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A mechanical strain-induced 1-aminocyclopropane-1-carboxylic acid synthase gene

(calcium/ethylene/signal transduction/touch/*Vigna radiata*)

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ABSTRACT **Ethylene production is observed in all higher plants, where it is involved in numerous aspects of growth, development, and senescence. 1-Aminocyclopropane-1-carboxylic acid synthase (ACC synthase; *S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14) is the key regulatory enzyme in the ethylene biosynthetic pathway. We are reporting an ACC synthase gene in *Vigna radiata* (mung bean) that is inducible by mechanical strain. The ACC synthase cDNA *AIM-1* was induced by mechanical strain within 10 min, reaching a maximum at 30 min, showing a dramatic reduction after 60 min, and showing no detectable message by 3 hr. The kinetics of induction for *AIM-1* was similar to a mechanical strain-induced calmodulin (*MBCaM-1*) in *V. radiata*, whereas the kinetics of its decline from maximum was different. When plants were subjected to calcium-deficient conditions, supplemental calcium, calcium chelators, calcium storage releasers, calcium ionophore, or calmodulin antagonists, there was no effect on *AIM-1*, indicating that the mechanical strain-induced *AIM-1* expression is a calcium-independent process. Induction of *MBCaM-1* in all cases behaved in the same way as *AIM-1*, suggesting that they share similar mechanically activated cis- and/or trans-acting elements in their promoter.**

Plants are exposed to strains induced by wind, vibrations, rain, plant parts rubbing against one another, and others. Some types of strains cause a decrease in rate of elongation and increase in lateral enlargement of plants. However, some mechanical strains also cause a decrease in photosynthesis, resulting in a reduction in growth and subsequent crop yield (1).

A close relationship exists between mechanical stimulation and ethylene responses in plants. Strain and ethylene induce similar alterations in growth (1). Braam and Davis (2) identified several transcripts in *Arabidopsis* that were elevated in response to mechanical stimulation. These cDNAs encode one calmodulin (CaM) and two calcium-modulated proteins. Interestingly, it was found that CaM mRNA undergoes a 100-fold elevation in response to touch. More recently, it has been shown that transcription was affected in response to mechanical strain stimulus (3). Two of the five mechanical strain-induced genes reported by Braam and Davis (2) are increased after exposure to ethylene; however, they specify that the response is delayed and weaker than a mechanical strain-induced response, suggesting that ethylene production is not the primary response to mechanical stimulation.

We have recently isolated from a *Vigna radiata* (mung bean) λgt11 library two different CaM cDNAs (*MBCaM-1* and *MBCaM-2*), which are 85% homologous inside the coding region but are highly divergent outside it and have different patterns of expression (4). *MBCaM-1*, which is the CaM probe used in the present study, is induced by mechanical strain, indoleacetic acid (IAA), and salt stress, albeit with different kinetics. In the absence of external stimuli, *MBCaM-1* exhibits day/night fluctuations with maximum expression occurring at the beginning of the dark period (4).

Ethylene production is observed in all higher plants, where it is involved in numerous aspects of growth, development, and senescence. Since *S*-adenosylmethionine (AdoMet) is involved in numerous other processes, the key regulatory step for ethylene formation is 1-aminocyclopropane-1-carboxylic acid synthase (ACC synthase; *S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14), which catalyzes the conversion of AdoMet to ACC (5). ACC can either be converted to ethylene via ACC oxidase or be modified by ACC *N*-malonyltransferase to produce malonyl-ACC, which is generally accepted to be an inactive end product (6). Recently, we have identified five different gene fragments for ACC synthase in *V. radiata* (7–9). One of them is a full-length cDNA (*AIM-1*), 1941 bp long, for an IAA-induced ACC synthase (8), which does not cross-hybridize with the others. There is little detectable *AIM-1* message found in nontreated *V. radiata* tissues; however, expression is apparent within 30 min after addition of 10 μM IAA, reaching a peak after ≈5 hr with a slight decrease in message after 12 hr. These changes in message correlate with changes in ACC levels found in these tissues after treatment with 10 μM IAA (8). In some species, mechanical strain has been shown to promote an increase in auxin, which promotes ethylene production via ACC (1). Although other hormones may play roles in mechanical strain-induced responses, evidence suggests that ethylene mediates mechanical strain-induced changes.

In contrast to plants, there is much known about mechanotransduction in animal cells and tissues. Mechanical stresses have been shown to stimulate various signal transduction mechanisms. Inositol phospholipid turnover and increases in inositol trisphosphate and diacylglycerol have been observed in endothelial cells subjected to shear stress due to blood flow (10). Adenylate cyclase activation and increase in cAMP have been shown in osteoblasts subjected to similar mechanical stress (11). Furthermore, shear stress-induced gene expression of platelet-derived growth factor (PDGF), *fos*, and *jun* has also been established (12, 13). Recently, it was shown that shear stress-induced PDGF expression was mediated by a cis-acting element in the promoter named shear stress responsive element (14). This element is also present in the promoter region of *fos* and *jun*. The exact nature of the mechanotransducing receptor is presently unknown. It is known, however, that some of the earliest mediators of the mechanical signal are guanine nucleotide binding proteins or G proteins (15, 16).

In this report, we describe the mechanical strain induction of ACC synthase (*AIM-1*) from *V. radiata* and signal trans-

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Abbreviations: CaM, calmodulin; ACC, 1-aminocyclopropane-1-carboxylic acid; IAA, indoleacetic acid.

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duction events involved in its induction. We show that an ACC synthase gene is regulated by mechanical strain, thereby paving the way to study the molecular regulation and signal transduction pathways involved in mechanical strain-induced ethylene.

MATERIALS AND METHODS

Plant Material and Treatments. *V. radiata* (Wilczek cv. Berken) seeds were imbibed for 24 hr in distilled water under aeration, sown in perlite, and grown at 25 ± 2°C for both the 16-hr day and 8-hr night period with cool white fluorescent and incandescent lamps. The perlite was carefully drenched every other day without touching the leaves with a solution containing 16 μM boric acid and 600 μM calcium nitrate. The concentration of calcium nitrate was reduced to 60 μM in experiments devoted to evaluate the effects of reduced calcium levels on *AIM-1* or *MBCaM-1* expression. Calcium levels in the leaves were determined by inductively coupled plasma emission spectroscopy at The Pennsylvania State University Agricultural Analytical Services Laboratory.

After 10 days, plants of uniform size were selected, and the roots and a portion of the hypocotyl were removed, leaving 8.5 cm below the two first true leaves. The cuttings were placed vertically in vials containing 5 ml of distilled water. After a 12-hr adaptation period, the remaining water was removed with small tubing and a syringe. Solutions containing signal transduction regulators were then applied to the basal portion of the stem. We have determined that under the same conditions it takes between 2 and 4 hr for compounds to move up the stem into the true leaves (4). Therefore, each regulator was allowed to be taken up for a period of 6 hr prior to the initiation of mechanical strain in order to be sure that the regulator being applied had sufficient time to reach its site of action. Mechanical strain, in the form of bending of each leaf by a force applied to the tip of the leaf, was applied to both leaves of the mung bean shoot. The two leaves were bent downward by ≈45° at the tip 30 times over a 30-sec period (at a frequency of 1 Hz). The force required to bend the leaves was ≈60 dynes (1 dyne = 0.1 μN). The average length of the leaves was 4 cm, and therefore the average bending moment was 240 dynes·cm. Leaves of the control plants were not subjected to such bending. Plants were placed in a growth chamber, and after the designated time period the leaves were removed, rapidly placed in liquid nitrogen, and stored at −80°C until analyzed (4). The elapsed time between cutting and freezing was <10 sec.

The effects of signal transduction regulators on *AIM-1* and *MBCaM-1* expression were tested on these 10-day-old mung bean plants. Regulators used in this study were 10 mM EGTA and 50 μM Quin 2-AM (calcium chelators); 1 μM thapsigargin (internal calcium storage releaser); 0.1 μM ionomycin (external calcium releaser); 50 μM A23187 (calcium ionophore), 10 mM $CaCl_2$ or $MgCl_2$; 10 μM W-7, 20 μM calmidazolium, 400 μM chlorpromazine, and 500 μM trifluoperazine (CaM inhibitors). All chemicals were purchased from either Sigma or Calbiochem.

Northern Blot Analysis. Northern blot analysis was carried out according to the procedure of Botella and Arteca (4). The membrane was first probed with *AIM-1*, stripped and reprobed with *MBCaM-1*, and stripped and reprobed using a pea ribosomal gene (17) to ensure that equal amounts of mRNA were present in each lane.

RESULTS

In a time course study outlined in Fig. 1, *AIM-1* mRNA levels of expression were detectable 10 min after mechanical stimulation; by 30 min there was a dramatic increase in message followed by a reduction in signal at 1 hr, with no detectable signal 3 hr after stimulation. Expression of *MBCaM-1* followed

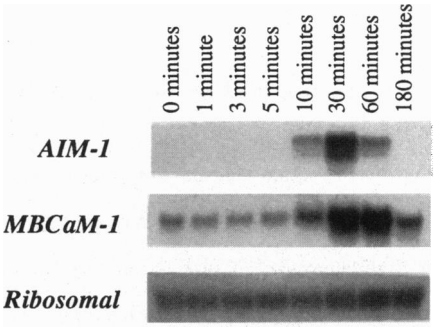

FIG. 1. Northern blot analysis of mechanical strain-induced ACC synthase (*AIM-1*) and CaM (*MBCaM-1*) gene expression over the time course indicated. Leaves from 10-day-old plants receiving no mechanical strain served as controls and others were mechanically stimulated for 30 sec; at the designated time interval, samples were taken and immediately placed in liquid nitrogen.

a pattern of induction similar to that of *AIM-1*. In contrast, however, *MBCaM-1* was always expressed at basal levels. Induction of *MBCaM-1* was apparent 10 min after mechanical stimulation and by 30 min maximum expression was observed, remained constant after 1 hr, but showed a decline 3 hr after mechanical stimulation.

To study the role of calcium in the mechanical strain induction of *AIM-1* and *MBCaM-1*, we evaluated the effects of EGTA, a known chelator of extracellular calcium. Surprisingly, EGTA was not able to block the induction of either gene (Fig. 2*A*). EGTA alone stimulated expression of *AIM-1* and *MBCaM-1* without mechanical strain but to a lower level than mechanical strain (Fig. 2*A*). This enhancement could have been due to stress induced by EGTA. In fact, *MBCaM-1* has been shown to be stimulated by salt stress (4). To determine the role of increases in intracellular calcium in the induction process, plants were treated with Quin 2-AM (13). Quin 2-AM was also ineffective in blocking mechanical strain-induced *AIM-1* and *MBCaM-1* (Fig. 2*B*). To evaluate the involvement of calcium without the use of inhibitors, plants were grown under reduced levels of calcium. Plants grown under low levels of calcium had 0.17% calcium by dry weight (dw) contained within their leaf tissue as compared to control plants, which had 1.55% calcium (dw), with the difference representing

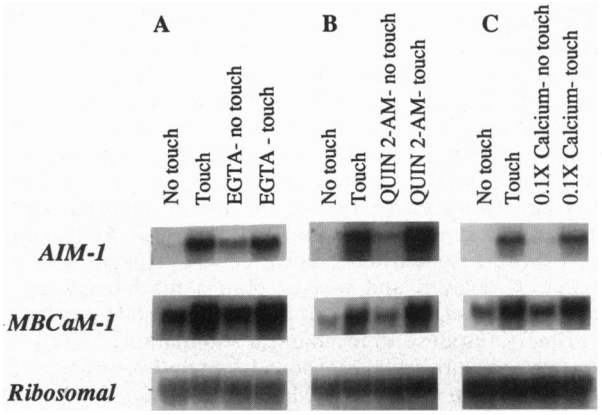

FIG. 2. Northern blot analysis of the effects of calcium chelators and calcium-poor plants on ACC synthase (*AIM-1*) and CaM (*MBCaM-1*) gene expression. Ten-day-old plants were treated with 10 mM EGTA (*A*) or 50 μM Quin 2-AM (*B*) for 6 hr; after treatment, plants were mechanically stimulated (Touch) for 30 sec or received no mechanical stimulation (No touch). Twenty minutes after stimulation, leaves were frozen in liquid nitrogen. Plants were grown with either standard calcium solutions or calcium-deficient solutions (0.1× Calcium) for 10 days and then mechanically stimulated for 30 sec; their leaves were collected and frozen after 20 min (*C*).

≈9-fold less calcium in plants grown under reduced calcium. There was no difference from the normal calcium control in the mechanical strain-induced expression of *AIM-1* or *MBCaM-1* in response to reduced calcium levels found in plant tissues, suggesting that their induction was calcium independent (Fig. 2*C*).

Since the use of calcium chelators and reduced calcium levels in plants failed to suppress the ability of mechanical strain to stimulate *AIM-1* and *MBCaM-1*, we evaluated different ways of increasing calcium within the plant. When plants were treated with $CaCl_2$, there was little or no difference in *AIM-1* and *MBCaM-1* expression (Fig. 3*A*). Plants treated with $MgCl_2$ as a control also showed no effect (Fig. 3*A*). Plants treated with thapsigargin (internal calcium storage releaser), ionomycin (external calcium storage releaser), or A23187 plus or minus calcium (calcium ionophore) also showed little or no difference in *AIM-1* or *MBCaM-1* expression (Fig. 3*A*). The question might be raised as to whether or not the compounds administered penetrated the cell, a point that can be raised with any exogenous applications. Others, however, have shown that these materials are effective in entering the cell (18). Our results with calcium chelators, calcium deficiency, and calcium mobilizing agents are all consistent with the hypothesis that calcium is not involved in mechanical strain induction of *AIM-1* and *MBCaM-1*.

To evaluate whether CaM was playing a role in the regulation of ethylene biosynthesis or its own expression, we evaluated the effects of four different CaM antagonists (chlorpromazine, trifluoperazine, calmidazolium, and W-7) at concentrations that have been shown to block CaM activity (19). None of the inhibitors tested was effective in blocking mechanical strain-induced *AIM-1* or *MBCaM-1* (Fig. 3*B*), once again supporting our other experiments showing that the process regulating these genes is calcium independent.

DISCUSSION

Plants are subject to a variety of environmental and endogenous mechanical forces and stresses. Mechanical stress has been postulated to mediate meristem morphogenesis (20). It has been suggested that the plant hormone ethylene mediates mechanical strain-induced morphogenetic changes. Little is known, however, about how mechanical strain induces ethylene production or the signal transduction process involved. In the present study, we utilized *V. radiata* plants because there is a large body of physiological information known about them. The use of mung bean plants also facilitated our physical and chemical treatments because of their morphology, their rapid growth rate, and our ability to obtain a large number of uniform plants and sufficient amounts of tissue to accomplish our objectives. ACC synthase protein is highly labile and found in low abundance in plants, making it difficult to accurately monitor its changes. In this study, we overcame this problem by monitoring expression of *AIM-1*, the gene encoding ACC synthase, thereby enabling us to evaluate the effects of mechanical stimulation on ethylene production.

Braam and Davis (2) identified five touch-induced (TCH) genes, three of which encoded either CaM or CaM-related proteins, suggesting that calcium ions and CaM are involved in the transduction of mechanical strain signals. These findings appear to be supported by additional research from the same group showing that the TCH genes in *Arabidopsis* cell cultures were stimulated in response to elevated levels of extracellular calcium (21). Whether there was an increase in intracellular calcium as a result of the increased extracellular calcium, however, was not determined. A subsequent study showed that wind-induced plant motion immediately elevated cytosolic calcium, also suggesting the involvement of calcium as a signal for mechanical strain (22). Although these studies suggest the involvement of calcium as a signal for mechanical strain, no clear role has been established at the present time. Our investigation, utilizing calcium chelators and ionophores, CaM antagonists, and calcium deprivation in a whole plant cutting, shows that intracellular calcium is not involved in mechanical strain-induced expression of two genes.

In summary, we are reporting that mechanical strain can induce the expression of an ACC synthase gene (*AIM-1*), which is the rate-limiting enzyme in the ethylene biosynthetic pathway. *MBCaM-1*, a gene encoding CaM, showed a striking parallel behavior to *AIM-1*, suggesting the existence of similar cis- or trans-acting factors controlling the expression of both genes, including mechanical factors. The evidence presented here strongly suggests the existence of a calcium-independent signal transduction pathway controlling the mechanical strain induction of *AIM-1* and *MBCaM-1*. Although the whole plant cutting model system used in this study has provided valuable information on the plants' response to mechanical strain at the whole plant level, it has limitations with respect to uptake of

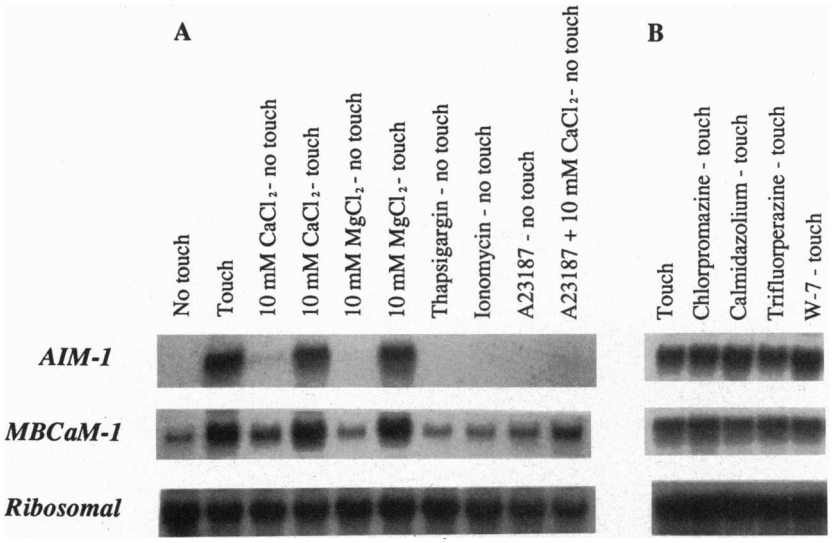

FIG. 3. Northern blot analysis of the effects of calcium, calcium storage releasers, calcium ionophore (*A*), and CaM antagonists (*B*) on ACC synthase (*AIM-1*) and CaM (*MBCaM-1*) gene expression. Ten-day-old plants were treated with 10 mM $CaCl_2$, 10 mM $MgCl_2$, 1 μM thapsigargin, 0.1 μM ionomycin, 50 μM A23187 with and without 10 mM $CaCl_2$, 400 μM chlorpromazine, 500 μM trifluoperazine, 20 μM calmidazolium, or 10 μM W-7 for 6 hr.

chemical agents and the ability to precisely define physical stimuli. Therefore, we are presently attempting to develop a cellular model system that will allow us to subject the cells to well defined physical forces. Using both experimental approaches will enable us to study the molecular regulation and signal transduction pathways involved in mechanical strain-induced ethylene production.

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