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Ethylene Biosynthesis in a Chilling-Sensitive *Arabidopsis* Mutant, *chs4-2*

Sun-A Kim[1], Seong-Ki Kim[2], Peter B. Kaufman[3], June Seung Lee[1]*, and Soo Chul Chang[4]*

[1]Department of Biological Science, Ewha Womans University, Seoul 120-750, Korea
[2]Department of Life Science, Chung-Ang University, Seoul 156-756, Korea
[3]Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA
[4]University College, Yonsei University, Seoul 120-749, Korea

We investigated chilling-induced changes in ethylene levels in *Arabidopsis* to find plants with distinct patterns of ethylene production in the cold-related biosynthetic pathway. The sensitive mutants identified here included *chs1-2*, *chs4-2*, and *chs6-2*. Among these, plants of the *chs4-2* mutant produced more ethylene than did the wild type after both were transferred from 4°C or 10°C to 22°C. This mutant also showed less freezing tolerance and more electrolyte leakage than the wild-type plants. Our results suggest a relationship between ethylene biosynthesis and chilling sensitivity in the mutant. To determine which of the enzymes involved in ethylene biosynthesis were induced by chilling, we tested the activities of ACC synthase and ACC oxidase in both mutant and wild-type plants, and found greater activity by ACC synthase as well as a higher ACC content in the mutants after all the plants were transferred from 10°C to 22°C. However, ACC oxidase activity did not differ between mutant and wild-type plants in response to chilling treatment. Therefore, we conclude that *chs4-2* mutants produce more ethylene than do other mutants or the wild type during their recovery from chilling conditions. Furthermore, we believe that ACC synthase is the key enzyme involved in this response.

Keywords: ACC synthase, *Arabidopsis*, chilling, *chs* mutant, ethylene

The ethylene biosynthetic pathway is well-established in higher plants. In this pathway, the precursor, methionine, is converted to S-adenosylmethionine (AdoMet) by AdoMet synthetase (EC 2.3.1.6) (Giovanelli et al., 1980). ACC synthase (EC 4.4.1.14) produces 1-aminocyclopropane-1-carboxylic acid (ACC) from AdoMet (Kende, 1989), and ethylene is formed from ACC by ACC oxidase (van der Straeten and van Montagu, 1991). Among these, ACC synthase is considered the key regulatory step for ethylene production because it is involved in various physiological events (Yang and Hoffman, 1984). However, both ACC synthase and ACC oxidase are also well-known rate-limiting steps for regulating ethylene biosynthesis in response to various signals (Fluhr and Mattoo, 1996). In addition, the fact that many genes encode these two enzymes suggests they belong to multigene families and, therefore, are capable of responding to many environmental and internal factors.

*Corresponding authors; fax +82-2-3277-3760, +82-2-3277-2385
e-mail schang@yonsei.ac.kr, jslee@ewha.ac.kr

Chilling damage in plants produces wilting, necrosis, chlorosis, or ion leakage from cell membranes (Lyons, 1973). These effects are due either to changes in the fatty acid composition of plant lipids, including phospholipids, or to the modified activity of enzymes involved in such physiological and metabolic events as growth and development, photosynthesis, and respiration (Graham and Patterson, 1982; Moon et al., 1993; Thomashow, 2001). Based on their responsiveness to low temperatures, plants are designated as either chilling-sensitive (i.e., unable to acclimate to cold) or chilling-resistant. *Arabidopsis*, which is chilling-resistant, increases its freezing tolerance after exposure to low temperatures (Gilmour et al., 1988). Based on its patterns of chilling response, Schneider et al. (1994) have identified four phenotypic classes of chilling-sensitive mutants of *Arabidopsis*. Class 1 mutants (*chs1-2*, *1-1*, *2-2*, *2-1*, and *3*) manifest yellow coloration, wilting, and death (Hugly et al., 1990). Class 2 mutants (*chs4-1*, *4-2*, and *4-3*) show the same color changes, but only in older leaves; younger leaves remain completely healthy. Class 3 mutants (*chs5*, *6-1*, *6-2*, and *6-3*) exhibit yellow patches only

in their leaf blades, whereas Class 4 mutants (*chs7*, *8*, *9*, *10*, *11*, *12*, *13*, *14*, and *15*) have partially yellow leaves at loci closest to the center of the rosette.

Relationships have been described between chilling and consequent changes in ABA (Leung and Giraudat, 1998) and ethylene levels (Field, 1984). For example, the induced production of ethylene in cucumber and bean is mediated by ACC synthase when plants are subjected to non-chilling temperatures after first being exposed to chilling conditions (Abeles et al., 1992). In the study presented here, our objective was to identify chilling-sensitive mutants of *Arabidopsis thaliana* that produce more ethylene than wild-type plants during their recovery from low-temperature treatment. We also investigated a possible role for ACC synthase in that response.

MATERIALS AND METHODS

Plant Materials and Chemicals

Seeds of wild-type plants, *Arabidopsis thaliana* L. cv. 'Columbia', and their mutants, *chs1-2*, *chs4-2*, and *chs6-2*, were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, USA). They were planted in plastic pots containing a 1:1:1 mixture of permiculite:vermiculite:sphagnum and reared in a controlled environment growth chamber (MRL-350HT, Sanyo, Japan) under continuous fluorescent illumination from cool white lamps (100 to 150 μmole $m^{-2}s^{-1}$). The plants were watered with a complete mineral nutrient solution; the temperature was set at 22°C and relative humidity was 40 to 50%. All chemicals were obtained from the Sigma-Aldrich Co. (St. Louis, MO, USA).

For the chilling treatment, 14-d-old plants were held for 24 h at either 4°C or 10°C; the control plants were incubated at 22°C. During this experiment, all plants were maintained under the same growing conditions as above.

Determination of Electrolyte Leakage and Freezing Tolerance

Electrolyte leakage was determined as described by Kim et al. (2002) and by using a slight modification of the method of Gilmour and Thomashow (1991). Detached leaves (0.5 g fresh weight) were collected and rinsed three times with deionized water to remove the electrolytes from injured cells and leaf surfaces. They were then cut into smaller pieces and incubated with shaking (60 rpm) for 3 h at 25 ± 1°C in 20 mL of distilled water (dH_20) in a water bath. Afterward, electrical conductivity of the solution was measured. To calculate relative values, the control electrolyte leakage, equivalent to total electrolyte leakage as reported by Gilmour and Thomashow (1991), was obtained by measuring the conductivity of bathing solutions that contained leaf samples first incubated with shaking (60 rpm) at 60 ± 1°C for 3 h, then cooled to 25°C in a water bath. Further exposure to 25 ± 1 or 60 ± 1°C for up to 24 h produced similar conductivity values. Relative electrolyte leakage for each experimental group was presented as a percentage of the control (total) leakage.

Freezing tolerance of our *Arabidopsis* leaves was defined according to the method of Gilmour et al. (1988). Temperature versus percent electrolyte leakage was plotted to determine the value for 50% electrolyte leakage, i.e., LT50. Excised leaves (0.5 g) were placed in sealed plastic bags and maintained in a low-temperature water bath (Masterline Model 2095; Forma Scientific, USA) set at -2°C. After 1 h of equilibration, the bath temperature was lowered manually every 30 min, in 2°C increments, to -12°C. Samples were withdrawn and placed on ice for 1 h, then incubated for 18 h at 4 ± 1°C. Freezing damage was estimated by the electrolyte leakage test described above. Each sample was transferred to a tube containing 20 mL d H_2O and shaken at 60 rpm for 30 min at 25°C. Conductivity of the resulting solution was measured with a conductance meter (TOA Electronics, Japan). A value for 100% leakage was obtained by incubating each sample at 65°C for 30 min and re-extracting with the original solution (Kim et al., 2002). Ion leakage of the non-frozen tissues was measured using samples incubated for 30 min at 25°C. Leakage was calculated by the following equation: Ion leakage (%) = $(EL_{frozen} - EL_{unfrozen})/(EL_{total} - EL_{unfrozen}) \times 100$.

Measurement of Ethylene Content

Leaf tissues were placed in vials containing Mes-Tris buffer (10 mM, pH 6.8) and 50 g mL^{-1} chloramphenicol, then shaken at 80 rpm in an incubator set at 25 ± 1°C. Afterward, 1 mL of air was withdrawn with a syringe and injected into a gas chromatograph equipped with a Porapac Q column (Shimadzu GC-8A, Japan).

Activity Assays for ACC Synthase and ACC Oxidase

ACC synthase activity was assayed as described by

Woeste et al. (1999), with slight modifications. Leaf tissues (3 g) were ground on ice with 6 mL of 100 mM Hepes-NaOH (pH 8.5) buffer containing 0.5 μM pyridoxal phosphate, 10 mM EDTA, 0.1 mM PMSF, and 4 mM DTT. This homogenate was kept for 4 min on ice, then centrifuged for 15 min at 15,000*g*. The supernatant was applied to a Sephadex G-25 column (3 × 11 cm; bed volume, 30 mL) and eluted with 2 mM Hepes-NaOH (pH 8.5) buffer containing 0.5 μM pyridoxal phosphate, 1 mM EDTA, and 0.5 mM DTT. The eluent was incubated for 1.5 h at 30°C in a solution comprising 50 mM Hepes-NaOH (pH 8.5), 0.2 mM AdoMet, and 0.5 μM pyridoxal phosphate. This was followed by incubation on ice for 10 min with a 1:1 mixture of 0.1 mL of 20 mM $HgCl_2$ and 0.1 mL of NaOH/NaOCl. The level of ethylene production from this mixture was used to determine ACC synthase activity (Lizada and Yang, 1979).

The assay for ACC oxidase was conducted *in vivo* according to the method of Wang and Woodson (1989). Leaf tissues were incubated for 1 h at 28 ± 1°C in a 250 mM Mes-Tris buffer (pH 6.8) containing 0.1 mM aminoethoxyvinylglycine (AVG). Afterward, the tissues were washed with dH_2O and infiltrated in the dark with 1 mM ACC for 2 h at 27 ± 1°C. The determination of ACC oxidase activity was based on values for ethylene produced in 1 h in the absence of ACC by leaf tissues in dH_2O.

Determination of ACC Contents

ACC contents were measured according to the methods of Lizada and Yang (1979). Leaf tissues were incubated in 80% ethanol at 80°C for 4 h. The extract was concentrated with a speed vacuum apparatus and the remaining pellet was dissolved in 2 mL of dH_20. This solution (0.8 mL) was mixed with 10 mM $HgCl_2$ (0.1 mL) and incubated for 15 min in a vial capped with a rubber stopper. NaOCl solution (0.1 mL, two volumes of 5% NaOCl, and one volume of saturated NaOH) was injected into the vial with a syringe, followed by incubation on ice for 10 min. One mL of air was then withdrawn and subjected to gas chromatography. ACC internal standards were added to produce a standard curve.

Replication of Experiments and Statistical Analysis

All experiments were performed at least three times and mean values were calculated according to Student's *t* test for significance.

RESULTS

The *chs4-2* Mutant Produced More Ethylene Following Chilling Treatment Than Wild-Type and Other Mutant Plants

Ethylene levels were measured hourly in wild-type and chilling-sensitive mutant (*chs1-2*, *4-2*, and *6-2*) plants during their recovery at 22°C following treatment at 22°C (control), 4°C, or 10°C. All plants produced greater amounts of ethylene in response to the two lower temperatures (Fig. 1). In addition, the greatest increase in ethylene content was achieved at 3 to 4 h after the initiation of recovery. However, when compared with the overall performance of our wild-type plants, the difference in ethylene production was less with mutants *chs1-2* and *6-2*, but greater with *chs4-2*. In those plants, ethylene levels during the first 4 h of recovery were 71.53 to 82.86 nL g FW^{-1} higher than that measured in the control (Fig. 1C). Chilling the wild-type plants at 4°C or 10°C also resulted in more ethylene production, although levels were only 40.65 to 41.21 nL g FW^{-1} greater than the control at the 4-h mark (Fig. 1A). Therefore, we were able to demonstrate that *chs4-2* plants were most sensitive to chilling treatment in terms of ethylene content and, thus, we selected them for further comparisons with the wild type.

Other experiments (data not shown) also showed that chilling at 10°C induced plants to produce more ethylene than when treatment was applied at 4°C. Based on those results, we selected incubation at 10°C as our standard chilling treatment.

chs4-2 Plants Exhibited a More Sensitive Response to Chilling Than Wild-Type Plants

To determine whether chilling sensitivity was correlated with ethylene production, we compared electrolyte leakage and freezing tolerance in wild-type and *chs4-2* plants, and found that the line for freezing tolerance in the latter was shifted to the left by 2°C (Fig. 2). This indicated that the mutants were less tolerant, leading us to conclude that those chilling-sensitive mutant plants had lower tolerance and exhibited greater electrolyte leakage upon chilling than did the wild-type plants.

Greater ACC Synthase Activity and ACC Content Observed in *chs4-2* Mutants Than in Wild-Type Plants

Based on the results shown in Fig. 1, we investi-

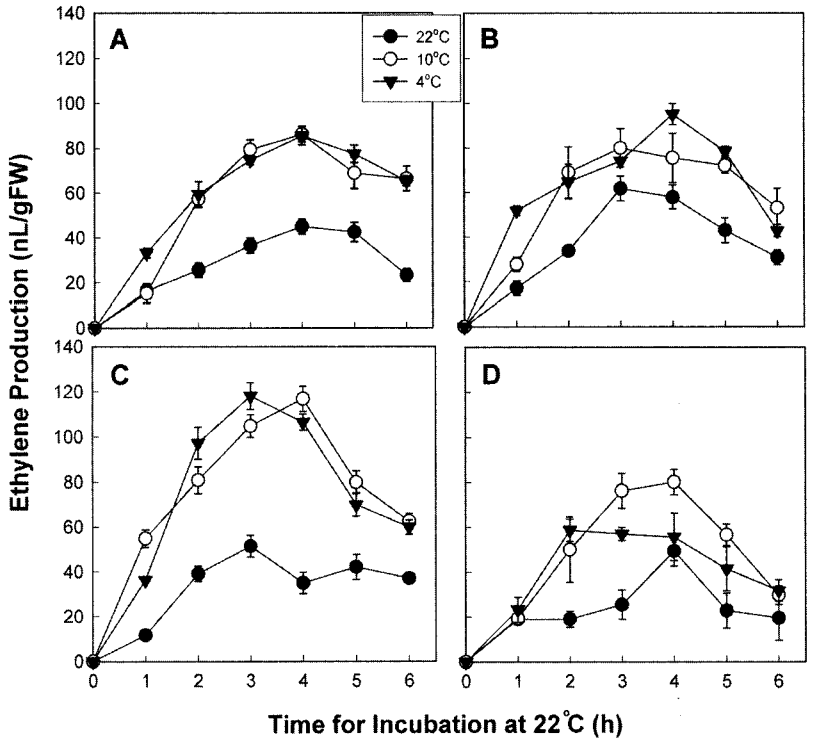

Figure 1. Effects of temperature on ethylene production in leaves of *Arabidopsis*. **A**, wild-type and mutant plants; **B**, *chs1-2*; **C**, *chs4-2*; and **D**, *chs6-2*. Whole plants were exposed to 22°C, 10°C, or 4°C for 24 h, then recovered at 22°C for up to 6 h. Rosette leaves, taken from plants immediately after cold treatment, were incubated at room temperature for various time periods in solutions containing 10 mM Mes-Tris buffer (pH 6.8) and 0.5 mg/L chloramphenicol. To measure ethylene content, 1 mL of air was withdrawn and subjected to gas chromatography. Bars in graph denote SE of four independent experiments using 0.5 g of leaf tissue.

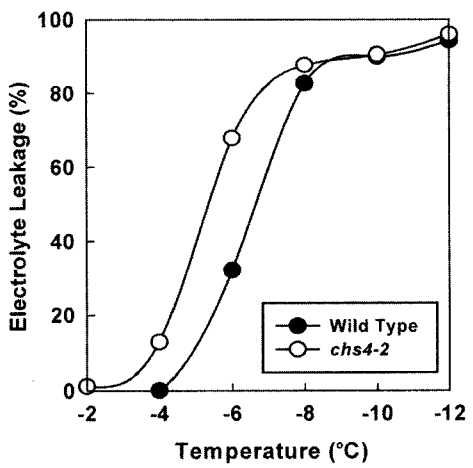

Figure 2. Comparison of freezing tolerance between wild-type and *chs4-2* mutant plants exposed to chilling. Temperature was lowered by 2°C at 30 min intervals, down to -12°C. Tolerance calculations were based on electrolyte leakage (see Materials and Methods). Conductance was then measured in collected leaves. Values for leakage were expressed as relative % of control (i.e., wild type exposed to 22°C) -- leakage in control was 8.1% . Bars in graph denote SE of four independent experiments using 0.5 g of leaf tissue.

gated whether ACC synthase and/or ACC oxidase were responsible for the increased ethylene production in *chs4-2* mutants after chilling treatment. Changes in ACC synthase activity were observed during this recovery period. For example, at 22°C, enzymatic activity did not differ significantly between the *chs4-2* and wild-type plants (Fig. 3A). However, following chilling treatment, activity in the mutant and wild-type plants increased by 7.29- and 1.87-fold, respectively. Thus, we believe that ACC synthase activity was apparently induced by chilling in those mutants.

To confirm that result, we measured ACC content in plants treated at the control temperature, and noted that ACC levels were more enhanced in our *chs4-2* plants (54% increase) than in the wild-type plants (43% rise; Fig. 3B).

ACC Oxidase After Chilling Treatment Does Not Respond Differently Between *chs4-2* and Wild-Type Plants

ACC oxidase contents were nearly the same in the control, wild-type, and *chs4-2* mutant plants after

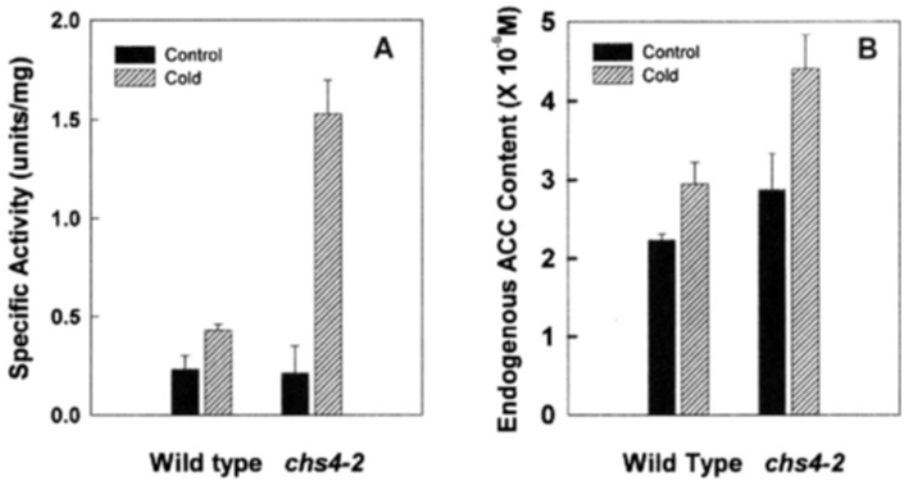

Figure 3. Effect of chilling on changes in **A**, ACC synthase activity, and **B**, ACC content. After wild-type and *chs4-2* mutant plants were incubated at 10°C for 24 h, leaves were collected for recovery in buffer at 22°C for 4 h (see Materials and Methods). **A**, ACC synthase activity; and **B**, ACC content. Bars in graphs denote SE of four independent experiments using 3 g of leaf tissue per treatment.

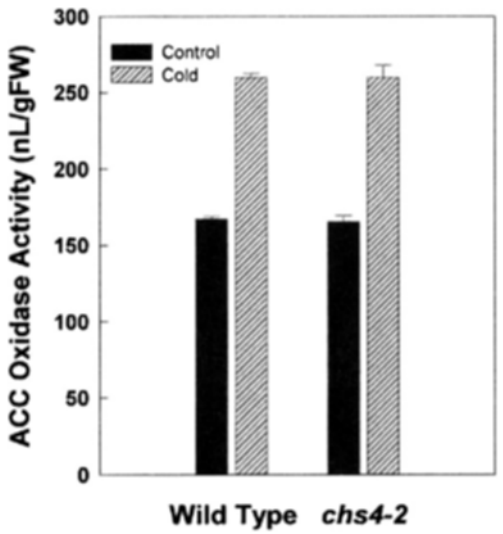

Figure 4. Effect of chilling on changes in ACC oxidase activity. Wild-type and *chs4-2* mutant plants were incubated at 10°C for 24 h, then leaves were collected for recovery at 22°C for 4 h. Tissue was incubated for 1 h in solution containing 250 mM Mes-Tris buffer (pH 6.8) and 0.1 mM AVG at 28 ± 1°C, then infiltrated in the dark for 2 h with 1 mM ACC at 27 ± 1°C. Ethylene production was measured and used to estimate ACC oxidase activity. Bars in graphs denote SE of four independent experiments using 3 g of leaf tissue per treatment.

chilling treatment (Fig. 4). Therefore, we concluded that this particular enzyme was not responsible for the difference in ethylene content between wild-type and *chs4-2* plants and, therefore, was not related to chilling-induced ethylene production.

DISCUSSION

Because ethylene is a well-known stress hormone in plants, we hypothesized that there may be a chilling-sensitive mutant(s) related to its production. Among the wild-type plants and three mutants tested -- *chs1-2*, *chs4-2*, and *chs6-2*-- *chs4-2* produced the greatest amount of ethylene during the recovery period. That chilling-sensitive mutant was then used to characterize where regulation occurs in the chilling-induced biosynthetic pathway for ethylene. However, because we tested only three out of the 21 mutants that belonging to Classes 1, 2, 3, and 4 (see Schneider et al., 1994), it is possible that additional mutants show other distinctive patterns of ethylene production. Nevertheless, we selected *chs4-1* and *chs4-3* for our initial comparisons because those mutants are relatives of *chs4-2*, and also because we did not believe such an investigation had previously been performed.

Our investigation of changes in the chilling-induced activity of ACC synthase and ACC oxidase -- two key enzymes in ethylene biosynthesis -- revealed that the former, a rate-limiting enzyme, responded as expected to low-temperature treatment in all wild-type and mutant plants. In particular, chilling caused the activity of ACC synthase to increase by 7.3-fold whereas the same stress treatment caused ACC content to rise by 1.5-fold in our mutants. We speculated that a large portion of the excess ACC was converted to N-malonyl-ACC, but we had to measure ACC N-malonyltransferase activity to prove this theory.

Exposure to low temperatures can have two possible effects on ethylene production. The first is to stimulate ACC synthesis, an action well-documented by many reports of its accumulation during chilling (Chen and Patterson, 1985; Wang, 1987; Chu and Lee, 1989; Cabrera and Saltveit, 1990; Abeles et al., 1992). This stress also causes a parallel increase in endogenous ACC when the degree of treatment is increased (Chen and Patterson, 1985). The second possible outcome is that cold stress can cause a decline in ethylene content through a reduction in its formation from ACC. Diminished ACC oxidase activity has been associated with increased ion leakage from cucumber fruit cells (Cabrera and Saltveit, 1990). In the current study, we confirmed that ACC synthase content increased in response to chilling in *chs 4-2* mutants as well as wild-type plants. Moreover, both the mutants and the wild-type plants exhibited increased ACC oxidase activity during the recovery period. Therefore, our data demonstrate the first possibility, but argue against the second.

In future studies, it should be possible to observe the role(s) of ethylene in regulating the effects of chilling, including necrosis and changes in electrolyte leakage and membrane composition. Although the *chs4-2* gene has not yet been cloned, it is considered EMS-mutagenized, so that its plants carry a point mutation in that gene (Schneider et al., 1994). Their phenotype suggests that the mutated gene is related to chilling-induced ethylene biosynthesis. More specifically, it is possible that this gene is related to the chilling-induced ACC biosynthetic process, which includes enhanced ACC synthase activity. If this gene is cloned, experiments can then be designed that will help explain its function in relation to the regulation of ethylene biosynthesis as a response to chilling.

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