Arabidopsis thaliana Responses to Mechanical Stimulation Do Not Require ETR1 or EIN2

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Plants exposed to repetitive touch or wind are generally shorter and stockier than sheltered plants. These mechanostimulus-induced developmental changes are termed thigmomorphogenesis and may confer resistance to subsequent stresses. An early response of Arabidopsis thaliana to touch or wind is the up-regulation of TCH (touch) gene expression. The signal transduction pathway that leads to mechanostimulus responses is not well defined. A role for ethylene has been proposed based on the observation that mechanostimulus of plants leads to ethylene evolution and exogenous ethylene leads to thigmomorphogenetic-like changes. To determine whether ethylene has a role in plant responses to mechanostimulus, we assessed the ability of two ethylene-insensitive mutants, etr1–3 and ein2–1, to undergo thigmomorphogenesis and TCH gene up-regulation of expression. The ethylene-insensitive mutants responded to wind similarly to the wild type, with a delay in flowering, decrease in inflorescence elongation rate, shorter mature primary inflorescences, more rosette paraclades, and appropriate TCH gene expression changes. Also, wild-type and mutant Arabidopsis responded to vibrational stimulation, with an increase in hypocotyl elongation and up-regulation of TCH gene expression. We conclude that the ETR1 and EIN2 protein functions are not required for the developmental and molecular responses to mechanical stimulation.

In response to mechanical stimuli such as wind or touch, plants undergo physiological and developmental changes that enhance resistance to subsequent mechanical stress. In general, plants that are grown in windy environments or exposed to repetitive touch stimulation are shorter, stockier, and often have altered flexibility. These changes in development in response to mechanostimulus are collectively known as thigmomorphogenesis (Mitchell, 1996; Ennos, 1997).

In Arabidopsis thaliana wind or touch stimulation results in the enhancement of expression of the touch (TCH) genes. TCH gene mRNAs accumulate very rapidly, within 10 min of touch stimulation of plants (Braam and Davis, 1990). TCH1 encodes CaM (Braam and Davis, 1990), TCH2 and TCH3 encode CaM-related proteins (Braam and Davis, 1990; Sistrunk et al., 1994; Khan et al., 1997), and TCH4 encodes a xyloglucan endotransglycosylase capable of modifying cell wall xyloglucans (Xu et al., 1995). Mechanostimulus regulation of TCH gene expression suggests that TCH protein function may contribute to the process of thigmomorphogenesis, e.g. by altering the properties of the cell wall (Antosiewicz et al., 1995; Braam et al., 1996; Xu et al., 1996).

The mechanostimulus signal transduction pathway leading to thigmomorphogenesis is not yet well defined. Changes in concentrations of cytoplasmic calcium may play the role of second messenger, transducing the mechanostimulus into an intracellular signal. Dramatic up-regulation of expression of the CaM and CaM-related TCH genes following mechanical stimulation implicates the involvement of Ca2+-binding proteins in touch responses of plants (Braam and Davis, 1990). In addition, rapid increases in concentrations of cytoplasmic calcium occur in plants subjected to touch or wind stimulation (Knight et al., 1991; Haley et al., 1995). Finally, externally applied Ca2+ (Braam, 1992) and Ca2+-channel antagonists (Polisensky and Braam, 1996) have been shown to affect the expression of touch-induced genes.

Recent evidence implicates the possible involvement of protein kinases in the mechanostimulus response pathway of plants. The activities of protein kinases have been shown to be rapidly increased following wounding or mechanical stimulation; in vitro phosphorylation assays demonstrate activation within 1 to 5 min of stimulation (Suzuki and Shinshi, 1995; Bøgre et al., 1996, 1997). In addition, expression levels of genes encoding mitogen-activated protein kinases and other protein kinases are increased within 30 min following wounding or mechanical stimulation (Seo et al., 1995; Mizoguchi et al., 1996; Bøgre et al., 1997). This suggests that mitogen-activated protein and other protein kinases, possibly Ca2+-dependent protein kinases (Piotrowski et al., 1996) and ribosomal protein S6 kinase (Mizoguchi et al., 1996), are involved in the mechanostimulus signal transduction pathway leading to gene expression.

For many years a role for the phytohormone ethylene in thigmomorphogenesis has been suspected. Touch stimulation results in a rapid increase in ethylene evolution (Goeschl et al., 1966; Poovaiah, 1974; Eisinger, 1983; Pressman et al., 1983; Biro and Jaffe, 1984; Takahashi and Jaffe, 1984), coinciding with a touch-induced increase in ACC synthase.

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Abbreviation: CaM, calmodulin.
activity (Biro and Jaffe, 1984), a key enzyme in ethylene biosynthesis. Mechanical impedance of maize roots also results in an increase in ethylene production, which is believed to be responsible for inducing root swelling and aerenchyma formation (Sarquis et al., 1991; He et al., 1996a). In addition, exogenous application of ethylene to plants often results in developmental and morphological changes that are similar to those occurring during thigmomorphogenesis (Goeschl et al., 1966; Brown and Leopold, 1972; Jaffe and Biro, 1979; Salveit et al., 1979; Erner and Jaffe, 1982; de Jaegher et al., 1987). For example, both exogenous ethylene treatment and touch stimulation lead to development of shorter tracheids (Biro et al., 1980; Telewski et al., 1983), alterations in cell shape (Jaffe and Biro, 1979), and changes in membrane fatty acid content (Erner and Jaffe, 1983).

Arabidopsis mutants defective in sensing or responding to ethylene are valuable tools for assessing the potential role of ethylene in plant responses to mechanical stimulation. The dominant etr1–3 (Bleecker et al., 1988; Chang et al., 1993) and recessive ein2–1 (Guzman and Ecker, 1990; Roman et al., 1995) mutants were identified because of their ethylene-insensitive growth and have been shown to be blocked in ethylene-induced gene transcription (Samac et al., 1990; Lawton et al., 1994). ETR1 is similar to the two-component His kinase receptors and most likely functions as an ethylene receptor (Bleecker et al., 1988; Chang et al., 1993; Schaller and Bleecker, 1995). The sequence and function of EIN2 have not yet been reported.

To determine whether ethylene response pathways are involved in controlling plant responses to mechanical stimuli, we examined whether etr1–3 and ein2–1 mutants display appropriate developmental alterations following mechanical stimulation. Furthermore, regulation of TCH gene expression was monitored to determine whether ethylene signaling is required for mechanostimulus-induced regulation of gene expression.

MATERIALS AND METHODS

Origin of Seed Stocks

ColO, etr1–3, and ein2–1 seeds were obtained from the Arabidopsis Stock Center (The Ohio State University, Columbus).

Treatment of Plants and Seedlings

For assessment of thigmomorphogenesis, plants were grown individually in pots under constant light at 22°C; at 14 d of age, plants were exposed to wind three times daily for 30 min using oscillating fans. Measurements were taken on plants throughout development, and final height measurements were determined when senescence was apparent (i.e. when the first silique turned brown). Measurements are reported as means ± se, and statistical significance was determined using Student’s t test.

Touch stimulation was performed on 14-d-old soil-grown plants (approximately 6–10 plants per 4-inch pot) by gently touching the rosette leaves and bending the plants back and forth 10 times. To generate sterile plants, seeds were treated briefly with 95% ethanol, suspended for 14 min in 5.25% sodium hypochlorite (Clorox), rinsed three times with sterile water, and then resuspended in sterile water before plating on a growth medium consisting of 0.5× minimal salts (Sigma), 1% Suc, and 1× Gamborg’s vitamins (Sigma), pH 5.7. After the seeds were plated, they were placed at 4°C in the dark for 4 d to enhance germination efficiency.

A vibration was applied by attaching the plates to a platform covering a 15-inch subwoofer speaker. The low-frequency vibration (50 Hz) was at approximately 90 dB at the speaker surface. For hypocotyl-length analysis, seedlings were grown in the dark on filter paper soaked with growth medium and were subjected to vibrational stimulation starting at the time of germination and continuing for 72 h. Measurements were made immediately after the cessation of stimulation. For vibration-induced gene expression analysis, light-grown 14-d-old seedlings grown on 0.8% agar in growth medium were subjected to 10 min of stimulation and then harvested at the indicated times following the cessation of stimulation.

RNA Analysis

Plants were harvested, placed immediately in liquid N2, and then ground to a powder with a mortar and pestle. RNA was isolated (Verwoerd et al., 1989), size separated on formaldehyde-agarose gels, and transferred to nylon membranes (Sambrook et al., 1989). Hybridization of the RNA blots was performed as described by Sambrook et al., (1989) using the radioactively labeled (Feinberg and Vogelstein, 1983) cloned cDNA fragments described by Braam and Davis (1990).

RESULTS

Growth Responses to Wind

The requirement for ethylene-regulated processes in the developmental responses of Arabidopsis to mechanical stimulation was investigated using the ethylene-insensitive mutants etr1–3 and ein2–1. The effects of wind on aspects of plant development were monitored to determine whether etr1–3 and ein2–1 plants responded similarly to the wild type.

Plants treated with wind showed a delay in the initiation of the inflorescence growth relative to control plants (Fig. 1A). Inflorescence emergence of wild-type plants was delayed by almost 6 d (25.6 ± 0.6 versus 31.3 ± 0.5 d for control and wind treatments, respectively), whereas emergence of etr1–3 and ein2–1 inflorescences was delayed by 3 (26.7 ± 0.3 versus 30.3 ± 0.3 d) and 8 d (28.7 ± 0.5 versus 37.0 ± 1.0 d), respectively. Wind treatment also retarded the rate of inflorescence elongation. In wild-type plants the initial rate of inflorescence elongation from inflorescence initiation to 15 cm in height was reduced from 22.0 ± 0.5 to 19.8 ± 0.6 mm/d (P < 0.01; data not shown). A more significant reduction of growth rate was evident when the increment of growth between 15 and 25 cm in height was...
assessed. Wild-type plants showed a reduction in growth rate from 22.8 ± 0.6 to 16.3 ± 0.4 mm/d (P < 0.001; Fig. 1B). The growth rate reduction, as a consequence of wind treatment, was also observed for both ethylene-insensitive mutants analyzed. From inflorescence emergence to elongation to 15 cm, etr1–3 growth rate was reduced from 24.3 ± 0.4 to 23.7 ± 1.1 mm/d (not significant) and ein2–1 elongation rate was changed from 23.9 ± 0.6 to 20.2 ± 0.6 mm/d (P < 0.001; data not shown). In addition, reductions in elongation rates between 15 and 25 cm were observed for the mutants. The growth rate of etr1–3 was reduced from 23.9 ± 0.8 to 15.6 ± 0.6 mm/d (P < 0.001) and that for ein2–1 was decreased from 25.3 ± 1.0 to 13.9 ± 0.8 mm/d (P < 0.001; Fig. 1B).

Individual inflorescences were considered mature when the first silique turned brown, and the heights of mature inflorescences were determined. Inflorescence height of wild-type plants was reduced from 397.4 ± 5.9 mm for control plants to 339.8 ± 5.2 mm for wind-treated plants (P < 0.001; Fig. 1C). The inflorescences of the ethylene-insensitive mutants were also reduced in height when plants were exposed to wind. For the etr1–3 mutant, mature inflorescence height was reduced from 395.2 ± 8.5 to 341.9 ± 5.5 mm (P < 0.001), and for ein2–1, the reduction was from 456.5 ± 9.1 to 338.4 ± 10.0 mm (P < 0.001).

Wind treatment of plants was also found to increase the number of rosette paraclades (secondary inflorescences) that formed when the primary inflorescences reached 25 cm.
cm in height. Most of the untreated wild-type plants had two or three rosette paraclades (average 2.6 ± 0.2), whereas most wind-treated wild-type plants had four paraclades (average 4.1 ± 0.1, P < 0.001; Fig. 1D). Increases in the number of rosette paraclades were observed for the ethylene-insensitive mutants. The calculated average of rosette paraclades for etr1–3 mutants was 3.1 ± 0.1 paraclades on untreated plants and 3.6 ± 0.1 paraclades on wind-treated plants (P < 0.01; Fig. 1D). Most untreated ein2–1 mutants had one or two paraclades (average 1.7 ± 0.2), whereas most wind-treated ein2–1 plants had four or five rosette paraclades (average 4.3 ± 0.2 P < 0.001; Fig. 1D).

**Touch-Induced Gene Expression**

To determine whether ETR1 and EIN2 protein functions are necessary for the rapid up-regulation of expression of the TCH genes by touch stimulation, we assayed TCH mRNA levels of control and touch-stimulated wild-type etr1–3 and ein2–1 plants. Northern analysis of etr1–3 and ein2–1 revealed that the TCH mRNAs increased in abundance following touch stimulation, with similar kinetics as in wild-type plants (Fig. 2). In all three genetic backgrounds, increases in the TCH mRNAs were detectable within 10 min following touch stimulation and peaked in abundance by 20 min after stimulation (Fig. 2). Therefore, signaling through ETR1 or EIN2 is clearly not necessary for the rapid up-regulation of the TCH genes following mechanical stimulation.

**Gene Expression and Growth Responses to Vibration**

Another method of delivering a mechanical stimulus to plants is to apply vibration. An advantage of a vibratory stimulus is that it can be quantified with respect to frequency and amplitude, thus making it feasible to provide an approximately reproducible stimulus.

A continuous, low-frequency (50 Hz) vibration was applied to 14-d-old seedlings, and expression levels of the TCH genes were monitored. Northern analysis indicated that the TCH genes were up-regulated in expression following vibration, with maximal RNA accumulation approximately 30 min after the initiation of stimulation (Fig. 3). The increases in TCH mRNA levels were much less dramatic than the up-regulation observed in touch-stimulated plants. This suggests that vibration provides a weaker mechanical strain than touch. In general, the kinetics and magnitude of vibration-induced TCH mRNA accumulation in the ethylene-insensitive mutants were similar to that of wild-type plants (Fig. 3). However, induction of TCH4 expression appears to be more prolonged in the etr1–3 mutant.

Takahashi et al. (1991) showed that treatment of germinating rice (Oryza sativa L.) or cucumber (Cucumis sativus L.) seeds with a 50-Hz vibration for 72 h results in a stimulation of the hypocotyl elongation rate. To test whether Arabidopsis plants also show a stimulatory growth response to vibration and whether any morphogenetic response to vibration requires the ETR1 or EIN2 proteins, we assessed the effects of a 50-Hz vibration on wild-type, etr1–3, and ein2–1 seedlings. Consistent with results seen for rice and cucumber, vibration of Arabidopsis seedlings for 72 h resulted in a stimulation of hypocotyl elongation (Fig. 4). At 72 h, stimulated wild-type seedling hypocotyls were 5.2 ± 0.1 mm in length, whereas untreated plant hypocotyls were approximately 3.6 ± 0.1 mm (P < 0.001). ETR1 and EIN2 are not required for this response because both ethylene-insensitive mutants showed increases in hypocotyl elongation (4.8 ± 0.1 to 7.1 ± 0.2 mm for etr1–3 and 3.9 ± 0.2 to 5.7 ± 0.2 mm for ein2–1; P < 0.001) after exposure to vibratory stimulation.

**DISCUSSION**

Mechanical stimuli such as wind bursts or direct contact are frequently encountered by plants. Plants have evolved the ability to sense and respond to these stimuli in ways that increase their resistance to such stresses. Arabidopsis plants have been shown previously to be delayed in bolting and display overall less elongation in response to repetitive touch stimulation (Braam and Davis, 1990). In addition, a
prominent and fast molecular response occurs in Arabidopsis; the *TCH* genes are up-regulated in expression in response to mechanical stimuli. The work described here takes a genetic approach to investigating the role of the ethylene-signaling pathway in controlling the developmental and molecular responses to mechanical stimulation. Definitive evidence demonstrates that *ETR1* and *EIN2*, two loci that act early in ethylene signaling, are not required for plants to sense and respond to different forms of mechanical stress. The *etr1–3* and *ein2–1* Arabidopsis mutants show a delay in inflorescence emergence, a reduced rate of inflorescence elongation, and a decrease in the height of mature inflorescences in response to wind treatment; these responses are similar to those of wind-treated wild-type plants (Fig. 1). Slight differences in the sensitivities between genotypes may be due to the overall structure and stature of the plants, making them differentially sensitive to mechanical perturbation. The critical result is that the ethylene mutants are clearly able to respond to the mechanical stimulus.

We also investigated whether Arabidopsis displays growth-response changes when subjected to the mechanical stimulus of vibration. The elongation rate of hypocotyls of etiolated Arabidopsis, like that of etiolated rice and cucumber hypocotyls (Takahashi et al., 1991), is enhanced when seedlings are subjected to a 50-Hz vibration (Fig. 4). This mechanoresponse also occurs in plants lacking *ETR1* or *EIN2* function. Therefore, the developmental and growth alterations that occur in Arabidopsis plants subjected to either wind or vibration do not require the functioning of *ETR1* or *EIN2*. *ETR1* and *EIN2* are not necessary for the up-regulation of *TCH* gene expression in response to touch or vibration (Figs. 2 and 3). Hence, ethylene is unlikely to be involved in either the molecular or developmental responses of plants to mechanical stimuli.

Previous observations support these conclusions. Plants treated with touch or shaking show a rapid (1–3 min) reduction in the rate of elongation, whereas evolution of ethylene is not detectable until 30 to 45 min following stimulation (Goeschl et al., 1966; Jaffe and Biro, 1979). Additional data that help rule out an early required role for ethylene biosynthesis in the mechanoresponse pathway is the recent finding that there is a very rapid phosphorylation of protein kinases following touch or wounding (Suzuki and Shinshi, 1995; Usami et al., 1995; Bögre et al., 1996, 1997). Furthermore, treatment of plants with some inhibitors of ethylene production or action have been shown to have no effect on thigmomorphogenesis (Takahashi and Suge, 1980; Boyer et al., 1983; Biddington, 1986) or to reduce radial expansion only, with no effect on touch-induced decreases in elongation growth (Biro and Jaffe, 1984). In contrast, however, Boyer et al. (1979, 1983, 1986) reported that ethylene action/biosynthesis inhibitors can reduce the thigmomorphogenetic effects on both radial expansion and elongation. Because Arabidopsis plants do not display a significant increase in radial expansion following mechanical stimulation, we are unable to test whether the ethylene response effectors *etr1–1* and *ein2–1* affect changes in radial expansion following mechanical stress.

In summary, the work presented here indicates that *ETR1* and *EIN2* protein functions are not required for the molecular and developmental responses of Arabidopsis to mechanical stress. A more direct mechanosignaling/transduction pathway must exist, perhaps involving the function of transmembrane proteins (He et al., 1996b; Brummell et al., 1997; Thonat et al., 1997) and/or stretch-activated channels (Falke et al., 1988; Zimmermann et al., 1997) as mechanosensors, Ca$^{2+}$ as a second messenger (Knight et al., 1991; Braam, 1992; Bush, 1995; Haley et al., 1995; Polisensky and Braam, 1996), and a kinase cascade (Suzuki and Shinshi, 1995; Usami et al., 1995; Bögre et al., 1997) to signal mechanoresponses, such as *TCH* gene expression (Braam and Davis, 1990), oxidative burst (Yahraus et al., 1995), cell wall changes (Bradley et al., 1992; Levine et al., 1994; Xu et al., 1995), and growth alterations (Jaffe, 1973; Jaffe and Forbes, 1993; Mitchell, 1996).

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**LITERATURE CITED**


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