Journal of Plant Biology, June 2001, 44(2) : 103-110

Differential Accumulation of Transcripts for ACC Synthase Homologs in Etiolated Mung Bean Hypocotyls in Response to Li^+

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In mung bean (*Vigna radiata*), 1-aminocyclopropane-1-carboxylate synthase (ACS) is encoded by a multigene family that comprises at least seven members. Their expression is induced by hormones, developmental signals, inhibition of protein synthesis, or stress. We performed an RT-PCR assay to understand the differential accumulation of transcripts for ACC synthase homologs as a response to Li^+ in etiolated mung bean hypocotyls. Treating the seedlings with LiCl caused inductions of the *ACS1*, *ACS4*, *ACS5*, *ACS6*, and *ACS7* genes. The induction level of *ACS7* was particularly high. This effect on *ACS7* expression was specific, lasting more than 8 h after the treatment. To understand the relationship of this Li^+ action with the phosphoinositide (PI) second messenger system, we examined the effect of Ca^{2+} treatment on Li^+-induction of the *ACS7* gene. Because Ca^{2+} did not reverse the Li^+ effect, we suggest that regulation of the *ACS7* gene by Li^+ in the mung bean may not involve the PI system.

Keywords: ACC synthase, differential accumulation, Li^+, multigene family, mung bean

Ethylene, a gaseous plant hormone, is involved in many aspects of plant growth, development, and senescence (Abeles, 1973). The rate of ethylene production is usually low in most tissues, but increases dramatically at certain developmental stages, including seed germination, leaf abscission, and fruit ripening (Yang and Hoffman, 1984). It also is induced by a variety of external factors, such as auxin applications, wounding, anaerobiosis, viral infection, elicitor treatment, chilling injury, drought, Cd^+, and Li^+ (Abeles, 1973). Methionine is the biological precursor of ethylene, being converted to S-adenosylmethionine (SAM) by SAM synthase. In a rate-limiting step, SAM is then converted to ACC by ACC synthase. Afterward, ACC is converted either to ethylene, CO_2, and HCN by ACC oxidase; or to N-malonyl-ACC by malonyl transferase (Adams and Yang, 1979).

In many species, ACC synthase, a short half-life and cytosolic enzyme (Kim and Yang, 1992), is the key regulatory enzyme in the ethylene biosynthetic pathway. This enzyme is encoded by a gene family whose members are differentially expressed due to various internal and external factors (Kende, 1993; Yu et al., 1998). For example, the enzyme in tomato is encoded by at least six genes that are differentially regulated in response to developmental, environmental, and hormonal factors (van der Straeten et al., 1990; Olson et al., 1991; Rottman et al., 1991; Yip et al., 1992; Olson et al., 1995). Several divergent members of a multigene family for ACC synthase have been cloned in *Arabidopsis* and rice (Liang et al., 1992; van der Straeten et al., 1992; Zarembinski and Theologis, 1993; Arteca and Arteca, 1999). Likewise, *ACS1, ACS2, ACS3* (Botella et al., 1992a, 1992b), *ACS4, ACS5* (Botella et al., 1993), *ACS6*, and *ACS7* (Kim et al., 1996) in mung bean also are members of a multigene family for ACC synthase; their *ACS1, ACS6*, and *ACS7* transcripts increase in response to auxin (Kim et al., 1992; Yi et al., 1999). Transcription of *ACS1* is also induced by mechanical stress (Botella et al., 1995), while that of *ACS5* is accumulated by OGA (Yu et al., 1998). However, little is known about the expression of other members of this multigene family.

Li^+ enhances the activity of ACC synthase in ripening tomato fruit (Boller, 1984), which leads to a large accumulation of the enzyme in treated tissues. The discovery of this effect has allowed researchers to purify this low-abundance plant protein from tomato and zucchini, and to clone the ACC synthase gene (Kende, 1993: Zarembinski and Theologis, 1994). Li^+, the smallest of the alkali metals, exerts profound neural and developmental influences in humans and other animals (Berridge et al., 1989; Birch, 1991). Although plants normally do not contain Li^+, they can absorb

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Abbreviations: ACC, 1-aminocyclopropaue-1-carboxylic acid; SAM, S-denosylmethionin.

those ions from their growth medium. Li^+ affects a plant's respiration; cellular transport; enzyme activities such as with peroxidase and ACC synthase (Laties, 1959, 1963; Boller, 1984; Wissocq et al., 1991); callus induction (Bagga et al., 1987); microtubule depolarization (Bartolo and Carter, 1992); and mitotic progression of stamen hair cells (Wolniak, 1987).

The molecular mechanism for Li^+ is not well understood, but Berridge et al. (1989) have proposed an "inositol depletion" hypothesis for expending its neural and developmental action. This hypothesis is based on the fact that Li^+ inhibits the phosphoinositide (PI) cycle, which is responsible for generating two second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). It does so by inhibiting the activity of inositol-phosphate phosphatases non-competitively, resulting in reduced IP3 release (Hallcher and Sherman, 1980). A gene for Li^+-sensitive inositol monophosphatase has been cloned from tomato (Gillaspy et al., 1995). In *Arabidopsis*, *ACS5* mRNA is expressed by Li^+; this action is, hypothetically, related to the PI cycle and Ca^{2+} (Liang et al., 1996). In mung bean, the PI metabolism also appears to be necessary for ethylene induction of the *ACO1* gene in root tissue (Jung et al., 2000).

Although it is one of the best model systems for studying ethylene biology, Li^+-action on ACC synthase genes has not been thoroughly examined in mung bean. In the current study, we first wanted to identify which members of the family of ACC synthase genes were regulated by Li^+, and determine whether Ca^{2+} treatment could reverse the Li^+-induced accumulation of transcripts for ACC synthase homologs. The objective was to broaden our understanding of the mechanism for Li^+-regulation in the expression of *ACS* genes.

MATERIALS AND METHODS

Plant Materials

Seeds of mung bean (*Vigna radiata* W.) were germinated and grown in vermiculite for three days, in the dark at 27°C.

Treatment of Mung Bean Hypocotyls with Various Stimuli

Unless otherwise indicated, all chemicals were obtained from Sigma, and were usually of molecular biology grade or high purity reagent grade.

For the various chemical treatments (including LiCl, CsCl, KCl, or NaCl), approximately 1-g samples of hypocotyl segments were cut 1 to 2 cm below the hook, then incubated in 2 mL of a medium containing the particular test chemical plus 10 mM MES/Tris buffer (pH 6.6) and chloramphenicol (50 μg mL^{-1}). The segments were placed in 15-mL vials, and continuously shaken in the dark for 60 min at 27°C. The samples were then stored at −70°C and used for the preparation of total RNA.

For the time-course experiments to monitor the accumulation of transcripts by Li^+, approximately 1-g samples of the excised hypocotyl fragments were incubated in 5-mL vials with 2 mL of a medium containing 10 mM MES/Tris buffer (pH 6.6), chloramphenicol (50 μL mL^{-1}), and 10 mM LiCl. The vials were shaken in the dark at 27°C for 5, 15, 30, 45, 60, 240, or 480 min.

To analyze the effect of Ca^{2+} on the accumulation of *ACS7* transcripts by Li^+, approximately 1-g samples of the excised hypocotyl fragments were incubated in 15-mL vials with 2 mL of a medium containing 10 mM MES/Tris buffer (pH 6.6), chloramphenicol (50 μL mL^{-1}), and 50 mL LiCl, plus 1, 10, or 50 mM $CaCl_2$. The vials were shaken in the dark for 60 min at 27°C.

Preparation of Total RNA

To isolate total RNA, mung bean hypocotyl fragments were ground with liquid nitrogen in a mortar. One hundred mg of the powder was transferred to a 1.5-mL microcentrifuge tube containing 1 mL of Tri-reagent (Molecular Research Center, Inc.), then held for 5 min at room temperature. Afterward, 200 μL of chloroform was added, mixed vigorously, and centrifuged at 12,000*g* for 15 min at 4°C. The aqueous phase was then transferred to a new tube into which was added 500 μL of isopropanol. This was mixed and centrifuged at 12,000*g* for 8 min at 4°C. The pellet was rinsed with 1 mL of 75% (v/v) ethanol, centrifuged at 7,500*g* for 5 min at 4°C, and air-dried for 5 to 10 min. This pellet was then dissolved in TE buffer and stored at −20°C. To remove the contaminating chromosomal DNA, RNase-free DNase I (Epicentre Technologies) was added at 0.2 units per 40 μg of RNA, and incubated at 37°C for 60 min. The reaction was stopped by the addition of 10 mM EDTA and a 0.2% (w/v) SDS mix. This was followed by a phenol:chloroform (1:1, v/v) extraction and an ethanol precipitation. The RNA pellet was resuspended in TE buffer and stored at −20°C.

Preparation of Internal Control RNA

To monitor the efficiency of both the cDNA conversion from RNA and the PCR amplification, 1,100 nt of in vitro-synthesized RNA from the luciferin-binding-protein (LBP) gene (Lee et al., 1993) was prepared and used as an internal control RNA. p1.1LBP, a plasmid DNA containing the *LBP* gene (Lee et al., 1993), was linearized by digestion with EcoRV and ClaI. In-vitro transcription was performed at 37°C for 2 to 4 h, in a

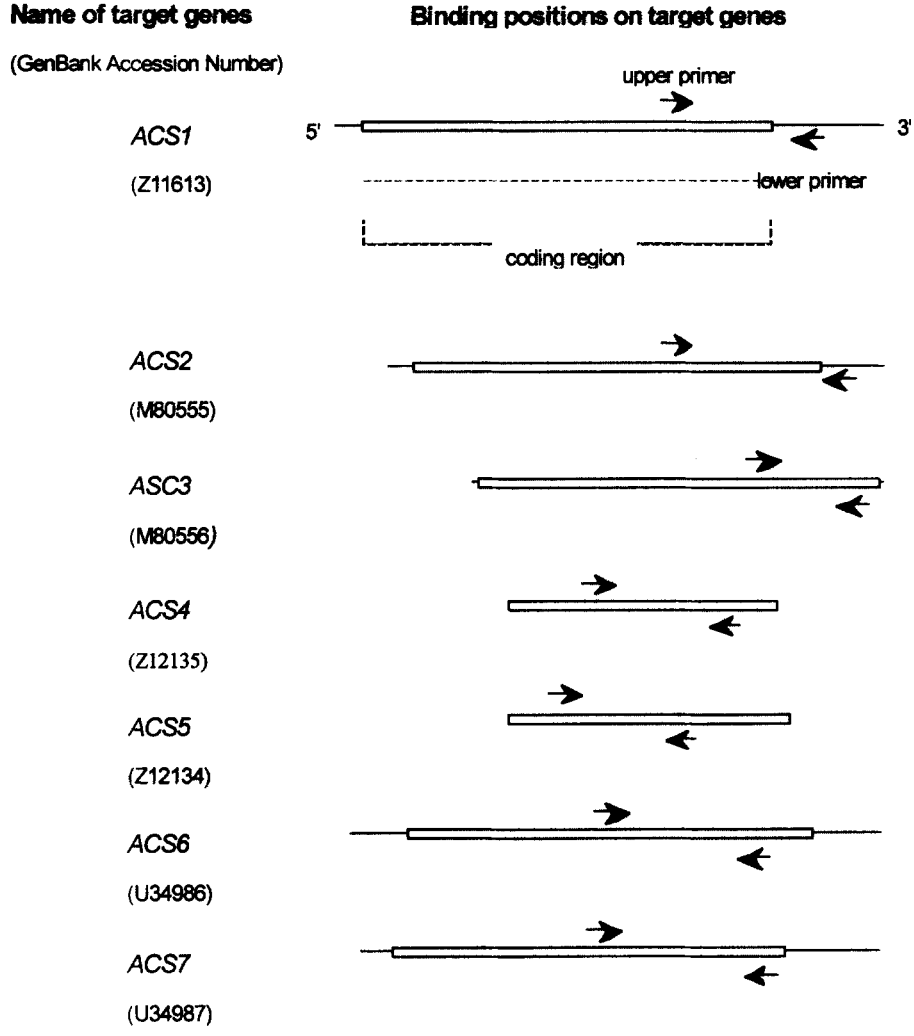

Figure 1. Binding positions of designated primers on target genes.

Table 1. Sequences of primers used for RT-PCR.

Target gene	Upper primer	Lowerprimer	Size of PCR product (bp)
	Sequence (5′ → 3′)	Sequence (5′ → 3′)	
ACS1	GTTGGCATAAAGTGCTTGCA	AAGCAGCACAACTTGTAGGA	584
ACS2	AGTGGTGATCAATGGGAGGA	ACAGATCTAGGACAACGTCA	691
ACS3	TCGCAAACTCAACACATGCT	CTCGAACAAGAGGTGATGTA	499
ACS4	AACCGGTCAACGCTCGATAT	CAAGAAGACCCCGGAGAGAT	563
ACS5	ACGCTTTACTAGTTCCTACT	GTTTCTGTATTGGCGACCCT	365
ACS6	CATTTGATCAGCGACGAGAT	CCGTTCCATGGCTAGGTTTA	606
ACS7	CATTTGATCAGCGACGAGAT	CGCGAGACGATAACCGGAAA	700
LBP	ATCAAGCAGCCCTGGAAGGT	TGGTCGACCACCCCACTTGTAA	840

20-μL reaction volume containing 2 μg of linearized plasmid DNA template; 1.25 mM each of ATP, CTP, GTP, and UTP; 80 mM Hepes-KOH (pH 7.5); 24 mM $MgCl_2$; 2 mM spermidine; 40 mM DTT; 600 units of T_7 RNA polymerase; 30 units of RNasin; and 0.38 units of yeast inorganic pyrophosphatase. The DNA template was removed using RNase-free DNase I (1 unit μg^{-1} of DNA) at 37°C for 15 min. RNA was recovered via phenol:chloroform:isoamylalcohol (25:24:1, v/v)) extraction, chloroform:isoamylalcohol (24:1, v/v)

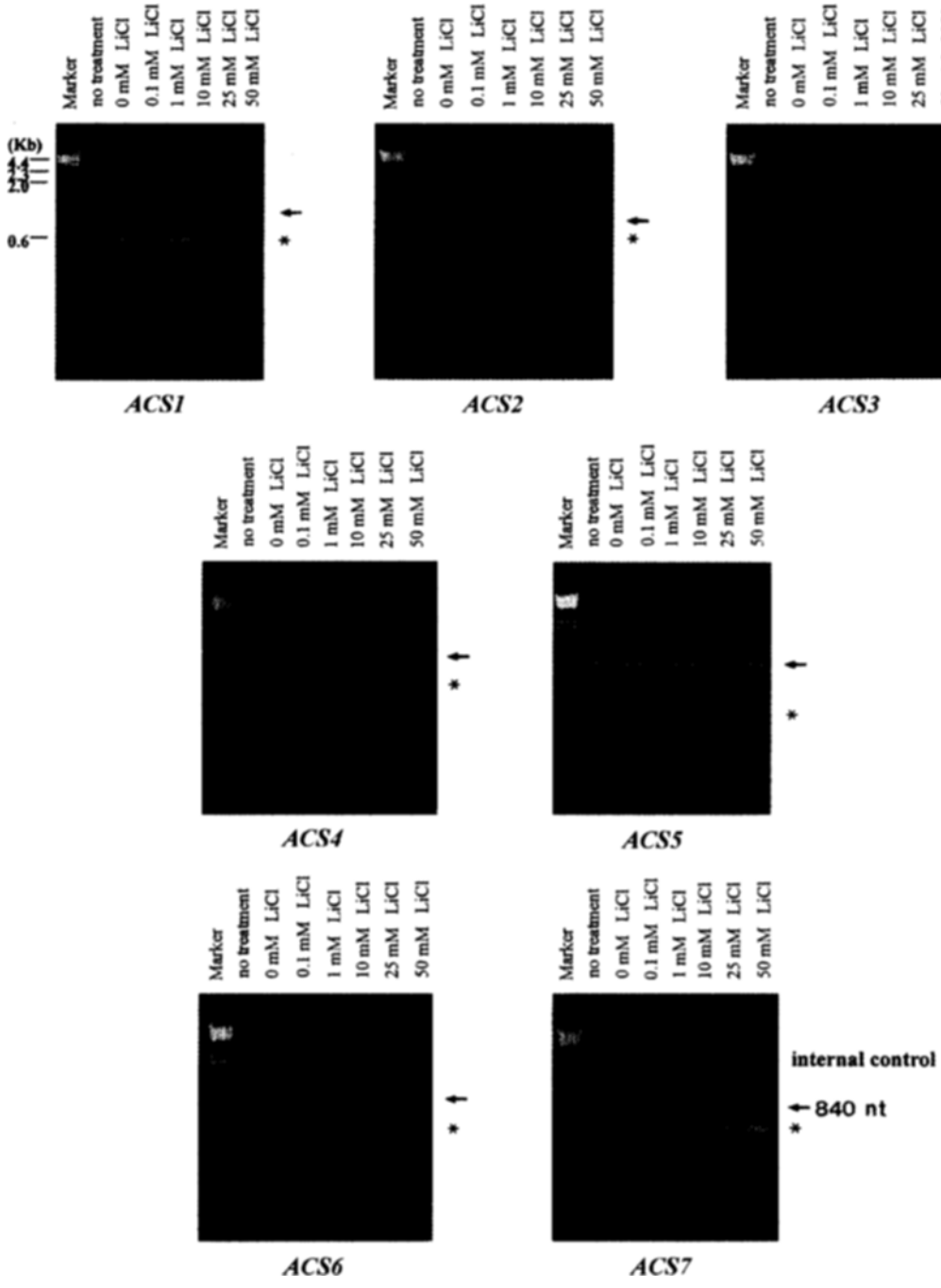

Figure 2. Differential accumulation of transcripts with increasing concentrations of Li^+. RNA for RT-PCR was from excised mung bean hypocotyls treated with 0.0 mM, 0.1 mM, 1.0 mM, 10.0 mM, 25.0 mM, or 50.0 mM for 60 min. Arrow indicates the RT-PCR product from the internal control (*LBP*) and asterisks indicate the RT-PCR product from the *ACS* gene.

extraction, and isopropanol precipitation. The RNA pellet was washed with 70% (v/v) ethanol, dried, and dissolved in TE buffer. Its purity and intactness were analyzed on a 1% agarose gel, and its concentration was determined by measuring A_{260} of the solution.

RT-PCR Assay

We conducted cDNA synthesis and PCR amplification to examine the transcripts that accumulated in response to our different stimuli. Oligonucleotides for RT-PCR were synthesized by either Bioneer, Inc., or the Central Laboratory of the College of Pharmacy, Ewha Womans University. The RiboMax™ Large-Scale RNA production for in-vitro transcription and the reverse transcriptase for cDNA synthesis, as well as the other restriction enzymes, were obtained from Promega. For cDNA synthesis, one pmole of oligo

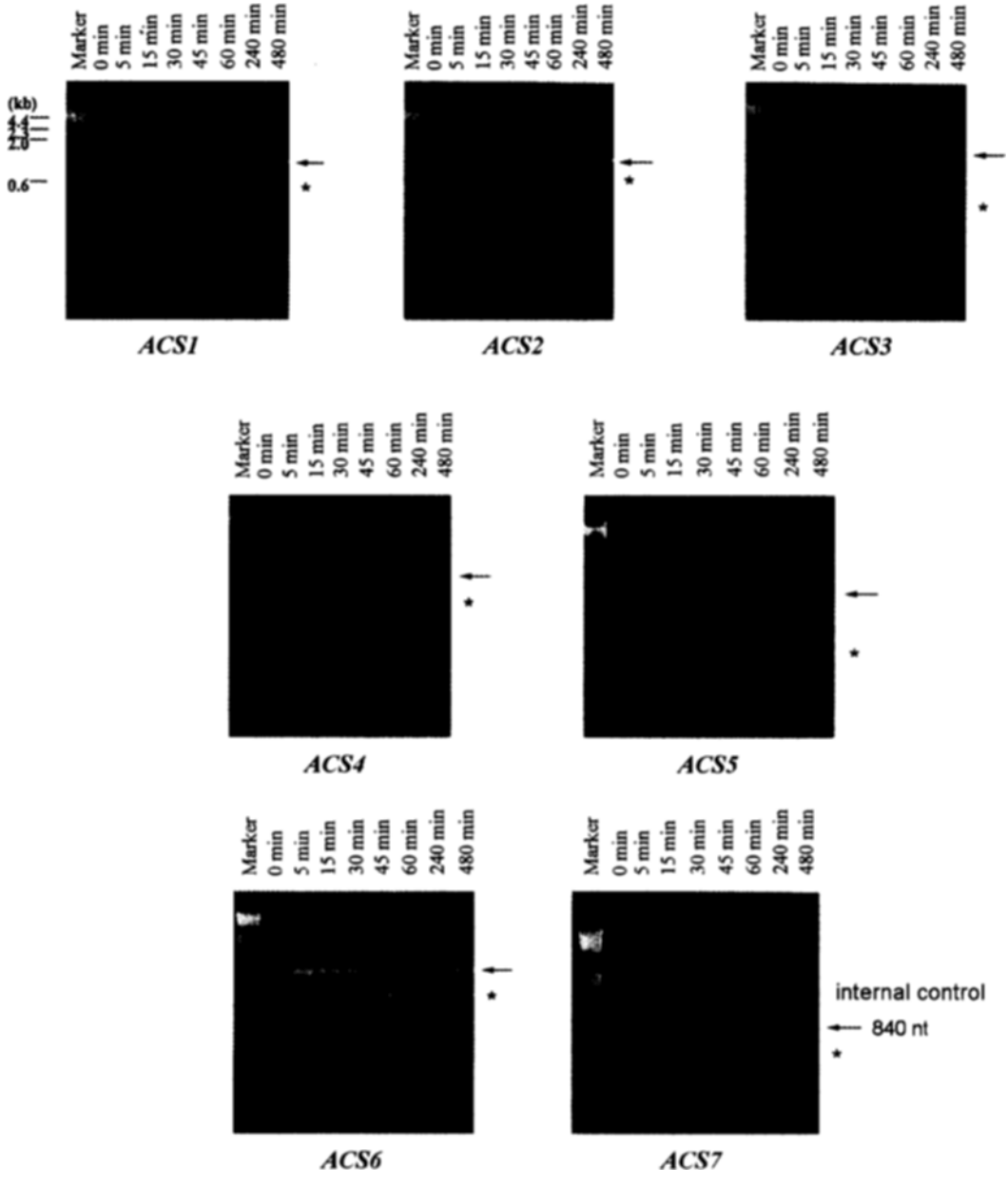

Figure 3. Time-course analysis of differential accumulation of transcripts by Li^+. RNA for RT-PCR was from excised mung bean hypocotyls treated with 10 mM LiCl for 0, 5, 15, 30, 45, 60, 240, or 480 min. Arrow indicates the RT-PCR product from the internal control (*LBP*) and asterisks indicate the RT-PCR product from the *ACS* gene.

$(dT)_{18}$, 2 μg of total RNA, and 8 pg of *LBP* synthetic mRNA in 5 μL were mixed, denatured at 70°C for 30 min, and annealed at 4°C for 2 min. Afterward, we added 5 μL of the cDNA master mixture that contained 1 mM dNTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 200 units of Moloney murine leukemia virus reverse transcriptase (RT). The complete mixture was incubated at 42°C for 1 h, and the reaction was stopped by incubation at 75°C for 10 min. DNA amplification was carried out in a 20-μL volume containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (w/v) Triton X-100, 1.6 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmole of each primer for the *ACS* genes, 10 pmole of each primer for *LBP*, 250 ng of the prepared cDNA mixture as template, and 0.8 unit of *Taq* DNA polymerase (Bioline). The procedure included 30 cycles at 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min. PCR amplification products were analyzed on a 1.4% (w/v) agarose gel. Table 1 and Figure 1 show the sequences of the designated primers and their binding positions on the target genes for ACC synthase, respectively.

RESULTS AND DISCUSSION

Li^+-Induced Expression of the ACC Synthase Gene Family

An inorganic ion, Li^+ promotes ethylene synthesis in plants (Zarembinski and Theologis, 1994), thereby influencing many physiological processes. To examine the effect of this ion on transcript accumulation, we treated excised mung bean hypocotyls with various concentrations of LiCl. The accumulation of transcripts for *ACS7* mRNA was greatly increased at levels of 10, 25, and 50 mM LiCl (Fig. 2). In contrast, no accumulation of transcripts for *ACS2* and *ACS3* was detected. Transcripts of *ACS4* were induced only slightly, remaining at nearly the same level regardless of the tested concentration. Accumulations of transcripts for *ACS1* and *ACS5* were highest in response to 1 mM of LiCl. *ACS6* showed the same expression pattern as for *ACS7*, although its transcription level was much lower.

For our time-course experiments, excised mung bean hypocotyls were treated with 10 mM of LiCl for 0, 5, 15, 30, 45, 60, 240, or 480 min. The RT-PCR assays that were performed to determine transcript accumulations showed that inductions varied among the *ACS* genes (Fig. 3). *ACS7* had the greatest change in dose response, being almost continuously induced during 30 min of LiCl treatment. In contrast, *ACS4* showed no distinct change in mRNA level over time. *ACS1* had a short-lived increase in the first 15 min, then decreased sharply after 60 min. Although not as dramatic as with *ACS1*, the accumulation of *ACS6* mRNA increased after 30 min, then decreased by 60 min. The accumulation of *ACS5* mRNA increased sharply at 480 min.

We compared the specificity of Li^+-induced accu-

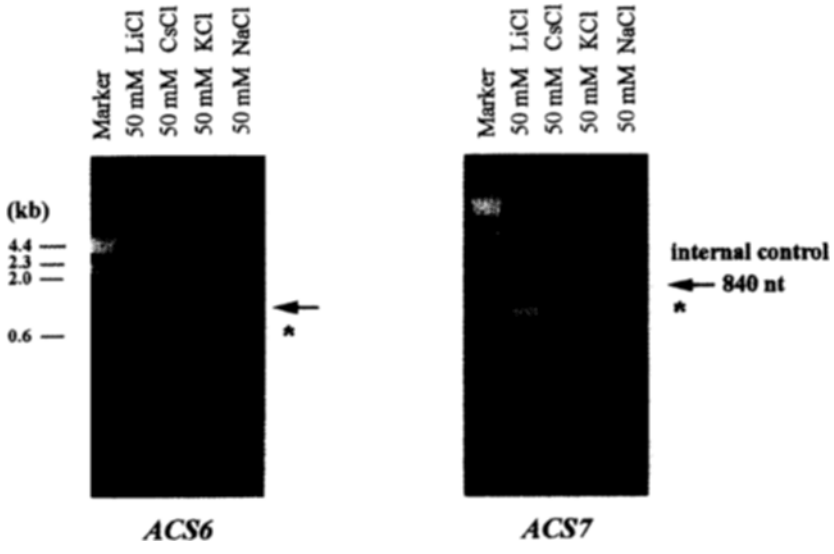

Figure 4. Specificity of the Li^+-induced accumulation of *ACS6* and *ACS7* mRNAs. RNA for RT-PCR was from excised mung bean hypocotyls treated with 50 mM of CsCl, KCl, or NaCl. Arrow indicates the RT-PCR product from the internal control (*LBP*) and asterisks indicate the RT-PCR product from the *ACS* gene.

mulations for *ACS6* and *ACS7* transcripts with that of other alkali ions in CsCl, KCl, or NaCl. For *ACS6*, accumulation appeared to be induced by salt stress, i.e., gene expression for samples treated with the other ions was the same as seen with LiCl (Fig. 4). In contrast, the transcription level of *ACS7* in response to the other alkali ions was much lower than with the same concentration of LiCl, suggesting that it was a LiCl-specific response.

Effects of Ca^{2+} on the Li^+-Inducibility of *ACS7*

Because Li^+ functions by interfering with the generation of the PI second messenger that regulates Ca^{2+} mobilization (Berridge et al., 1989; Keizer et al., 1995), its effects usually can be reversed with intermediates of the PI cycle or by Ca^{2+} (Wissocq et al., 1991). Ca^{2+} treatment interrupts the accumulation of *ACS5* transcripts induced by Li^+ in *Arabidopsis* (Liang et al., 1996). In the current study, we examined the effects of Ca^{2+} on LiCl induction of the *ACS7* gene. Expression of *ACS7* was increased for hypocotyls treated only with Ca^{2+} (Fig. 5). However, its level of induction was similar to those in the CsCl, KCl, or NaCl treatments (Fig. 4), suggesting that salt stress may have prompted the accumulation. Nevertheless, treatment with $CaCl_2$ (i.e., external Ca^{2+}) did not alter the Li^+-inducibility of *ACS7*, implying that this treatment could not reverse the effect of Li^+. These results clearly demonstrate that the mechanism of Li^+-action on *ACS7* expression is not similar to that for *ACS5* expression in *Arabidopsis*, where the PI cycle pathway may be involved in regulating gene expression.

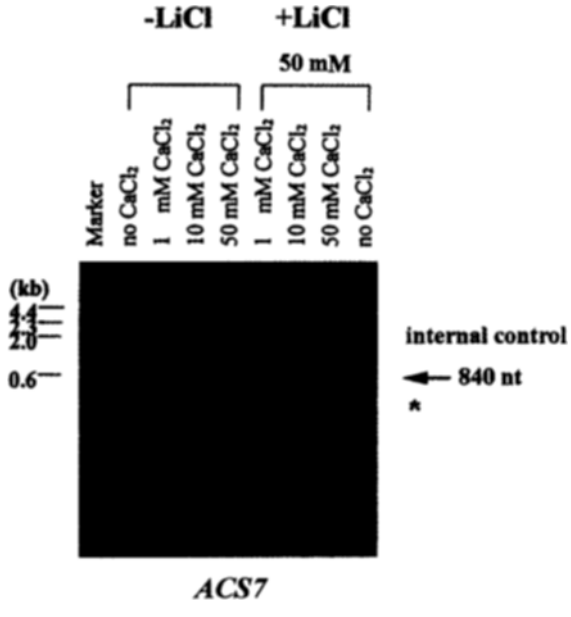

Figure 5. Effect of Ca^{2+} on Li^+-induced *ACS7* gene expression. RNA for RT-PCR was from excised mung bean hypocotyls treated with 1 mM, 10 mM, or 50 mM $CaCl_2$, plus 50 mM LiCl or without LiCl for 60 min. Arrow indicates the RT-PCR product from the internal control (*LBP*) and asterisks indicate the RT-PCR product from the *ACS7* gene.

ACKNOWLEDGEMENT

This work was supported in part by a grant from the Korea Research Foundation (1997), and by a grant from the Brain Korea 21 Project (2001).

Received May 15, 2001; accepted June 7, 2001.

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