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Arabidopsis *TCH3* Encodes a Novel Ca^{2+} Binding Protein and Shows Environmentally Induced and Tissue-Specific Regulation

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The Arabidopsis touch (*TCH*) genes are up-regulated in response to various environmental stimuli, including touch, wind, and darkness. Previously, it was determined that *TCH1* encodes a calmodulin; *TCH2* and *TCH3* encode calmodulin-related proteins. Here, we present the sequence and genomic organization of *TCH3*. *TCH3* is composed of three repeats; remarkably, the first two repeats share 94% sequence identity, including introns that are 99% identical. The conceptual *TCH3* product is 58 to 60% identical to known Arabidopsis calmodulins; however, unlike calmodulin, which has four Ca^{2+} binding sites, TCH3 has six potential Ca^{2+} binding domains. TCH3 is capable of binding Ca^{2+}, as demonstrated by a Ca^{2+}-specific shift in electrophoretic mobility. 5′ Fragments of the *TCH3* locus, when fused to the β-glucuronidase (*GUS*) reporter gene, are sufficient to confer inducibility of expression following stimulation of plants with touch or darkness. These *TCH3* sequences also direct expression to growing regions of roots, vascular tissue, root/shoot junctions, trichomes, branch points of the shoot, and regions of siliques and flowers. The pattern of expression of the *TCH3/GUS* reporter genes most likely reflects expression of the native *TCH3* gene, because immunostaining of the TCH3 protein shows similar localization. The tissue-specific expression of *TCH3* suggests that expression may be regulated not only by externally applied mechanical stimuli but also by mechanical stresses generated during development. Consequently, TCH3 may perform a Ca^{2+}-modulated function involved in generating changes in cells and/or tissues that result in greater strength or flexibility.

INTRODUCTION

The five touch (*TCH*) genes of Arabidopsis were originally isolated as a result of their inducibility following treatment with stimuli such as touch, wind, rain, wounding, and darkness (Braam and Davis, 1990). This regulation is fast and dramatic; 10 to 30 min following stimulation, *TCH* mRNAs increase 10- to 100-fold (Braam and Davis, 1990). Partial sequencing revealed that *TCH1* is a calmodulin gene, and *TCH2* and *TCH3* encode calmodulin-related proteins (Braam and Davis, 1990). Calmodulin is a ubiquitous, highly conserved eukaryotic protein that functions to modulate the activity of a variety of target enzymes in a calcium ion (Ca^{2+})–dependent manner (for reviews, see Klee and Vanaman, 1982; Roberts et al., 1986; Cohen and Klee, 1988; Allan and Hepler, 1989; Roberts and Harmon, 1992). Calmodulin also has essential Ca^{2+}-independent functions, at least in *Saccharomyces cerevisiae* (Geiser et al., 1991). In addition to the *TCH* genes, Arabidopsis has at least six more calmodulin-related genes (Bartling et al., 1993; Boguski et al., 1993; Ling and Zielinski, 1993) and five other expressed calmodulin genes (Ling et al., 1991; Perera and Zielinski, 1992; Gawienowski et al., 1993), of which at least three are touch inducible in expression (Ling et al., 1991; Perera and Zielinski, 1992).

The finding that calmodulin and calmodulin-related genes have striking regulation of expression suggests that cytoplasmic Ca^{2+} may play a role in the signal transduction pathways activated by the inductive stimuli. Indeed, many of these stimuli that result in the induction of *TCH* gene expression cause an immediate rise in the cytoplasmic Ca^{2+} levels (Knight et al., 1991, 1992; D.H. Polisensky and J. Braam, unpublished data). Furthermore, increases in external Ca^{2+}, which cause increases in cytoplasmic Ca^{2+} (D.H. Polisensky and J. Braam, unpublished data), are sufficient to induce expression of *TCH2*, *TCH3*, and *TCH4* (Braam, 1992), thus implicating a role for Ca^{2+} itself in the regulation of calmodulin-related genes.

Here, we report that *TCH3* encodes a novel calmodulin-related protein that binds Ca^{2+} and contains six potential Ca^{2+} binding sites. The *TCH3* gene may have evolved relatively recently, as the 5′ region of the gene (including sequences encoding the first two potential Ca^{2+} binding sites and containing the first intron) is a nearly identical duplication of the region encoding the second pair of potential Ca^{2+} binding

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sites. In addition, we have localized DNA sequences sufficient to confer touch and darkness inducibility. *TCH3* expression also appears to be developmentally regulated; *TCH3* reporter gene fusions are expressed and TCH3 protein accumulates in the vascular tissue, near tips of roots, at the junctions of shoots and roots, in trichomes, and at branching points.

RESULTS

The Tripartite Nature of the *TCH3* Gene

We determined the full-length sequence and genomic organization of the *TCH3* gene. A partial *TCH3* cDNA (Braam and Davis, 1990) was used as a probe to identify overlapping *TCH3* genomic clones and a poly(A)-containing cDNA of ~1200 bp. The locus was sequenced in its entirety, and the full 1522-bp sequence of the transcription unit is shown in Figure 1A. The cDNA and genomic sequences were compared to identify the intron/exon boundaries and poly(A) addition site. *TCH3* has three introns of 100, 100, and 101 bp, respectively. All three introns are found after the first nucleotide in the codon encoding a conserved glycine within a potential Ca^{2+} binding site of TCH3; introns of calmodulin genes are often found in this location, including those of Arabidopsis (Perera and Zielinski, 1992; Chandra and Upadhyaya, 1993), Chlamydomonas (Zimmer et al., 1988), and Aspergillus (Rasmussen et al., 1990). The *TCH3* intron/exon junctions follow the GT/AG rule (Mount, 1982).

A remarkable feature of the *TCH3* gene is that it is composed of a 367-bp region repeated three times (Figure 1B) followed by an unrelated 350-bp 3′ end. The first two repeats are 94% identical in sequence, and the third repeat is 76% identical to the first two repeats. Each of these repeats encodes a pair of potential Ca^{2+} binding sites. The amino acids encoded by these repeats are aligned in Figure 1C. Again, the first and second repeats share greatest identity (87%), whereas the third repeat is 58 and 55% identical, respectively, to repeats one and two. Interestingly, the introns also show striking sequence identity; the first two are 99% identical, whereas the third is 86% identical to both introns one and two (Figure 1B). This gene arrangement is not due to a cloning artifact, as both genomic and cDNA clones contain the repeats. Furthermore, hybridization of genomic DNA digested with restriction enzymes that cut outside the repeated region revealed bands of the predicted size (data not shown). The first ATG codon is flanked by the characteristic nucleotides of plant initiator codons (Lutcke et al., 1987) and is present at the start of the longest open reading frame. Thus, this codon is probably used to initiate translation.

To determine the start site of transcription, a radioactively labeled 300-bp genomic DNA fragment, consisting of sequences upstream of the longest cDNA and ending 20 nucleotides 5′ to the proposed translation initiation codon, was hybridized with total RNA and treated with S1 nuclease. The protected DNA fragments were analyzed by gel electrophoresis. In addition to the labeled 300-bp DNA fragment, two protected bands were detected. As shown in Figure 2A, lane 3, the longest fragment protected from digestion by 100 units per mL of nuclease is ~60 bp. The smaller bands, ~25 bp, may have resulted from the labeled input DNA hybridizing to an internal region of the *TCH3* mRNA due to the presence of repeated sequences or to an mRNA of a related gene. Protection of specific DNA fragments was not evident when RNA was omitted in the hybridization reaction (lane 4). These results indicate that the 5′ end of the *TCH3* mRNA extends ~80 bases upstream of the proposed translation initiation codon.

To verify the results of the S1 nuclease protection experiment and to determine more precisely the 5′ end of the *TCH3* mRNA, an oligonucleotide complementary to the sequence beginning 17 nucleotides upstream from the proposed translation initiation codon was used to prime reverse transcription of total RNA. As indicated in Figure 2B, lane P, two extended products were detected. The major band, indicated by the large arrow, indicates the presence of *TCH3* mRNA with a 5′ end 80 bases upstream of the proposed initiation codon and is most likely identical to that determined by S1 nuclease protection (Figure 2A, lane 3). The full-length mRNA, not including a poly(A) tail, would therefore be 1221 bp long, consistent with the size of *TCH3* RNA detected on RNA gel blots (data not shown). Primer extension also revealed a second, much less abundant, extended product (Figure 2B, lane P, small arrow), indicating the possibility of a minor transcriptional start site 41 bp farther upstream. We did not detect a corresponding protected fragment with S1 nuclease protection; however, it is possible that the presence of nonspecific fragments (Figure 2A, lanes 3 and 4) would make a minor fragment of the expected size difficult to detect.

TCH3 Encodes a Novel Calmodulin-Related Protein

TCH3 encodes a 324–amino acid protein with highest sequence identity to calmodulin proteins. Figure 3 shows the deduced amino acid sequence of TCH3 in its best fit alignment with the amino acid sequences encoded by Arabidopsis calmodulin and calmodulin-related genes (Ling et al., 1991; Perera and Zielinski, 1992; Chandra and Upadhyaya, 1993; Gawienowski et al., 1993; Ling and Zielinski, 1993). TCH3 is 58 to 60% identical in amino acid sequence to the Arabidopsis calmodulins. In addition, another Arabidopsis calmodulin-related gene, *CaBP-22*, which encodes a 22-kD Ca^{2+} binding protein (Ling and Zielinski, 1993), shares 50% amino acid identity with TCH3. The *TCH3*-encoded protein is significantly longer than the calmodulins; TCH3 has an N-terminal extension, a C-terminal extension, and an internal insertion. The N terminus of TCH3 that extends beyond that aligned with calmodulin represents one of the three repeats present in the *TCH3* gene and encodes two potential Ca^{2+} binding sites. Thus, TCH3 has the potential to bind six Ca^{2+} ions, in contrast to calmodulin and CaBP-22, which may bind up to four Ca^{2+} ions. In

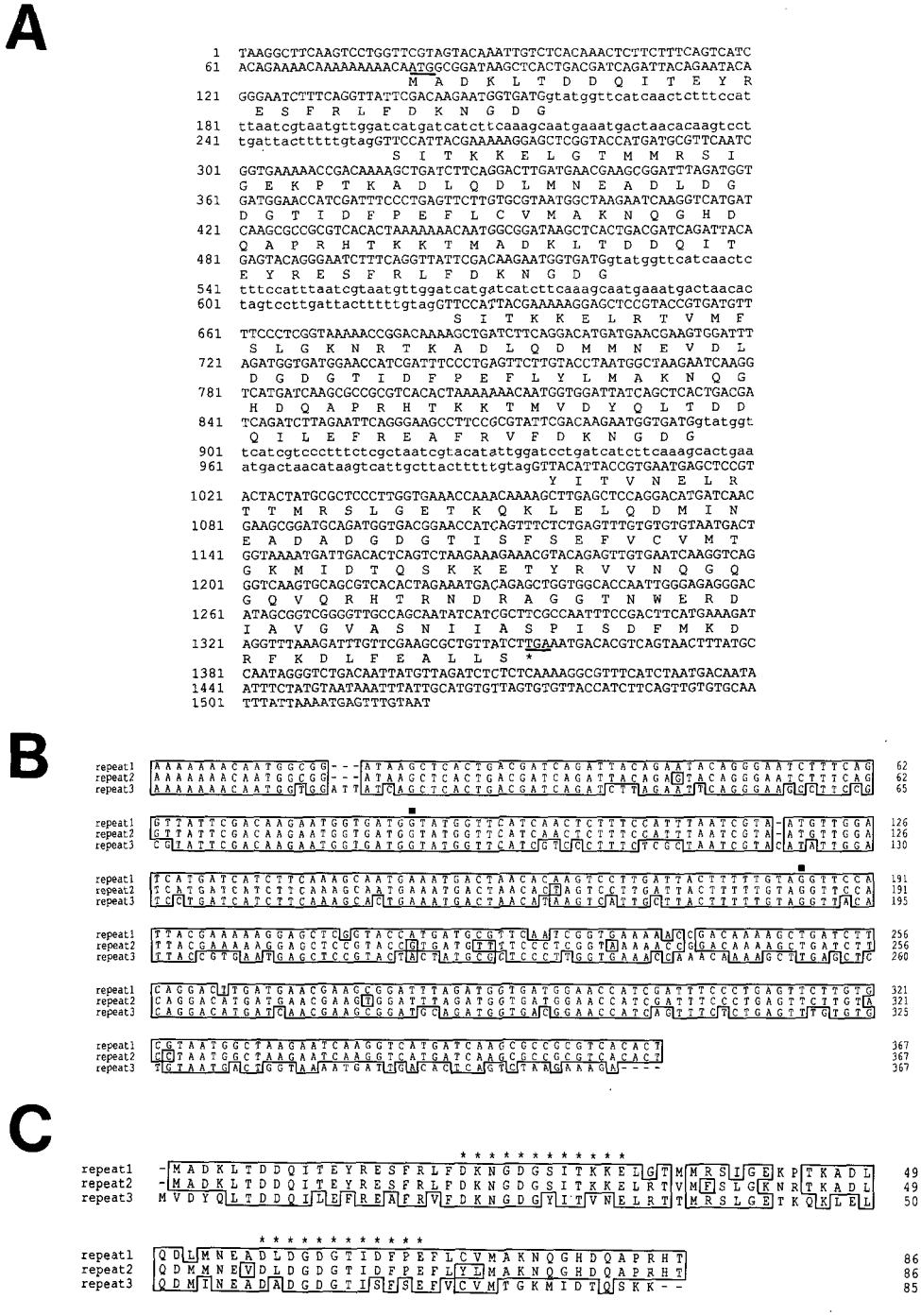

Figure 1. Nucleotide Sequence of *TCH3* and Sequence Comparison of Internal Repeats.

(A) The sequence of the *TCH3* gene. The sequence starts at the major transcriptional start site (see Figure 2) and ends with the last nucleotide before the poly(A) tail addition site. The start and stop codons are indicated by underlining. Introns are in lowercase letters. The GenBank accession number is L34546.

(B) Nucleic acid sequence alignment of the *TCH3* repeats. The three 367-bp repeats are aligned. Identical nucleotides are boxed. Dashes indicate gaps inserted to maximize alignment. The closed squares indicate the beginnings and the ends of introns.

(C) Amino acid sequence alignment of the TCH3 repeats. The three amino acid repeats are aligned. Identical amino acids are boxed. Dashes indicate gaps to maximize alignment. Asterisks indicate potential Ca^{2+} binding sites.

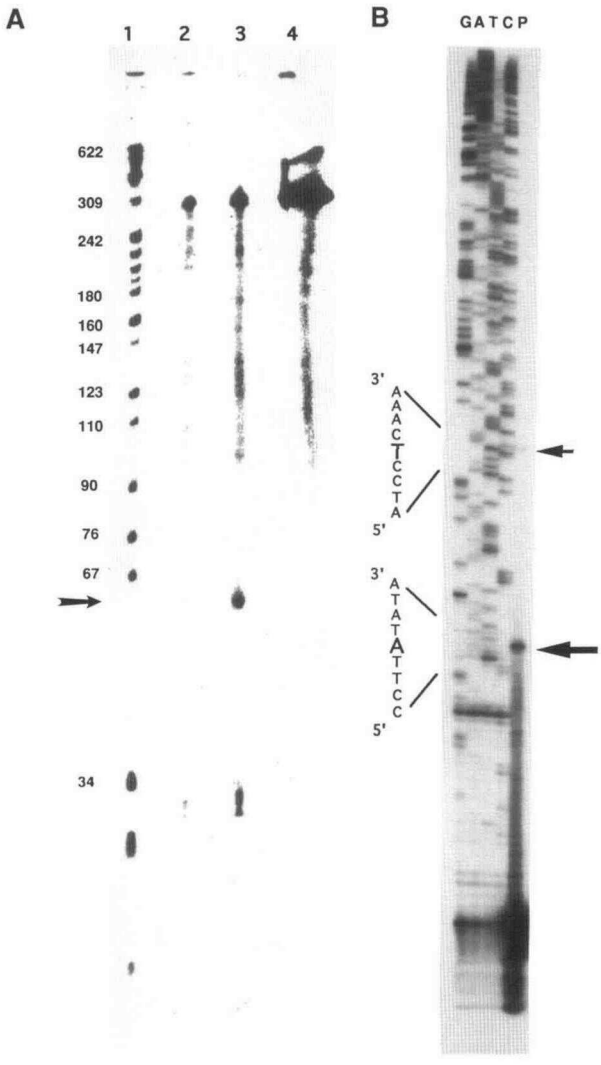

Figure 2. Determination of the Transcriptional Initiation Site(s) of *TCH3*.

(A) S1 nuclease protection. Labeled DNA probe was hybridized to total RNA, digested with 500 units/mL (lane 2) or 100 units/mL (lane 3) of S1 nuclease, and subjected to electrophoresis. The control reaction lacked RNA during hybridization and was digested with 100 units/mL of S1 nuclease (lane 4). Molecular mass markers are shown in lane 1 and are given in nucleotide base pairs at left. The major protected fragment is indicated by an arrow. The major band of ~300 bp is the labeled input DNA.

(B) Primer extension of *TCH3* mRNA. The primer-extended products, in lane P, indicate the major (bold arrow) and minor (small arrow) transcriptional start sites. Sequencing of genomic DNA using the identical primer is shown in lanes GATC. The prominent bands near the bottom of the lanes are the unextended primers.

addition, TCH3 has a 70–amino acid C-terminal extension that is absent in the calmodulin proteins and is 64% identical to the C-terminal extension of CaBP-22. Finally, the regions between pairs of potential Ca^{2+} binding sites in TCH3 are extended by 17 amino acids relative to the analogous region of calmodulin and CaBP-22.

TCH3 Binds Ca^{2+}

To determine whether TCH3 is capable of binding Ca^{2+}, we assayed for a Ca^{2+}-dependent mobility shift of TCH3 by SDS-PAGE. This method has been used to assay Ca^{2+} binding of calmodulin (Burgess et al., 1980) and calmodulin-related proteins (e.g., see Ling and Zielinski, 1993). The mobility of TCH3 was increased in the presence of $CaCl_2$ relative to its mobility in the presence of EGTA, a Ca^{2+}-specific chelator, as shown in Figure 4 (compare first and second lanes). In the presence of Ca^{2+}, TCH3 migrated as a doublet. The significance of multiple bands is unknown, although it is possible that TCH3 molecules with different amounts of Ca^{2+} bound display distinct mobilities. Multiple bands on protein gels have also been detected for calmodulin and, in some cases (e.g., see Williams et al., 1984), may also be a result of different amounts of Ca^{2+} bound. The mobility shift of TCH3 is specific to Ca^{2+}, as no shift was detectable in the presence of Mg^{2+}, another divalent cation (Figure 4, third lane). This Ca^{2+}-dependent mobility shift indicates that the TCH3 protein binds Ca^{2+}.

5′ Fragments of the *TCH3* Locus Confer Inducibility by Touch and Darkness

The *TCH3* gene has been shown to be regulated by a variety of environmental stimuli (Braam and Davis, 1990; Braam, 1992). To begin to identify sequences responsible for this regulation, we fused extensive regions of the *TCH3* gene locus to the *Escherichia coli* reporter gene β-glucuronidase (*GUS*) and assayed for inducibility of the fusion genes in transgenic Arabidopsis plants. Figure 5 is a schematic diagram of the two *TCH3/GUS* fusion genes. *−1350/+843* is a translational fusion containing 843 bp downstream of the major transcriptional start site of the *TCH3* gene, including the first two introns. *−2150/+57* is a transcriptional fusion containing ~2200 bp of the *TCH3* locus, including 57 bp of the major transcription unit. These fusion genes were transformed into Arabidopsis cells, and transgenic plants were generated. DNA gel blot analysis was used to identify transgenic plants that resulted from independent transformation events (data not shown).

We tested whether these regions of the *TCH3* gene are able to confer touch and darkness inducibility of expression onto the *GUS* reporter gene. Independent transgenic plants harboring the fusion genes were stimulated with either touch or darkness and then harvested 30 min after stimulation. Total RNA was purified, and the accumulation of RNA containing *GUS* sequences was compared with *TCH3* RNA accumulation. Both fusion genes were clearly inducible in expression by touch (Figure 6A) and darkness (Figure 6B). There was, however, variability in the magnitude of induction among the

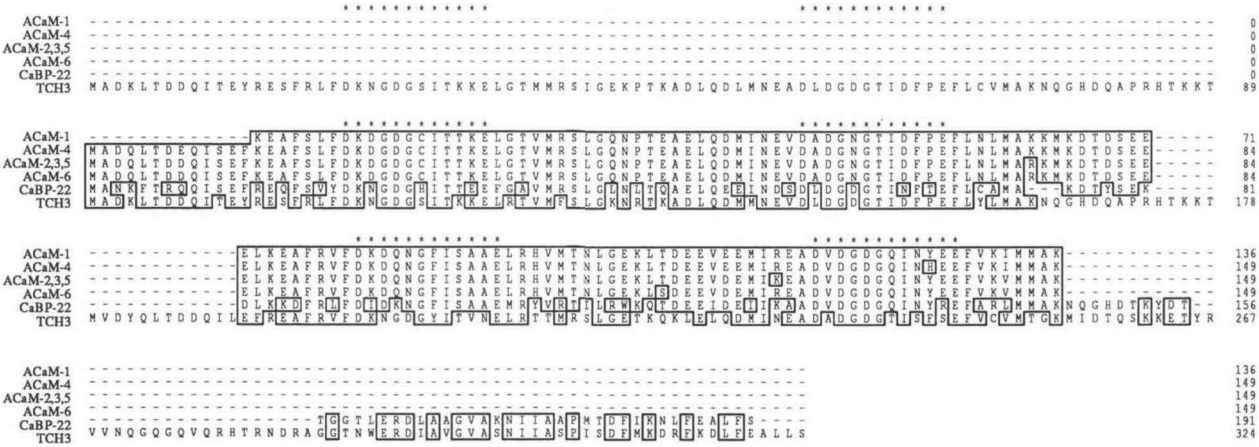

Figure 3. TCH3 Is Closely Related to Calmodulin and CaBP-22.

The deduced amino acid sequences of Arabidopsis TCH3, calmodulins, and CaBP-22 are aligned. Identical amino acids are boxed. Dashes indicate gaps introduced to maximize alignment. Asterisks indicate the Ca^{2+} binding domains of calmodulin.

independent transgenics. For example, transgenics 2 and 3 containing construction *−2150/+57* showed relatively low levels of mRNAs derived from the fusion gene after stimulation (Figures 6A and 6C, lanes 2+ and 3+ of *−2150/+57*). This variability may be due to the sites of insertion or number of active transgene copies.

The induction of expression of the *TCH3* gene is rapid and transient (Braam and Davis, 1990). We tested whether expression of the *TCH3/GUS* fusion genes maintained the kinetics of induction similar to the endogenous *TCH3* gene. The kinetics of expression of independent transformants *−1350/+843-1* and *−2150/+57-2* after touch (Figure 7A) and darkness (Figure 7C) stimulation were comparable to the pattern of endogenous *TCH3* expression. Other independent lines showed similar kinetics (data not shown).

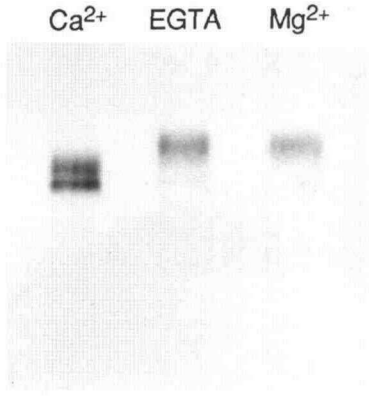

Figure 4. TCH3 Binds Ca^{2+}.

Total plant protein was incubated in the presence of 10 mM $CaCl_2$ and 2 mM EGTA (Ca^{2+}), 10 mM EGTA (EGTA), or 10 mM $MgCl_2$ and 2 mM EGTA (Mg^{2+}), separated by SDS-PAGE, and analyzed by protein gel blotting with anti-TCH3 antibodies.

Tissue-Specific Expression Pattern of *TCH3*

Transgenic plants expressing the *TCH3/GUS* fusion genes were examined for tissue-specific GUS activity. The patterns of expression, examples of which are shown in Figure 8, were indistinguishable for the two gene fusions. Control plants harboring the *GUS* gene driven by either the relatively constitutive 35S cauliflower mosaic virus promoter (Caspar and Quail, 1993) or no promoter indicate that the staining patterns are due to the regulation of *GUS* gene expression by the *TCH3* sequences and are not a property of *GUS* itself or the availability of substrate (data not shown). GUS activity was found in the roots, primarily at the growing regions (Figure 8A) but usually excluding the distal root tip (Figure 8B), and in the vascular tissue near emerging lateral roots (Figure 8C). The root/shoot junction was strongly stained by GUS activity (Figure 8D). Activity was also detected in the vascular tissue of the shoot (Figure 8E) and often within leaf trichomes (Figure 8F). Various branching points of the shoot had prominent GUS activity; for example, as shown in Figure 8G, staining was seen at the attachment points of cauline leaves. In siliques, GUS activity was restricted to the tips and bases (Figure 8H), and in the flower, *TCH3*-driven GUS expression was found in the vasculature of the sepals and within the anthers and stigma (Figure 8I). The pattern of GUS activity was not altered significantly following touch or darkness stimulation (data not shown). The expression pattern of the *TCH3/GUS* fusion genes most likely reflects the expression pattern of the endogenous *TCH3* gene because direct immunolocalization of the TCH3 protein

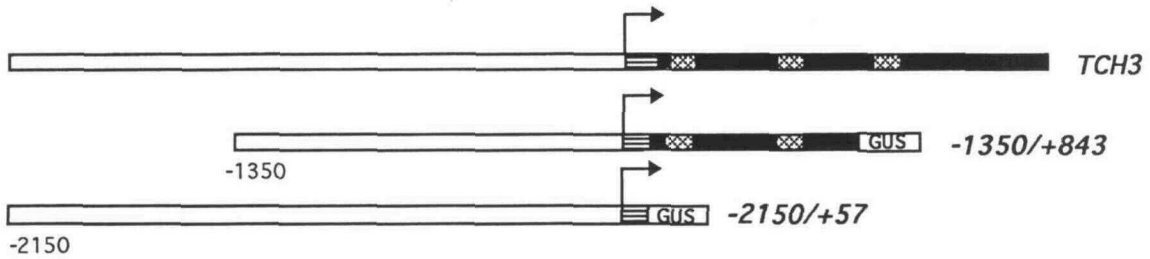

Figure 5. Schematic Diagram of *TCH3/GUS* Fusion Genes.

The genomic organizations of *TCH3*, the *−1350/+843* fusion, and the *−2150/+57* fusion are illustrated. The arrows represent the major transcriptional start site. Open box, upstream region; horizontally striped box, 5′ untranslated region; closed box, exon; cross-hatched box, intron.

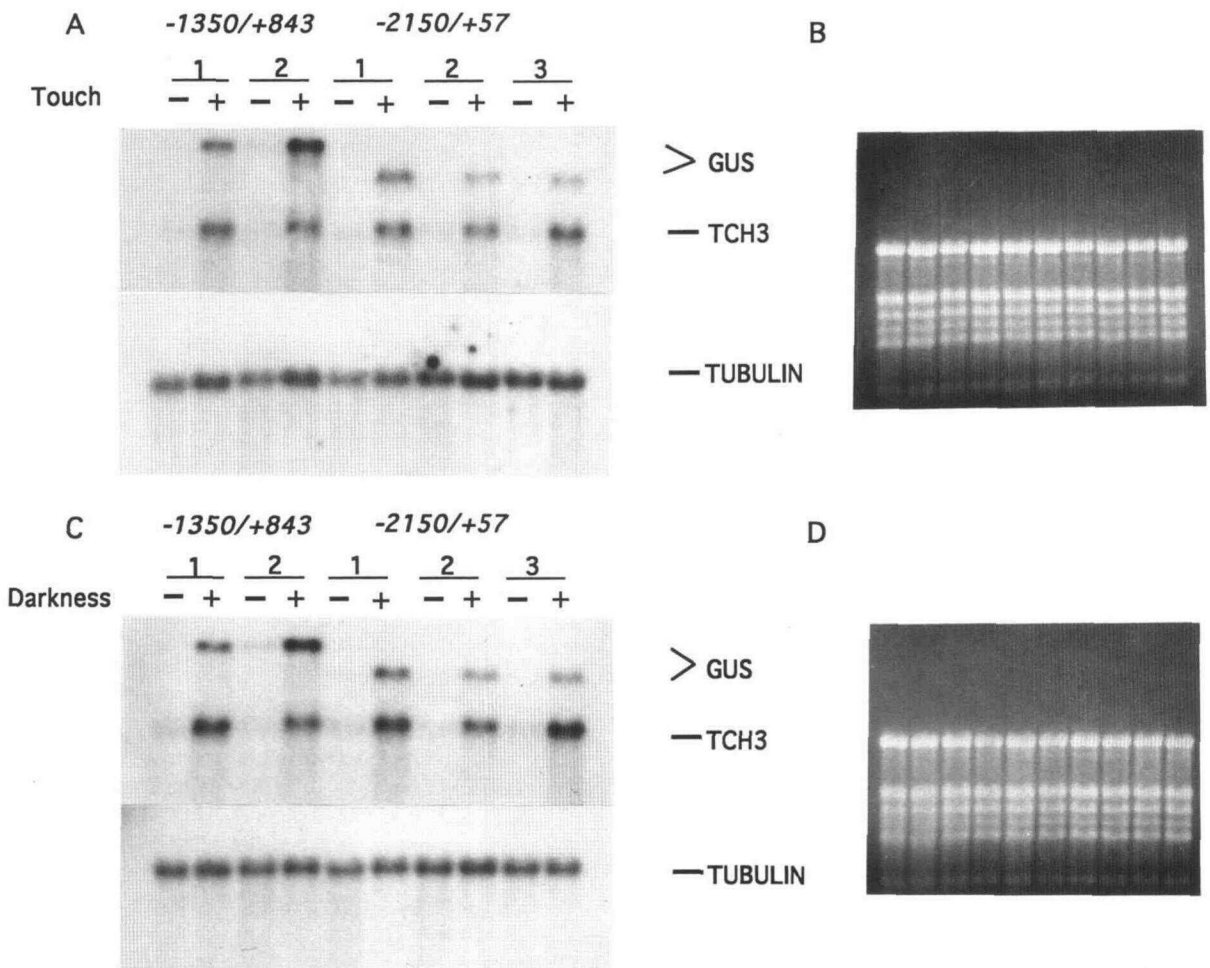

Figure 6. *TCH3/GUS* Fusion Genes Are Induced in Expression by Touch and Darkness.

(A) and **(C)** RNA gel blots of RNA from touch- and darkness-treated plants.
(B) and **(D)** Ethidium bromide–stained gels for **(A)** and **(C)**, respectively.
Independent transgenic plants (indicated by 1, 2, or 3) harboring either the *−1350/+843* or the *−2150/+57 TCH3/GUS* fusion gene were left alone as controls (−), or stimulated (+) with touch **(A)** or darkness **(C)**. Plants were harvested 30 min after stimulation, and RNA was purified, subjected to formaldehyde gel electrophoresis, transferred to filters, and hybridized sequentially with the probes indicated at the right.

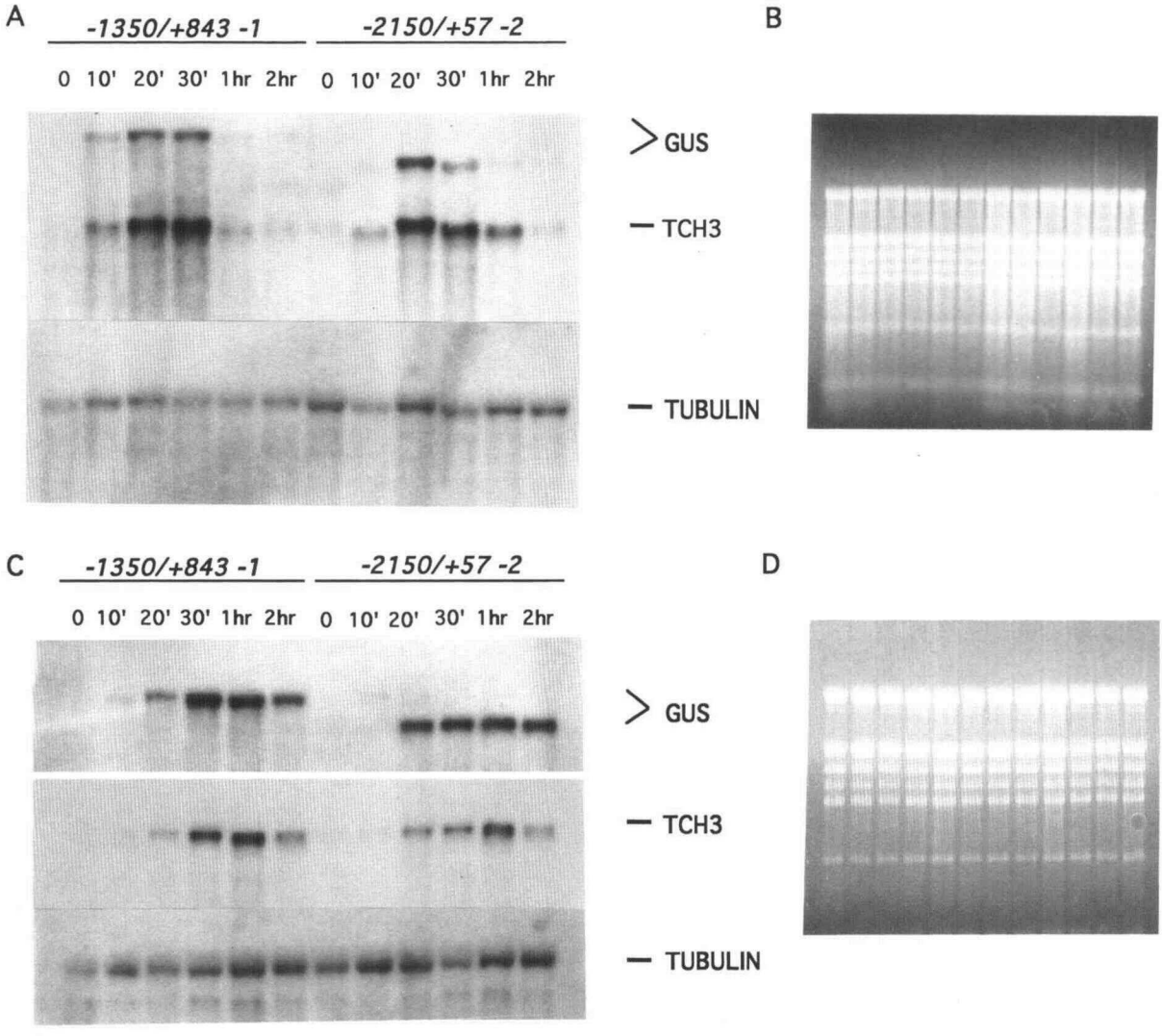

Figure 7. Expression of the *TCH3/GUS* Fusion Genes Shows Similar Kinetics of Induction by Touch and Darkness.

(A) and **(C)** RNA gel blot analysis of the kinetics of induction by touch and darkness.
(B) and **(D)** Ethidium bromide–stained gels for **(A)** and **(C)**, respectively.
Independent transgenics harboring the *−1350/+843* construct (independent transgenic No. 1) or the *−2150/+57* construct (independent transgenic No. 2) were treated with either touch **(A)** or darkness **(C)**. Plants were harvested at the indicated time points, and RNA was purified, subjected to formaldehyde gel electrophoresis, blotted to filters, and hybridized sequentially with the probes indicated at the right. Prime (') stands for minutes.

reveals a very similar developmental pattern (Figures 8J to 8P; D.M. Antosiewicz, D.H. Polisensky, and J. Braam, manuscript in preparation).

DISCUSSION

The Arabidopsis *TCH3* gene encodes a novel Ca^{2+} binding protein. The deduced amino acid sequence indicates that TCH3 has six potential Ca^{2+} binding sites of the EF hand type (Kretsinger, 1987). Each site is composed of the appropriate amino acids required to coordinate Ca^{2+} (Strynadka and James, 1989). TCH3 is capable of binding Ca^{2+}, as evidenced by a Ca^{2+}-specific mobility shift during electrophoresis. The number of sites filled under these conditions is uncertain; the presence of multiple bands (Figure 4) may suggest that the protein can exist with a subset of Ca^{2+} binding sites filled. TCH3 is the first example of a calmodulin-related protein in plants with greater than four EF hands; however, such molecules have been previously identified in animal cells (reviewed in Moncrief et al., 1990).

Calmodulin modulates the activity of a variety of target

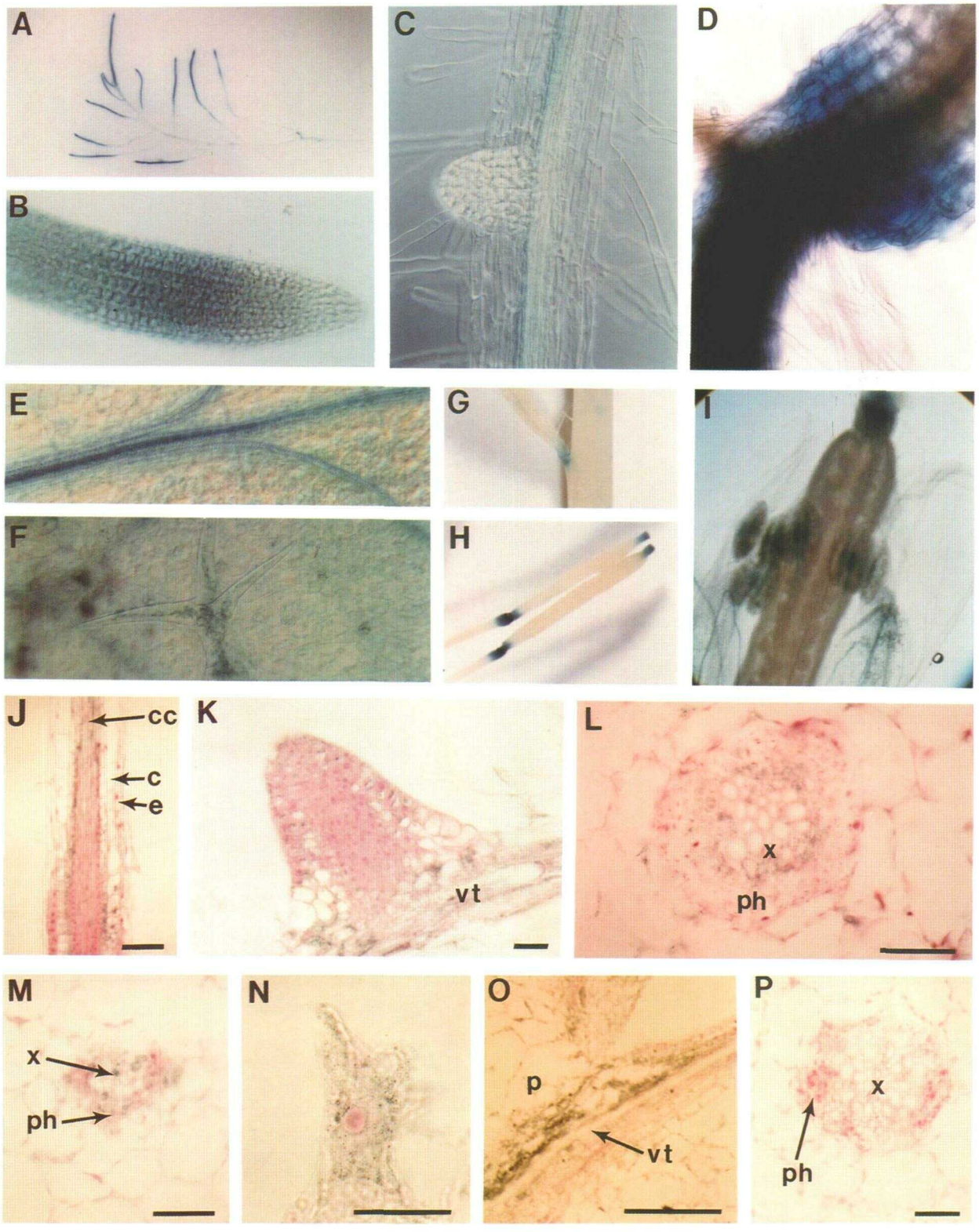

Figure 8. Developmental and Tissue-Specific Expression of the *TCH3/GUS* Fusion Genes and Localization of TCH3 Protein.

enzymes in a Ca^{2+}-dependent manner and in this way is thought to mediate cellular responses to alterations in cytoplasmic Ca^{2+} levels (Klee and Vanaman, 1982; Roberts et al., 1986; Cohen and Klee, 1988; Allan and Hepler, 1989; Roberts and Harmon, 1992). The function of the novel TCH3 protein is currently unknown. Sequence divergence suggests that if TCH3 also modulates target protein activities, these targets would likely be distinct from those of calmodulin. It is also possible that TCH3 has distinct, but presumably Ca^{2+}-dependent, functions. For example, it may bind Ca^{2+} so as to sequester or transport the ion; these roles have been attributed to some other calmodulin-related proteins (reviewed in Heizmann and Hunziker, 1991).

Based on sequence analysis, the Arabidopsis *TCH3*, *CaBP-22*, and calmodulin genes most likely evolved from a common ancestor containing one or two Ca^{2+} binding sites. However, because of the significant divergence in the sequence of the linker region that connects pairs of EF hands, it is probable that *TCH3* did not evolve directly from genes encoding calmodulin or CaBP-22. Furthermore, the striking identity between sequences encoding the first and second pairs of EF hands in *TCH3* suggests that a relatively recent duplication gave rise to the sequences encoding the N-terminal pair of binding sites of TCH3.

Regulation of *TCH3* gene expression is complex. Multiple stimuli result in the accumulation of *TCH3* mRNAs (Braam and Davis, 1990; Braam, 1992). Whereas several of the inductive stimuli share mechanical properties, such as touch, wind, and wounding, other inductive stimuli, such as darkness, do not. How these unrelated stimuli cause a common molecular response is unknown. One possibility is that the different stimuli act through distinct signal transduction pathways and distinct *cis* and *trans* regulatory elements to confer *TCH3* gene induction. Conversely, the signal transduction pathways may converge at a point prior to *TCH3* gene regulation. There is evidence to suggest that an increase in cytoplasmic Ca^{2+} concentration may be sufficient for induction of *TCH3* expression (Braam, 1992). Therefore, one possibility is that a variety of signal transduction pathways share the use of Ca^{2+} as a second messenger, and an increase in free cytoplasmic Ca^{2+} may result in an increased expression of *TCH3*. To begin to determine the mechanism of regulation and the *cis* and *trans* regulatory elements, we have tested the ability of 5′ fragments of the *TCH3* gene to confer inducibility of expression to a reporter gene. A 1400-bp region, including 57 bp of an untranslated leader region, is shared by both constructions tested and therefore is most likely sufficient to confer both touch and darkness inducibility. It will be interesting to further dissect the regulatory region and to identify the minimal sequences necessary and sufficient for induction of *TCH3* expression by touch and darkness. Thus, it will be possible to ascertain whether a single regulatory element acts to confer inducibility by multiple stimuli or whether distinct elements control *TCH3* gene expression.

Another interesting characteristic of the regulation of *TCH3* expression is that the accumulation of mRNAs is transient, indicating that the *TCH3* mRNAs are unstable. The similar kinetics of transgene transcript accumulation, in particular those derived from the *−2150/+57* construct, suggest that sequences sufficient for rapid mRNA degradation are localized within the first 57 nucleotides of the *TCH3* mRNA.

Reporter gene activity in transgenic plants demonstrates that the *TCH3*-derived DNA also confers tissue-specific regulation of expression. These sequences most likely control tissue-specific regulation of *TCH3* expression because the native TCH3 protein accumulates in a similar tissue-specific pattern (Figure 8; and D.M. Antosiewicz, D.H. Polisensky, and J. Braam, manuscript in preparation). After stimulation, there is no detectable change in the localization of *TCH3*-directed GUS activity or TCH3 protein, suggesting that *TCH3* expression is always restricted to specific locations, even when up-regulated in response to external stimulation. Thus, if TCH3 plays a role in modifying cells or tissues in response to environmental stimuli (see below), its function must be limited to specific regions.

Interestingly, several of the sites of accumulation of *TCH3*-controlled GUS activity and TCH3 protein appear to be locations where significant mechanical stress would be expected. For example, when a secondary branch or a cauline leaf emerges from the primary stem, the attachment points are likely to experience mechanical strain due to the weight of the branching structure. In addition, the vascular tissue is subjected to

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Figure 8. (continued).

(A) to **(I)** Photographs of transgenic Arabidopsis plants harboring *TCH3/GUS* fusion genes stained for GUS activity. Plants were ~3 weeks old; exceptions are indicated. Blue color indicates the presence of GUS activity.

(J) to **(O)** Photographs of sections showing immunolocalization of TCH3 in nontransgenic plants. Plants were ~10 days old. Black particles indicate the presence of TCH3 antigen.

(P) Photograph of a cross-section of the root/shoot junction treated with preimmune serum.

(A) Roots. **(B)** Distal root tip. **(C)** Young lateral root. **(D)** Root/shoot junction. **(E)** Vascular tissue of the petiole. **(F)** Leaf trichome. **(G)** Branch point of cauline leaf (6-week-old plant). **(H)** Siliques (6-week-old plant). **(I)** Flower (6-week-old plant). **(J)** Longitudinal section of elongation and part of differentiation zones of root. **(K)** Longitudinal section of young lateral root. **(L)** Cross-section of root/shoot junction. **(M)** Cross-section of petiole. **(N)** Leaf trichome. **(O)** Longitudinal section of branch point of rosette leaf.

c, cortex; cc, central cylinder; e, epidermis; p, parenchyma; ph, phloem; x, xylem; vt, vascular tissue. Bars in **(J)**, **(K)**, **(L)**, **(M)**, **(N)**, **(O)**, and **(P)** = 100 μm.

pressure or tension. Thus, it is possible that *TCH3* expression is up-regulated not only in response to exogenous mechanical stimuli but also in response to mechanical stress that occurs during normal development.

The expression pattern of the *TCH3/GUS* reporter genes and the localization of the TCH3 protein presented in this report may give insight into the possible physiological roles of TCH3. One possibility is that TCH3 has a role in generating changes in cells and/or tissues that result in greater strength or flexibility. Most of the regions with significant *TCH3* expression may require alterations in the mechanical properties of cell walls. A clear example is the vascular tissue; the walls of cells that give rise to the xylem and phloem are thickened and provide reinforcement (Aloni, 1987). *TCH3* expression near emerging lateral roots may reflect the differentiation of vascular bundles that will be directed into the lateral root. Cell walls of trichomes are also significantly thicker than epidermal cell walls (e.g., see figure 7B in Hülskamp et al., 1994). Branch points and junctions, such as the base of the silique and the point where the shoot joins the root, would likely require strengthening and/or increased flexibility of the tissue to withstand compressive and tensile forces generated by the weight of the structures.

Finally, it is possible that touch induction of *TCH3* expression plays a role in thigmomorphogenesis, the physiological changes that occur in plants following mechanical stimulation (reviewed in Jaffe and Forbes, 1993). Thigmomorphogenesis generally results in shorter, stockier plants that are more resistant to mechanical rupture (Jaffe and Forbes, 1993). At least in some plant species, the altered mechanical properties may result in part from an increase in the number and a decrease in length of xylem tracheids (Telewski and Jaffe, 1986a, 1986b). The phloem is also affected by mechanical stimulation in that there are immediate but transient blockages of phloem transport (Jaeger et al., 1988). Darkness may also result in pressure changes within the vasculature, as a decrease in transpiration may be accompanied by continual water uptake, thus decreasing tension while elastically increasing xylem cell expansion (Schulze et al., 1985). Thus, the sequence and localization pattern of TCH3 suggest that TCH3 may perform a Ca^{2+}-modulated function involved in modifying cell and/or tissue mechanical properties both in response to external and developmentally produced mechanical stimulation.

METHODS

Plant Manipulations

Arabidopsis thaliana plants were maintained at 24°C in 65 to 75% humidity under constant light. For generation of transgenic plants, fusion constructs *−1350/+843* and *−2150/+57* were transformed into *Agrobacterium tumefaciens* LBA4404 via electroporation. *A. thaliana* ecotypes No-O or RLD seed were transformed according to Valvekens et al. (1988). Independent transformants were determined by DNA gel blot hybridization. Analyses were performed on third generation seeds that were homozygous for kanamycin resistance.

Touch and darkness treatments of plants were conducted as described previously (Braam and Davis, 1990).

Transgenic plants were stained for β-glucuronidase (GUS) activity essentially according to Gallagher (1992). Briefly, plants were vacuum-infiltrated in 2% paraformaldehyde, 100 mM sodium phosphate, pH 7.0, and 1 mM EDTA for 2 min and then left on ice for 30 min. After a washing in 100 mM sodium phosphate, pH 7.0, the plants were submerged and vacuum-infiltrated in 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc; gift from Molecular Probes, Eugene, OR) in 50 mM sodium phosphate, pH 7.0, 0.5% Triton X-100 for 2 min and subsequently incubated at 37°C for several hours. After stopping the reaction with water, the plants were bleached by washing with 70% ethanol several times. Expression patterns of *TCH3/GUS* fusion genes were similar in seven independent lines containing *−1350/+853* and three independent lines containing *−2150/+57*.

DNA and RNA Manipulations

A partial *TCH3* cDNA (from approximately +80 to +645) was labeled with digoxigenin using the Boehringer Mannheim (Indianapolis, IN) Genius kit and used to probe Arabidopsis genomic and cDNA libraries (Sambrook et al., 1989). Restriction mapping and subcloning were performed using methods according to Sambrook et al. (1989). DNA sequence analysis was determined by the dideoxynucleotide chain-termination method (Sanger et al., 1977) or was carried out by the Nucleic Acids Core in the Department of Molecular and Human Genetics at Baylor College of Medicine (Houston, TX) using automated fluorescence DNA sequencing techniques (Smith et al., 1986). Both DNA strands were sequenced in their entirety. Sequence alignments and analyses were performed using software from Genetics Computer Group (Version 8; University of Wisconsin, Madison, WI; Devereux et al., 1984) and the BLAST network service from the National Center for Biotechnology Information (Altschul et al., 1990).

S1 analysis and primer extension were performed essentially as described by Sambrook et al. (1989). For S1 nuclease protection, a 300-bp genomic DNA fragment (which spans the end of the longest cDNA covering largely upstream sequences and including 20 nucleotides 5′ of the proposed initiation codon) was dephosphorylated and gel purified before labeling with T4 polynucleotide kinase. Sixty nanograms of radioactively labeled DNA probe was hybridized at 47°C for 16 hr to no RNA (as a control) or to 0.5 μg of total RNA from plants stimulated by touch. S1 nuclease digestion was conducted at 37°C for 2 hr at 500 units/mL or 100 units/mL of S1 nuclease. The control with no RNA was incubated with 100 units/mL of S1 nuclease. The protected fragments were purified and subjected to electrophoresis on a 6% polyacrylamide/urea gel. Products were detected using a phosphorimager (Fuji, Tokyo, Japan). The radiolabeled DNA marker was made by filling in recessed 3′ ends of MspI-cut pBR322 with radiolabeled nucleotides using the Klenow fragment of DNA polymerase I.

For primer extension, a synthetic oligonucleotide, 5′-CTGTGATGACTGAAAGAAGAGTTTGTGAGAC-3′ complementary to the sequence beginning 20 bp from the 5′ end of the longest cDNA, was radiolabeled with T4 polynucleotide kinase (U.S. Biochemical Corp., Cleveland, OH) according to the manufacturer's specifications. Two picomoles of primer were extended by Moloney murine leukemia virus reverse transcriptase (U.S. Biochemical Corp.) in the presence of 50 μg of total

RNA from plants stimulated by darkness. The oligonucleotide was also used for sequencing (Sequenase kit, U.S. Biochemical Corp.). Reaction products were subjected to electrophoresis through a 6% polyacrylamide/urea gel, and autoradiography was performed.

For RNA gel blots, total RNA was purified (Verwoerd et al., 1989), subjected to electrophoresis on formaldehyde gels, and transferred to filters, as previously described (Braam and Davis, 1990). Filters were probed with hexamer-labeled DNA fragments (Feinberg and Vogelstein, 1983). Probes were derived from a 2.2-kb BamHI-SacI fragment of *GUS* from the vector pBI101 (Jefferson et al., 1987; Clontech, Palo Alto, CA), a partial *TCH3* cDNA (Braam and Davis, 1990), and a BamHI-KpnI fragment of an Arabidopsis β-tubulin (Marks et al., 1987).

The fusion genes were constructed as follows. A 2.2-kb BglII fragment of pMS2, a *TCH3* genomic DNA subclone, was ligated into the BamHI site of the GUS reporter vector pBI101.3 (Jefferson et al., 1987; Clontech) and called *−1350/+843*. Subcloning of 2.2 kb upstream of *TCH3* was conducted by polymerase chain reaction using the two primers (1) 5′-CCTCGAGGTCGACGGTATCG-3′ and (2) 5′-GTGACGTCGACGACTGAAAGAAG-3′, and linearized pMS2 was used as template. Primer (1) anneals to the sequence within the pBluescript II KS+ vector (Stratagene, La Jolla, CA) of pMS2. Restriction sites HindIII and SalI, respectively, were engineered into the primers for cloning purposes, and the polymerase chain reaction product was ligated into pBI101.1 (Jefferson et al., 1987; Clontech) and called *−2150/+57*.

Antibody Production and Immunoblotting

A fragment from the coding region of *TCH3* (approximately +80 to +640) was cloned into pWR590 (Guo et al., 1984), and TCH3 was produced as a fusion with β-galactosidase. The IgG fraction of antisera raised against this protein was purified using an Econo-Pac serum IgG purification column (Bio-Rad) according to the manufacturer's directions. IgG was concentrated by Centriprep 30 and 100 filtration units (Amicon, Inc., Beverly, MA).

To isolate total plant protein for immunoblots, frozen shoot tissue was ground to a fine powder with a mortar and pestle, dounced in the presence of an equal volume of 4% SDS, 20% glycerol, 120 mM Tris, pH 6.8, and centrifuged at 12,000*g* for 5 min. Supernatants were transferred to new tubes, and total protein concentration was estimated using the Pierce (Rockford, IL) Micro BCA kit.

Before loading on 12% SDS-PAGE (pH 9.3) gels, 2-mercaptoethanol was added to a final concentration of 5% and bromophenol blue was added to 0.01%, and samples were adjusted to 10 mM $CaCl_2$ + 2 mM EGTA, 10 mM EGTA, or 10 mM $MgCl_2$ + 2 mM EGTA. (2 mM EGTA was added to the $MgCl_2$ sample to chelate trace amounts of Ca^{2+} in the cell extracts and $MgCl_2$. To enable comparison, the $CaCl_2$ sample was treated identically; that is, EGTA was added to 2 mM.) Protein (12 μg) was loaded in each lane. After electrophoresis, protein was transferred to nitrocellulose as described (Harlow and Lane, 1988), 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 vacuum to enhance retention of calmodulin and calmodulin-related proteins. All antibody dilutions were in 150 mM NaCl, 10 mM Tris, pH 7.5, 0.1% Tween 20, 1% nonfat milk. Horseradish peroxidase–conjugated secondary antibody was detected by chemiluminescence (ECL kit; Amersham, Arlington Heights, IL). The antibody recognizes a single band on standard protein gel blots that is ~36 kD, the predicted size of TCH3, and there is no detectable cross-reaction with calmodulin (~17 kD) or Ca^{2+} binding protein 22 (CaBP-22; 22 kD) (data not shown).

Immunolocalization

Small pieces of roots and epicotyls (~2 to 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 hr at room temperature before incubation overnight (12 to 14 hr) at 4°C. After fixation, the tissue was washed for 1 hr in buffer, dehydrated in a graded series of ethyl alcohol, and embedded in Steedman's Wax (Norenburg and Barrett, 1987). Sections of 6 to 14 μm were cut for the immunoreaction, then affixed to slides precoated with a high molecular weight poly-L-lysine.

The entire immunogold labeling procedure was carried out at room temperature. The embedding medium was removed from sections with ethyl alcohol. After rehydration, sections were incubated in PBS for 10 min before incubation in blocking solution A (2% dried milk, 0.15 M NaCl, 0.1 M NH_4 in PBS). Primary anti-TCH3 antiserum diluted 1:100 with solution A was applied for 1.5 hr followed by three 10-min washes: once in solution A and twice in solution B (2% dried milk, 0.1% Tween 20, 0.05% sodium azide in PBS). Secondary antibody (colloidal gold 5 nm-linked goat anti–rabbit IgG; Amersham) was diluted 1:50 with solution B and applied for 1 hr. After amplification of colloidal gold label by the Silver Enhancer Kit (Sigma, St. Louis, MO), the sections were stained with 0.05% basic fuchsin in 5% ethyl alcohol. Sections were observed and photographed with the Photo Microscope III (Carl Zeiss, Inc., Oberkoken, Germany). Controls included omission of primary antibody, omission of immunogold reagent, and use of preimmune rabbit serum. Under the control conditions, little or no background labeling was detected.

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