Mechanically Stimulated *TCH3* Gene Expression in Arabidopsis Involves Protein Phosphorylation and EIN6 Downstream of Calcium[1]

Andrew J. Wright, Heather Knight, and Marc R. Knight*

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, United Kingdom

Mechanical signals are important both as environmental and endogenous developmental cues in plants. Among the quickest measurable responses to mechanical stimulation (MS) in plants is the up-regulation of specific genes, including *TCH3*, in Arabidopsis. Little is known about the signaling events and components that link perception of mechanical signals to gene expression in plants. Calcium has been identified previously as being potentially involved, and a role for ethylene has also been suggested. Using the protein kinase inhibitor staurosporine, we determined that MS up-regulation of *TCH3* expression requires protein kinase activity in young Arabidopsis seedlings. Our data from studies on the Arabidopsis *ein6* mutant demonstrate that the EIN6 protein is also required, but that its role in mechanically induced *TCH3* expression appears to be independent of ethylene. Challenge of seedlings with protein phosphatase inhibitors calyculin A and okadaic acid stimulated *TCH3* expression even in the absence of MS, implying protein phosphatase activity acting to negatively regulate *TCH3* gene expression. This phosphatase activity acts either downstream or independently of EIN6. EIN6 and protein kinase activity, on the other hand, operate downstream of calcium to mediate mechanically stimulated *TCH3* expression.

Plant development is receptive to and influenced by mechanical signals, both internal and external. It has been speculated that cellular tension and compression within tissues during normal plant cell growth may act as a signal to alter the direction planes of cell division and possibly affect cell differentiation (Biro et al., 1980; for summary, see Trewavas and Knight, 1994). Environmental cues such as wind, touching, rubbing, and growth against objects are perceived by plants, and induce specific responses. These involve alterations in the growth of plants to cope and compensate for these mechanical variables in processes known as thigmomorphogenesis (Jaffe and Forbes, 1993) and thigmotropism (Okada and Shimura, 1990). Plant growth regulators, e.g. auxin, abscisic acid, and ethylene, have been implicated as being involved in the processes of thigmomorphogenesis (Jaffe and Biro, 1979; Biro and Jaffe, 1984; Erner and Jaffe, 1982) and thigmotropism (Okada and Shimura, 1990). However, knowledge of the signaling pathways leading from perception (the nature of which is itself not understood) of mechanical signals to such growth responses is very limited.

Calcium has been postulated to be involved in mechanical stimulation (MS) signaling by several strands of evidence. The treatment of soybean (*Glycine max*) plants with calcium antagonists has been shown to inhibit the growth responses during thigmomorphogenesis (Jones and Mitchell, 1989). In addition, MS in the form of touch and wind has been shown to cause rapid elevations in cytosolic-free calcium concentration ($[Ca^{2+}]_{cyt}$) in several plant species, including Arabidopsis (Knight et al., 1991, 1992, 1995; Haley et al., 1995). As well as these rapid changes in $[Ca^{2+}]_{cyt}$, one of the earliest of responses of plants (apart from specialized plants such as the Venus flytrap [*Dionaea muscipula*] and *Mimosa pudica*) to mechanical signals, often measurable after just a few minutes of stimulation (Braam and Davis, 1990), is the up-regulation of specific genes (Braam and Davis, 1990; Botella et al., 1995; Mizoguchi et al., 1996). In Arabidopsis, for instance, several touch (*TCH*) genes have been identified, including *TCH3* (Braam and Davis, 1990). *TCH3* is greatly up-regulated in response to a variety of mechanical signals, with peak expression (as measured by steady-state transcript levels) occurring 30 min after stimulation (Braam and Davis, 1990). It is interesting that calcium again has been implicated in the expression of these genes. Addition of exogenous calcium to Arabidopsis cell suspension cultures (Braam, 1992) causes induction of *TCH3* expression in the absence of a primary (mechanical) signal. Additionally, *TCH3* expression is inhibited in the presence of calcium antagonists when the gene is induced in response to cold (Polisensky and Braam, 1996). In addition to calcium, there is the potential for ethylene to be involved in MS signaling. It is interesting that ethylene can induce the expression of *TCH3* in the absence of a primary (mechanical) signal (Sistrunk et al., 1994). However, some other evidence would suggest

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* Corresponding author; e-mail marc.knight@plants.ox.ac.uk; fax 44–1865–275023.

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that ethylene is not actually used in planta to mediate MS up-regulation of *TCH3* (Johnson et al., 1998).

This current study was aimed at determining whether there was evidence of components other than calcium in the signaling pathway(s) leading from perception of MS to *TCH3* gene up-regulation specifically in Arabidopsis seedlings. Having identified such potential signaling components, this study also aimed to determine where they acted (upstream/downstream) relative to calcium and to each other in these signal transduction pathway(s).

RESULTS

Protein Kinase and Protein Phosphatase Inhibitors Affect Mechanically Stimulated Expression of *TCH3*

To test for the potential involvement of protein phosphorylation events in the pathway leading from MS to *TCH3* gene expression, we tested the effect of the protein kinase inhibitor, staurosporine, and the protein phosphatase inhibitors, okadaic acid and calyculin A, on mechanically stimulated *TCH3* expression in Arabidopsis seedlings (Fig. 1). At a concentration of 10 μM, staurosporine significantly inhibited mechanically stimulated *TCH3* expression. Okadaic acid and calyculin A (both at 1 μM) produced significantly enhanced *TCH3* expression in both mechanically stimulated and control (non-stimulated) samples. The induction of *TCH3* expression by okadaic acid and calyculin A did not seem to depend at all on the involvement of the primary signal, i.e. MS. To examine the dose dependency of these inhbitors, the effect of different concentrations of staurosporine, okadaic acid, and calyculin A on *TCH3* expression was tested (Fig. 2). Staurosporine showed inhibition of mechanically stimulated *TCH3* expression at all the concentrations tested (0.1, 1, and 5 μM), with the severity of inhibition increasing with increasing staurosporine concentration (Fig. 2A). The induction of *TCH3* expression by calyculin A was first detectable at a concentration of 0.1 μM, where significantly greater expression of *TCH3* was detected in the inhibitor-treated, but nonmechanically stimulated, sample

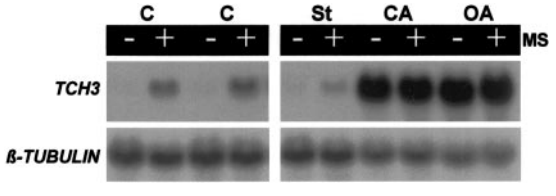

Figure 1. Effect of protein kinase and phosphatase inhibitors on mechanically stimulated *TCH3* gene expression in Arabidopsis seedlings. Seedlings in flasks were treated for 4 h in either 10 μM staurosporine (St), 1 μM calyculin A (CA), 1 μM okadaic acid (OA), or 1% (v/v) dimethyl sulfoxide (DMSO) as a control in duplicate (C), after which time flasks were shaken for 60 s (+MS) or left undisturbed (−MS). Thirty minutes after shaking, tissue was harvested, total RNA extracted, and *TCH3* and *β-tubulin* mRNA levels detected by RNA-blot hybridization.

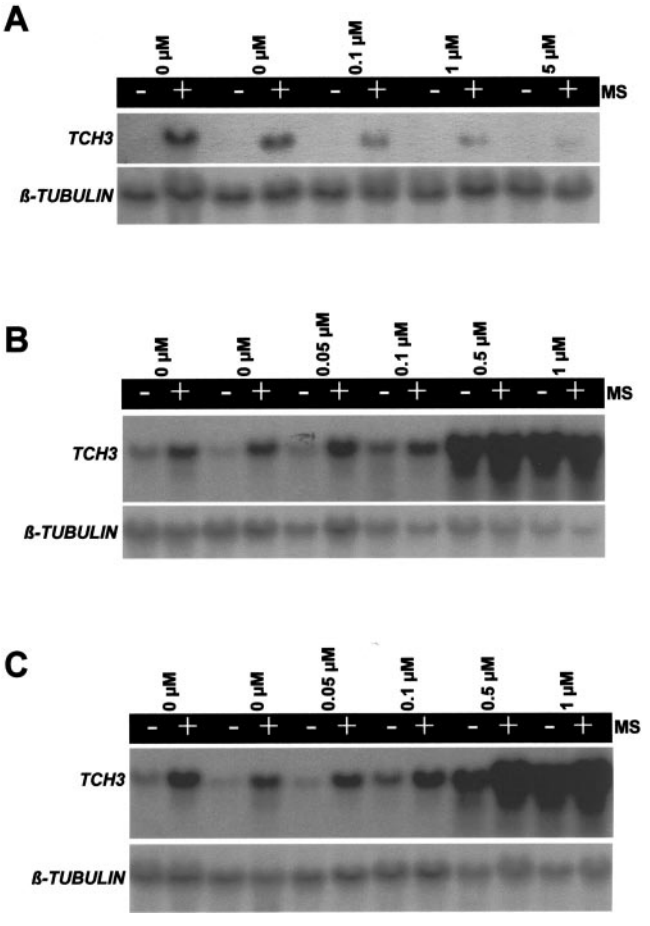

Figure 2. Dose dependence of the effect of staurosporine, calyculin A and okadaic acid on *TCH3* gene expression in Arabidopsis seedlings. Seedlings were treated and experiments performed exactly as described in the figure legend for Figure 1 with 0, 0.1, 1, and 5 μM staurosporine (A); 0, 0.05, 0.1, 0.5, and 1 μM calyculin A (B); and 0, 0.05, 0.1, 0.5, and 1 μM okadaic acid (C) being added. All 0 μM controls are presented in duplicate.

than in the corresponding unstimulated control sample (Fig. 2B). This effect became very clear at 0.5 μM calyculin A, where it appeared to be maximal (there was no greater effect at 1 μM; Fig. 2B), suggesting that the calyculin A effect saturates at 0.5 μM. The situation with okadaic acid (Fig. 2C) was very similar as for calyculin A, except that *TCH3* induction in non-induced plants at 0.5 μM okadaic acid was significantly less than for 0.5 μM calyculin A. As a consequence, for okadaic acid, 1 μM produced a greater effect on *TCH3* expression than did 0.5 μM. This implies that the okadaic acid effect saturates at 1 μM or greater concentrations of this particular inhibitor.

Mechanically Stimulated Expression of *TCH3* Is Reduced in the *ein6* Mutant of Arabidopsis

Ethylene has for some time been strongly implicated in thigmomorphogenesis (Jaffe and Biro, 1979; Biro and Jaffe, 1984) and *TCH3* expression specifi-

cally has been shown to be inducible by the application of exogenous ethylene gas (Sistrunk et al., 1994). However, even taking these data together, it cannot be concluded that in planta, ethylene is actually used as a component of a chain of events leading from MS to *TCH3* expression. The fact that the molecular (including *TCH* gene expression) and physiological responses to MS have been reported to be unaffected in *ein2* and *etr1* ethylene-insensitive mutants of Arabidopsis would support this view (Johnson et al., 1998). To test whether Arabidopsis actually uses ethylene in planta to mediate MS induction of *TCH3*, we examined the mechanically stimulated *TCH3* expression in a number of ethylene-related mutants of Arabidopsis. These mutants included ethylene-insensitive mutants *eir1-1*, *ein2-1*, *ein3-1*, *ein4*, *ein5-5*, *ein6*, *ein7*, *hls1-1*, and *etr1-1* (Chao et al., 1993, 1997; Roman et al., 1995; Alonso et al., 1999; Raz and Ecker, 1999); ethylene overproducing mutants *eto1-1*, *eto2*, and *eto3* (Guzman and Ecker, 1990; Woeste et al., 1999); and the ethylene-constitutive mutant *ctr1-1* (Kieber et al., 1993; Roman et al., 1995). The uninduced levels of *TCH3* transcript appeared similar in all mutants, even the *ctr1* and the ethylene-overproducing mutants. All mutants showed significant mechanically stimulated *TCH3* expression comparable with wild type (Fig. 3), except for *ein6* (Fig. 3, A and B). The *ein6* mutant consistently showed a greatly reduced, or no, mechanically stimulated *TCH3* expression.

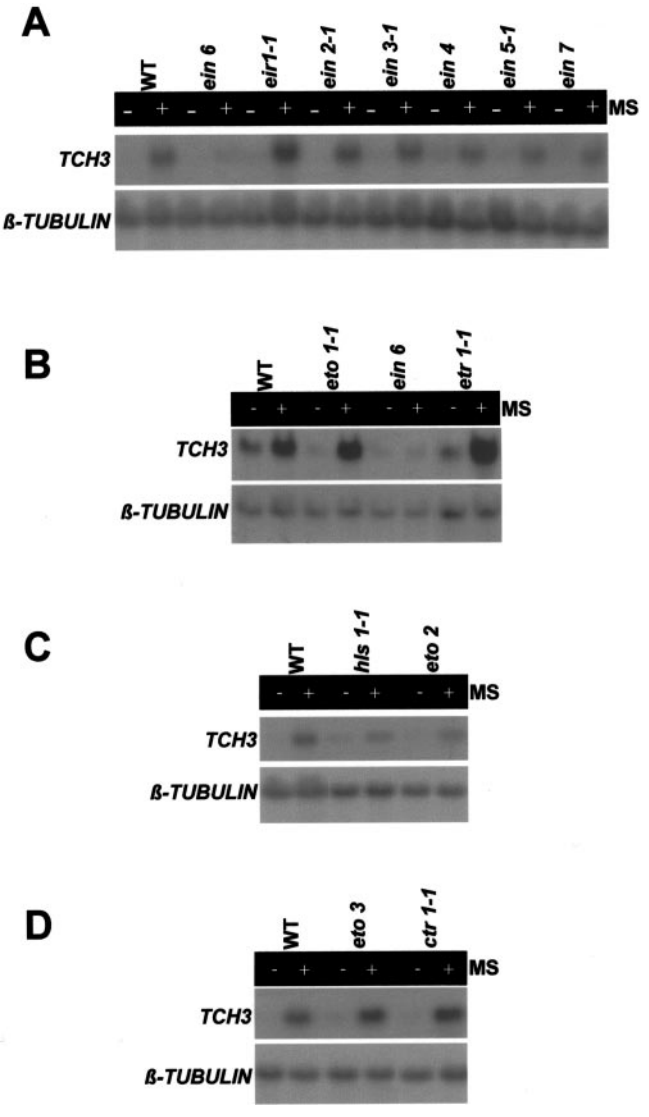

Figure 3. Mechanically stimulated *TCH3* gene expression in seedlings of ethylene mutants of Arabidopsis. Wild-type and mutant seedlings were grown on agar plates and replicate plates either mechanically stimulated for 10 s (+MS) or left undisturbed (−MS). Thirty minutes after shaking, tissue was harvested, total RNA extracted, and *TCH3* and *β-tubulin* mRNA levels detected by RNA-blot hybridization. Mutants analyzed included ethylene-insensitive mutants *eir1-1*, *ein2-1*, *ein3-1*, *ein4*, *ein5-1*, *ein6*, and *ein7* (all A); *hls1-1* (C), and *etr1-1* (B); ethylene-overproducing mutants *eto1-1* (B), *eto2* (C), and *eto3* (D); and the ethylene-constitutive mutant *ctr1-1* (D).

Calcium-Induced *TCH3* Expression Is Inhibited by Staurosporine and the *ein6* Mutation

Calcium has been implicated as a second messenger involved in signaling pathways leading to *TCH3* gene expression. In Arabidopsis cell suspension cultures, the addition of extracellular calcium induces the expression of *TCH3* (Braam, 1992) and mechanically stimulated transient elevations in $[Ca^{2+}]_{cyt}$ have been detected in plants including Arabidopsis (Knight et al., 1991, 1992, 1995; Haley et al., 1995). To see if the inhibitory effects of staurosporine and *ein6* were either upstream or downstream of calcium, it was desirable to measure calcium-induced *TCH3* expression in whole seedlings. First of all, we tested whether externally added calcium chloride could in fact induce *TCH3* in whole seedlings in our experimental system (Fig. 4). In these experiments, we mechanically desensitized the seedlings overnight as described in "Materials and Methods." This was necessary to allow the observation of the effect of extracellular calcium addition, which would not be possible with the high background of *TCH3* expression because of MS provoked by the addition itself. Magnesium chloride (isoosmotic to the calcium chloride) was used as a control for the calcium ion, and also to control for the possible effect of osmotic shock on *TCH3* expression. The addition of 100 mM $MgCl_2$ caused a relatively small elevation of *TCH3* expression. In contrast, 100 mM $CaCl_2$ caused a much more substantial induction of *TCH3* expression. Thus, the inducing effect specifically attributable to the calcium ion was clear in this experimental setup. The small induction caused by the $MgCl_2$ compared with the water control was most likely because of an osmotic response. Using this experimental system of calcium-induced *TCH3* expression in whole Arabidopsis seedlings, we tested the effects of staurosporine and the *ein6* mutation. The data in Figure 5 show that at

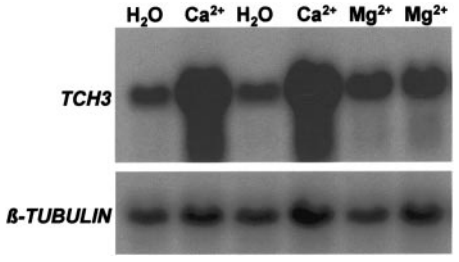

Figure 4. Calcium induction of *TCH3* expression in Arabidopsis seedlings. Seedlings in flasks were desensitized to MS by maintaining shaking up to and beyond the point of addition of compounds. Ca^{2+}, Mg^{2+} (both added to a final concentration of 100 mM) or the same volume of water was added. Thirty minutes after addition, tissue was harvested, RNA extracted, and *TCH3* and *β-tubulin* mRNA levels detected by RNA-blot hybridization.

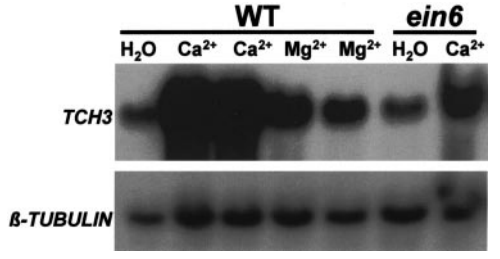

Figure 6. Inhibition of calcium induction of *TCH3* expression in the *ein6* mutant. Seedlings in flasks were treated exactly as described in the legend to Figure 4. Both wild-type (WT) and *ein6* seedlings were treated with either 100 mM Ca^{2+}, 100 mM Mg^{2+}, or water. Thirty minutes after addition of Ca^{2+}/Mg^{2+}/water, tissue was harvested, RNA extracted, and *TCH3* and *β-tubulin* mRNA levels detected by RNA-blot hybridization.

a concentration of 10 μM, staurosporine inhibited calcium-induced *TCH3* expression in Arabidopsis seedlings, similar to its effect on mechanically stimulated *TCH3* expression (Figs. 1 and 2). Treating seedlings with both calcium and phosphatase inhibitor did not produce *TCH3* expression in excess of levels seen when seedlings were given these two treatments separately (data not shown). The data in Figure 6 show that the *ein6* mutation also inhibits calcium-induced *TCH3* expression, similar to its effect on mechanically stimulated *TCH3* expression (Fig. 3, A and B). It is interesting that with *ein6*, there is still a small amount of induction of *TCH3* after treatment with $CaCl_2$, the level of which is very similar to that obtained with $MgCl_2$ in the wild type.

We also examined whether the okadaic acid and calyculin A induction of *TCH3* was affected by the *ein6* mutation (Fig. 7). The data show that both okadaic acid and calyculin A at concentrations of 0.5 μM caused similar levels of induction of *TCH3* in *ein6* as in the wild type. These data also confirmed the finding (Fig. 2, B and C) that at a concentration of 0.5 μM, calyculin A is more potent than okadaic acid in terms of its effect on *TCH3* expression.

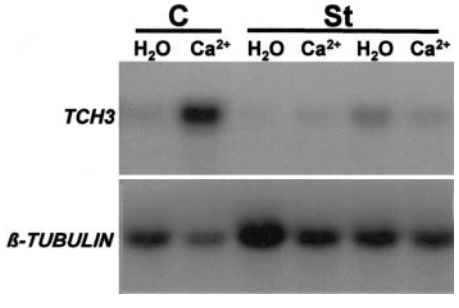

Figure 5. Staurosporine inhibition of calcium-induced *TCH3* expression in Arabidopsis seedlings. Seedlings in flasks were treated exactly as described in the legend to Figure 4, except that 4 h before addition of Ca^{2+} or water, some samples were treated with 10 μM staurosporine (St; in duplicate) and others with 1% (v/v) DMSO as a control (C). Thirty minutes after addition of Ca^{2+}/water, tissue was harvested, total RNA extracted, and *TCH3* and *β-tubulin* mRNA levels detected by RNA-blot hybridization.

Finally, we examined the effect of kinase inhibition, using staurosporine, upon the calyculin A-mediated induction of *TCH3* expression (Fig. 8). As can be seen, treatment with staurosporine under the same conditions that inhibited MS-induced *TCH3* expression also inhibited the induction of *TCH3* expression caused by calyculin A addition.

DISCUSSION

The signal transduction of MS in plants is a poorly understood process. Primary stimuli (touching, rubbing, shaking etc.) lead to a battery of growth responses, which collectively form the process known as thigmomorphogenesis (Jaffe and Forbes, 1993) and the specialized thigmotropic responses in organs such as tendrils and roots (Okada and Shimura, 1990; Klüsener et al., 1995). Many plant genes are upregulated in response to MS, and the best characterized of these are the *TCH* genes of Arabidopsis (Braam and Davis, 1990). One of these genes, *TCH3*, encodes a calmodulin-like protein, of as yet undeter-

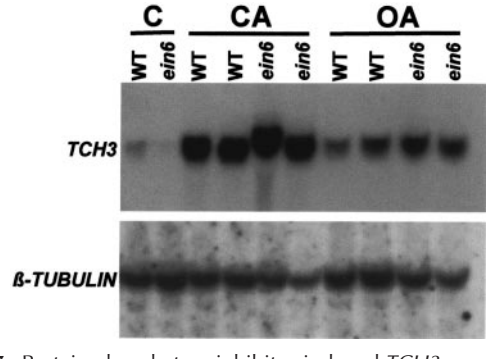

Figure 7. Protein phosphatase inhibitor-induced *TCH3* expression in seedlings of the *ein6* mutant of Arabidopsis. Seedlings in flasks were treated for 4.5 h in either 0.5 μM calyculin A (CA), 0.5 μM okadaic acid (OA), or 1% (v/v) DMSO as a control (C), during which time the samples were left undisturbed. Both wild-type (WT) and *ein6* (*ein6*) seedlings were used. Tissue was harvested, RNA extracted, and *TCH3* and *β-tubulin* mRNA levels detected by RNA-blot hybridization.

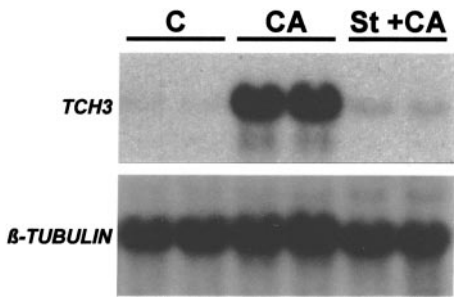

Figure 8. Inhibition of calyculin A induction of *TCH3* expression by staurosporine. Seedlings in flasks were treated exactly as described in the legend to Figure 4, except that 4 h before addition of calyculin A or 1% (v/v) DMSO, some samples were treated with 10 μM staurosporine (St+CA) (in duplicate) and others with 1% (w/v) DMSO as a control (CA and C). Four hours after addition of 0.5 μM calyculin A (St+CA and CA) or 1% (v/v) DMSO (C), tissue was harvested, total RNA extracted, and *TCH3* and *β-tubulin* mRNA levels detected by RNA-blot hybridization.

mined function, which is rapidly (peak of expression 30 min after MS) induced (Braam and Davis, 1990; Sistrunk et al., 1994) by MS. It is known that this gene is also induced by ethylene, auxin, cold, and extracellular calcium (Braam, 1992a; Sistrunk et al., 1994; Antosiewicz et al., 1995; Polisensky and Braam, 1996).

In terms of MS signaling, the second messenger calcium is as yet the only potential component identified. The evidence available includes the fact that MS provokes rapid $[Ca^{2+}]_{cyt}$ increases in plants including Arabidopsis (Knight et al., 1991, 1995) and addition of extracellular calcium in the absence of MS in Arabidopsis cell suspension cultures induces *TCH3* expression (Braam, 1992). Furthermore, in response to cold, *TCH3* expression is inhibited by the calcium channel blockers lanthanum and gadolinium and the calcium chelator 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (Polisensky and Braam, 1996).

The research described in this paper was aimed at obtaining evidence of new components in MS signal transduction, leading to *TCH3* expression, and placing these (upstream/downstream) relative to calcium in a signal transduction pathway in Arabidopsis seedlings.

Staurosporine has been shown to inhibit other signal transduction pathways in plants, e.g. the calcium-regulated expression of *CAB* in response to red light in soybean suspension cells and tomato (*Lycopersicon esculentum*) hypocotyl cells (Bowler et al., 1994a, 1994b) and the calcium-regulated expression of *KIN2* in response to abscisic acid in Arabidopsis hypocotyl cells (Wu et al., 1997). The data presented here are consistent with the involvement of staurosporine-sensitive protein kinase activity in the transduction of MS leading to *TCH3* expression. This activity may be the result of one or more kinases. It appears that this kinase activity is necessary for MS induction of *TCH3* expression. MAP kinase cascade activation has been demonstrated in response to MS (Bogre et al., 1996), so it is possible that the target for staurosporine is one or more of the kinase components of these cascades.

Because inhibition of protein phosphatase activity (Figs. 1 and 2, B and C) results in increased expression of *TCH3*, it seems that protein phosphatase activity is negatively regulating *TCH3* expression. This phosphatase activity must act upstream of, or in concert with, the staurosporine-sensitive kinase activity (Fig. 8). One possible mechanism could be that this protein phosphatase is acting antagonistically to a protein kinase that positively regulates *TCH3* expression, in a similar way as is proposed for kinase induction of EIN3 activity in ethylene signaling in Arabidopsis (Bowler and Chua, 1994). If the effect of okadaic acid and calyculin A inhibition of protein phosphatase activity is to release the activity of such a reciprocal kinase, then this implies (as in the ethylene/EIN3 example) that the MS signaling pathway leading to *TCH3* gene expression is constitutively switched on, and is inhibited from acting when there is no MS. In such a scheme, MS would release the inhibition (by inhibiting the protein phosphatase activity) of the signal transduction pathway and greater flux would occur through the pathway leading to *TCH3* expression. This may be possible, but our present study only allows the conclusion that protein phosphatase activity may be involved as a negative regulator of the signaling pathway leading from MS to *TCH3* expression. The staurosporine-sensitive kinase activity is required for mechanically stimulated *TCH3* expression; therefore, we conclude that this kinase activity is part of the signaling pathway leading from MS to *TCH3* up-regulation. It is still possible that the protein phosphatase activity, whereas definitely negatively regulating *TCH3* expression (as shown in Figs. 1 and 2), is not involved in MS signal transduction specifically, i.e. this activity is not inhibited by MS to lead to induced *TCH3* expression, but is used by the plant for induction of *TCH3* gene expression in response to another factor. However, the fact that the effect of inhibiting this phosphatase activity can be blocked by staurosporine gives credence to the idea that this phosphatase activity is actually involved in MS signaling.

Ethylene is strongly implicated in the mechanical responses of plants leading to thigmomorphogenesis (Biro et al., 1984; Jaffe and Forbes, 1993). The artificial application of ethylene gas has also been shown to induce *TCH3* (Sistrunk et al., 1994). All ethylene mutants we tested, apart from *ein6*, showed a wild-type *TCH3* response to MS when RNA loading was taken into account by examining constitutive tubulin expression (Fig. 3). It is notable that the dominant ethylene-insensitive mutants *etr1-1* and *ein4* showed normal expression because these genes encode parts of the ethylene receptor in Arabidopsis, and the *ein4* and *etr1-1* mutants show reductions in all other ethylene responses tested (Chang et al., 1993; Roman et

al., 1995; Hua et al., 1998). Therefore, if ethylene were used in planta to mediate mechanically stimulated *TCH3* up-regulation, one would expect reduced, or no, *TCH3* induction in *ein4* and *etr1-1* (but this is clearly not the case; Fig. 3). Our data are consistent with the observation by Johnson et al. (1998) that *ein2* and *etr1* mutants show wild-type MS responses. In addition, it can be seen in Figure 3 that the basal levels of *TCH3* expression in the ethylene-overproducing mutants (*eto 1-1*, *eto2*, and *eto3*) and the constitutive ethylene signaling mutant *ctr1* are not elevated, despite the fact that such mutants show elevated levels of expression of bona fide ethylene-regulated genes (Kieber et al., 1993; Ecker, 1995). Taken together, this seems to be strong evidence that in planta, ethylene is not used to mediate mechanically stimulated *TCH3* gene expression, even though application of exogenous ethylene can induce *TCH3* gene expression (Sistrunk et al., 1994).

In light of these observations, it seems that the (as yet uncloned) *ein6* mutation is exerting its effect on *TCH3* expression independently of the involvement of ethylene. In other words, EIN6 is involved in both ethylene signaling and MS signaling, similar to the way in which HLS1 affects both ethylene and auxin signaling (Ecker, 1995). The possibility that *ein6* is simply a mutation in the *TCH3* gene itself, thus leading to reduced expression, is discounted as in response to other stimuli *TCH3* expression reaches wild-type levels in *ein6* (e.g. Fig. 7). Also arguing against this possibility is the fact that *EIN6* resides on chromosome III of Arabidopsis (Roman et al., 1995), whereas *TCH3* resides on chromosome II (Lin et al., 1999). The data presented here (Fig. 3, A and B) suggest that in wild-type Arabidopsis seedlings, the EIN6 protein plays a role in the mediation of the MS signal leading to *TCH3* expression.

As discussed above, calcium appears to be a component in MS signaling leading to *TCH3* expression. To establish whether the effects of staurosporine and *ein6* were upstream or downstream of calcium, the effect of either inhibitor or mutation, respectively, on calcium induction of *TCH3* expression was measured. Addition of extracellular calcium chloride caused a significant induction of *TCH3* expression in Arabidopsis seedlings, much greater than isoosmolar magnesium chloride, suggesting this effect was largely calcium specific (Fig. 4). Our experiments were performed upon MS-desensitized plants for technical reasons outlined in "Results." Thus, the data obtained relating to calcium activation of *TCH3* expression may not be specific to MS signaling and may also relate to signaling from other stimuli that lead to *TCH3* expression, and which involve calcium, e.g. low temperature. Calcium induction of *TCH3* expression has been similarly demonstrated in Arabidopsis cell suspension cultures (Braam, 1992). It is interesting that magnesium caused a slight induction, likely to be as a result of osmotic stress. We have shown previously that this level of osmoticum can induce osmotically regulated genes (Knight et al., 1997). This slight induction appeared to be unaffected by the *ein6* mutation (Fig. 6), suggesting that osmotically induced *TCH3* expression occurs via an EIN6-independent pathway. Both staurosporine (Fig. 5) and *ein6* (Fig. 6) significantly inhibited the calcium induction of *TCH3*. These data imply that the staurosporine-sensitive kinase and the EIN6 protein act downstream of calcium in MS signaling leading to *TCH3* expression. Staurosporine treatment did not affect MS-induced $[Ca^{2+}]_{cyt}$ responses in Arabidopsis seedlings (data not shown), also consistent with the kinase activity acting downstream of MS-induced $[Ca^{2+}]_{cyt}$. A combined treatment of calcium and phosphatase inhibitor did not show an amplified response in terms of *TCH3* expression. This implies that increased flux through the calcium part of the signaling pathway does not lead to enhanced flux through the phosphatase-sensitive part of the pathway. This leads to the conclusion that either calcium is not upstream of the phosphatase activity, or that under these conditions the pathway has already achieved maximal flux. Thus, it is not possible to conclude the hierarchy of calcium and protein phosphatase activity in the MS signaling pathway. To investigate whether the okadaic acid- and calyculin A-sensitive protein phosphatase activity was potentially upstream or downstream of EIN6, we compared levels of *TCH3* induction promoted by these two inhibitors in *ein6* and wild type (Fig. 7). Our data suggest that the negatively regulating protein phosphatase activity acts either downstream of EIN6 in the MS signal transduction pathway leading to *TCH3* expression in Arabidopsis seedlings or independently of EIN6.

In conclusion, *TCH3* expression can be negatively regulated by protein phosphatase activity. This appears to be independent or downstream of EIN6, but whether it is specifically involved in MS up-regulation of *TCH3* expression is not yet known. This study also indicates the necessity for both EIN6 and staurosporine-sensitive protein kinase activity to mediate mechanically stimulated *TCH3* expression in Arabidopsis. Both of these components seem to act downstream of calcium, and may well be involved in transducing mechanically stimulated $[Ca^{2+}]_{cyt}$ signals to effect *TCH3* up-regulation. In the future, it will be very interesting to clone the EIN6 locus to be able to begin to understand the fundamentals of its role in mechanical signaling of Arabidopsis.

MATERIALS AND METHODS

Plant Materials and Chemicals

All experiments were performed using Arabidopsis seedlings grown on 0.8% (w/v) agar plates containing full-strength Murashige and Skoog nutrient medium (Murashige and Skoog, 1962) with a 16-h photoperiod as pre-

viously described (Knight et al., 1997). Arabidopsis wild-type seeds were supplied by Lehle Seeds (Round Rock, TX). Ethylene mutant seeds were supplied by the Nottingham Arabidopsis Stock Centre (Nottingham, UK), contacted via the Arabidopsis Information Management System (http://aims.cps.msu.edu/aims/aims.html), and were sown, selfed, and seeds harvested. Seedlings were either 5 or 11 d old at the beginning of experiments, depending on the particular experiment (see below). Calbiochem-Novabiochem Ltd. (Nottingham, UK) supplied staurosporine and calyculin A. LC Laboratories (Woburn, MA) supplied okadaic acid. These inhibitors were all dissolved in DMSO from Sigma (Poole, UK) to produce stock solutions (as described below). All other chemicals were obtained from BDH (Poole, UK).

MS of Plants

For analysis of ethylene mutants, 11-d-old seedlings were stimulated by applying a whole agar plate of seedlings for 10 s to a vortex mixer at its maximum setting (Rotamixer, Hook and Tucker Instruments Ltd., Croydon, UK) for 10 s. For experiments involving inhibitors, seedlings aged 5 d were transferred to 4 mL of one-half-strength Murashige and Skoog nutrient medium in a 25-mL conical flask. The flasks were covered with foil and plants left to recover in the growth cabinet overnight. For calcium induction experiments, to achieve mechanical desensitization of the seedlings, after transfer into the liquid induction system, the seedlings were incubated overnight on an orbital shaker set at a speed of 120 rpm in the growth cabinet. The following day, calyculin A, staurosporine, or okadaic acid at the desired concentration were added to the system in a total volume of 40 μL of DMSO (1% [v/v] final). The system was mixed briefly (approximately 2–3 s) by manual shaking. The seedlings were then left free from MS to recover. Four hours later, the seedlings were mechanically stimulated by manually shaking the flask for 60 s. The seedlings were harvested a further 30 min after the stimulus (time point confirmed as having maximal *TCH3* expression by RNA gel-blot hybridization; data not shown). The seedlings were blotted dry with tissue paper and frozen in liquid nitrogen. The entire harvesting process was designed to take less than 2 min to avoid any unwanted mechanically induced gene expression. For experiments with exogenous $CaCl_2$, Arabidopsis seedlings aged 5 d were desensitized to MS by shaking (120 rpm) flasks overnight on a shaker in the growth room. The following day, the desired inhibitors were added to the flask and incubated for 4 h before the addition of 1 mL of 0.5 M $CaCl_2$ or 0.5 M $MgCl_2$. The seedlings were harvested 30 min after the addition of these latter solutions.

RNA Gel-Blot Hybridization

Approximately 20 to 25 mg of wild-type or mutant Arabidopsis seedlings were treated as described above, and harvested into microcentrifuge tubes. Total RNA was prepared from seedling tissue using RNeasy plant RNA mini-preps (Qiagen, Dorking, UK). For RNA gel-blot hybridizations, total RNA samples (10 μg per lane) were electrophoresed through 1.0% (w/v) agarose (Life Technologies, Paisley, UK) formaldehyde gels (Sambrook et al., 1989). RNA was transferred to nylon membranes (Böehringer Mannheim, Mannheim, Germany) by capillary action. Blots were prehybridized and hybridized in 50% (v/v) formamide at 42°C. Blots were washed twice in each of the following successively: 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 0.1% (w/v) SDS, followed by 1× SSC and 0.1% (w/v) SDS, and finally 0.1× SSC and 0.1% (w/v) SDS at 42°C. Probes for β-tubulin were prepared from the products of PCR using specific primers as described previously (Knight et al., 1999). Probe for *TCH3* was prepared in the same way, using the primers *TCH*-L (5-TCAAGATAACAGCGCTTCGAA-3) and *TCH*-R (5-AACAATGGTGGATTATCAGCTC-3; Genosys, Cambridge, UK).

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