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Genetic Analysis of the Regulation of *TCH* Gene Expression

The goals of this work were to gain insight into how *TCH* gene expression is regulated by diverse environmental and hormonal stimuli. This work resulted in the following publications and submitted manuscripts.

Iliev, E., Xu, W., Polisensky, D.H., Oh, M.-H., Torisky, R.S., Clouse, S.D., and Braam, J., Transcriptional and post-transcriptional regulation of Arabidopsis *TCH4* expression by diverse stimuli: Roles of *cis* regions and brassinosteroids, *Plant Physiol*., **130**, 770-783 (2002).

Rose, J.K.C., Braam, J., Fry, S.C., and Nishitani, K., The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolases: Current perspectives and a new unifying nomenclature, *Plant & Cell Physiol*., **43**, 1421-1435 (2002).

McCormack, E., and Braam, J., Calmodulins and related potential calcium sensors of Arabidopsis, *New Phytol.*, **159**, 585-598 (2003).

Lee, D., Polisensky, D.H., and Braam, J., Genome-wide identification of touch- and darkness-regulated Arabidopsis genes: a focus on calmodulin-like and *XTH* genes, *New Phytol.*, **165**, 429-444 (2005).

Braam, J., Tansley Review, In touch: plant responses to mechanical stimuli, *New Phytol.,* **165**, 373-389 (2005).

Delk, N., Johnson, K.A., Chowdhury, N.I., and Braam, J., *CML24*, regulated in expression by diverse stimuli, encodes a potential Ca^{2+} sensor that functions in responses to ABA, day length and ion stress, *Plant Physiol*., **139**, 240-253 (2005).

McCormack, E., Tsai,Y.-C., and Braam, J., Handling calcium signaling: Arabidopsis CaMs and CMLs, *Trends Plant Sci*., **10**, 383-389 (2005).

McCormack, E., Velasquez, L., Delk, N., and Braam, J., Touch-responsive behaviors and gene expression in plants, In *Communication in Plants: Neuronal Aspects of Plant Life* (Eds: Frantisek Baluska, Stefano Mancuso, Dieter Volkmann), Springer Press, pp. 249-261 (2006).

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Tsai, Y.-C., Delk, N., Chowdhury, N. and Braam, J. Arabidopsis potential calcium sensors regulate nitric oxide levels and the transition to flowering, Plant Signaling and Behavior, 2: 446-454 (2007).

Braam, J. and Phillips, D. Induction of plant gene expression by mechanical force, *Proceedings of the 4th International Plant Biomechanics Conference* (Eds: FW Telewski, L Kohler, FW Ewers), Michigan State University Printing Services, East Lansing, MI, in press.

Tewari, J., Olek, A., Mezzari, M., Becnel, J., Braam, J., Carpita, N.C. and McCann, M.C. Identification and classification of novel cell wall phenotypes of Arabidopsis mutants, *Plant J*., revision under review.

Chehab, E.W., Eich, E., Braam, J. Thigmomorphogenesis: A compex plant response to mechano-stimulation, Accepted, pending revision.

Summary:
The Arabidopsis *TCH* genes have complex regulation of expression. The *TCH* genes are very rapidly upregulated in expression with an increase in transcript level detectable by northern blots within five minutes. The expression is strong and transient, with a return to basal levels within one to two hours. The *TCH* genes, originally isolated as a consequence of their upregulation in response to the mechanical stimulus of touch, are also upregulated by a variety of seemingly disparate environmental and hormonal stimuli. To gain insight into the complexities of *TCH* gene regulation, we took a number of approaches.

We sought to identify whether there was a common cis element that conferred the diverse aspects of *TCH4* regulation of expression or whether distinct elements could be identified. We found that induction of expression by the diverse stimuli of touch, darkness, cold, heat and brassinosteroids is conferred to reporter genes by the same 102 bp 5' untranscribed *TCH4* region. This result is consistent with the idea that shared regulatory elements are employed by diverse stimuli. Distal regions influence magnitude of kinetics of expression and likely harbor regulatory elements that are redundant with those located more proximal to the transcriptional start site. Substitution of the proximal regulatory region sequences in the context of distal elements does not disrupt inducible expression. *TCH4* expression induction is transcriptional, at least in part because 5'-untranscribed sequences are sufficient to confer this regulation. However, 5' untranslated sequences are necessary and sufficient to confer the marked transience of *TCH4* expression, most likely through an effect on mRNA stability. Perception of BR is not necessary for *TCH4* induction by environmental stimuli because regulation is intact in the BR-insensitive mutant, *bri1-2*. The full response to auxin, however, requires the functioning of *BRI1*. Developmental expression of *TCH4* is unlikely to mediated by BR because *TCH4::GUS* is expressed in BR perception and biosynthetic mutants *bri1-2* and *det2-1*, respectively.

Because the 102 bp region shown to be sufficient for most aspects of *TCH4* regulation is related to sequences identified as responsible for regulation of the cold- and touch-inducible *CBF* genes (Iliev et al., 2002; Zarka et al., 2003), we examined whether *TCH* gene expression is appropriately regulated in the *ice1* mutant (supplied in collaboration with Zhu). ICE1 is the putative regulatory factor responsible for cold induction of *CBF* expression (Chinnusamy et al., 2003). Results indicated that inducibility of expression is intact in the *ice1* mutant although there may be differences in the transience of the induced *TCH* expression.

In collaboration with Hirt, we examined the potential role of Map kinases in the signal transduction pathways that function in *TCH* expression regulation. *MKK2* overexpressor and *mkk2* null plants were examined for *TCH* gene expression in stimulated and control plants. These lines, described in Teige *et al.* (2004), were obtained by driving a constitutively active *MKK2-EE* with the 35S CaMV promoter or by a T-DNA insertion generating an mRNA null knock out mutant. Results indicated that *MKK2* is not required for inducible *TCH2*, *TCH3* or *TCH4* expression.

TCH::LUC transgenic plants were generated to gain insight into spatiotemporal aspects of *TCH* expression. We found that although the *TCH* genes encode proteins as distinct as calcium-binding proteins and cell wall modifying enzymes, their overall expression patterns, including distribution in plant tissues and kinetics, are highly similar. However, the distribution of *TCH* expression in stimulated plants is distinct depending upon the specific stimulus applied. These distinctions in spatial expression may indicate complexities in regulation that may explain how diverse stimuli can result in distinct physiological responses and yet result in the overall upregulation of the same genes. The specificity of the response may lie in the tissue/organ localization of expression (Chehab, submitted).

Touch-inducible gene expression appears to be much more common that generally appreciated. We sought to determine the prevalence of touch-inducible genes in the Arabidopsis genome. In addition, we sought to assess whether all touch-inducible genes are similar to the *TCH* genes in that they are also inducible by other stimuli, or whether there are genes that are responsive only to touch. Furthermore, we wanted to gain insight into the physiological relevance of touch-induced gene expression. Identification of all touch-regulated genes should shed light on the types of cellular processes that may be altered in response to mechanical stress perturbations. We used microarray analysis to screen the 22810 genes represented on the Affymetrix Arabidopsis gene chip for inducibility of expression (Lee et al., 2005). We chose to use darkness as the second stimulus because it appears to be very different from the direct mechanical perturbation and can be delivered to plants uniformly by simply removing light. Three separate sets of plants were harvested for each stimulus. Strikingly, 589 genes had touch-inducible expression; 171 had reduced expression. Darkness increased expression of 461 genes and decreased expression of 72 genes. Over half of the touch-inducible genes resemble the *TCH* genes in that they are also upregulated by darkness; 67% of those darkness-inducible were also touch inducible. Expression of 12 calmodulin-like (*CML*) genes and four genes encoding xyloglucan endotransglucosylase/hydrolases (XTHs) were elevated by touch. Three *XTHs* had reduced expression. In darkness-treated plants, 10 *CMLS* and nine *XTHs* had increased expression and one *XTH* was repressed. Over 2.5% of total genes were touch-inducible. Many were also darkness upregulated, consistent with the hypothesis that these stimuli have partially overlapping signal transduction pathways. Regulated gene identities suggest that calcium and kinase signaling, wall modification, disease resistance and downstream transcription responses may be altered in response to mechanostmulation or darkness.

A long-term goal of our proposed work was to identify mutants defective in *TCH* gene regulation and isolate the genes responsible for the mutant phenotypes. We isolated a number of potential mutants that were defective in *TCH4::LUC* expression by identifying plants with higher and/or more sustained luciferase activity after heat stimulation than the parental *TCH4::LUC* plants under similar conditions. We focused primarily on mutants with higher than parental *TCH4::LUC* activity because we did not want to identify plants that were epigenetically gene silenced. Most of these mutants also had altered endogenous *TCH4* expression using both northern and quantitative real-time RT-PCR, providing evidence that the change in luciferase expression was not due to a mutation to the *LUC* gene itself but was a consequence of an alteration in the functioning of the *TCH4* regulatory region. Unfortunately, however, when endogenous *TCH*

expression was compared among the mutants, the parental *TCH4::LUC* and the Col-O background control, we discovered that all the transgenic lines had lower expression than the wild type control, most likely resulting from co-suppression of the starting parent line. Thus our potential mutants were likely to not be defective in *TCH* gene regulation but instead were reversing the gene silencing that had occurred in the parental line.

We then conducted the screen with a different *TCH4::LUC* line that maintained wild type levels of endogenous *TCH4* expression. Seeds from this line have been mutagenized with EMS and with T-DNA insertions (in collaboration with Bressan and Hasegawa, Purdue University).

We have also begun to investigate characteristics of expression of the *TCH2* gene, also called *CML24*, which encodes a calmodulin (CaM)-like (CML) protein (Delk et al., 2005). CML24 shares over 40% amino acid sequence identity with CaM, has 4 EF hands and undergoes a Ca^{2+}-dependent change in migration rate through denaturing gel electrophoresis, indicating that CML24 binds Ca^{2+} and, as a consequence, undergoes conformational changes. We have found that *CML24* expression occurs in all major organs and is induced from 2- to 15-fold in plants subjected to touch, darkness, heat, cold, hydrogen peroxide, abscisic acid (ABA) and indole-3-acetic acid. The putative *CML24* regulatory region confers reporter expression at sites of predicted mechanical stress, in regions undergoing growth, in vascular tissues and various floral organs and in stomata, trichomes and hydathodes. *CML24* underexpressing transgenics are resistant to ABA inhibition of germination and seedling growth, defective in long-day induction of flowering, and have enhanced tolerance to $CoCl_2$, molybdic acid, $ZnSO_4$ and $MgCl_2$. $MgCl_2$ tolerance is not due to reduced uptake nor to elevated Ca^{2+} accumulation. Together these data present evidence that *CML24*, a gene expressed in diverse organs and responsive to diverse stimuli, encodes a potential Ca^{2+} sensor that may function to enable responses to ABA, day length and presence of various salts. Further investigation of CML24 function and regulation led us to find a critical role for CML24 in nitric oxide regulation. Because of the potential for cross reactivity and instability of the epigenetic silencing in the underexpressing lines, we used tilling to identify true genetic lesions within the CML24 gene. We found that distinct alleles encoding mutant forms of the Arabidopsis potential calcium sensor CML24 also cause alterations in flowering time. CML24 can act as a switch in the response to day length perception; loss-of-function *cml24* mutants are late flowering under long days, whereas apparent gain of CML24 function results in early flowering (Tsai et al., 2007). We identified at which steps in the flowering transition regulatory pathways, CML24 functions. We found that CML24 is required for proper *CONSTANS* (*CO*) expression whereas components upstream of *CO* in the photoperiod pathway are largely unaffected in the *cml24* mutants. Because of potential redundancy with the related CML23 gene, we identified and characterized T-DNA insertion mutants of CML23. We found that CML23 and CML24 have overlapping, though nonidentical expression and function (Tsai et al., 2007). Together, CML23 and CML24 inhibit *FLOWERING LOCUS C* (*FLC*) expression and therefore impact the autonomous regulatory pathway of the transition to flowering. Nitric oxide levels are elevated in *cml23/cml24* double mutants and are largely responsible for *FLC* transcript accumulation. Therefore, *CML23* and *CML24* are potential calcium sensors regulate nitric oxide accumulation. In turn, NO levels influence the transition to flowering through both the photoperiod and autonomous regulatory pathways.

Understanding how the CML proteins control nitric oxide accumulation will be an important future research goal.

We also completed two family-wide analyses on XTH gene function. First, in collaboration with McCann and Carpita, we used fourier transform infrared spectroscopy (FTIR) to assess, verify and classify wall architectural changes that occur as a result of single XTH insertion mutations (Tewari et al., submitted). Thirty-four homozygous mutant lines of Arabidopsis representing 21 members of the xyloglucan endotransglucosylase/hydrolase gene family provided a set of mutants in which altered cell wall phenotypes are expected but are uncharacterized. While there were few obvious changes in sugar composition of mutant lines compared to wild type, novel phenotypes were identified by infrared spectroscopy, indicating several distinct altered wall architectures. Various non-allelic *xth* mutant lines were deficient in methyl ester but enriched in cellulose compared with wild type. We used Kohonen networks to classify cell wall architectures of *xth* mutant lines and previously characterized cell wall mutants. The *xth* spectroscopic phenotypes were distinct from those of *rsw1*, a temperature-sensitive cellulose synthase mutant, and seven *murus* mutants having specific defects in matrix polysaccharide composition. We compared the relative similarities between spectra derived from populations of different genotypes. We conclude that *xth* mutants have chemical changes in their cell walls not detectable as phenotypic growth and development changes, consistent with the existence of feed-back loops that modify wall composition in response to a life-long deficiency of a cell wall enzyme.

Furthermore, to gain insight into the potential physiological relevance of the distinct members of this family, *GUS* reporter fusion genes were constructed, and plants expressing these transgenes were characterized to reveal spatial and temporal patterns of expression (Becnel et al., 2006). In addition, Genevestigator sources were mined for comprehensive and comparative *XTH* expression regulation analysis (Becnel et al., 2006). These data revealed that the Arabidopsis *XTHs* are likely expressed in every developmental stage from seed germination through flowering. All organs showed *XTH::GUS* expression and most, if not all, are found to express multiple *XTH::GUS* genes. These data suggest that XTHs may contribute to morphogenesis at every developmental stage and in every plant organ. Different *XTHs* have remarkably diverse and distinct expression patterns indicating that paralogous genes have evolved differential expression regulation perhaps contributing to the maintenance of the large gene family. Extensive overlap in *XTH* expression patterns is evident; thus, XTHs may act combinatorially in determining wall properties of specific tissues or organs. Knowledge of gene-specific expression among family members yields evidence of where and when gene products may function and provides insights to guide rational approaches to investigate function through reverse genetics.