The Plant Journal (1995) 8(5), 623–636

Cellular localization of the Ca^{2+} binding TCH3 protein of *Arabidopsis*

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Summary

TCH3 is an *Arabidopsis* touch (*TCH*) gene isolated as a result of its strong and rapid upregulation in response to mechanical stimuli, such as touch and wind. *TCH3* encodes an unusual calcium ion-binding protein that is closely related to calmodulin but has the potential to bind six calcium ions. Here it is shown that TCH3 shows a restricted pattern of accumulation during *Arabidopsis* vegetative development. These data provide insight into the endogenous signals that may regulate *TCH3* expression and the sites of TCH3 action. TCH3 is abundant in the shoot apical meristem, vascular tissue, the root columella and pericycle cells that give rise to lateral roots. In addition, TCH3 accumulation in cells of developing shoots and roots closely correlates with the process of cellular expansion. Following wind stimulation, TCH3 becomes more abundant in specific regions including the branchpoints of leaf primordia and stipules, pith parenchyma, and the vascular tissue. The consequences of *TCH3* upregulation by wind are therefore spatially restricted and TCH3 may function at these sites to modify cell or tissue characteristics following mechanical stimulation. Because TCH3 accumulates specifically in cells and tissues that are thought to be under the influence of auxin, auxin levels may regulate *TCH3* expression during development. *TCH3* is upregulated in response to low levels of exogenous indole-3-acetic acid (IAA), but not by inactive auxin-related compounds. These results suggest that TCH3 protein may play roles in mediating physiological responses to auxin and mechanical environmental stimuli.

Introduction

Plants have the unusual capacity to undergo developmental alterations in response to environmental conditions, thus enabling them to acclimate to the conditions of their environment. For example, plants subjected to a variety of mechanical perturbations, such as wind or touch, show a growth response called thigmomorphogenesis which generally includes a decrease in longitudinal growth and an increase in radial growth (reviewed in Jaffe and Forbes, 1993). These changes often make plants more resistant to additional mechanical perturbations. The signal transduction pathways that lead to alterations in plant development in response to environmental stimuli are largely unknown.

In *Arabidopsis*, there are a set of genes, the *TCH* genes, which are upregulated in expression 10- to 100-fold following treatment with a variety of environmental stimuli, including touch, wind, darkness and heatshock (Braam, 1992; Braam and Davis, 1990). The induction of expression of these genes is rapid and transient; maximal levels of expression occur 10–60 min following stimulation and return to basal levels by 1–3 h (Braam, 1992; Braam and Davis, 1990). By determining how these genes are regulated and the roles of the gene products, we may identify the signaling pathways activated following environmental stimulation and the physiological significance of the regulation.

Stimuli such as touch, wind, cold shock and fungal elicitors which result in an increase in *TCH* gene expression (Braam and Davis, 1990; Polisensky and Braam, unpublished results) cause increases in cytoplasmic calcium ion concentration ($[Ca^{2+}]$) (Knight *et al.*, 1991; 1992; Polisensky and Braam, unpublished results). In addition, increasing the external $[Ca^{2+}]$, which leads to a cytoplasmic $[Ca^{2+}]$ increase (Polisensky and Braam, unpublished results), is sufficient to upregulate at least a subset of the *TCH* genes, including *TCH3* (Braam, 1992). These data have led to the suggestion that Ca^{2+} itself may be the signal which controls *TCH* gene expression (Braam, 1992).

At least three of the *TCH* gene products appear to be Ca^{2+}-modulated proteins. *TCH1* encodes an *Arabidopsis* calmodulin (CaM) and *TCH2* and *TCH3* encode CaM-related proteins (Braam and Davis, 1990; Sistrunk *et al.*, 1994). CaM is a critical Ca^{2+} receptor in eukaryotic cells (reviewed in Allan and Hepler, 1989; Cohen and Klee, 1988; Klee and Vanaman, 1982; Roberts and Harmon, 1992; Roberts *et al.*, 1986). As a result of increased cytoplasmic $[Ca^{2+}]$, CaM binds Ca^{2+} and becomes activated to influence the activity of numerous target enzymes. In this way, CaM functions to mediate cellular responses to fluctuations in cytoplasmic $[Ca^{2+}]$. *Arabidopsis* has at least six expressed CaM genes (Braam and Davis, 1990; Gawienowski *et al.*, 1993; Ling *et al.*, 1991; Perera and Zielinski, 1992). In addition to *TCH1*, three other *Arabidopsis* CaM-genes (*ACaM1-3*) are also inducible in expression by touch stimulation (Ling, *et al.*,

Received 5 April 1995; revised 14 July 1995; accepted 16 August 1995.
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1991; Perera and Zielinski, 1992). *TCH3* encodes a novel Ca^{2+} binding protein with 58–60% amino acid identity to known *Arabidopsis* CaMs and has the potential to bind six Ca^{2+} ions (Sistrunk *et al.*, 1994). Because of the sequence divergence from CaM, TCH3 function is likely distinct from that of CaM.

Here we present the findings that TCH3 is abundant in specific tissues, including the shoot apical meristem, vascular tissue, a variety of cells undergoing cell expansion, the root columella, and pericycle cells that appear to give rise to lateral roots. Because this pattern of TCH3 accumulation correlates with regions of high auxin content or known auxin action, we tested whether auxin influences *TCH3* expression and find that exogenous auxin leads to an increase in *TCH3* mRNA accumulation. Following wind stimulation of plants, the pattern of TCH3 accumulation is also restricted to specific regions, but is altered in comparison with non-stimulated plants. We discuss the significance of these findings with respect to the developmental regulation of *TCH3* expression and the physiological roles of TCH3 protein during development and in response to environmental stress.

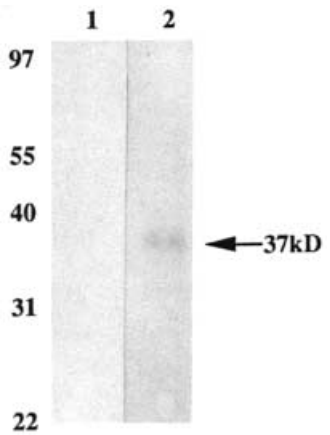


Figure 1. Immunoblotting of TCH3 protein from plants.
Total plant proteins were analyzed by immunoblotting using pre-immune (lane 1) or TCH3 antiserum (lane 2). The arrow indicates the 37 kDa TCH3 protein. Molecular mass markers are indicated at left.

Results

Specificity of the anti-TCH3 antibody

The TCH3 antiserum is specific in that only a single band of the predicted size of TCH3 (37 kDa) is recognized by the antiserum on Western blots (Figure 1, lane 2). At the same dilution (1:4000, data not shown) and even at greater concentration (1:2000 dilution, Figure 1, lane 1), pre-immune sera shows no immunoreaction.

TCH3 localizes to the meristematic rib zone and vasculature of Arabidopsis *epicotyls*

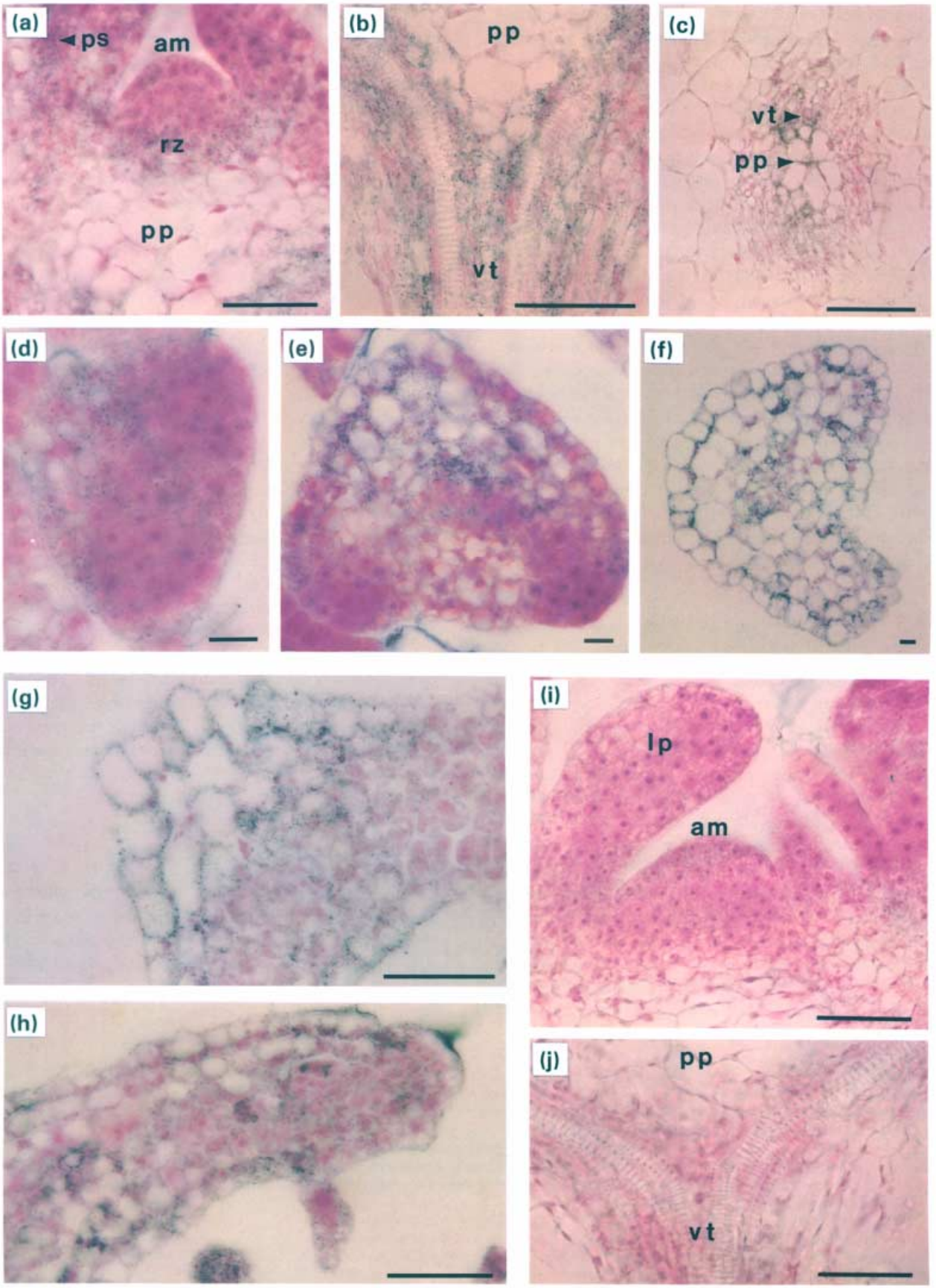
In *Arabidopsis* epicotyls, TCH3 is found in the rib zone of the meristem (Figure 2a). TCH3 is also abundant within the vascular tissue and the pith parenchyma of the cotyledonary node where vascular bundles divert to cotyledons (Figure 2b and c). In contrast, TCH3 is not detectable in the epicotyl pith parenchyma (Figure 2a and b). Sections probed with the pre-immune serum show no significant immunoreactivity (Figure 2i and j).

TCH3 accumulation in developing leaves correlates with cellular expansion

During leaf development TCH3 accumulates preferentially in enlarging cells undergoing vacuolization. In primordia of approximately 200 μm in height, TCH3 is not at the tip but is approximately 50 μm below (Figure 2d). Only cells which appear to be in the process of expanding and that contain vacuoles show significant TCH3 immunostaining. Cells located more proximally are more differentiated and more vacuolated (Figure 2e). Again, the non-vacuolated cells, located in the primordium flanks, are virtually unlabeled; in contrast, TCH3 is in most of the enlarging cells (Figure 2e).

In the proximal region of older primordia, most cells contain large vacuoles. These cells, which are differentiating into the epidermis, parenchyma and vascular tissue,

Figure 2. Localization of TCH3 antigen in the epicotyl and developing leaves.
Black grains indicate the presence of TCH3 antigen.
(a) Shoot apex (longitudinal section). Signals are restricted to the rib zone, leaf primordia and provascular strands.
(b) Epicotyl—the cotyledonary node area (longitudinal section). TCH3 is in the vascular tissue and in the pith parenchyma where vascular strands diverge, but absent in the pith parenchyma above.
(c) Cotyledonary node (transection). Pith parenchyma and vascular tissue are immunolabeled.
(d) Leaf primordium (transection below the tip). Immunoreaction is restricted to enlarging, vacuolating cells mainly at the abaxial part of the primordium.
(e) Leaf primordium (transection through the base). TCH3 is in enlarging and vacuolating cells.
(f) Older leaf primordium (transection through the base). TCH3 is in all differentiating tissues.
(g) Young leaf blade (transection). TCH3 is restricted mainly to the enlarging cells on the abaxial part of the blade within the midrib area.
(h) Young leaf blade (transection). TCH3 is in an irregular pattern outside the midrib area.
(i) Shoot apex (longitudinal section). Pre-immune serum shows no significant immunoreaction.
(j) Cotyledonary node area of epicotyl (longitudinal section). Pre-immune serum shows no significant immunoreaction.
Abbreviations: am, apical meristem; lp, leaf primordium; pp, pith parenchyma; ps, provascular strand; rz, rib zone; vt, vascular tissue.
In (a)–(c) and (g)–(j), bars = 100 μm; in (d)–(f), bars = 10 μm.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contain TCH3 (Figure 2f). Older primordia (Figure 2f) are less intensely immunostained than younger primordia (Figure 2e) indicating that TCH3 is more abundant in leaf primordia cells undergoing extensive expansion than cells in a more expanded state.

During leaf blade formation, TCH3 is most abundant in the midrib area within actively enlarging and differentiating epidermal cells as well as the adjacent parenchymatic cells (Figure 2g). Outside the midrib area, cells are labeled irregularly (Figure 2h) perhaps reflecting the fact that meristematic activity occurs throughout the blade (Pyke *et al.*, 1991) and cells in different stages of expansion may be found in close proximity.

Within the differentiating epidermis, the presence of TCH3 again correlates with the state of cell expansion. Enlarging cells are the most intensely immunostained; this can be seen within rows of cells (Figure 3a and b) and in isolated cells (Figure 3c and d). TCH3 also accumulates in developing leaf trichomes (Figure 3d and e) which are single cells that undergo extensive expansion.

In mature leaves TCH3 accumulation is restricted primarily to the xylem region of the vascular bundles of leaf blades (Figure 3g and h). The phloem is less intensely immunostained (Figure 3h).

TCH3 localization in the developing root

In root tips, TCH3 immunostaining occurs primarily in the proximal three storeys of the columella (Figure 4a). TCH3 is not in the meristematic zone (Figure 4a) but accumulates in all three meristems of the elongation zone with the intensity of immunostaining increasing proximally (Figures 4b and c). These meristems will give rise to epidermis, cortex, endodermis, and the central cylinder which consists of the pericycle and primary vascular tissue. In the specialization zone (beginning from about 500 μm from the initial cells of the primary meristems), TCH3 levels decrease in outer layers with TCH3 abundant only in the central cylinder (Figure 4d). Where primary root differentiation is almost complete, TCH3 is in only the youngest and still differentiating central metaxylem vessel (Figure 4e).

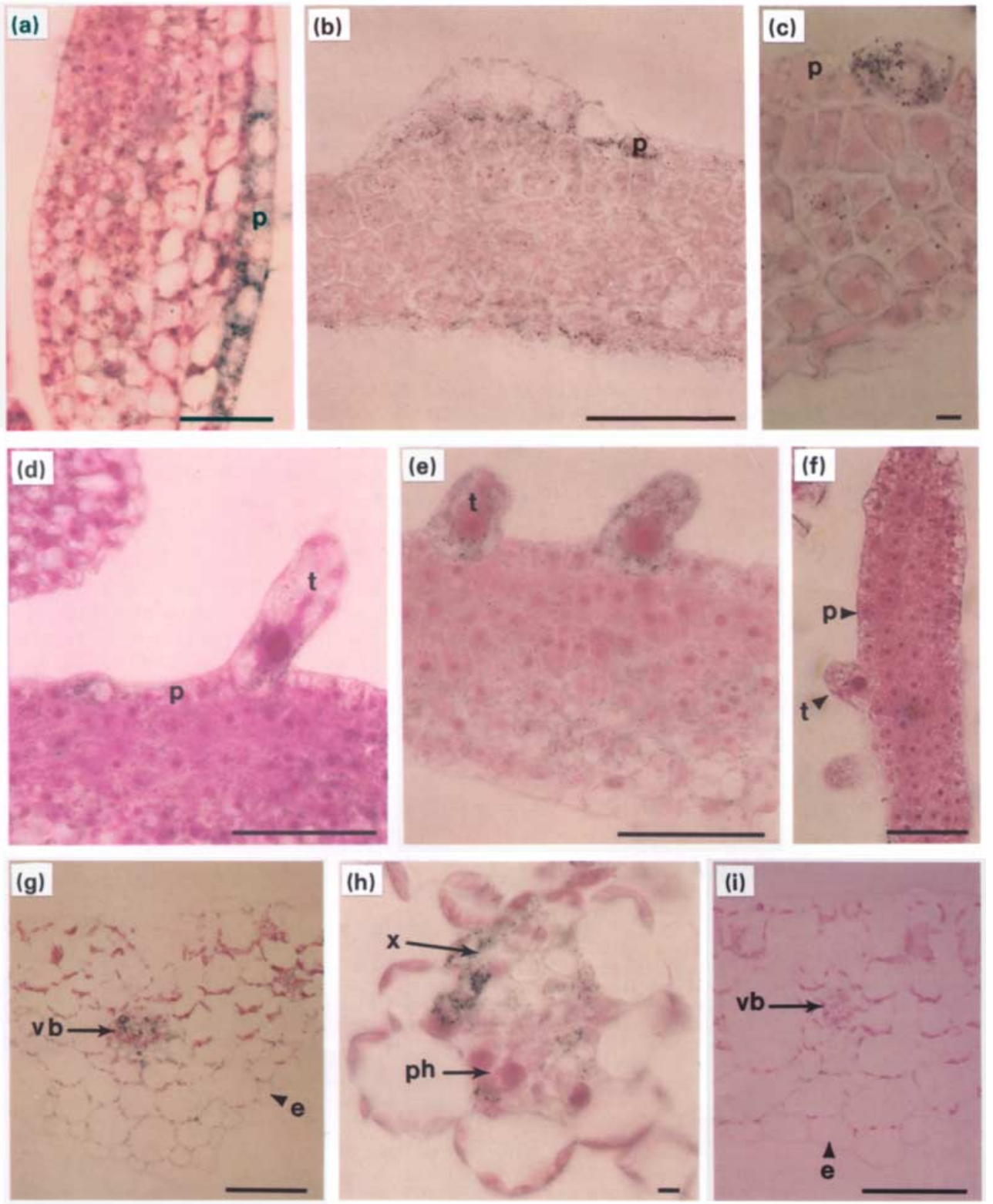
TCH3 accumulation in older parts of the main root is primarily associated with two developmental events: (i) secondary growth and (ii) initiation and growth of lateral roots. Secondary root thickening begins with division of cells located between the primary xylem and phloem that form the vascular cambium (Dolan *et al.*, 1993). During the early cell divisions, TCH3 is present in cells located close to the primary xylem vessels (Figure 4f). Later in root development when cambial cells are actively dividing, TCH3 is most abundant in developing xylem, primarily in immature vessels, and less abundant in the phloem (Figure 4g). TCH3 is also detected in the pericycle (Figure 4g) being most abundant in pericycle cells that contribute to lateral root formation. During the early development of lateral root primordia, TCH3 is abundant in dividing cells originating from the pericycle that are at the base of the primordium (Figure 4h and i). In addition, pericycle cells located opposite older lateral roots are also labeled; often TCH3 accumulation is limited to only two or three cells adjacent to the opposite protoxylem pole (Figure 4j).

As secondary growth of the main root progresses, the epidermis, cortex, and endodermis are in the process of being lost and the formation of the periderm begins. During this process, TCH3 is predominantly in the cambial zone and its derivatives that are differentiating into secondary xylem elements (Figure 5a). Fewer cells are labeled in the differentiating secondary phloem. In addition, elevated levels of TCH3 are in the developing periderm. TCH3 is not located around the entire circumference and is restricted to the most internal one or two cells of the periderm (Figure 5a and b). TCH3 accumulates to high levels in the developing periderm in the regions of lateral root branches (Figure 5c) and is most abundant in cells located opposite to the branched lateral root.

TCH3 accumulates in the vascular tissue of the root-hypocotyl junction and hypocotyl

TCH3 is very abundant at the base of the hypocotyl (Figure 5d), primarily localizing within the differentiating xylem derivatives of the cambium and to a lesser extent within the phloem derivatives (Figure 5e and f). Within mature xylem, TCH3 is detected in xylem parenchyma (Figure 5f). In contrast, TCH3 is absent from the cortex

Figure 3. Localization of TCH3 antigen in leaf primordia, immature and mature leaf blades.
Black grains indicate the presence of TCH3 antigen.
(a) Leaf primordium (longitudinal section). TCH3 is abundant in enlarging protodermal cells.
(b)–(e) Young leaf blade during differentiation (transections). TCH3 is abundant in enlarging protodermal cells and young trichomes.
(f) Leaf primordium probed with the preimmune serum shows no significant immunoreaction (longitudinal section).
(g) Mature leaf (transection). TCH3 is in vascular bundles.
(h) Vascular bundle of the mature leaf (transection). TCH3 is primarily in xylem.
(i) Mature leaf blade probed with pre-immune serum shows no significant immunoreaction.
Abbreviations: e, epidermis; p, protodermis; ph, phloem; t, trichome; vb, vascular bundle; x, xylem.
In (a) and (b) and (d)–(g) and (i), bars = 100 μm; in (c)–(h), bars = 10 μm.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layers of the hypocotyl (Figure 5d). Although there is much less advanced secondary thickening close to the cotyledonary node than in the base of the hypocotyl, the pattern of TCH3 immunostaining remains similar (Figure 5g).

Changes in TCH3 localization after mechanical stimulation

The level of *TCH3* mRNA increases sharply within 30 min following mechanical stimulation (Braam and Davis, 1990). Therefore, we determined whether mechanical stimulation affects the level and pattern of accumulation of TCH3 in plants. No significant difference in TCH3 accumulation is detectable in epicotyls of plants that were stimulated by bending back and forth several times (data not shown). However, after constant wind stimulation, approximately 40% of plant epicotyls show modification in the level and pattern of TCH3 immunostaining. Only in wind-blown plants, TCH3 is present at the bases of leaf primordia and stipules, in the pith parenchyma and within the central zone of the apical meristem (Figure 5h). In addition, the level of TCH3 accumulation is significantly increased within the vascular tissue (Figure 5h). Thus, the increase in TCH3 transcripts following wind stimulation is transduced into a change in TCH3 protein accumulation. The change in localization pattern suggests that TCH3 functions to modify these cells or tissues in response to the environmental stimulation.

TCH3 is upregulated by low levels of exogenous auxin

The pattern of TCH3 accumulation in *Arabidopsis* plants correlates with tissues or cells in which auxin is thought to accumulate, act, or be transported in a polar fashion. Therefore, we tested whether expression of *TCH3* is affected by exogenous auxin. We find that within 30 min of exposure of *Arabidopsis* plants to indole-3-acetic acid (IAA) at concentrations as low as 1 μM, there is a significant increase in *TCH3* mRNA levels (Figure 6a). Tryptophan and indoyl-3-aldehyde, inactive auxin-related compounds, have no significant effects on *TCH3* expression levels (Figure 6b). Low levels of induction of expression by solvent (Figure 6a, lane EtoH and 6b, Control) and possibly the high levels of expression in response to high concentrations of IAA (Figure 6a, 100 μM) may be a result of response to stress. Overall, however, these results indicate that *TCH3* expression can be significantly induced by exogenous auxin.

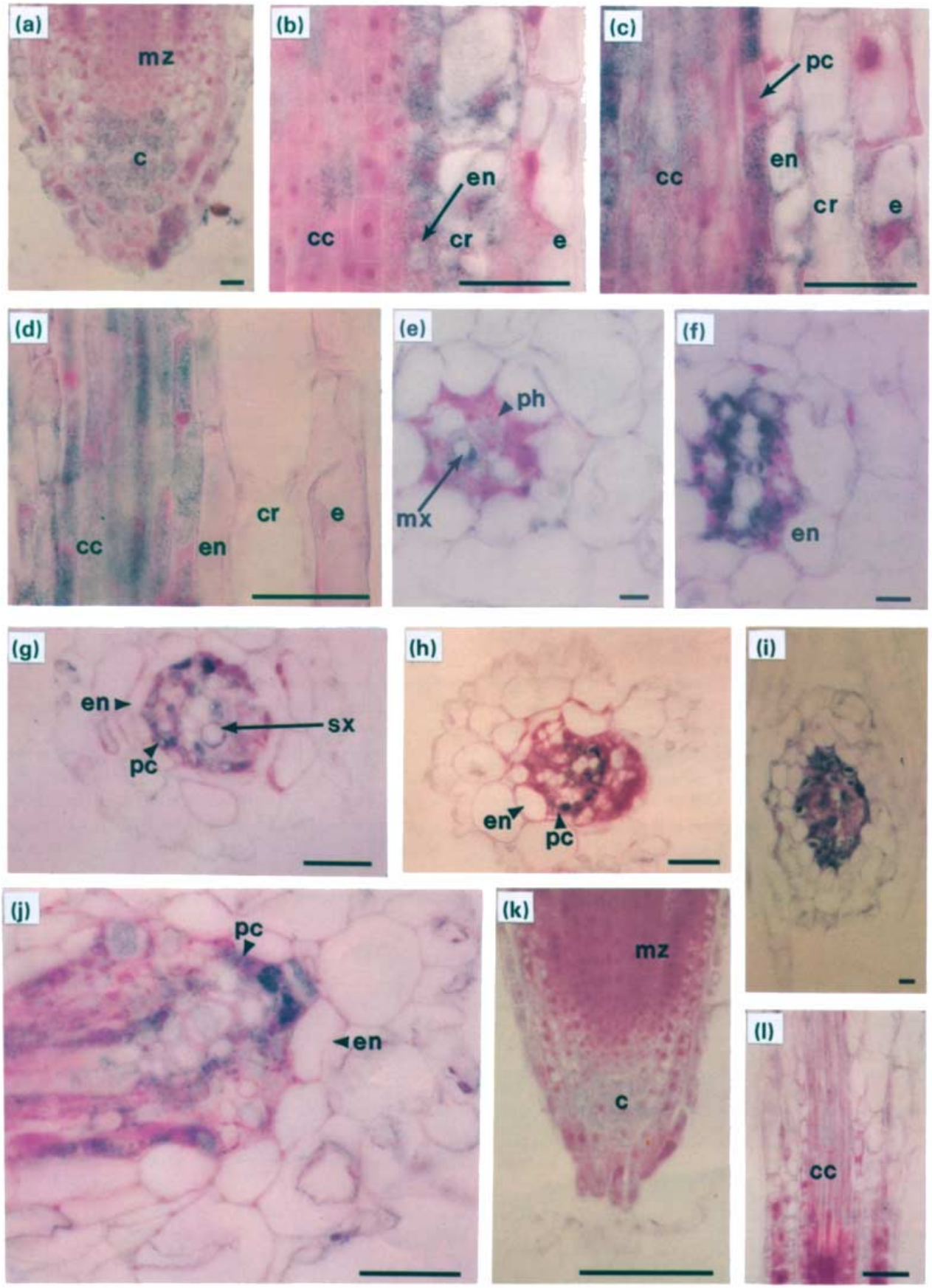
Discussion

We have shown that TCH3, a calmodulin-related Ca^{2+} binding protein (Sistrunk *et al.*, 1994), accumulates in specific cells of various tissues throughout vegetative development of *Arabidopsis*. This pattern of TCH3 protein accumulation gives insight into (i) the signals that may regulate *TCH3* expression during development and (ii) the possible physiological functions of the TCH3 protein.

Regulation of expression of the TCH3 gene and localization of the TCH3 protein

We find a correlation between sites of TCH3 accumulation and cells and tissues whose behavior may be under the influence of auxin. That is, auxin is thought to promote vascular tissue differentiation (Aloni, 1995) and lateral root formation (Torrey, 1950; Wightman *et al.*, 1980), regulate the rate of cell expansion (Cleland, 1995) and division (Evans, 1985), and be transported in a polar fashion in dicots through parenchyma cells associated with vascular tissue (Morris and Thomas, 1978; Lomax *et al.*, 1995). TCH3 is abundant in cells of these types or cells undergoing these processes. For example, TCH3 accumulates in the root pericycle cells, especially in their derivatives at the

Figure 4. TCH3 localization in the distal region of root.
Arrows point to black silver grains which indicate the presence of TCH3 antigen.
(a) Root tip (longitudinal section). TCH3 is located in the columella.
(b)–(c) Elongation zone (longitudinal sections). TCH3 antigen is in all differentiating cells.
(d) Differentiation zone (longitudinal section). Immunolabeling is restricted primarily to the central cylinder.
(e) Primary root (transection). TCH3 is detected within only the youngest metaxylem vessel.
(f) Region of secondary growth initiation (transection). TCH3 is present where cambium begins to be active.
(g) Secondary growth of the main root—early stage (transection). Immunolabeling is present in the differentiating vascular elements and in the pericycle.
(h) Lateral root formation (transection through the median part of newly initiated root primordium). Cells originating from the pericycle at the base of the root primordium are heavily immunostained.
(i) Lateral root formation (longitudinal tangent section through the base of the primordium). Cells originating from the pericycle at the base of the root primordium are heavily immunostained.
(j) The branch of 1–2 cm long lateral root (transection through the main root). TCH3 is in the pericycle cells located opposite to the primordium and in the differentiating vascular tissue.
(k) Apical meristem (longitudinal section). Pre-immune serum shows only a very weak immunoreaction.
(l) Elongation and differentiation zones (longitudinal section). Pre-immune serum shows no significant immunoreaction.
Abbreviations: c, columella; cr, cortex; cc, central cylinder (pierome that differentiates into the pericycle and vascular bundles); e, epidermis; en, endodermis; mz, meristematic zone; mx, metaxylem; pc, pericycle; ph, phloem; sx, secondary xylem.
In (b)–(d) and (g)–(L) bars, = 100 μm; in (a), (e), (i) and (f), bars = 10 μm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base of the emerging lateral roots; these cells give rise to lateral root primordia. The protein is found in leaf and root cells undergoing expansion (Figures 2d–h; 3a–e; 4b–d). TCH3 is also abundant in procambial strands (Figure 2a), regions of differentiating primary and secondary xylem elements (Figures 4d and e; 5a and d–g), and xylem parenchyma cells that may compose the mature secondary xylem (Figure 5f). The pattern of TCH3 accumulation within the differentiating and mature vasculature is also very similar to the pattern of ^{3}H-IAA staining in pea seedlings which is thought to show the pathway of auxin transport and localization of IAA metabolites (Morris and Thomas, 1978). Additional support for a relationship between TCH3 and auxin comes from the finding that the *TCH3* gene is upregulated in expression in plants exposed to low levels of exogenous auxin, but not by inactive auxin-related compounds (Figure 6).

Genes that are rapidly altered in expression levels by auxin have been isolated from a number of different plants, including *Arabidopsis* (Ainley *et al.*, 1988; Alliotte *et al.*, 1989; Conner *et al.*, 1990; Gil *et al.*, 1994; Hagen, 1995; Hagen *et al.*, 1984; Jena *et al.*, 1989; McClure *et al.*, 1989; Oeller *et al.*, 1993; Takahashi *et al.*, 1989; Theologis, 1986; Theologis *et al.*, 1985; Walker and Key, 1982; Yamamoto *et al.*, 1992; van der Zaal *et al.*, 1987). Expression of these genes is affected by exogenous auxin, and therefore developmental expression of these genes may normally be controlled by endogenous auxin (e.g. Gee *et al.*, 1991; McClure and Guilfoyle, 1989).

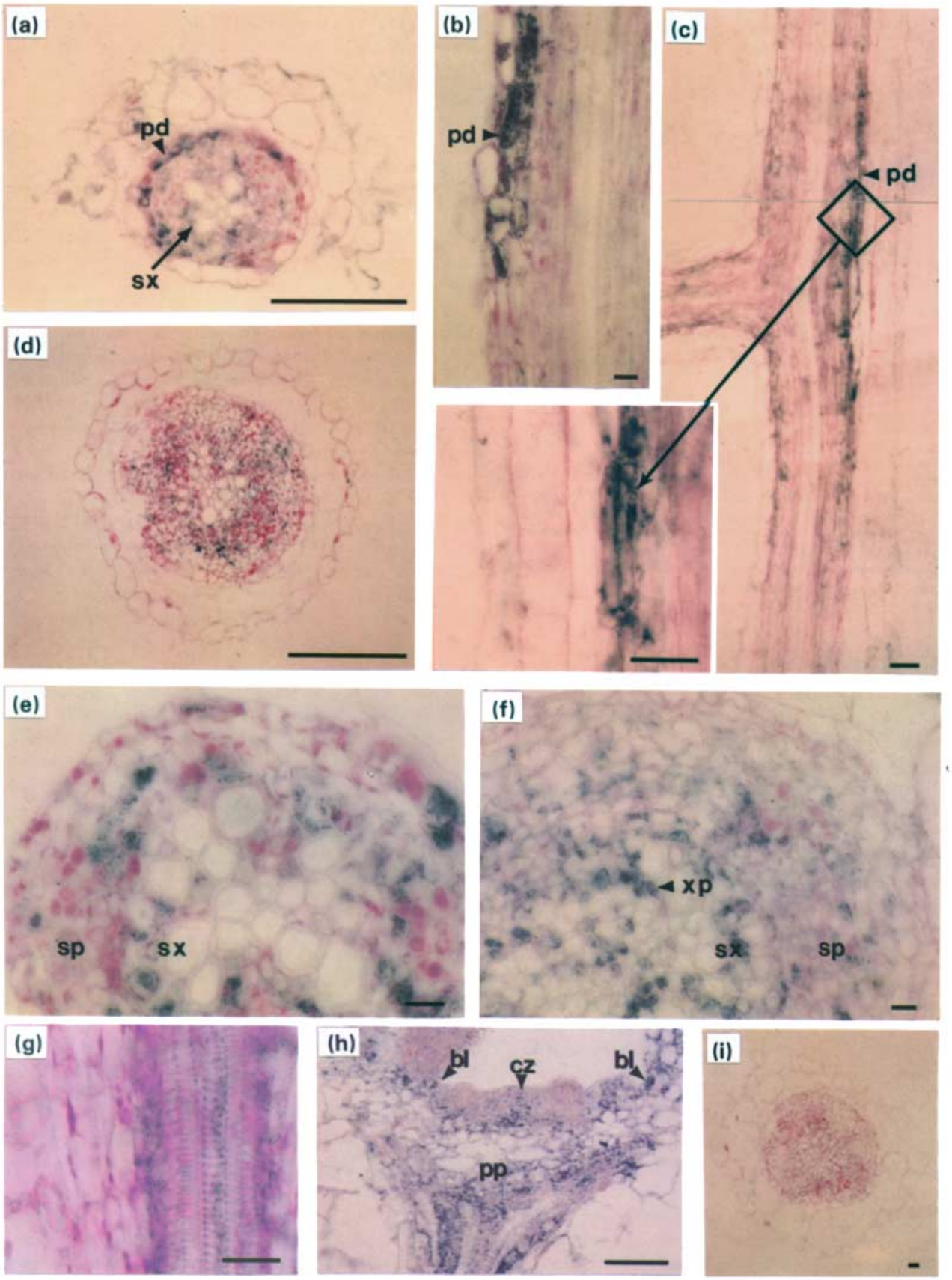
Some auxin-induced genes are regulated by other stimuli as well (for example, see Czarnecka *et al.*, 1984; Dominov *et al.*, 1992; Hagen *et al.*, 1988). *TCH3* also shows complex regulation in that expression is also induced following touch, wind, darkness and temperature shocks (Braam, 1992; Braam and Davis, 1990; Polisensky and Braam, unpublished results). How diverse stimuli cause a common molecular response is not understood. It is possible that distinct signal transduction pathways and *cis* regulatory elements are used in response to different stimuli or that the signaling pathways converge at some point prior to gene regulation. Indeed, there is some evidence to suggest that the *TCH* genes are regulated by increases in cytosolic [Ca^{2+}]; such increases have been detected following many of the inducing stimuli such as touch, wind, cold, heat and auxin (Felle, 1988; Gehring *et al.*, 1990; Knight *et al.*, 1991, 1992; Polisensky and Braam, unpublished results). Therefore, different stimuli may result in *TCH3* gene up-regulation through increases in cytosolic Ca^{2+}. This is perhaps more likely than a possible scenario whereby mechanical stimulation may cause fluctuations in auxin that then influence TCH3 gene expression. Dissection of the *cis* regulatory elements and the use of mutants unable to respond to specific stimuli, like exogenous auxin, should shed light on the relationship between hormonal and environmental regulation of *TCH3* expression.

Possible physiological functions of TCH3

TCH3 encodes a Ca^{2+} binding protein that shares significant amino acid sequence identity (58–60%) to the known *Arabidopsis* CaMs (Sistrunk *et al.*, 1994). TCH3 differs from CaM in having six potential Ca^{2+} binding sites, extended linker regions between pairs of Ca^{2+} binding sites, and a carboxyl-terminal extension. The biochemical function and subcellular location of TCH3 remain to be determined. However, based on sequence similarity to CaM, it is possible that TCH3 acts similarly to CaM and may mediate responses to changes in cytoplasmic [Ca^{2+}] by regulating the activity of target proteins. Alternatively, TCH3, by binding Ca^{2+}, may function to sequester the ion to limit its availability. That is, TCH3 may have a role in either sensing [Ca^{2+}] fluxes or modifying such fluctuations.

The immunolocalization data presented here provide insight into the role(s) the Ca^{2+} binding protein TCH3 plays in *Arabidopsis* physiology and development. For example, a common feature of cells in which TCH3 accumulates, including leaf primordia cells, midrib cells, trichomes and cells of the elongation zone of roots, is that they appear to be undergoing expansion. This suggests that TCH3 may be involved in the process of cellular expansion. It is

Figure 5. TCH3 localization in roots undergoing secondary thickening and in the hypocotyl.
Black grains indicate the presence of TCH3 antigen.
(a) Region of advanced secondary thickening of root (transection). TCH3 is in the forming periderm and the differentiating vascular tissue.
(b) Proximal part of the main root (longitudinal section). TCH3 is most abundant in the periderm.
(c) Branch of 1–2 cm long lateral root (longitudinal section through the main root). TCH3 is most abundant in the periderm opposite to the root branch.
(d) Base of the hypocotyl (transection). TCH3 is present primarily in the vascular tissue.
(e) and (f) Vascular tissue of the hypocotyl (transections). TCH3 is in differentiating xylem elements, in the mature xylem parenchyma and, to a lesser extent, within the secondary phloem.
(g) Upper part of the hypocotyl (longitudinal section) TCH3 is in the vascular tissue.
(h) Wind-stimulated epicotyl (longitudinal section). TCH3 is in the bases of developing leaves, pith parenchyma and meristem central zone and increases in abundance in vascular tissue.
(i) Hypocotyl (transection). Pre-immune serum shows no significant immunoreaction.
Abbreviations: cz, central zone; bl, base of developing leaf; pd, periderm; pp, pith parenchyma; sx, secondary xylem; sp, secondary phloem; xp, xylem parenchyma.
In (a) and (c), (d), (g)–(i), bars = 100 μm; in (b), (e) and (f), bars = 10 μm.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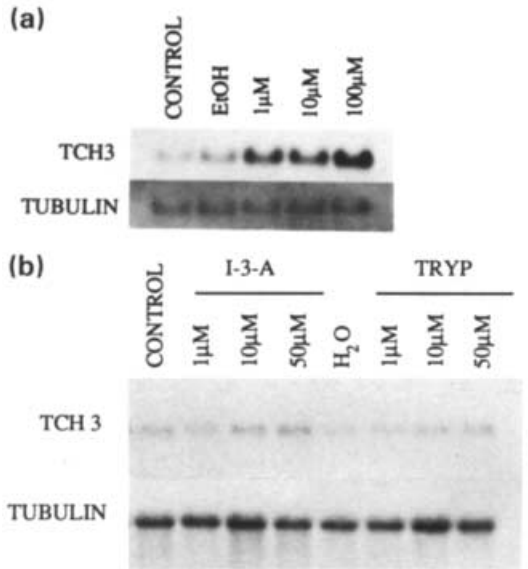

Figure 6. *TCH3* induction of expression by exogenous auxin.
(a) Plants were left untreated (Control), treated with 0.1% ethanol (EtOH) or treated with 1 or 10 μM IAA (in 0.01% ethanol) or 100 μM IAA (in 0.1% ethanol).
(b) Plants were treated with 0.05% ethanol solvent (Control), with an equal volume of water (H_2O), or with 1 or 10 μM I-3-A (in 0.01% ethanol) or 50 μM I-3-A (in 0.05% ethanol) or the indicated concentrations of tryptophan (in water).
Plants were harvested 30 min after treatment. RNA was purified, subjected to gel electrophoresis and blotted to a filter. The filter was hybridized with *TCH3* or tubulin sequences as indicated at left.

thought that both auxin, an inducer of *TCH3* expression, and Ca^{2+}, the TCH3 ligand, may influence cell expansion. Auxin can promote extension (Cleland, 1995), whereas external Ca^{2+} appears to inhibit cell expansion (Demarty *et al.*, 1984). Auxin is thought to act by causing proton secretion into the cell wall which leads to cell wall loosening and cell expansion (Cleland, 1995). How external Ca^{2+} restricts cell expansion is uncertain. Increases in external $[Ca^{2+}]$ may inhibit cell wall loosening by modulating the availability of free protons and thus modifying local pH or by cross-linking the pectin component of the cell wall (reviewed in Demarty *et al.*, 1984). Increases in cytoplasmic $[Ca^{2+}]$ may also be necessary for secretion of cell wall polysaccharides during growth (Griffing and Ray, 1979; Steer, 1988). Drugs that block CaM function can inhibit auxin-induced cell expansion (Elliot *et al.*, 1983; Raghothama *et al.*, 1985). However, because CaM inhibitors have been shown to block functions of other proteins (reviewed in Roberts *et al.*, 1986), inhibition of cell expansion may be due to blocking the action of CaM-related proteins such as TCH3. Ca^{2+} can also play a role in regulating auxin transport (de la Fuente 1984; Allan and Rubery, 1991) and therefore may affect expansion indirectly by modulating auxin availability. TCH3 protein is abundant in the parenchyma cells associated with the vascular bundles which may be the cells responsible for polar auxin transport (Morris and Thomas, 1978). In summary, it is possible that auxin results in increased TCH3 production and this protein may function to modulate cytoplasmic or apoplastic $[Ca^{2+}]$ and thus influence the process of cell expansion.

Auxin, calcium, and CaM have also been implicated in controlling tropic growth responses in plants that have been reoriented with respect to the gravity vector (Evans, 1991; Lee *et al.*, 1983; Moore and Evans, 1986). Interestingly, TCH3 is abundant in the first three storeys of the columella cells which are thought to play a critical role in perceiving the gravitational signal (Olsen *et al.*, 1984). This specific localization of TCH3 suggests the possibility that TCH3 is involved in root graviperception.

Cell expansion and cell wall modification are two processes that take place during the differentiation of vascular tissue. TCH3 accumulation in differentiating vascular cells and the increase in TCH3 levels following wind stimulation suggest that TCH3 may function in vascular tissue development and modification. Auxin, Ca^{2+} and CaM have been implicated in vascular tissue differentiation. Auxin regulates vasculature development *in vivo* (Aloni, 1995) and is essential for both induction and differentiation of *Zinnia* tracheary elements *in vitro* (Fukuda, 1992). Higher levels of Ca^{2+} are seen in differentiating cells (Roberts and Haigler, 1989) and the presence of Ca^{2+} channel blockers can block auxin-induced vascular strand differentiation *in vivo* (Soumelidou *et al.*, 1994) and results in thinner tracheary element cell walls *in vitro* (Roberts and Haigler, 1990). Experiments using pulses of inhibitors of CaM function indicate possible roles for CaM, or other CaM-like proteins that may be affected by the inhibitors, early in the differentiation process (Roberts and Haigler, 1990) and also in secondary wall formation (Kobayashi and Fukuda, 1994).

Mechanical stimulation also leads to vascular tissue modification which contributes to thigmomorphogenetic radial expansion (Telewski and Jaffe, 1986). An increase in ethylene production occurs following mechanical stimulation, and the ethylene is thought to block the basipetal flow of auxin. As a result of accumulated auxin, cambial activity and xylem production are increased and ethylene production is further enhanced (Roberts, 1988). It is also possible that if the mechanical perturbation causes damage to the phloem, auxin would be released from the sieve tubes (Benayoun *et al.*, 1975) and would result in repair or enhanced production of vascular tissue (Aloni, 1995). Thus, both auxin and ethylene have been implicated in the thigmomorphogenetic responses of plants. In addition, Jones and Mitchell (1989) showed that Ca^{2+} and CaM may be involved in modulating plant responses to mechanical stimuli. Treatment with EDTA and CaM antagonists inhibits mechanical stimulus-induced growth reduction. Addition of external Ca^{2+} plus an ionophore resulted in growth inhibition accompanied by an increase in radial stem expansion (Jones and Mitchell, 1989) similar to the

developmental changes observed after mechanical stimulation. The findings that *TCH3* is an auxin- and mechanostimulus-induced, CaM-related gene whose product localizes to developing vascular cells and increases in abundance in vascular tissue following wind stimulation strongly support a hypothesis that TCH3 may function in vasculature development and alterations that take place during thigmomorphogenesis. Touch-induced developmental changes can increase the strength or flexibility of the plant tissues and likely acclimate mechanically perturbed plants to environmental stimuli such as wind gusts (Biddington, 1986; Jaffe and Forbes, 1993).

Furthermore, TCH3 localizes to regions of organs that would likely be under mechanical stress and the protein abundance can be enhanced in plants subjected to wind. For example, TCH3 is found at the attachment points of secondary branches of the shoot (Sistrunk *et al.*, 1994), branching points of leaves (Sistrunk *et al.*, 1994), and leaf primordia and stipules of wind-blown plants (Figure 5h). The protein is also abundant at the base of hypocotyls (Sistrunk *et al.*, 1994, Figure 5h) and the abscission zone of siliques (Sistrunk *et al.*, 1994). The cells in these tissues are likely to be under mechanical strain due to the weight of the structure or the movement caused by wind. The localization of TCH3 at these sites is consistent with a function in altering tissue strength or flexibility that is induced by mechanical strains generated both during plant development and by external environmental conditions.

Experimental procedures

Plant material and treatments

Arabidopsis thaliana plants, ecotype Columbia, were cultivated in a plant growth room under continuous light at 24°C in 65–75% humidity. Plants grown in soil were used for shoot material. For growth of sterile roots, plants were grown in petri plates with 0.5× MS salts (Sigma, St Louis, MO), 1× Gamborgs vitamins (Sigma) and 1% sucrose, pH 5.7.

Plants to be treated with IAA or related compounds were grown for 12 days in liquid medium (0.5× MS salts with 1× Gamborgs vitamins) on a rotary shaker at 120 r.p.m. in 24 h light at 24°C. Twelve to 16 h before the experiment, the medium was replaced with 20 ml fresh medium. Compounds were added to the media without disturbing the flasks. Samples were collected after 30 min by decanting media and plunging plants into liquid nitrogen. Samples were stored at –80°C prior to RNA purification.

Touch treatments were done by gently touching the rosette leaves and bending the plants back and forth several times. Plants were harvested 1.5 h after treatment. Wind stimulation was conducted using a fan for 4 days beginning from the seventh day from planting.

Antibody production and immunoblotting

A fragment from the coding region of *TCH3* (approximately +80 to +640) was cloned into the pATH2 (Koerner *et al.*, 1991) expression vector using standard techniques (Sambrook *et al.*, 1989), and TCH3 was produced as a trpE fusion protein (Koerner *et al.*, 1991).

To isolate TCH3 protein, bacteria were pelleted by centrifuging at 5000 r.p.m. for 5 min at 4°C and then stored at –20°C. Pellets were washed in 50 mM Tris pH 7.5, 5 mM EDTA (TE), centrifuged at 5000 r.p.m. for 5 min and then resuspended in TE. Lysozome (3 mg ml^{-1} was added, and the cells were incubated on ice for 2 h. NaCl (5 M) was added to a final concentration of 300 mM. Ten per cent NP40 was added to a final concentration of 0.75%, and the sample was kept on ice for 30 min. After sonication for 10 sec to shear the DNA, the sample was centrifuged at 10 000 r.p.m. for 10 min at 4°C. Inclusion bodies were washed by resuspending in 4 ml 10 mM Tris pH 7.5, 1 M NaCl; a dounce was used to facilitate resuspension. Finally, the sample was centrifuged again at 10 000 r.p.m. for 10 min at 4°C, and the pellet was resuspended in 0.2 ml 10 mMTris pH 6.8.

Protein was diluted with an equal volume 2× Laemmli sample buffer (Laemmli, 1970) and loaded in a single well of a 10% resolving, 5% stacking SDS polyacrylamide gel. The gel was run at 40 V overnight. Following electrophoresis, the gel was washed with three or four changes of de-ionized water, and stained in aqueous 0.05% Coomassie brilliant blue R-250 for 10 min at room temperature. The gel was washed with numerous changes of water over several hours. When the protein band was visible, it was excised with a scalpel.

Protein was sent to Bethyl Laboratories, Inc. (Montgomery, TX) for innoculation into rabbits. Serum was stored at –80°C.

Frozen shoot tissue was ground with a pestle and mortar, and plant protein was extracted by lysing the cells by douncing in 4 M urea, 2% SDS, 50 mM Tris pH 6.8, 1 mMEDTA and 5% 2-mercaptoethanol on ice. Extracts were centrifuged at 12 000 ***g*** for 5 min, and supernatants were stored at –20°C.

Laemmli sample buffer was added to a final concentration of 1× and the extracts were then boiled for 3 min. $CaCl_2$ was added to a final concentration of 5 mM, and approximately 150 μg of protein were loaded on to a 10% resolving, 5% stacking SDS polyacrylamide gel. Following electrophoresis, protein was electrophoretically transferred to nitrocellulose (Towbin *et al.*, 1979) with the addition of 2 mM $CaCl_2$ to the transfer buffer to promote transfer of low molecular-weight proteins (McKeon and Lyman, 1991). The blots were baked overnight at 65°C under a vacuum to enhance retention of CaM and CaM-related proteins.

Sera were diluted in 150 mM NaCl, 10 mM Tris pH 7.5, 0.1% Tween 20, 1% dried non-fat milk, 0.02% NaN_3. Alkaline phosphatase conjugated anti-rabbit secondary antibody was detected colorimetrically using 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt and 0.48 mM nitroblue tetrazolium in 10 mM Tris pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$.

Immunocytochemistry

Small pieces of plant material (about 2–4 mm^2) were fixed in a solution of 4% paraformaldehyde, 0.5% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.3, for 2 h at room temperature and then continued overnight (12–14 h) at 4°C. Samples were washed in 50 mM sodium phosphate buffer and dehydrated in a graded series of ethanol. Tissues were then embedded in Steedman's wax (Norenburg and Barrett, 1987). Sections (6–14 μm) were affixed to slides precoated with high molecular-weight poly-L-lysine.

An immunogold silver staining detection method was used to locate the antigen/antibody complexes. All reactions were performed at room temperature. Sections were rehydrated in

ethanol and then incubated in PBS for 10 min. PBS with 2% dry milk, 15 mM NaCl, 100 mM NH_4Cl was used to block non-specific binding. Primary anti-TCH3 antibody (diluted 1:100 in blocking solution) was incubated with the sectioned tissue for 1.5 h. The tissue was then washed for 10 min in blocking solution followed by two 10 min washes in PBS with 2% dry milk, 0.1% Tween, 0.05% sodium azide (= solution A). Secondary antibody (colloidal gold 5 nm linked goat anti-rabbit IgG, Amersham, Arlington Heights, IL) was diluted 1:50 with solution A and applied for 1 h. To enhance detection of the colloidal gold, the Silver Enhancer Kit (Sigma) was used. Sections were stained with 0.05% basic fuchsin in 5% ethanol and photographed with a Photo Microscope III (Zeiss). Controls, consisting of omission of primary antibody, omission of immunogold reagent and, use of pre-immune rabbit serum (1:100 dilution), gave little or no background staining.

RNA manipulations

RNA purification and Northern analyses were conducted as described previously (Braam and Davis, 1990; Verwoerd *et al.*, 1989). Hybridization of tubulin mRNA (Marks *et al.*, 1987) is a control to show that similar amounts of RNA are loaded in each lane.

Acknowledgments

We would like to thank the members of the Braam laboratory for continual discussions on this work and input on the manuscript. We also thank Mark Jacobs and Juan Botas for critical review of the manuscript. This work was supported by grants from the National Institutes of Health (No. R29 GM46346), National Science Foundation (No. MCB-9118661) and National Aeronautics and Space Administration (No. NAGW-3139) to J.B.

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