Planta (1997) 203: S35–S41

Planta
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Plant responses to environmental stress: regulation and functions of the *Arabidopsis TCH* genes

Janet Braam, Melissa L. Sistrunk, Diana H. Polisensky, Wei Xu, Mary M. Purugganan, Danuta M. Antosiewicz, Paul Campbell, Keith A. Johnson

Department of Biochemistry and Cell Biology, Rice University, 6100 S. Main, Houston, TX 77005-1892, USA

Received: 27 June 1996 / Accepted: 31 July 1996

Abstract. Expression of the *Arabidopsis TCH* genes is markedly upregulated in response to a variety of environmental stimuli including the seemingly innocuous stimulus of touch. Understanding the mechanism(s) and factors that control *TCH* gene regulation will shed light on the signaling pathways that enable plants to respond to environmental conditions. The TCH proteins include calmodulin, calmodulin-related proteins and a xyloglucan endotransglycosylase. Expression analyses and localization of protein accumulation indicates that the potential sites of TCH protein function include expanding cells and tissues under mechanical strain. We hypothesize that at least a subset of the TCH proteins may collaborate in cell wall biogenesis.

Key words: *Arabidopsis* – Calcium – Calmodulin – Cell wall – Gene regulation (*TCH* genes) – Xyloglucan endotransglycosylase

Introduction

Plants are very sensitive to environmental stimuli and can undergo changes in physiology and development that acclimate them to their particular surroundings. For example, in response to gravistimulation, plant roots will turn to grow towards the gravity vector, while shoots will turn away. A more overall growth response occurs following mechanical stimuli such as touch and wind. Plants generally grow shorter and stockier and often become stronger or more flexible. These developmental changes, called thigmomorphogenesis, enable plants to withstand further environmental challenges, such as wind gusts.

How plants perceive most environmental stimuli is not understood. Nor are the signal transduction pathways that elicit cellular responses characterized. Furthermore, it is unclear how the environmental stimulus response pathways are integrated with genetically programmed morphogenesis.

To begin to investigate how plants sense and respond to various environmental stimuli, we focus on the regulatory and functional characteristics of the *Arabidopsis* touch (*TCH*) genes. These genes are powerful molecular tools that enable an investigation into the mechanism(s) and signaling components involved in regulating plant gene expression in response to external stimuli. In addition, the regulatory behavior of these genes indicates that the *TCH* gene products may function to alter physiology and development in ways to make plants more adapted to their environment. In this report, progress in the study of the regulation and functions of the *Arabidopsis TCH* genes is summarized. This review was presented as part of the International Workshop on Plant Biology in Space (Bonn, Germany) and is very similar to the review published elsewhere (Braam et al. 1996).

Complexity of *TCH* gene regulation of expression

The *TCH* genes were discovered serendipitously through experiments designed to identify differentially expressed genes (Braam and Davis 1990). Control experiments led to the discovery that these genes were upregulated in expression by seemingly innocuous mechanical stimuli, like touch, wind and water spray. Subsequently, it was found that *TCH* gene expression is also induced by stimuli that do not appear to share mechanical properties, such as darkness and temperature shocks (Braam and Davis 1990; Braam 1992; Polisensky and Braam 1996). Expression of at least a subset of the *TCH* genes is also upregulated by applying auxin and/or brassinosteroid to plants (Antosiewicz et al. 1995; Xu et al. 1995). Regulation of *TCH* gene expression by the various inducing stimuli has three salient characteristics, as

Abbreviations: CaM = calmodulin; *TCH* = touch gene; XET = xyloglucan endotransglycosylase (syn. EXT = endoxyloglucan transferase); *XTR* = *XET*-related cDNA

Correspondence to: J. Braam; Fax: 1(713)2855154;
E-mail: braam@bio.rice.edu

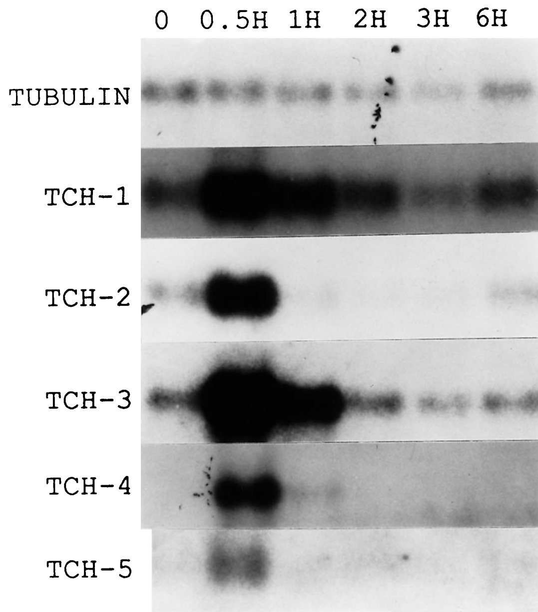

Fig. 1. Rapid, strong and transient upregulation of *TCH* gene expression. Plants were left alone as controls (*lane 0*) or stimulated by spraying with water and harvested at the time points indicated above each lane. The RNA was purified, size-separated on formaldehyde gels, blotted to filters and hybridized with the cDNA probes listed *at left*. Tubulin is used as a control to demonstrate that similar amounts of RNA are loaded in each lane. From Braam and Davis 1990; reprinted with permission from Cell Press

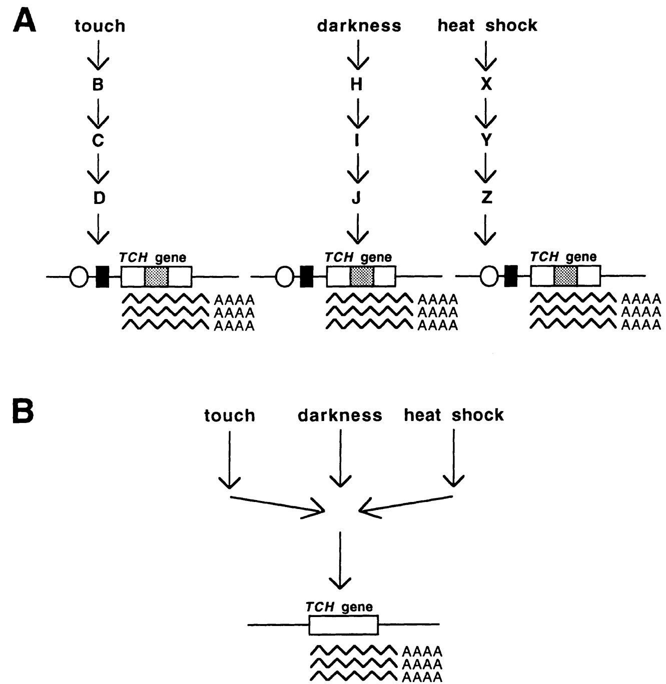

Fig. 2A,B. Two models illustrating how diverse stimuli could lead to the common response of *TCH* gene regulation of expression. **A** Perception of different stimuli leads to the activation of distinct signal transduction pathways that act to upregulate *TCH* gene expression through different *cis* regulatory elements. **B** Perception of different stimuli leads to the activation of signal transduction pathways that converge at a point prior to *TCH* gene regulation of expression

illustrated in the example shown in Fig. 1. (i) The increase in *TCH* mRNA levels following stimulation is of high magnitude. (ii) *TCH* gene induction of expression occurs very rapidly in stimulated plants; however, the kinetics of expression vary depending upon the particular *TCH* gene and the inducing stimulus. Increased levels of *TCH* mRNAs are apparent by Northern analysis within 10 min (the shortest time assayed) of treating plants with stimuli (Braam and Davis 1990; Braam 1992). (iii) Induction of expression is transient; in the example shown, *TCH* mRNAs return to steady-state levels by 1–3 h. This behavior indicates that the *TCH* mRNAs must have high turnover rates. Therefore, in response to unrelated stimuli, *TCH* gene expression is strongly and rapidly turned on and, at least in part as a consequence of rapid RNA turnover, gene expression is rapidly turned off.

Regulation of *TCH* gene expression is complex, in part, because this regulation occurs in response to multiple, apparently unrelated, stimuli. Two possible models shown in Fig. 2 portray how unrelated stimuli may lead to the upregulation of *TCH* gene expression. Each inducing stimulus may be perceived through a specific receptor which activates a distinct signal transduction pathway resulting in the activation of *TCH* gene expression (Fig. 2A). In this scenario, different *cis* regulatory elements would be expected to control the response of *TCH* gene expression to each inducing stimulus. The mechanism of regulation could also vary depending on the stimulus; for example, as illustrated in Fig. 2A, touch may activate a *trans*-acting factor that binds to 5′ sequences to increase the rate of transcription initiation, whereas darkness could act through sequence elements in the transcribed region to modulate mRNA stability. An alternative model, shown in Fig. 2B, is that transduction pathways activated by different stimuli may converge prior to the regulation of *TCH* gene expression. It is possible that there is a common second messenger activated in response to the different stimuli that signals upregulation of *TCH* gene expression. A combination of these two models could also be true; pathways activated in response to a subset of stimuli converge whereas other stimuli may act through parallel and independent pathways. These models can be distinguished by two approaches: (i) determination of whether there is a common step in the signal transduction pathways activated by different stimuli, and (ii) identification of the *cis* regulatory element(s) controlling *TCH* gene expression and determining whether there is a single or multiple element(s). As described below, we have initiated both approaches.

Changes in Ca^{2+} as a potential common messenger

Fluctuation in the levels of free cytosolic calcium ion (Ca^{2+}) was strongly suspected as a possible second messenger mediating *TCH* gene induction for a variety of reasons. Cells often experience changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]$) to signal that an external stimulus has been perceived (Bush 1995). In

addition, as described more fully below, three of the *TCH* genes encode calmodulin (CaM) or CaM-related proteins. Calmodulin is a major Ca^{2+} receptor in eukaryotic cells and is thought to mediate many cellular responses to Ca^{2+} signals (Roberts and Harmon 1992). Therefore, one possibility is that a variety of signal transduction pathways share the use of $[Ca^{2+}]$ fluxes as a second messenger, and an increase in free cytoplasmic $[Ca^{2+}]$ may result in the induction of *TCH* gene expression.

If changes in $[Ca^{2+}]$ mediate *TCH* gene regulation, it would be expected that the stimuli which induce *TCH* gene expression cause increases in cytosolic free $[Ca^{2+}]$. Until recently, monitoring changes in cytosolic $[Ca^{2+}]$ in plant cells has been a technically demanding task. However, Knight, Trewavas and colleagues have reported the success of a novel approach for monitoring intracellular $[Ca^{2+}]$ in plant cells (Knight et al. 1991). These researchers have demonstrated that expression of the jellyfish apoaequorin gene, which encodes a Ca^{2+}-dependent luminescent protein, in tobacco plants can result in detectable luminescence when intracellular $[Ca^{2+}]$ increases. Several stimuli known to lead to upregulation of *TCH* gene expression, such as touch, cold shock and heat shock, have been shown to result in immediate increases in cytosolic free $[Ca^{2+}]$ using this technology (Knight et al. 1991, 1992, 1993, 1996; Haley et al. 1995; Polisensky and Braam 1996).

Are changes in $[Ca^{2+}]$ responsible for regulation of *TCH* gene expression? In the absence of any known inducing stimulus, exposure of cultured *Arabidopsis* cells to a sudden increase of external $[Ca^{2+}]$, which causes an immediate rise in cytosolic $[Ca^{2+}]$ (unpublished results), is sufficient to induce expression of at least a subset of the *TCH* genes (Braam 1992). Treatment with Mg^{2+} under similar conditions has no effect on *TCH* gene expression (Braam 1992). These data are consistent with the possibility that changes in $[Ca^{2+}]$ are sufficient for *TCH* gene upregulation of expression. However, it has not been ruled out that increased external $[Ca^{2+}]$ has other effects on cells, in addition to causing an immediate rise in cytosolic $[Ca^{2+}]$, which influence *TCH* gene expression levels.

The availability of extracellular Ca^{2+} is clearly important for induction of expression of the *TCH* genes. When EGTA or BAPTA [1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid] are used to chelate Ca^{2+} during heat and cold shocks, respectively, there is a significant inhibition of *TCH* gene expression (Braam 1992; Polisensky and Braam 1996). Expression of *TCH3*, however, can be enhanced by exposing cells to BAPTA even in the absence of an inducing stimulus (Polisensky and Braam 1996). This effect may be due to potential alterations in $[Ca^{2+}]$ homeostasis as a consequence of a lowered availability of external Ca^{2+}.

Thus, there is a good correlation between increases in cytosolic $[Ca^{2+}]$ and *TCH* gene upregulation of expression, and data indicate that the increases in $[Ca^{2+}]$ may be necessary and sufficient for stimulus-induced *TCH* gene expression. Therefore, Ca^{2+} may be an important second messenger which mediates *TCH* gene upregulation of expression in response to environmental stimuli.

Identification of *cis* regulatory regions

We are isolating and characterizing the *TCH* gene sequences that control regulation of *TCH* gene expression as another approach to determining how different environmental stimuli lead to the strong and transient increases in *TCH* mRNA levels. If, for example, the model in Fig. 2A is correct, one would expect that there are distinct *cis* regulatory elements responsible for upregulating *TCH* gene expression in response to different stimuli. A single *cis* regulatory element would be expected if the model in Fig. 2B is correct. As a first step in this analysis, we have isolated relatively large regions of genomic DNA harboring sequences of *TCH3* and *TCH4* and fused these DNA fragments to the *uidA* reporter gene encoding β-glucuronidase (GUS). When one to two kilobasepairs (kb) of *TCH3* and *TCH4* genomic sequences are fused to *uidA*, the fusion gene shows inducibility of expression by environmental stimuli (Sistrunk et al. 1994; Xu et al. 1995). These transgenes also behave similarly to the endogenous *TCH* genes in magnitude and kinetics of induction. Thus, the *TCH* gene fragments are sufficient to confer *TCH* gene-like regulation of expression on an unrelated reporter gene. Because the *uidA* reporter-gene transcripts accumulate transiently in a manner similar to that of the *TCH* mRNAs, the sequences responsible for rapid RNA degradation are likely present in these *TCH*-reporter gene fusions. Interestingly, the transcripts derived from the transgene contain only a short 5′ portion of the *TCH* gene sequences, strongly suggesting that short elements located at the 5′ ends of *TCH3* and *TCH4* transcripts are responsible for mRNA instability. To identify sequence elements responsible for *TCH* gene regulation of expression and to determine whether single or multiple *cis* regulatory elements function to induce expression of the *TCH* genes, the regulatory sequences are being further delineated by deletion analysis. These studies will lead, in turn, to the isolation and characterization of the *trans*-acting factors and other components of the signal transduction pathways used by *Arabidopsis* to respond to diverse environmental stimuli.

Identities and functions of the *TCH* gene products

TCH1 encodes calmodulin. The *TCH* genes are rapidly and dramatically upregulated by environmental stimuli; therefore, the *TCH* gene products may have roles in the developmental and physiological responses of *Arabidopsis* to the environment. Sequencing of the *TCH* cDNAs revealed that *TCH1* encodes one of the *Arabidopsis* CaMs (Braam and Davis 1990). Zielinski and colleagues (Ling et al. 1991; Perera and Zielinski 1992; Gawienowski et al. 1993) have found that there are at least six expressed CaM genes in *Arabidopsis*, and at least three of these are inducible by touch stimulation (Braam and

Davis 1990; Ling et al. 1991; Perera and Zielinski 1992). Calmodulin has four Ca^{2+} binding sites and is a major Ca^{2+} receptor in cells (for review, see Roberts and Harmon 1992). When cytoplasmic [Ca^{2+}] increases, CaM binds Ca^{2+} and undergoes conformational changes such that it interacts with and modifies the activity of a variety of target proteins. In this way, CaM is thought to mediate many of the cellular changes evoked in response to [Ca^{2+}] flux. Thus, *TCH1* and the other *Arabidopsis* CaMs likely have roles in cellular signal transduction.

TCH3 is a novel Ca^{2+}-binding protein. We deduced the TCH3 amino acid sequences from the *TCH3* cDNA (Sistrunk et al. 1994) and found that TCH3 has six potential Ca^{2+} binding sites of the EF-hand type. Each site is composed of the appropriate amino acids required to coordinate Ca^{2+} (Strynadka and James 1989). Using a Ca^{2+} specific electrophoretic mobility-shift assay, we demonstrated that TCH3 is capable of binding Ca^{2+} (Sistrunk et al. 1994).

The amino acid sequence of TCH3 is significantly different from that of CaM, indicating that if TCH3 functions by modulating target protein activities, these targets would likely be distinct from those of CaM. It is possible that TCH3 has other, presumably Ca^{2+}-dependent, functions. For example, it may bind Ca^{2+} so as to sequester or transport the ion; these activities have been attributed to other CaM-related proteins (for review, see Heizmann and Hunziker 1991).

To gain insight into the physiological roles of TCH3, we investigated the expression pattern of the *TCH3-uidA* reporter gene constructs and the immunolocalization of the TCH3 protein. The patterns of *TCH3*-driven *uidA* expression and TCH3 protein accumulation were very similar, indicating that the results from these analyses most likely reflect endogenous *TCH3* expression patterns (Sistrunk et al. 1994; Antosiewicz et al. 1995).

Several of the sites of accumulation of *TCH3*-controlled GUS activity and TCH3 protein are locations where significant mechanical strain would be expected. For example, *TCH3-uidA* (Sistrunk et al. 1994) is expressed and TCH3 protein accumulates (Antosiewicz et al. 1995) where secondary branches and cauline leaves emerge from the primary stem; these attachment points likely experience mechanical strain due to the weight of the branching structure. In addition, *TCH3-uidA* expression (Sistrunk et al. 1994) and TCH3 protein accumulation (Antosiewicz et al. 1995) are strong in the cells of the vascular tissue; these tissues are subjected to pressure and tension. Therefore, it is possible that *TCH3* expression is upregulated not only in response to exogenous mechanical stimuli, but also in response to mechanical stress that develops during morphogenesis. Furthermore, TCH3 may function to generate changes in cells and/or tissues that result in greater strength or flexibility. Touch-induction of *TCH3* expression may also play a role in thigmomorphogenesis, the physiological changes that occur in plants following touch or wind stimulation (for reviews, see Jaffe and Forbes 1993; Mitchell and Myers 1995).

The *TCH3-uidA* reporter gene is also expressed in regions of growth (Sistrunk et al. 1994) and TCH3 protein accumulates in cells undergoing expansion (Antosiewicz et al. 1995). The protein TCH3 accumulates in developing trichomes and in expanding cells that give rise to the midrib area of developing leaves. In the roots, *TCH3-uidA* is most strongly expressed in the elongation zone.

Based on these data, we propose the hypothesis that TCH3 performs a Ca^{2+}-influenced function involved in cell or tissue reinforcement and cell expansion. A unifying idea is that TCH3 has a role in modifying the plant cell wall. How could a cytosolic Ca^{2+}-binding protein function in cell wall metabolism? One possibility is that TCH3 may play a role in vesicular traffic regulating the secretion of structural components to the wall. Our current experiments are aimed at testing this hypothesis.

TCH4 encodes a cell wall-modifying enzyme. Based on sequence similarity and in-vitro activity of a recombinant TCH4 protein, TCH4 was identified as a xyloglucan endotransglycosylase (XET; Xu et al. 1995). Since XETs are capable of modifying a major component of the plant cell wall, they may play important functions in altering wall properties in response to environmental conditions. Indeed, because the cell wall is a primary determinant of cell and organ shape, alterations in morphogenesis would most likely require cell wall modification.

Cellulose microfibrils embedded in a matrix of hemicellulose (heterogeneous and usually branched polysaccharides), pectin, and proteins (reviewed in Carpita and Gibeaut 1993) comprise the plant primary cell wall. The major hemicellulose in dicotyledonous species is xyloglucan. Xyloglucans consist of (1–4)-β-linked D-glucosyl residues of which approximately 75% have single xylosyl residue side chains, disaccharides of xylosyl and galactosyl, or trisaccharides of xylosyl, galactosyl, and fucosyl residues. Xyloglucans can form tight hydrogen bonds with the cellulose microfibrils and form tethers between adjacent microfibrils, which are coiled around the cell (Fry 1989; Hayashi 1989; McCann et al. 1990; Hayashi et al. 1994). The XETs, also called endoxyloglucan transferases (EXTs; Okazawa et al. 1993), cleave xyloglucan polymers internally and ligate the newly generated reducing end to another xyloglucan chain (Farkas et al. 1992; Fry et al. 1992; Nishitani and Tominaga 1992; Fanutti et al. 1993). Therefore, by modifying xyloglucan polymers, XETs may have critical roles in determining properties of the wall, such as extensibility, tensile strength and integrity (Fry 1989; Fry et al. 1992; McCann et al. 1992; Talbott and Ray 1992; Redgwell and Fry 1993). Furthermore, XET activity may be important for incorporation of xyloglucan into the cell wall, perhaps ligating short xyloglucan subunits that may be secreted (McCann et al. 1992; Nishitani 1995; Xu et al. 1996).

Sequence and genomic Southern analyses indicate that *TCH4* is a member of a multigene family in *Arabidopsis* (Xu et al. 1996). Using an azuki bean

XET-encoding gene as a probe, Okazawa et al. (1993) isolated an *Arabidopsis EXT*, and *Meri-5* was isolated as a gene expressed in meristems (Medford et al. 1991) and was later found to share significant sequence similarity to *XETs* (de Silva et al. 1993, 1994; Okazawa et al. 1993). Comparison of the *TCH4* sequence to Expressed Sequence Tags (EST) characterized by the *Arabidopsis* sequencing project (Höfte et al. 1993; Newman et al. 1994) led to the identification of additional *XET*-related (*XTR*) cDNAs (Xu et al. 1996). We determined the full-length coding sequences of five novel *XTRs* and found that the *Arabidopsis* XTRs share between 35% and 84% amino acid identity (Xu et al. 1996). All but one of the XTRs have the conserved DEIDFEFLG sequence found among known XETs and this sequence is also very similar to the proposed active site region in Bacillus β-glucanase (Borriss et al. 1990). Because plant and bacterial proteins share strong sequence relatedness in this region, it has been suggested that these amino acid residues may be critical for the cleavage of (1–4)-β-glycosyl linkages (de Silva et al. 1993; Okazawa et al. 1993). The XTR proteins have a stretch of hydrophobic amino acids at the N-terminus which may function as a signal sequence, a potential N-linked glycosylation site and, in the C-terminal portion of the protein, either two or four cysteine residues which may form disulfide bridges. Because the potential N-terminal signal sequences show significantly less conservation than sequences predicted to be within the mature proteins, it is likely that the remarkable conservation of the mature protein sequences is due to evolutionary selection rather than to very recent gene duplications.

Although XET enzymatic activity has been demonstrated only for the TCH4 member of the XTR family of *Arabiodpsis* proteins (Xu et al. 1995), these proteins are very similar in primary sequence, and we would therefore predict that the XTRs have related biochemical activities. What is the significance of an extensive *XET*-related gene family? Part of the explanation for distinct gene family members may be that the *XTRs* are differentially regulated in expression (Xu et al. 1996). Northern analysis measuring changes in overall mRNA accumulation from the different *XTRs* indicates that *TCH4* is unique in its regulation; none of the other *XTRs* are induced in expression by all the stimuli tested: touch, darkness, heat shock, cold shock, auxin and brassinosteroid. However, each of the genes is responsive to at least one of the stimuli. Based on the frequency and source of mRNA used to make the EST libraries, it is also likely that the *XTR* genes are differentially regulated in expression during development (Xu et al. 1996). Thus, the multiple *XTRs* may have evolved so that specific subsets of proteins may function to alter cell wall properties during development and/or in response to particular hormonal and environmental stimuli.

It is also possible that XTRs are distinguished by subtle differences in enzymatic properties. For example, one class of XTRs may catalyze transglycosylation of only xyloglucan polymers of particular length or that harbor specific sugar side chains. Some may preferentially carry out hydrolysis rather than transglycosylation

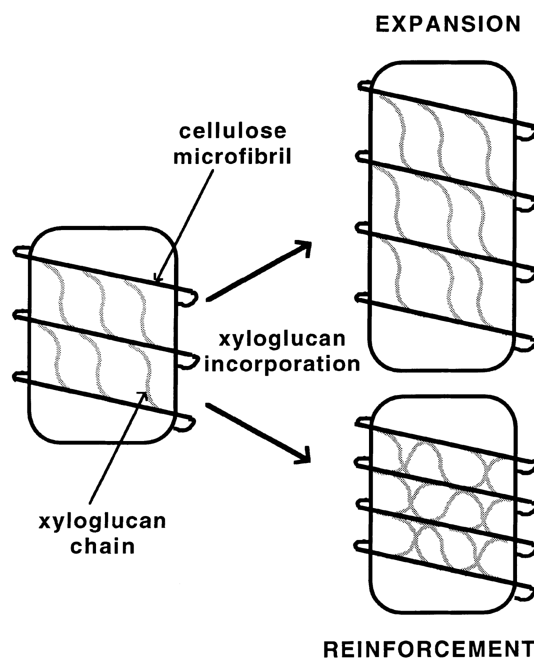

Fig. 3. Scheme showing how TCH4 may function to incorporate xyloglucan into cell walls. During both cell expansion (*top right*) and cell wall reinforcement of nongrowing cells (*bottom right*), nascent xyloglucan may be incorporated into the cell wall. We propose that the TCH4 XET functions in transglycosylating xyloglucan during incorporation into the wall during these two physiological processes

or may have distinct pH or temperature optima. Alternatively, the enzymes may have identical enzymatic activities, but the consequences of the activities could be distinct. For example, some enzymes may contribute to cell wall degradation, whereas others may act to assemble nascent cell walls or reinforce established walls. Functional consequences could be determined spatially; that is, some of the *XTRs* may be expressed only in specific tissues or cells, or the XTR proteins may be specifically targeted to distinct regions of the cell wall. To gain a fuller understanding of the functions of different *XET*-related genes in *Arabidopsis*, the in-vitro enzymatic activities of the encoded proteins and the organ- and cell-type-specific gene expression and XTR protein accumulation remain to be determined.

Analysis of *TCH4* expression leads to insight into the possible developmental and physiological functions of the *TCH4*-encoded XET. We find a strong correlation of TCH4 with expansion because *TCH4* expression is upregulated by the growth-promoting hormones auxin and brassinosteroid and in-situ *TCH4-uidA* expression is prominent in regions where cellular expansion is likely occurring (Xu et al. 1995). That is, the *TCH4-uidA* reporter gene is strongly expressed in young expanding leaves, trichomes, and lateral root primordia. Under low-light conditions, *TCH4-uidA* is expressed throughout the elongated hypocotyl. Cells likely undergoing cell wall modification, such as those in the developing vascular tissue and abscission zones, also express the *TCH4-uidA* reporter gene.

The correlation of *TCH4* expression with growth and expansion was surprising in light of the fact that *TCH4*

was originally identified because of its upregulation of expression in response to mechanical stimuli which result in decreased elongation growth in *Arabidopsis*. How could *TCH4* be involved in these apparently opposite processes? There may be a common alteration of cell walls mediated by TCH4 both in response to mechanical stress and in cells undergoing expansion. We propose that the *TCH4*-encoded XET activity is involved in incorporating xyloglucan into cell walls (Fig. 3). Transglycosylation of short polymers within the wall could generate the long xyloglucan wall polymers and intertwine them among the cellulose microfibrils. This activity would lead to increased xyloglucan crosslinks with microfibrils and hence cell wall reinforcement of nongrowing cells stimulated by touch or wind (Fig. 3, bottom right); whereas, in expanding cells, xyloglucan incorporation will lead to hemicellulose replacement to maintain wall thickness and integrity concomitant with the increase in cell size (Fig. 3, top right). Because expression levels of *TCH3* and *TCH4* are regulated very similarly, it is possible that TCH3 collaborates with TCH4 in some manner during cell wall biogenesis.

Summary

Regulation of *TCH* expression may be a common and rapid response to diverse stimuli that could represent one of the earliest steps in plant adaption to environmental stress. Determining the mechanism(s) and regulatory factors involved in *TCH* gene expression and understanding the biochemical and cellular functions of the *TCH* gene products will shed light on how plants which are bombarded by diverse and changing environmental stimuli undergo appropriate physiological alterations that enable survival.

This work was supported by grants from the National Science Foundation (No. MCB-9118661 and IBN-9418750), National Institutes of Health (No. R29 GM46346) and National Aeronautics and Space Administration (No. NAGW-3139 and NAGW-5007).

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