorylation sites (Figure 4D, bottom panel), indicating that phosphorylation of S480, S483, and S488 is independent of each other. S400, which is conserved in most ACSs, is not a phosphorylation site for MPK6. Mutation of all three corresponding Ser residues in ACS2 (Figure 4A) also abolished the MPK6 phosphorylation (data not shown).

Gain-of-Function *ACS6^{DDD}* Transgenic Plants Overproduce Ethylene

Phosphorylated ACS6 has similar V_{max} (390 μmol/h/mg) and K_m for its substrate *S*-adenosyl-L-Met (49 μM) as the unphosphorylated ACS6 (V_{max}, 332 μmol/h/mg; K_m, 42 μM). Mutant ACS proteins shown in Figure 4D have similar specific activities as the

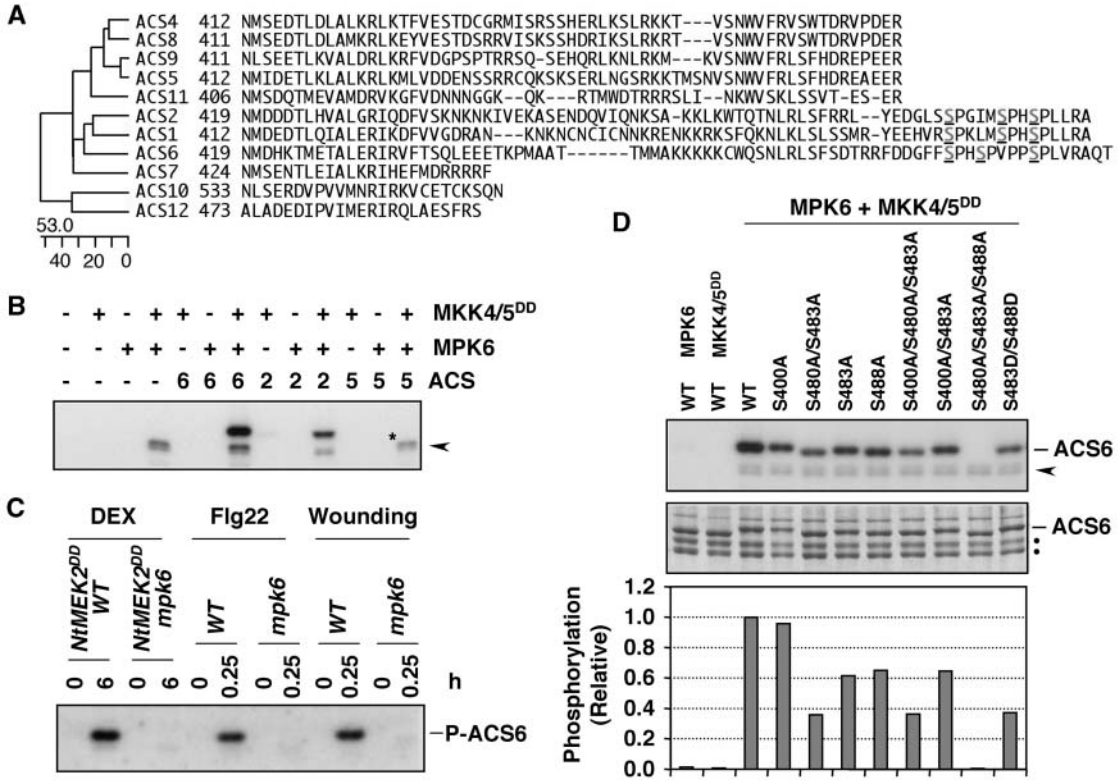

Figure 4. Phosphorylation of ACS2 and ACS6 by MPK6.

(A) One group of Arabidopsis ACS isoforms represented by ACS1, ACS2, and ACS6 contains conserved MAPK phosphorylation sites (underlined) at their C termini.

(B) Phosphorylation of recombinant ACS2 and ACS6 by MPK6 in vitro. Recombinant MPK6 was first activated as described in Methods. Active MPK6 was then used to phosphorylate His-tagged ACS2, ACS5, and ACS6 in the presence of [γ-^{32}P]ATP. After electrophoresis, the phosphorylated proteins were visualized by autoradiography. Reactions with various components omitted (−) were used as controls. The lower band (indicated by an arrowhead) was a result of phosphorylation of MPK6 by MKK4^{DD}/MKK5^{DD}. The asterisk indicates the position of ACS5 protein.

(C) Phosphorylation of ACS6 by native MPK6. ACS6 phosphorylation activity in extracts from *NtMEK2^{DD}* Arabidopsis after DEX treatment (2 μM for 6 h) or nontransgenic plants after Flg22 (0.2 μM for 0.25 h) or wounding treatment (0.25 h) in both wild-type and *mpk6* mutant backgrounds was determined by an in-gel kinase assay with recombinant ACS6 as the embedded substrate.

(D) S480, S483, and S488 at the C terminus of ACS6 are three independent MPK6 phosphorylation sites. Phosphorylation of ACS6 proteins with single, double, and triple mutations by MPK6 was performed as in **(B)**. The levels of phosphorylation were quantitated using a Fuji Film FLA-5000 imaging system (top). The lower band (indicated by an arrowhead) was the phosphorylated MPK6 as in **(B)**. After being normalized to the amount of proteins as determined by Coomassie blue staining (middle), the relative levels of phosphorylation were plotted (bottom). Dots indicate the partially degraded ACS6 with the phosphorylation sites missing.

wild-type protein as well, suggesting that phosphorylation of ACS6 by MPK6 does not alter its enzymatic activity. As a result, we speculated that the phosphorylation of ACS6 and ACS2 by MPK6 might change their stability in vivo, similar to members in the ACS group represented by ACS5 and ACS9 (Chae et al., 2003; Wang et al., 2004). To test this, we transformed both wild-type and *NtMEK2^{DD}* Arabidopsis with Flag-tagged *ACS6* and its mutants, *ACS6^{AAA}* (S480A/S483A/S488A) and *ACS6^{DDD}* (S480D/S483D/S488D) (Figure 5A). With all three phosphorylation sites missing, ACS6^{AAA} may lose the regulation by MPK6 in vivo. By contrast, with the negative charge on Asp, ACS6^{DDD} may behave like a phosphorylated ACS6 and lead to the constitutive overproduction of ethylene in *ACS6^{DDD}* transgenic plants. To avoid the difference in the levels of transgene expression as a result of positioning effects, we used pooled T1 seedlings for the experiments so that we can compare the stability of the three forms of ACS6 directly.

Ethylene production rates in *ACS6^{WT}* and *ACS6^{AAA}* transgenic seedlings were very low, similar to that in the vector control. By contrast, *ACS6^{DDD}* plants produced ~40 times more ethylene (Figure 5B). Transgene expression in pooled *ACS6^{WT}*, *ACS6^{AAA}*, and *ACS6^{DDD}* seedlings was comparable, which was ~25-fold of that found in the vector control (Figure 5C). Immune complex ACS assays using anti-Flag antibody revealed significant accumulation of ACS activity in the pooled *ACS6^{DDD}* transgenic seedlings, which correlated with the accumulation of ACS6^{DDD} protein (Figure 6A). By contrast, Flag-tagged ACS6^{WT} and ACS6^{AAA} were not detected in untreated seedlings (Figure 6A). These results demonstrate that by replacing Ser residues with Asp, which mimics the phosphorylated form of ACS6, ACS6^{DDD} protein becomes more stable,

resulting in elevated cellular ACS activity and ethylene biosynthesis. This phenotype was also observed in T2 and T3 *ACS6*^{DDD} transgenic plants (data not shown).

MPK6 Phosphorylation Sites at the C Terminus of ACS6 Are Required for MPK6-Induced ACS6 Accumulation in Arabidopsis

To determine if S480/S483/S488 are essential for MPK6 phosphorylation-dependent protein accumulation, we treated pooled

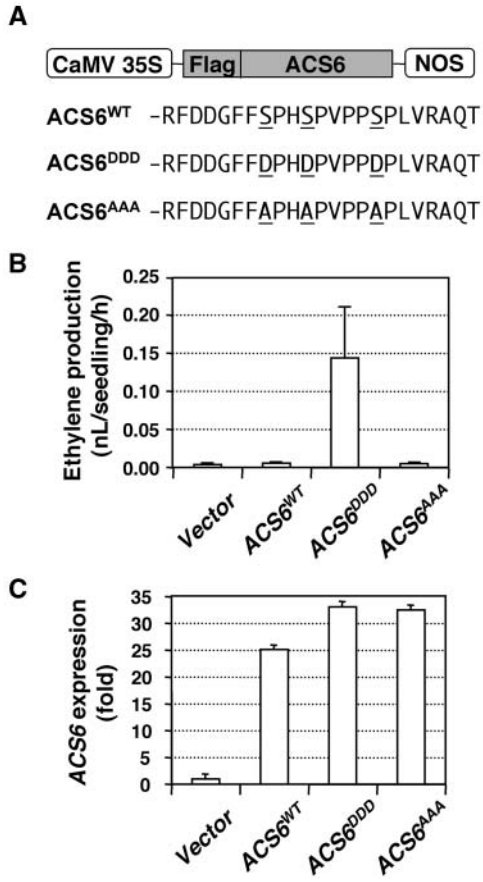

Figure 5. Gain-of-Function *ACS6*^{DDD} Transgenic Seedlings Constitutively Overproduce Ethylene.

(A) *ACS6* constructs used for transformation. The C-terminal sequences of ACS6 and its mutants are shown. MPK6 phosphorylation sites and mutated amino acid residues are underlined. CaMV 35S, 35S promoter of *Cauliflower mosaic virus*.

(B) Gain-of-function *ACS6*^{DDD} transgenic seedlings constitutively overproduce ethylene. After selection on kanamycin plates, T1 transgenic seedlings were transferred to 50-mL GC vials with 6 mL of medium (20 seedlings/vial). When the seedlings were 12 d old, the GC vials were flushed and capped. Ethylene levels in the headspace were determined 24 h later. Error bars indicate standard deviation ($n = 6$).

(C) Transgene expression in pooled T1 *ACS6*^{WT}, *ACS6*^{DDD}, and *ACS6*^{AAA} seedlings is similar. The levels of *ACS6* mRNA (endogenous plus transgene) in pooled seedlings (>100) from **(A)** were determined by quantitative RT-PCR, which were normalized to that in vector control transgenic seedlings. Error bars indicate standard deviation ($n = 3$).

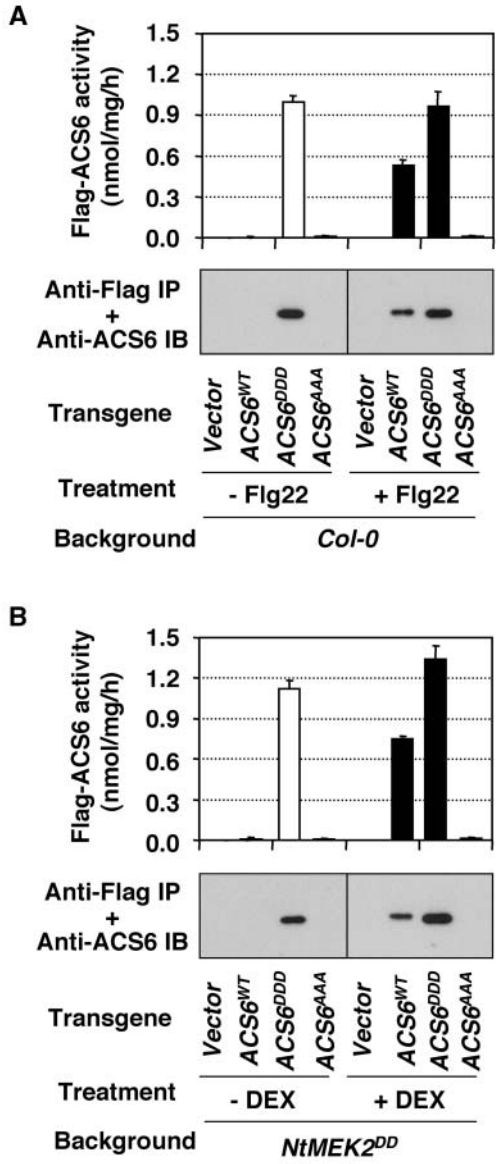

Figure 6. Phosphorylation of ACS6 by MPK6 Results in the Accumulation of ACS6 Protein and the Increase in Cellular ACS Activity.

(A) Elevated levels of ACS6 activity and protein in *ACS6*^{DDD} plants and *ACS6*^{WT} plants after Flg22 treatment. The activity of Flag-tagged ACS6 and its mutants in protein extracts prepared from pooled seedlings before and after Flg22 treatment was determined by the anti-Flag immune complex ACS assay. Error bars indicate standard deviation ($n = 3$). ACS6 protein was detected by coupled immunoprecipitation and immunoblot analysis. Anti-Flag antibody and anti-ACS6 antibody were used for immunoprecipitation and immunoblot analysis, respectively.

(B) Elevated levels of ACS6 activity and protein in *ACS6*^{DDD} plants and *ACS6*^{WT} plants in *NtMEK2*^{DD} transgenic background before and after DEX treatment. The activity of Flag-tagged ACS6 and its mutants in protein extracts prepared from pooled seedlings in *NtMEK2*^{DD} transgenic background before and after DEX treatment was determined by immune complex ACS assay using anti-Flag antibody. Error bars indicate standard deviation ($n = 3$). ACS6 protein was detected by coupled immunoprecipitation and immunoblot analysis as in **(A)**.

transgenic seedlings in *NtMEK2*^{DD} background with DEX. Immune complex ACS assays using anti-Flag antibody revealed enhanced levels of Flag-tagged ACS activity in *ACS6*^{WT} seedlings (Figure 6B). By contrast, ACS activity from the Flag-tagged *ACS6*^{AAA} transgenic seedlings remained undetectable. The enhanced ACS activity in *ACS6*^{WT} seedlings after MPK6 activation correlated with the accumulation of ACS6^{WT} protein, whereas ACS6^{AAA} failed to accumulate (Figure 6B). Treatment of *ACS6*^{WT} transgenic seedlings with Flg22, which activates MPK6 (Nühse et al., 2000; Asai et al., 2002) (see Figure 10B), led to the accumulation of ACS6^{WT} protein as well (Figure 6A). By contrast, we never detected ACS6^{AAA} protein.

ACS6^{WT} protein immunoprecipitated from *ACS6*^{WT} seedlings after treatment showed a slight upshift in size (Figure 6). This phenomenon is associated with the phosphorylation of ACS6 by MPK6 in vitro (Figure 4D), indicating that ACS6^{WT} detected after MPK6 activation may be in a phosphorylated form. Consistent with this, alkaline phosphatase treatment of ACS6^{WT} protein immunoprecipitated from *ACS6*^{WT} transgenic seedlings reversed the band upshift (Figure 7), confirming that the upshift of ACS6^{WT} protein is indeed caused by phosphorylation. Taken together, these results demonstrate that phosphorylation of S480/S483/S488 by MPK6 is essential for the accumulation of ACS6 in vivo.

Gain-of-Function *ACS6*^{DDD} Transgenic Plants Show Ethylene-Induced Phenotypes

Gain-of-function *ACS6*^{DDD} transgenic Arabidopsis showed several ethylene-induced phenotypes. More than 90% of the *ACS6*^{DDD} T1 seedlings had hairy roots (Figure 8A). Elongation of the main root was greatly inhibited (Figure 8B). Soil-grown plants showed varying degrees of dwarfism, similar to the constitutive ethylene-response mutant, *constitutive-triple-response1 (ctr1)* (Figure 8C) (Kieber et al., 1993; Hall and Bleecker, 2003). These phenotypes are associated with the constitutive production of ethylene in the *ACS6*^{DDD} plants. All of these phenotypes are heritable. Similar results were obtained with *ACS2*^{WT}, *ACS2*^{DDD}, and *ACS2*^{AAA} transgenic plants (data not shown), suggesting that ACS2 is regulated through a similar mechanism.

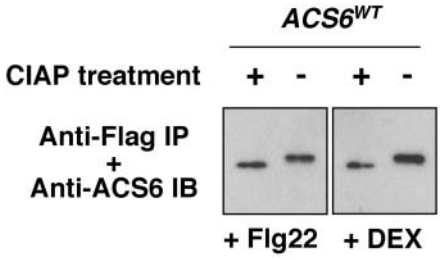

Figure 7. Phosphatase Treatment Reverses the Upshift of ACS6^{WT} Protein from Seedlings after MPK6 Activation.

Flag-tagged ACS6^{WT} protein was immunoprecipitated from *ACS6*^{WT} transgenic seedlings treated with DEX (in *NtMEK2*^{DD} background) or Flg22 (in Col-0 background). Half of the immune complex was treated with calf intestinal alkaline phosphatase (CIAP), which was then resolved in a 10% SDS-polyacryamide gel along with the untreated portion. The ACS6^{WT} protein was detected by immunoblot analysis using an ACS6-specific antibody.

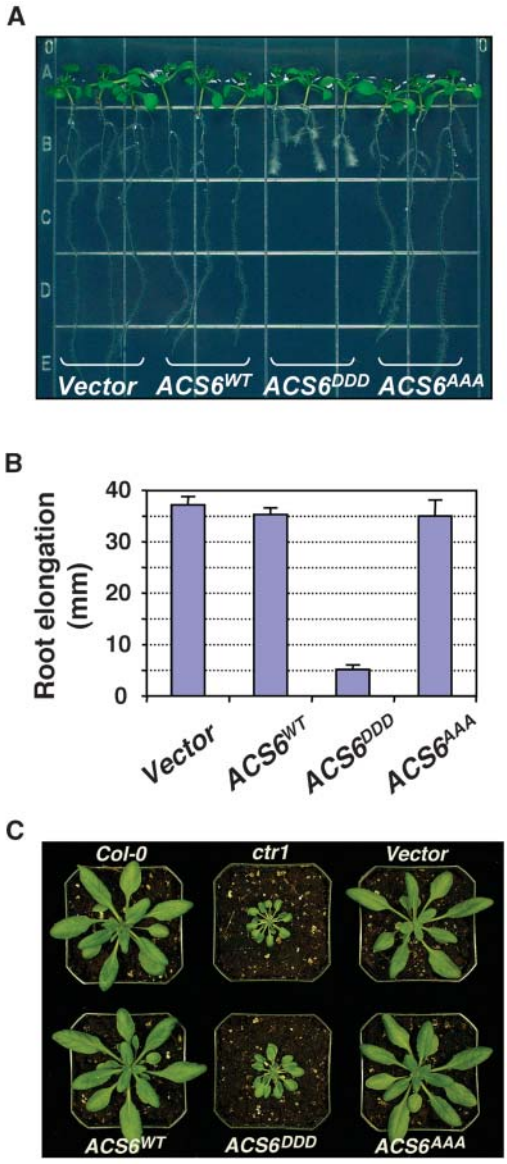

Figure 8. Ethylene-Induced Morphology in Gain-of-Function *ACS6*^{DDD} Transgenic Plants.

(A) Hairy root phenotype of *ACS6*^{DDD} transgenic seedlings.
(B) Reduced root elongation of *ACS6*^{DDD} transgenic seedlings. Error bars indicate standard deviation ($n = 12$).
(C) Soil-grown *ACS6*^{DDD} transgenic plants showed a *ctr1*-like phenotype.

ACS2 and ACS6 Are Required for High-Level Ethylene Induction after MPK6 Activation

To provide genetic evidence that ACS2 and ACS6 function downstream of MPK6, we crossed the *NtMEK2*^{DD} transgene into *acs2* and *acs6* knockout mutants. The induction of ethylene by

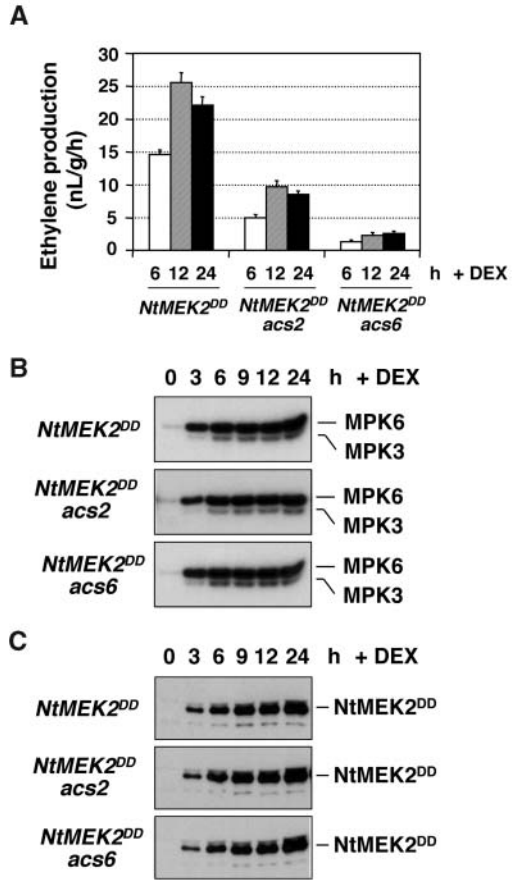

Figure 9. *ACS2* and *ACS6* Are Required for High-Level Induction of Ethylene Biosynthesis in *NtMEK2^{DD}* Plants.

(A) Ethylene production in *NtMEK2^{DD}*, *NtMEK2^{DD}/acs2*, and *NtMEK2^{DD}/acs6* seedlings after DEX treatment. Twelve-day-old homozygous F3 seedlings grown in 50-mL GC vials were treated with DEX (2 μM). Ethylene levels in the headspace were determined at various times.
(B) MAPK activation in *NtMEK2^{DD}*, *NtMEK2^{DD}/acs2*, and *NtMEK2^{DD}/acs6* seedlings after DEX treatment as determined by in-gel kinase assay using MBP as a substrate.
(C) NtMEK2^{DD} induction in *NtMEK2^{DD}*, *NtMEK2^{DD}/acs2*, and *NtMEK2^{DD}/acs6* seedlings as determined by immunoblot analysis.

NtMEK2^{DD} in the F3 homozygous seedlings was greatly compromised when either *ACS* gene is mutated (Figure 9A). In-gel kinase assays and immunoblot analyses revealed comparable levels of NtMEK2^{DD} induction and MPK6/MPK3 activation (Figures 9B and 9C). The major reduction of ethylene induction in *NtMEK2^{DD}/acs2* and *NtMEK2^{DD}/acs6* plants after DEX treatment provided genetic evidence that these two ACS isoforms are involved in the MPK6-induced ethylene production. Apparently, ACS6 plays a more important role than ACS2 in the induction of ethylene biosynthesis after MPK6 activation. In *NtMEK2^{DD}/acs2* plants, the ethylene production is ~40% of that in *NtMEK2^{DD}* plants, whereas in *NtMEK2^{DD}/acs6* plants, the ethylene production is only ~11% of that in *NtMEK2^{DD}* plants. Because the reduction of ethylene biosynthesis in both *NtMEK2^{DD}/acs2* and *NtMEK2^{DD}/acs6* plants is >50%, we conclude that ACS2 and ACS6 act synergistically in the process. The molecular mechanism underlying this phenomenon is currently unknown. One possibility is that the heterodimer of ACS2 and ACS6 has a higher activity in vivo (Tsuchisaka and Theologis, 2004).

An Additional Pathway Is Involved in the Induction of Ethylene in Plants under Stress

To determine if the MPK6 cascade is the only pathway that regulates the ethylene induction in plants under stress, we examined the ethylene production in *mpk6* mutant seedlings treated with Flg22. As shown in Figure 10A, we found that ethylene production induced by Flg22 was only partially blocked (~50%) in the *mpk6* mutant. The smaller reduction in ethylene induction in *mpk6* plants treated with Flg22 (~50%) in comparison to that in *NtMEK2^{DD}/mpk6* plants treated with DEX (>90%) suggests the involvement of additional pathway(s) besides the MPK6 cascade in Flg22-induced ethylene production. This MPK6-independent pathway is likely to go through ACS isozymes other than ACS6 because, in the *mpk6* mutant, the Flg22-induced ACS6 activity is blocked (Figure 3C).

In *mpk6* mutant seedlings treated with Flg22, the activation of the 48-kD MPK6 was absent, whereas the activation of two other kinases, MPK3 and a 40-kD kinase, was not affected (Figure 10B). The identity of the 40-kD kinase is currently unknown. In

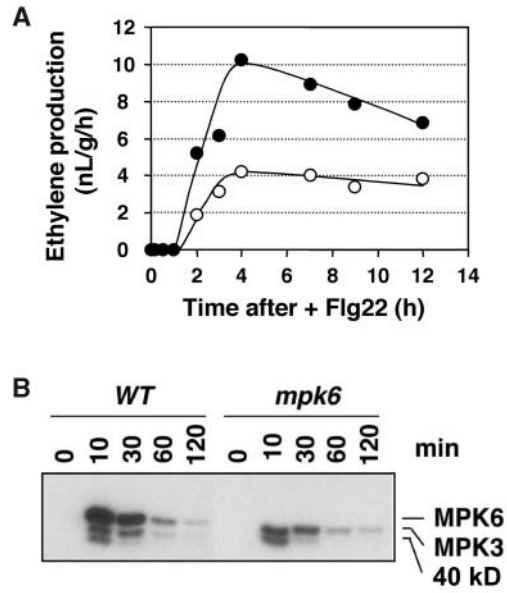

Figure 10. Mutation in *MPK6* Only Partially Blocks Flg22-Induced Ethylene Production.

(A) Two-week-old wild-type (closed circles) and *mpk6* (open circles) seedlings grown in 50-mL GC vials were treated with Flg22 (0.2 μM). Ethylene levels in the headspace were determined at various times.
(B) MAPK activation in wild-type and *mpk6* mutant seedlings treated with Flg22. Two-week-old wild-type and *mpk6* seedlings grown in 50-mL GC vials were treated with Flg22 (0.2 μM), and samples were taken at various times. MAPK activity in the total protein extracts was determined by in-gel kinase assay using MBP as a substrate.

MKK4^{DD}, *MKK5*^{DD}, and *NtMEK2*^{DD} Arabidopsis, DEX treatment does not activate this 40-kD kinase (Figure 1) (Ren et al., 2002), suggesting that it is not a downstream component of the same MAPKK as the MPK6 and MPK3. At this stage, it is unknown whether this 40-kD kinase is involved in the MPK6-independent ethylene induction in Arabidopsis treated with Flg22.

ACC Treatment Does Not Activate MPK6 in Arabidopsis Seedlings

It was reported recently that MPK6 functions downstream of CTR1 in the ethylene signaling pathway, and *ctr1* mutant plants have constitutively high MPK6 activation (Ouaked et al., 2003). This would be an unprecedented MAPK cascade because loss-of-function of the upstream MAPKKK should result in the loss of MPK6 activation rather than constitutive activation of MPK6. In light of our findings in this report, we tested whether ACC treatment can indeed activate MPK6 in Arabidopsis seedlings. As shown in Figure 11, ACC treatment failed to activate MPK6 in Col-0 and various ethylene mutant seedlings, although high levels of ethylene were produced after ACC feeding. In addition, we did not observe higher basal levels of MPK6 activity in *ctr1*, a constitutive ethylene response mutant, or *ethylene-overproducing 1* (*eto1*), a mutant that produces elevated levels of ethylene. Based on these results, we conclude that ethylene cannot activate MPK6 in Arabidopsis seedlings, and MPK6 does not function downstream of CTR1 in the ethylene signaling pathway.

ACC is a weak acid. Our initial ACC treatment experiments were performed using ACC stock solutions with an adjusted pH of 5.8, which is the same as the pH of the medium for growing Arabidopsis seedlings. It was documented that some weak acids can disturb the cellular pH and cause the activation of MAPK (Tena and Renaudin, 1998). As a result, we also performed the experiments using ACC stock solutions with an unadjusted pH. Again, we did not see the activation of MPK6 after the seedlings were fed with ACC (Figure 11A, bottom panels). We consistently observed lower ethylene production (lower in vivo ACO activity) in ethylene insensitive mutants and higher ethylene production (higher in vivo ACO activity) in *ctr1* and *eto1* mutants after the addition of ACC (Figure 11B). Without exogenous ACC, none of the genotypes produced detectable levels of ethylene within 1 h. Within 24 h, *eto1* seedlings produced ~10 times more ethylene than Col-0 seedlings.

DISCUSSION

Ethylene, a gaseous plant hormone, plays important roles in regulating plant growth, development, and responses to the environment (Abeles et al., 1992; Kende, 1993; Zarembinski and Theologis, 1994; Chang and Shockey, 1999; Bleecker and Kende, 2000; Schaller and Kieber, 2002; Wang et al., 2002). Ethylene-regulated processes are initiated by an increase in ethylene biosynthesis, which is under the control of environmental and endogenous cues. In comparison with the major advances in our understanding of the ethylene biosynthetic pathway and signaling components downstream of ethylene, relatively little is known about the signal transduction pathways that modulate ethylene biosynthesis in plants (Kende, 2001).

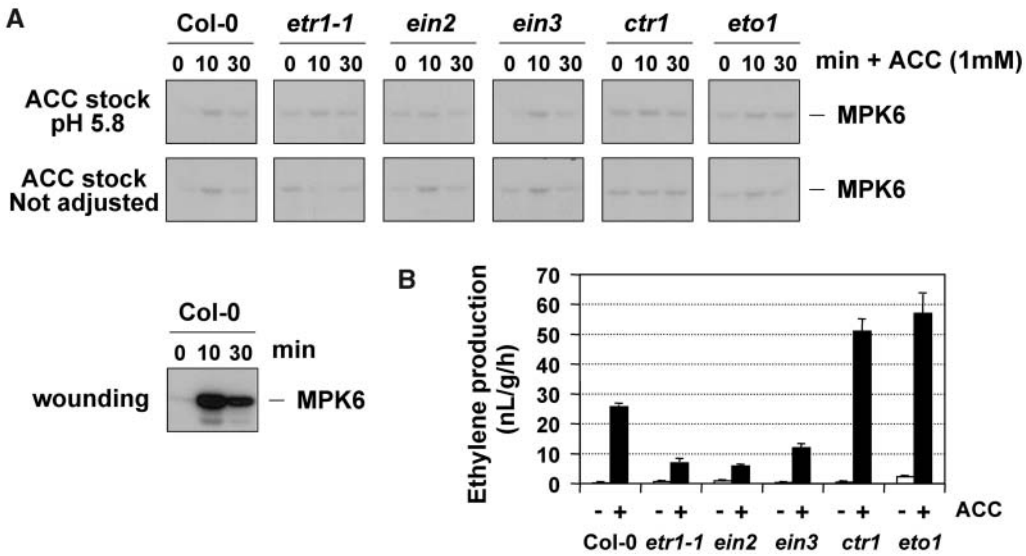

Figure 11. No Significant MPK6 Activation Was Observed in Arabidopsis Seedlings Treated with ACC.

(A) Two-week-old wild-type and mutant seedlings grown in 50-mL GC vials were treated with ACC (1 mM final concentration). The pH of ACC stock solutions was either adjusted to 5.8 or unadjusted. Samples were taken before the addition of ACC (0 min) and 10 and 30 min after the addition of ACC. Wounding treated samples were used as positive controls. MAPK activity in the total protein extracts was determined by in-gel kinase assay using MBP as a substrate.

(B) Ethylene production in Arabidopsis seedlings in the absence and presence of exogenous ACC. Two-week-old Arabidopsis seedlings grown in 50-mL GC vials were treated with 1 mM ACC (+) or an equal volume of water (−) as controls. The vials were flushed and then capped. Ethylene levels in the headspace were determined after 1 h for vials received ACC or after 24 h for vials received no ACC. Error bars indicate standard deviation ($n = 3$).

MAPK cascades are known to function downstream of the sensors/receptors in eukaryotic cells. Recently, we demonstrated the involvement of tobacco SIPK in the regulation of ethylene biosynthesis (Kim et al., 2003). In this report, we reveal how the activation of this stress-responsive MAPK cascade induces ethylene biosynthesis (Figure 12). The identification of ACS2/ACS6 as the substrates of MPK6 links this MAPK cascade directly to the induction of ethylene biosynthesis in plants under stress.

Stress is known to activate multiple signaling pathways. The gain-of-function *NtMEK2^{DD}* transgenic system used in this study allowed us to look at the responses regulated by this MAPK pathway specifically. In this system, the *NtMEK2^{DD}* transgene is under the control of an inducible promoter, which allows us to mimic the activation of this MAPK cascade by exogenous stimulus. More importantly, it provides a timeline for us to follow after the activation of MPK6/MPK3. Using *NtMEK2^{DD}/mpk6* plants, we demonstrated that ethylene induction by NtMEK2^{DD} requires the endogenous MPK6 (Figure 1). Results from this cross also suggest that MPK3 plays a less important role in the induction of ethylene in this gain-of-function system.

It is known that different stresses induce different levels of ethylene with different kinetics. This is also the case for stress-induced MPK6/SIPK activation. For instance, wounding of leaf tissue only induces a transient activation of SIPK/MPK6, which correlates with a transient ethylene production (Abeles et al., 1992; Zhang and Klessig, 1998b; Tatsuki and Mori, 1999) (data not shown). By contrast, gene-for-gene interactions or elicitors that induce hypersensitive cell death induce a long-lasting activation of SIPK/MPK6, which correlates with a longer-lasting ethylene induction (de Laat and van Loon, 1982, 1983; Abeles et al., 1992; Zhang and Klessig, 1998a; Zhang et al., 1998). In *NtMEK2^{DD}* transgenic plants, the application of DEX induces a long-lasting activation of SIPK/MPK6, which is associated with a long-lasting induction of ethylene biosynthesis (Figure 1) (Kim et al., 2003).

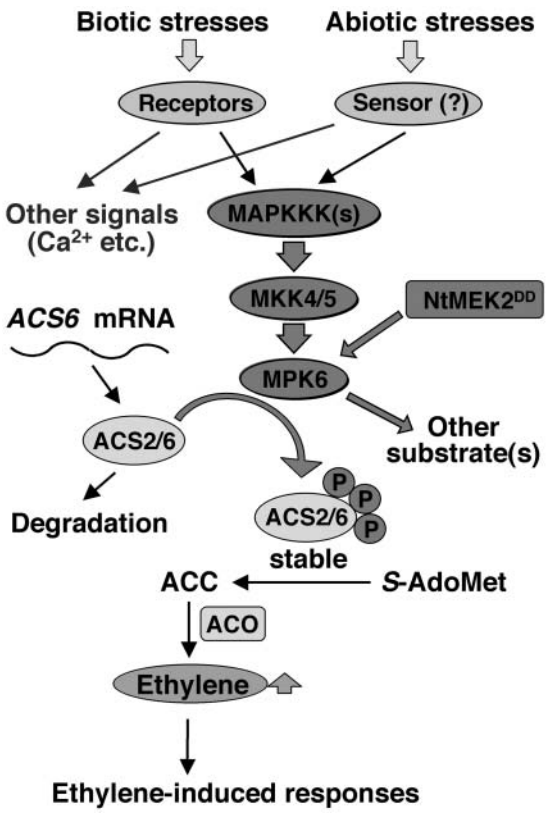

Figure 12. A Model Depicts the Role of MPK6 Cascade in Stress-Induced Ethylene Production.

The stimulation of ethylene production by stress occurs within 10 to 30 min (Yang and Hoffman, 1984; Abeles et al., 1992). Stress-induced activation of SIPK/MPK6 happens within one to several minutes, preceding the ethylene induction (Zhang and Klessig, 2001). As illustrated in Figure 12, the events necessary for the induction of ethylene biosynthesis are all posttranslational. This mechanism ensures the rapid induction of ethylene and the deployment of ethylene-induced responses in plants under stress. The *ACS6* gene is known to be induced at the transcriptional level by stress (Vahala et al., 1998; Wang et al., 2002). Our analyses demonstrated that *ACS2* is induced as well, though at a lower level (data not shown). It is likely that the transcriptional activation of these two genes contributes to the increase in ACS activity in plants under stress. However, the phosphorylation event is a more critical determinant. In the absence of MAPK activation, the newly synthesized ACS is rapidly degraded, resulting in no net increase in the ACS protein. Previously, we observed elevated levels of *ACO* gene expression in tobacco after SIPK/WIPK activation (Kim et al., 2003). Because the *ACO* gene activation falls behind the ethylene induction in tobacco, we concluded that it is not involved in the ethylene induction, at least at the initial stage. In this report, we measured the ACO activity in vivo and found that ACO activity is not the limiting step in the gain-of-function Arabidopsis plants (Figure 2B).

Based on studies using general kinase and phosphatase inhibitors, protein phosphorylation/dephosphorylation was implicated in the regulation of ethylene induction in plants under stress (Spanu et al., 1994; Tuomainen et al., 1997; Felix et al., 2000). In addition, it was shown that ACS is the target of such regulation. Possible mechanisms underlying the phosphorylation and dephosphorylation regulation of ACS include either the change in ACS activity, the change in ACS stability, or both (Spanu et al., 1994). Recently, tomato LeACS2 was shown to be phosphorylated by an unidentified calcium-dependent protein kinase (CDPK) from pericarp tissue (Tatsuki and Mori, 2001). LeACS2 is in the same subgroup as ACS2 and ACS6, which has the MAPK phosphorylation sites at the C terminus (see Supplemental Figure 1 online). The identified CDPK phosphorylation site in LeACS2 is conserved in most ACS members, including Arabidopsis ACS2 and ACS6. As a result, this subgroup of ACS may be under the regulation of two kinase pathways. One is the MPK6 cascade identified in this report and the other is the unidentified CDPK pathway reported by Tatsuki and Mori (2001). Wounding induces a long-lasting ethylene induction in tomato pericarp tissue but only a transient induction of ethylene in the leaf tissue (Tatsuki and Mori, 1999). This phenomenon suggests the presence of tissue-specific pathway(s). Could the unidentified

CDPK from pericarp be such a pathway? Much research is needed to address this complex question.

ACS6 protein is present at extremely low abundance in Arabidopsis seedlings, even after induction. Calculations based on the specific activity of ACS in total protein extracts (Figure 2A) and that of the purified recombinant ACS6 protein revealed that there is only ~1.5 ng of ACS6 in every 100 μg of total protein extract. In addition, the molecular mass of ACS6 (~55 kD) is the same as the highly abundant ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit. As a result, it is not possible for us to detect ACS6 in total protein extract directly by immunoblot analysis using the ACS6 antibody. In this study, we used the coupled immunoprecipitation-immunoblot analysis to detect the ACS6 protein. We also took advantage of the extremely high specific activity of ACS6 and the highly sensitive ACS assay to detect ACS6 activity specifically using the immune complex ACS6 assay.

Because of the very low uptake of ^{32}P-orthophosphate by Arabidopsis seedlings and the low abundance of ACS6 protein, we failed to detect directly the phosphorylation of ACS6 by in vivo ^{32}P labeling. Instead, we used the upshift of ACS6^{WT} protein after MPK6 activation (Figure 6) as an indication of ACS6 phosphorylation in vivo. Phosphatase treatment of immunoprecipitated ACS6^{WT} reversed the upshift (Figure 7), confirming that the band upshift is attributable to phosphorylation. In addition, we showed that the MPK6 phosphorylation sites in ACS6 are essential for ACS6 accumulation after MPK6 activation in vivo (Figure 6). Together with the in vitro phosphorylation assays (Figure 4) and data from the gain-of-function *ACS6^{DDD}* transgenic Arabidopsis (Figures 5 and 6), we conclude that MPK6 regulates ACS6 protein accumulation by direct phosphorylation. Because of the lack of an ACS2-specific antibody, some of the analyses could not be done with the *ACS2^{WT}*, *ACS2^{DDD}*, and *ACS2^{AAA}* transgenic plants. However, based on the similar phenotypes of *ACS2^{DDD}* transgenic plants (data not shown), we conclude that ACS2 is regulated similarly by MPK6.

Based on biochemical and genetic analyses, we placed the MPK6 cascade upstream of the ethylene biosynthetic pathway (Figure 12). Recently, MPK6 was reported to function downstream of CTR1 in the ethylene signaling pathway (Ouaked et al., 2003). Because it is difficult to generate a model that can accommodate both results, we tested whether ACC can indeed activate MPK6 in Arabidopsis. We failed to detect the activation of MAPK in Arabidopsis after ACC treatment, even though feeding ACC to Arabidopsis leads to high levels of ethylene production (Figure 11). One major flaw in the Ouaked et al. (2003) report is that AOA was mistakenly used as an ACO inhibitor. As a matter of fact, AOA is an ACS inhibitor (Abeles et al., 1992). This makes their finding that AOA blocks ACC-induced MAPK activation really questionable because AOA cannot block the conversion of ACC to ethylene in Arabidopsis. Based on our results, the MPK6 pathway functions upstream of ethylene, not downstream of CTR1.

Preliminary studies using proteasome inhibitors indicated that ubiquitin-proteasome pathway is involved in the degradation of ACS6. It is likely that the phosphorylation of ACS6 by MPK6 prevents or slows down this process, resulting in elevated levels of cellular ACS activity and ethylene biosynthesis. ACS6^{DDD} mutant protein can mimic the phosphorylated form of ACS6 in vivo. We also transformed Arabidopsis with ACS6 containing a single S-to-D mutation (at all three positions). None of them overproduce ethylene (data not shown), suggesting that the phosphorylation of multiple Ser residues is required to stabilize ACS6. This may be the reason why no gain-of-function ACS6 mutants were identified in the genetic screens that generated *eto2* and *eto3* mutants (Vogel et al., 1998; Woeste et al., 1999; Chae et al., 2003). *eto2* and *eto3* have mutations in the C terminus of *ACS5* and *ACS9*, respectively, which results in the stabilization of ACS5 or ACS9 protein and the overproduction of ethylene (Chae et al., 2003). Recently, ETO1 was shown to encode a protein that interacts with ACS5 and regulates its activity through two mechanisms: (1) directly inhibiting ACS5 activity and (2) targeting ACS5 protein to degradation (Wang et al., 2004). Phosphorylation was proposed to play a role in the process by regulating the interaction between ACS5 and ETO1. Because ACS5 is not a substrate of MPK6, it is likely that another unidentified kinase, possibly the CDPK reported by Tatsuki and Mori (2001), is involved in the regulation of ACS5 stability/activity.

The identification of the first substrate of plant MAPKs reveals an important mechanism by which MPK6 regulates plant stress responses. Gain-of-function *ACS6^{DDD}* plants phenotypically mimic plants under stress, with an overall reduced stature, smaller leaf blades, a shortened main root, and an increased number of root hairs (Figure 8), suggesting that some of the morphological alterations in plants under stress might be under the control of the MPK6 cascade. Numerous studies have demonstrated that stresses from both biotic and abiotic sources rapidly activate SIPK/MPK6 (Tena et al., 2001; Zhang and Klessig, 2001; Jonak et al., 2002; MAPK Group, 2002). It was speculated that plant stress-responsive MAPKs may phosphorylate transcription factors, similar to their counterparts in animals and yeast (Widmann et al., 1999; Davis, 2000; Chang and Karin, 2001; Tena et al., 2001; Zhang and Klessig, 2001; Hazzalin and Mahadevan, 2002; Jonak et al., 2002; MAPK Group, 2002). The identification of a plant-specific enzyme as the substrate of MPK6 reveals a new mode of MAPK action in plants. More importantly, it uncovers a signaling pathway that controls the biosynthesis of ethylene in plants under stress. During evolution, plant MAPKs adopted unique substrates, despite the fact that the structure of the MAPK cascade itself is highly conserved.

METHODS

Plant Growth and Treatments

Arabidopsis thaliana seedlings were grown in 50-mL GC vials with 6 mL of half-strength MS medium situated in a growth chamber at 22°C under continuous light (70 $\mu E/m^{-2}\ s^{-1}$). Unless indicated otherwise, 12-d-old seedlings were used for the experiments. DEX and Flg22 was used at final concentrations of 2 and 0.2 μM, respectively. Soil-grown plants were maintained at 22°C in a growth chamber with a 14-h light cycle (100 $\mu E/m^{-2}\ s^{-1}$). Wounding treatments were performed by either punching out leaf discs using a cork borer or cutting seedlings into small pieces using a razor blade. Samples were frozen in liquid nitrogen and stored at −80°C until use.

Selection of T-DNA Insertion Mutants and Generation of Crosses

T-DNA insertional mutants were obtained from the Wisconsin Arabidopsis Knockout Facility (*mpk6-1* in Ws-0 background, deposited to ABRC as CS31099) and ABRC (*mpk6-2*, Salk_073907; *mpk6-3*, Salk_127507; *acs2*, Salk_025672; *acs6*, Salk_090423; all in Col-0 background) (Sussman et al., 2000; Alonso et al., 2003). Homozygous F3 plants from various crosses were used for experiments. The steroid-inducible promoter:*NtMEK2*^{DD} transgene was followed by hygromycin resistance, and T-DNA insertions were followed by PCR of genomic DNA. All three alleles of *mpk6* were crossed into *NtMEK2*^{DD} transgenic background. Similar results were obtained, and data from *NtMEK2*^{DD}/*mpk6-1* are shown. For Flg22 treatment, *mpk6-2* and *mpk6-3* were used, and data from *mpk6-2* are shown.

Preparation of Recombinant Proteins

A *Nde*I site was introduced in front of the ATG start codon of ACSs by PCR, which was then ligated in frame into the pET-28a(+) vector (Novagen, Madison, WI) and sequenced. Mutations were introduced by QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA) and confirmed by sequencing. BL21(DE3) cells transformed with pET-28a(+) constructs were induced with 0.25 mM isopropylthio-β-galactoside for 3 h. His-tagged proteins were purified using nickel columns (Pharmacia, Piscataway, NJ) and concentrated using Centricon-10 (Millipore, Bedford, MA).

In Vitro Phosphorylation of ACS

Recombinant His-tagged MPK6 (7.5 μg) was activated by incubation with recombinant MKK4^{DD} and MKK5^{DD} (equal mixture, 0.25 μg) in the presence of 50 μM ATP in 50 μL of reaction buffer (20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT) at 22°C for 1.5 h. Activated MPK6 was then used to phosphorylate recombinant ACS proteins (1:20 enzyme substrate ratio) in the same reaction buffer with 25 μM ATP and [γ-^{32}P]ATP (0.1 μCi per reaction). The reactions were stopped by the addition of SDS-loading buffer after 20 min. The phosphorylated ACS was visualized by autoradiography after being resolved in a 10% SDS-polyacrylamide gel. The levels of phosphorylation were quantitated using a Fuji Film FLA-5000 imaging system (Tokyo, Japan). The relative amounts of ACS proteins in the Coomassie Brilliant Blue R 250–stained gels were quantitated using the NIH Image program. For enzymes used in the ACS activity assay, phosphorylation was performed without the addition of [γ-^{32}P]ATP. The completeness of phosphorylation was determined by 100% upshift of the ACS proteins in a 10% SDS-polyacrylamide gel after Coomassie Brilliant Blue staining.

Agrobacterium tumefaciens-Mediated Transformation

ACS genes and their mutants with a *Nde*I site added before ATG were first cloned in frame into an intermediate vector with a Flag-epitope tag at the 5′-end. The inserts were then moved into a modified pBI121 binary vector with *Xho*I and *Spe*I cloning sites. Agrobacterium GV3101 carrying different constructs was grown overnight in LB medium containing 25 μg/mL of gentamycin and 50 μg/mL of kanamycin. Transgenic Arabidopsis plants were generated using the flower-dipping method (Clough and Bent, 1998). T1 transformants were selected in the presence of kanamycin. After selection (5 to 6 d), the seedlings were either transplanted to soil for morphological observation and setting seeds, or transferred to 50-mL GC vials with 6 mL of half-strength MS medium (20 seedlings per vial). At least six of these vials were used for each construct in each experiment when the seedlings were 12 d old.

Assay of Ethylene Biosynthesis Rates

The GC vials with Arabidopsis seedlings were flushed and capped immediately after treatment or without treatment. At indicated times, ethylene levels in the headspace of the GC vials were determined by gas chromatography as previously described (Kim et al., 2003). Seedlings were then harvested, weighed, and frozen in liquid nitrogen for future analyses.

Protein Extraction, Immunoblot Analysis, and In-Gel Kinase Activity Assay

Protein was extracted from seedlings and stored at −80°C (Yang et al., 2001). The concentration of protein extracts was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with BSA as the standard. Immunoblot detection of Flag-tagged NtMEK2 and HisACS was performed as previously described (Yang et al., 2001). Either MBP (0.1 mg/mL) or His-tagged ACS6 (0.02 mg/mL) was used as the substrate in the in-gel kinase assay (Zhang and Klessig, 1997; Yang et al., 2001).

To detect Flag-tagged ACS6 proteins in *ACS6*^{wt}, *ACS6*^{DDD}, and *ACS6*^{AAA} transgenic Arabidopsis, 50 μL of anti-Flag M2 affinity gel (Sigma, St. Louis, MO) were used to immunoprecipitate Flag-tagged proteins from 800 μg of the total extracts. The levels of ACS6 proteins in the immune complexes were determined by immunoblot analysis using a goat anti-ACS6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Dephosphorylation of Flag-tagged ACS6^{WT} was performed by incubating the immune complex with five units of alkaline phosphatase at 37°C for 30 min.

ACS Activity Assay and Immune Complex ACS Activity Assay

Total proteins were extracted in two volumes (w/v) of ACS extraction buffer (100 mM Hepes, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 1 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerolphosphate, 10 μM pyridoxal 5′-phosphate, 10% glycerol, 10 μM MG115, 10 μM PSI, and Complete protease inhibitors [EDTA-free, Roche Molecular Biochemicals, Indianapolis, IN]). ACS activity in 300 μg of total extract was determined as previously described (Kim et al., 2003).

For immune complex ACS activity assay using the goat anti-ACS6 antibody (Santa Cruz Biotechnology), 6 μg of antibody were mixed with 300 μg of the total extracts in 1 mL of immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 μM pyridoxal 5′-phosphate, and Complete protease inhibitors) in the absence or presence of peptide competitor (6 μg). After incubation at 4°C for 1.5 h, protein G-agarose (30 μL) was added, and the incubation was continued for another 3 h. After washing three times with 1.5 mL of immunoprecipitation buffer each, the ACS activity in the immune complex was determined by the ACS assay described above. Immune complex ACS activity assays using anti-Flag antibody were done similarly except anti-Flag M2 affinity gel (Sigma) was used.

In Vivo ACO Activity Assay

In vivo ACO activity in *NtMEK2*^{DD} Arabidopsis seedlings was determined by measuring ethylene production in the presence of exogenously added ACC (Felix et al., 1991). Briefly, two sets of *NtMEK2*^{DD} seedlings grown in 50-mL GC vials were treated with DEX (2 μM). At various times, ACC (1 mM final concentration) was added to one set of the vials. The other set received no ACC and was used as controls. The GC vials were flushed and then capped. Ethylene levels in the GC vials were determined 1.5 h later. Higher levels of ethylene production in the presence of ACC indicate higher ACO activity. No ethylene was detected in vials with only medium plus ACC, and ethylene production in vials stopped after the seedlings were removed.

Quantitative RT-PCR Analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After an additional ethanol precipitation and DNase treatment, 2 μg of total RNA were used for RT. Quantitative PCR analysis was performed using an Optican 2 real-time PCR machine (MJ Research, Watertown, MA). Primers (forward 5′-GTTCCAACCCCTTATTATCC-3′ and backward 5′-CCGTAATCTT-GAACCCATTA-3′) were used to amplify the *ACS6* transcripts (from both endogenous and transgene). After normalized to the ubiquitin control (forward 5′-CACACTCCACTTGGTCTTGCGT-3′ and backward 5′-TGGTCTTTCCGGTGAGAGTCTTCA-3′), the relative levels of *ACS6* transcripts were calculated.

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