

# Transcriptional and Posttranscriptional Regulation of Arabidopsis *TCH4* Expression by Diverse Stimuli. Roles of cis Regions and Brassinosteroids<sup>1</sup>

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The Arabidopsis *TCH4* gene is up-regulated in expression by diverse environmental and hormonal stimuli. Because *TCH4* encodes a xyloglucan endotransglucosylase/hydrolase, this change in expression may reflect a recruitment of cell wall-modifying activity in response to environmental stress and growth. How diverse stimuli lead to the common response of *TCH4* expression regulation is not known. Here, we show that induction of expression by the diverse stimuli of touch, darkness, cold, heat, and brassinosteroids (BRs) 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 and 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::GUS* 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 *BR11*. Developmental expression of *TCH4* is unlikely to be mediated by BR because *TCH4::GUS* is expressed in BR perception and biosynthetic mutants *bri1-2* and *det2-1*, respectively.

Plants are sensitive to a number of abiotic environmental stimuli including light, wind, and temperature. Changes in these environmental conditions often result in rapid and dramatic alterations in plant gene expression, and these molecular responses likely aid plants in acclimating to or withstanding the potential stresses of the environment.

There are sets of genes that change their expression level in response to light stimuli (Ma et al., 2001), others that show elevated expression in extreme heat (Sung et al., 2001), and others that are induced in expression by cold (Thomashow, 1999). The existence of distinct gene sets that respond to different stimuli suggests that specific receptors and signal transduction pathways are utilized in response to alterations in light and different temperature extremes to drive distinct gene expression changes.

In addition to genes whose expression is regulated in response to a single stimulus, there are genes that are induced in expression by multiple, diverse stimuli. For example, the *TCH4* gene of Arabidopsis was originally discovered because of its dramatic response to the seemingly innocuous stimulus of touch (Braam and Davis, 1990). *TCH4* encodes a xyloglucan endotransglucosylase/hydrolase (XTH, formerly abbreviated XET; Xu et al., 1995; Campbell and Braam, 1998). *TCH4* is also up-regulated by darkness, heat shock, and cold shock (Braam and Davis, 1990; Braam, 1992; Polisensky and Braam, 1996). In addition, *TCH4* expression is elevated by brassinosteroids (BRs) and auxin (indole-3-acetic acid [IAA]; Xu et al., 1995). Because *TCH4* expression is strongly influenced by environmental and hormonal stimuli and the encoded protein acts on a major component of the

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plant cell wall, we hypothesize that TCH4 plays a role in cell wall modifications in response to environmental stress and during morphogenesis (Xu et al., 1995, 1996; Campbell and Braam, 1999). How these diverse stimuli lead to the common molecular response of TCH4 regulation of expression is unknown.

One possibility is that the TCH4 regulatory region may contain separate cis-acting elements, with each responding to activation of a separable signal transduction pathway. For example, the TCH4 locus, being elevated in expression by both heat and cold, would harbor the heat shock element and dehydration-responsive element, cis-elements defined to drive expression by heat and cold, respectively, in Arabidopsis (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Schöffl et al., 1998). Alternatively, genes such as TCH4 may be controlled by a single cis-element that is responsive to multiple stimuli. In this scenario, various signal transduction pathways may converge at some point before the induction of TCH4 transcriptional activity. For example, various environmental stimuli could cause increases in an endogenous growth regulator, such as BRs, which could serve to mediate gene expression changes. Furthermore, there may exist multiple signal transduction pathways with various degrees of shared and separate components. One way to distinguish between these possible scenarios is to identify the region responsible for TCH4 expression regulation and determine if separate regulatory cis-elements exist. In addition, mutants defective in hormone biosynthesis and/or response can be used to investigate the potential roles of hormones in mediating gene expression responses to environmental stimuli.

We tested the transcriptional activity of sequences found within the TCH4 locus to localize regions controlling induction of TCH4 expression. We found that both transcriptional and posttranscriptional mechanisms are involved in TCH4 gene regulation. In addition, using BR biosynthesis and response mutants, we assessed the role of BR as a mediator of TCH4 regulation.

## RESULTS

### The Regulatory Region of TCH4

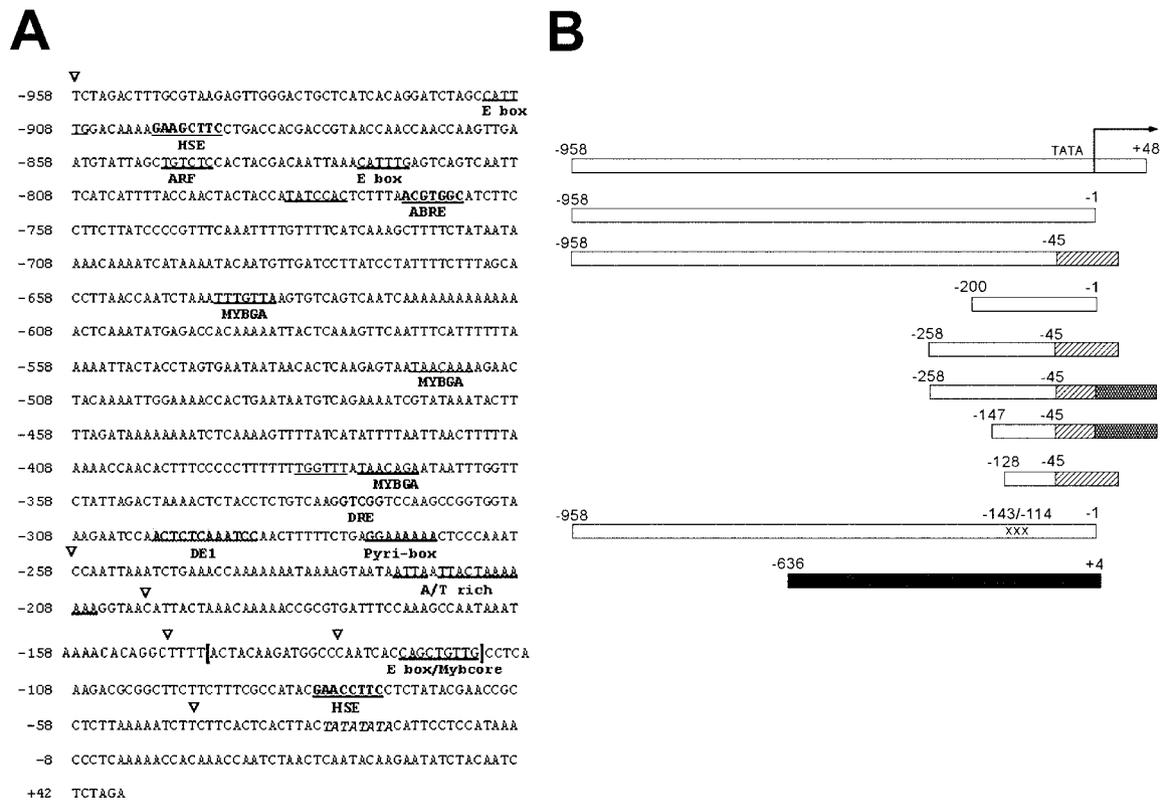
Approximately 1 kb of 5'-non-coding sequences, including the 5'-untranslated region (UTR), of the TCH4 locus were shown to confer upon a reporter gene the regulatory properties of TCH4 (Xu et al., 1995). To begin identifying the DNA sequences responsible for TCH4 regulation, we compared the TCH4 5' sequences with known regulatory motifs (Fig. 1A). At position -299/-288 relative to the transcriptional start site (+1), 10 of 12 bases match a reverse complement DE1 element (GGATTTTACAGT) shown to be sufficient for darkness inducibility of expression (Inaba et al., 2000). There are three E

box motifs (CANNTG) at positions -912/-907, -826/-821, and -122/-117. E boxes in eukaryotic genomes act as binding sites of basic helix-loop-helix transcription factors (Massari and Murre, 2000). The E box at position -122/-117 is overlapping with CTGTTG, a reverse complement of an MYB core motif (YAACNG). There are three additional MYB-related motifs [YAACA(A/G)A], found at -642/-636, -520/-514, and -376/-371. These sequences are similar to those important for the functioning of the GA-responsive element (Lovegrove and Hooley, 2000). GA-responsive elements can be found associated with a pyrimidine box (also called box 2) and a TATCCAC motif, elements thought to enhance regulation (Lovegrove and Hooley, 2000). Interestingly, sequences related to all three of these components are present in the upstream region of TCH4 but are in a different relative placement and spacing from the transcriptional start site than would be expected based on analyses of GA-regulated genes (Gubler and Jacobsen, 1992; Lanahan et al., 1992). A pyrimidine box is present at -275/-268 and a TATCCAC box resides at -794/-788. At -848/-843, there is an auxin response factor-binding site consensus sequence (TGTCTC) that is found in early auxin response genes (Ulmasov et al., 1997). An ACGT-containing abscisic acid (ABA) response element (Busk and Pages, 1998) resides at -771/-765. However, full ABA inducibility requires the presence of multiple copies of ABA response element or a coupling element (Shen and Ho, 1995; Shen et al., 1996; Hobo et al., 1999). Located at -328/-324 is an inverted copy CCGAC, a core sequence of the dehydration-responsive element (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994) found in many cold- and drought-responsive genes. Two potential heat shock elements (Schöffl et al., 1998), each consisting of two copies of alternating units of nGAAn, are located at -899/-892 and -81/-74. However, because efficient binding of heat shock factor requires at least three tandem nGAAn units (Barros et al., 1992), it is unclear whether these sites would be sufficient to confer heat shock regulation.

To test the functional relevance of these sequences and identify potential cis-regulatory regions, we generated subregions (Fig. 1B), fused them to the reporter genes encoding  $\beta$ -glucuronidase (GUS) and firefly luciferase (LUC), introduced them into Arabidopsis plants, and assayed gene activity in response to environmental and hormonal stimuli.

### Role of TCH4 5'-UTR in the Transience of Induced Gene Expression

TCH4 induction of expression is remarkably transient in nature (Braam and Davis, 1990), indicating that if TCH4 is regulated by transcriptional induction, transcription initiation must cease rapidly and the TCH4 transcripts must be unstable. Previously,

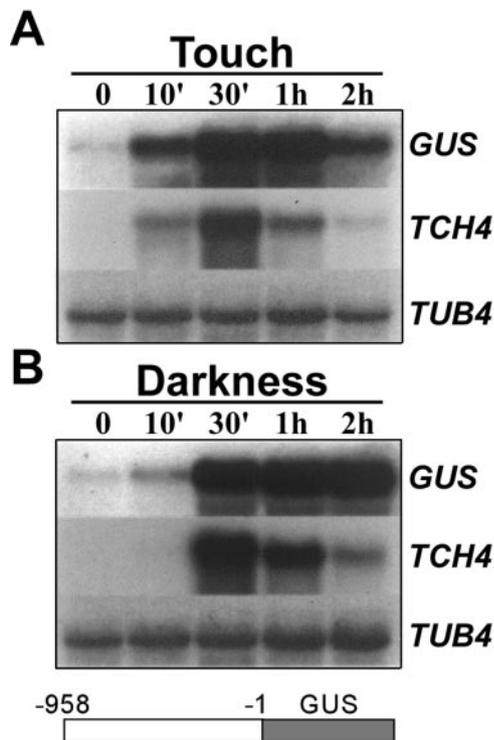


**Figure 1.** 5' *TCH4* genomic sequence and schematic representation of *TCH4* regions generated and tested for transcriptional regulatory activity. A, Genomic sequence of *TCH4* upstream region sufficient to confer up-regulation of expression. The inverted triangles indicate the 5' positions of *TCH4* regions generated and tested for transcriptional regulatory activity (see B). Sequences related to defined regulatory motifs in the sense orientation are underlined, whereas inverted motifs are shaded in gray (motifs are defined in text). The putative TATA box is indicated in italics. Numbering refers to distance from transcriptional start site designated as +1. B, White rectangles represent *TCH4* sequences between -958 and +48 and subregions fused to the *GUS* and *LUC* reporter genes. The numbering refers to distance relative to the transcriptional start site (+1). The arrow indicates the start position and direction of transcription. For some constructs, the endogenous promoter was replaced with -90- or -46-bp cauliflower mosaic virus (CaMV) 35S promoter (hatched, size indicated by rectangle length); some constructs also have a 143-bp region of a 5'-UTR from tobacco etch virus (TEV) (cross hatched). The penultimate rectangle from the bottom represents a construct containing *TCH4* sequences from -958 to -1 that has substituted nucleotides between -143 and -114. The black rectangle at the bottom represents the sequences present from the *UBQ10* locus for *UBQ10::LUC*. These representations of transgenes are not drawn to scale.

we showed that *GUS* transgenes containing -958 to +48 *TCH4* sequences, which include the 5'-UTR, are up-regulated in expression with similar magnitude and kinetics as endogenous *TCH4* (Xu et al., 1995). This result indicates that the *TCH4* -958 to +48 sequences are sufficient to confer transient expression to a reporter gene. Because the only *TCH4* sequences present in the transcript are the 48-base 5'-UTR, it is likely that this UTR is sufficient to result in mRNA instability. We further tested this possibility by analyzing the mRNA accumulation kinetics from a *GUS* transgene fused to only the *TCH4* upstream -958 to -1 sequences. Removal of the 48-base UTR does not affect the inducibility of the transgene but results in a prolonged accumulation of *GUS* mRNAs relative to that of the endogenous *TCH4* transcripts in plants stimulated by touch (Fig. 2A), darkness (Fig. 2B), or heat (Fig. 3A, III). These results are consistent with a role for the 5'-UTR of *TCH4* in mRNA insta-

bility. Furthermore, these data indicate that only 5'-untranscribed sequences, and not sequences in the mRNA, are required for conferring touch, darkness, and heat inducibility to transgenes. Therefore, *TCH4* is most likely increased in expression by transcriptional up-regulation and not by posttranscriptional mechanisms acting through the mRNA.

Figure 3 illustrates data relevant to identifying the 5'-regulatory sequences of *TCH4* (to be discussed in the next section) and cis-elements affecting mRNA accumulation kinetics (addressed here). Surprisingly, transcripts with 5'-untranslated sequences from the TEV, which we added to the transgenes in efforts to enhance translation (Carrington and Freed, 1990; Nunberg et al., 1994), also accumulate only transiently. Figure 3, B and C, compare the accumulation kinetics of *GUS* mRNA derived from transgenes harboring the -258 to -45 *TCH4* region and a CaMV 35S minimal promoter region with (transgene IV) or



**Figure 2.** The loss of 48 bp of the 5'-UTR of *TCH4* affects the transient accumulation of mRNA after touch or darkness. A and B, Total RNA was isolated from plants after no stimulus (0) or after touch (A) or darkness (B). Plants were harvested at the indicated times. Four micrograms of total RNA was size fractionated on gels, blotted onto nylon membranes, and hybridized sequentially with the probes shown at right. The prime indicates minutes; "h" stands for hour(s). *TUB4* (*Tubulin*) is shown as a loading and transfer control. The transgene assayed is represented by the rectangle at the bottom (not drawn to scale); see Fig. 1 legend for details.

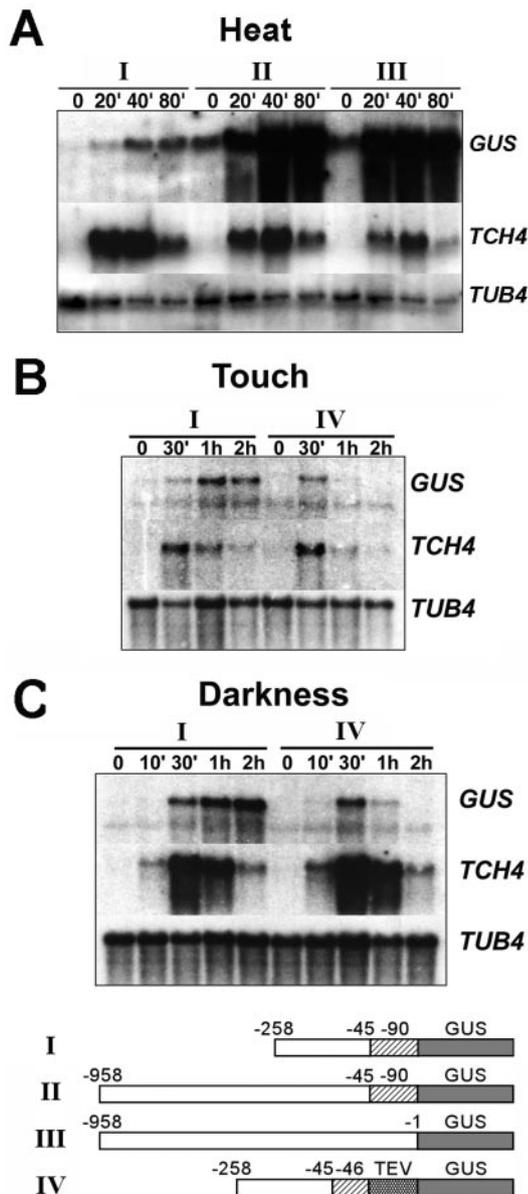
without (transgene I) the TEV 5'-UTR. For historical reasons, the transgenes without the TEV 5'-UTR have 90 bp of the CaMV 35S promoter, whereas transgenes with the TEV 5'-UTR have 46 bp of the CaMV 35S promoter. The  $-90/+8$  CaMV 35S region was originally thought to serve as a minimal promoter; subsequently, it was determined that only the  $-46/-1$  region is required for minimal promoter activity and lacks an element between  $-83$  and  $-63$  that can confer root-specific expression (Benfey et al., 1989; Lam et al., 1989). In response to touch (Fig. 3B) and darkness (Fig. 3C), *TCH4* expression is transiently up-regulated with a prominent decrease in transcript accumulation by 2 h. Transcripts derived from the *TCH4::GUS* transgenes that harbor the TEV 5'-UTR have accumulation kinetics closely comparable with that of *TCH4* transcripts (Fig. 3, BIV and CIV). The response of the *TCH4::GUS* transgenes lacking a *TCH4* or TEV 5'-UTR is more prolonged with an abundance of *GUS* transcripts present up to 2 h post-stimulation (Fig. 3, AI, BI, and CI). There are two possible explanations for this result. First, the TEV 5'-UTR may result in *GUS* mRNA instability. Alter-

natively, because the *TCH4::GUS* transgenes differ not only in the presence of the TEV 5'-UTR but also in the length of the CaMV 35S minimal promoter region, it is also possible that transcriptional initiation fails to shut off efficiently in the context of the  $-90$  CaMV 35S promoter. Overall, these results indicate that the *TCH4* and, most likely, the TEV UTRs, can confer mRNA instability to the *GUS* mRNA. The *TCH4* 5'-UTR, therefore, most likely plays a role in the transient nature of *TCH4* induction of expression.

#### The Sequences between $-258$ and $-45$ Are Sufficient to Confer Response to Heat, Touch, and Darkness

We tested transgenic plants harboring subregions of the *TCH4* sequences (Fig. 1B) fused to reporter genes for the ability to confer up-regulation of expression in response to stimuli known to lead to an increase in expression of *TCH4*. Subjecting plants to  $35^{\circ}\text{C}$  results in an increase in *TCH4* mRNA within 20 min (Fig. 3A, *TCH4*). The level of *TCH4* expression induction by heat can vary among plants (Fig. 3A, *TCH4*); therefore, we compared reporter gene expression levels with those of the native *TCH4* gene. The *GUS* gene driven by the  $-258$  to  $-45$  region of the *TCH4* locus and including the  $-90/+8$  CaMV promoter is up-regulated in expression by high temperature, similar to *TCH4*; however, there is a delay in *GUS* mRNA accumulation compared with that of the endogenous *TCH4* mRNA (Fig. 3A). This transgene is identical to that used for assessment of touch and darkness inducibility shown in Figure 3, B, I; and C, I. The alteration in accumulation kinetics of this transgene's transcripts is also seen after touch and darkness stimuli, as discussed below. *GUS* mRNAs derived from *TCH4::GUS* transgenes harboring additional *TCH4* sequences ( $-958$  to  $-45$  and  $-958$  to  $-1$ ) accumulate to much higher levels than those derived from the  $-258/-45$  *TCH4::GUS* transgenes (Fig. 3, A, II and III, *GUS*). Similar reductions in magnitude are seen for cold inducibility of *TCH4::GUS* transgenes harboring  $-258/-45$  *TCH4* sequences as compared with those with additional distal sequences (data not shown). With respect to BR induction, the kinetics and magnitude of response to BR treatment of transgenic plants harboring a  $-958/-1$  *TCH4::GUS* construct are nearly identical to that of the endogenous *TCH4*, whereas a  $-200/-1$  *TCH4::GUS* transgene shows dramatic reduction in expression levels while still retaining BR inducibility (data not shown). These results suggest that sequences between  $-958$  and  $-258$  affect the magnitude of mRNA accumulation, perhaps acting as quantitative enhancers or due to the presence of redundant functional motifs. Therefore, although there are regulatory elements within the  $-258$  to  $-45$  region, these 213 bp do not represent the complete *TCH4* control region.

The  $-258$  to  $-45$  region of *TCH4* is also sufficient to confer touch and darkness inducibility of expres-

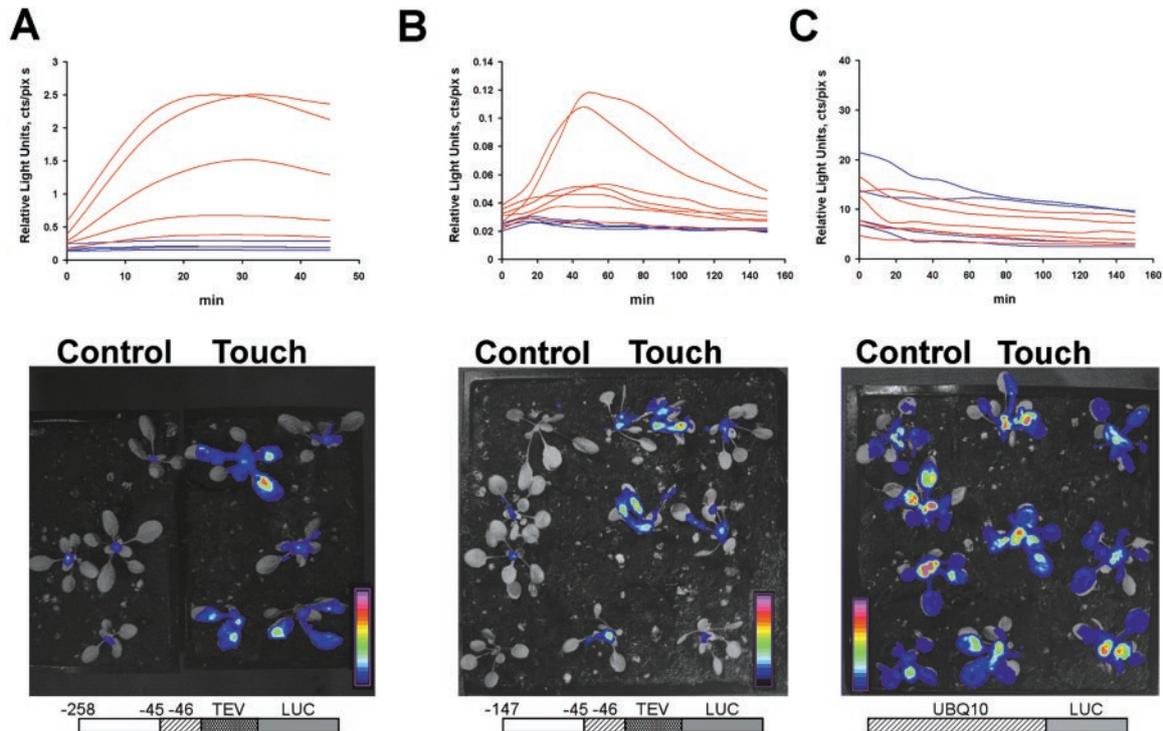


**Figure 3.** *TCH4* sequences between  $-258$  and  $-45$  are sufficient to confer up-regulation of expression in response to heat, touch, and darkness. A, Plants harboring transgenes with *TCH4* sequences between  $-258$  and  $-45$  (I),  $-958$  and  $-45$  (II) and the  $-90$  CaMV 35S minimal promoter fused to *GUS*, and  $-958$  and  $-1$  (III) fused to *GUS* were unstimulated (0) or placed at  $35^{\circ}\text{C}$ . B and C, Plants harboring transgenes composed of *TCH4* sequences between  $-258$  and  $-45$  and the  $90$  bp of the CaMV 35S promoter (“I”) or *TCH4* sequences between  $-258$  and  $-45$ , the  $46$  bp of the CaMV 35S promoter, and the TEV leader (“IV”) were left unstimulated (0) or stimulated with touch (B) or darkness (C). A through C, Plants were harvested at the indicated times. Eight micrograms of total RNA was size fractionated on gels, blotted to nylon filters, and hybridized sequentially to probes listed on the right. The lower band in the *GUS* panel in B and C is frequently observed and is nonspecific. The prime indicates minutes; h indicates hour(s). *TUB4* is shown as a loading and transfer control. The transgenes assayed are represented by rectangles at the bottom of the figure (not drawn to scale); see Fig. 1 legend for details.

sion upon the *GUS* reporter gene (Fig. 3, B and C, respectively). The transgene with the  $-46$ -bp CaMV 35S promoter and the TEV 5'-UTR has expression kinetics that closely reflect that of the endogenous *TCH4* (Fig. 3, BIV and CIV); whereas transcripts derived from the transgene with the  $-90$ -bp CaMV 35S promoter and lacking a 5'-UTR region are delayed in both up- and down-regulation (Fig. 3, BI and CI). The observed delay in accumulation of mRNA is unlikely to be due to loss of transcriptional regulatory sequences because the same *TCH4* region is sufficient to up-regulate expression with the rapid kinetics of the endogenous *TCH4* when in the context of the  $-46$ -bp CaMV minimal promoter and the TEV 5'-UTR. Therefore, the delay in kinetics may be due to the combination of the  $-90$ -bp CaMV 35S promoter region and the short  $-258$  to  $-45$  *TCH4* regulatory region in transgene I; the longer minimal promoter may impact the functioning of these *TCH4* regulatory sequences, perhaps by placing them too far from the transcriptional start site.

Assessment of the regulatory activity of subregions of the  $-258$  to  $-45$  sequences using *GUS* reporter gene fusions and northern analysis was not feasible because the amount of *GUS* mRNA generated by these gene fusions was below the level of detection (data not shown). Therefore, we employed firefly *LUC* as a more sensitive reporter and monitored expression as real-time activity displayed as luminescence. *LUC* activity can be detected at very low levels and because *LUC* activity, unlike *GUS*, has a relatively short half-life (Millar et al., 1992), it is a good tool for reporting rapid and relatively transient changes in gene expression.

To verify that *LUC* activity reflects gene expression, we monitored luminescence emission over time of transgenics harboring the  $-258$  to  $-45$  *TCH4::LUC* transgene. As shown in Figure 4A, the touched transgenics (individual plants represented by red traces in top panel, right portion of bottom panel) emit higher levels of light than the untouched control population (blue traces in top panel, left portion of bottom panel). There is basal *LUC* activity in unstimulated transgenic plants, primarily detected from the shoot apex (Fig. 4A, left, bottom). Similarly, *TCH4::GUS* activity is found in young expanding leaves in the shoot apex (Xu et al., 1995). In comparison, touched transgenics show *LUC* activity primarily at sites directly mechano-stimulated such as leaves and petioles, while retaining expression at the shoot apex (Fig. 4A, right, bottom). Independent transgenics show similar responses (data not shown). The touch-induced increases in luminescence are due to the function of the *TCH4* regulatory sequences, because a similar *LUC* transgene driven by the *UBQ10* regulatory region (Sun and Callis, 1997) shows no significant change in activity over time after comparable stimulation (Fig. 4C). *UBQ10* expression has been reported to be relatively constitutive (Sun and Callis, 1997). This is an important



**Figure 4.** Inducible in vivo LUC activity conferred by the  $-258$  to  $-45$  and  $-147$  to  $-45$  *TCH4* sequences in response to touch stimulation. Plants harboring  $-258$  to  $-45$  *TCH4*::*LUC* (A),  $-147$  to  $-45$  *TCH4*::*LUC* (B), or *UBQ10*::*LUC* (C) transgenes, were sprayed with 1 mM luciferin and 50 mM sodium citrate, pH 5.6, and allowed to absorb the substrate for 30 min. The plants were then placed in a low-light imager and an initial luminescence reading was obtained before stimulation. Control plants were left undisturbed, whereas others were touch stimulated by gently bending them back and forth 20 times. Fifteen-minute (A and B) or 5-min (C) luminescence readings were collected, and the data were extracted and analyzed with Excel (Microsoft, Redmond, WA). The graphs at the top represent the response profiles of individual plants. Traces of control plants are shown in blue; touch-stimulated plants are shown in red. Bottom, Computer-generated false-color overlays of the light emission at 45 min poststimulation. The color bar inset represents the dynamic range of light levels, where minimum light is indicated with dark blue and maximum light is shown in magenta. The transgenes assayed are represented by rectangles below each panel (not drawn to scale); see Figure 1 legend for details.

control because luminescence from transgenics with constitutively expressed *LUC* genes have been seen to increase after wounding under some conditions, apparently due to increased uptake of the luciferin substrate (Nass and Scheel, 2001; D.H. Polisensky, E. Iliev, and J. Braam, unpublished results). We found that the use of young plants, sodium citrate (pH 5.6) as a solvent for luciferin, and a 30-min absorption time improves the uptake of luciferin and results in no significant changes in *UBQ10*::*LUC* activity (Fig. 4C; D.H. Polisensky, E. Iliev, and J. Braam, unpublished data).

#### The *TCH4* Upstream Region between $-147$ and $-45$ Is Sufficient to Confer Responses to Touch, Temperature Shocks, Darkness, and 24-Epibrassinolide (24-epiBL)

To further define the potential cis-regulatory element(s) conferring *TCH4* up-regulation of expression in response to environmental and hormonal stimuli, we tested subregions of the  $-258$  to  $-45$  sequences of *TCH4*. Figure 4B illustrates that the  $-147$  to  $-45$  *TCH4* sequences confer touch-inducible expression to

*LUC* (Fig. 4B). Similar to that of the  $-258$  to  $-45$  *TCH4*::*LUC* transgene, the  $-147$  to  $-45$  *TCH4*::*LUC* expression in touched plants is observed in leaves, petioles, and shoot apex, whereas unstimulated plants show active LUC primarily in the shoot apex. However, the basal and induced expression levels conferred by the shorter 102 bp of the  $-147$  to  $-45$  *TCH4* region are lower (approximately 10-fold reduction) than that conferred by the  $-258$  to  $-45$  region. In addition, activity peaks at approximately 60 min when regulation is conferred by the shorter regulatory region, whereas the  $-258$  to  $-45$  *TCH4*::*LUC* transgenics show a maximal response at approximately 30 min. Independent transformants harboring the  $-147$ / $-45$  transgene show similar expression behaviors with respect to magnitude and kinetics of induction (data not shown). These results indicate that the *TCH4* sequences between  $-147$  and  $-45$  are sufficient to confer touch-induced up-regulation of expression; however, additional sequences, residing between  $-258$  and  $-147$ , play a role in controlling the magnitude of basal and induced expression in addition to enabling the rapidity of enhanced trans-

scription initiation. We find that this 102-bp region between  $-147$  and  $-45$  may be approaching the minimal length for assaying regulatory activity conferred upon reporter genes. When we removed 19 additional bases to generate a  $-128$  to  $-45$  *TCH4::GUS* reporter, we were unable to detect activity even under induced conditions in multiple independent transgenics (data not shown).

To test whether the  $-147$  to  $-45$  *TCH4* genomic sequences are sufficient to confer full regulatory properties of *TCH4* to a reporter gene, we subjected transgenic plants to other inducing stimuli, allowed the newly synthesized LUC to accumulate for 1 to 3 h (as indicated), and then applied luciferin and monitored levels of LUC activity. The luminescence data were collected and binned according to relative levels, and the numbers of individual plants per bin are reported in Figure 5 (bar graphs in top panels). False-color representations of luminescence overlaid on photographs of plants are shown in the lower panels of Figure 5. Similar results were obtained with an additional independent transgenic (data not shown).

The  $-147$  to  $-45$  *TCH4::LUC* transgenic plants emit increased luminescence in response to a 10-min cold treatment (Fig. 5A), 1 h at  $37^{\circ}\text{C}$  (Fig. 5B), 2 h of darkness (Fig. 5C), and 3 h after application of 24-epiBL, a commonly used form of synthetic brassinolide (Fig. 5D). There is inherent variation in luminescence from control and stimulated plants; however, in all cases, the differences in luminescence between control and stimulated plants are apparent. The induced responses are strongest for darkness and heat shock. Similar assays for auxin (IAA) induction of the  $-147$  to  $-45$  *TCH4::LUC* expression showed no detectable response in multiple transgenics (data not shown). Transgenic plants with the *UBQ10* regulatory region driving *LUC* were used as controls for these experiments to assess whether any of the treatments affect *LUC* activity and/or luminescence production. *UBQ10::LUC*-generated luminescence levels after treatments with touch, darkness, or 24-epiBL application used here were comparable with that of untreated plants (Fig. 4C, additional data not shown). After the cold and heat treatments administered in these experiments, slight decreases in luminescence from *UBQ10::LUC* plants are observed (data not shown). Reductions in luminescence after transgenic plant exposure to temperature extremes may reflect reduced photosynthetic rates and ATP availability. Overall, the data in Figures 4B and 5 indicate that the *TCH4* genomic sequences between  $-147$  and  $-45$  harbor cis-regulatory element(s) sufficient to confer up-regulation of expression in response to mechanical stimulation, cold shock, heat shock, darkness, and 24-epiBL.

We compared sequences between  $-147$  and  $-45$  to potential regulatory regions of other touch-inducible genes, including *TCH1* (Braam and Davis, 1990), *TCH2* (Braam and Davis, 1990; Khan et al., 1997),

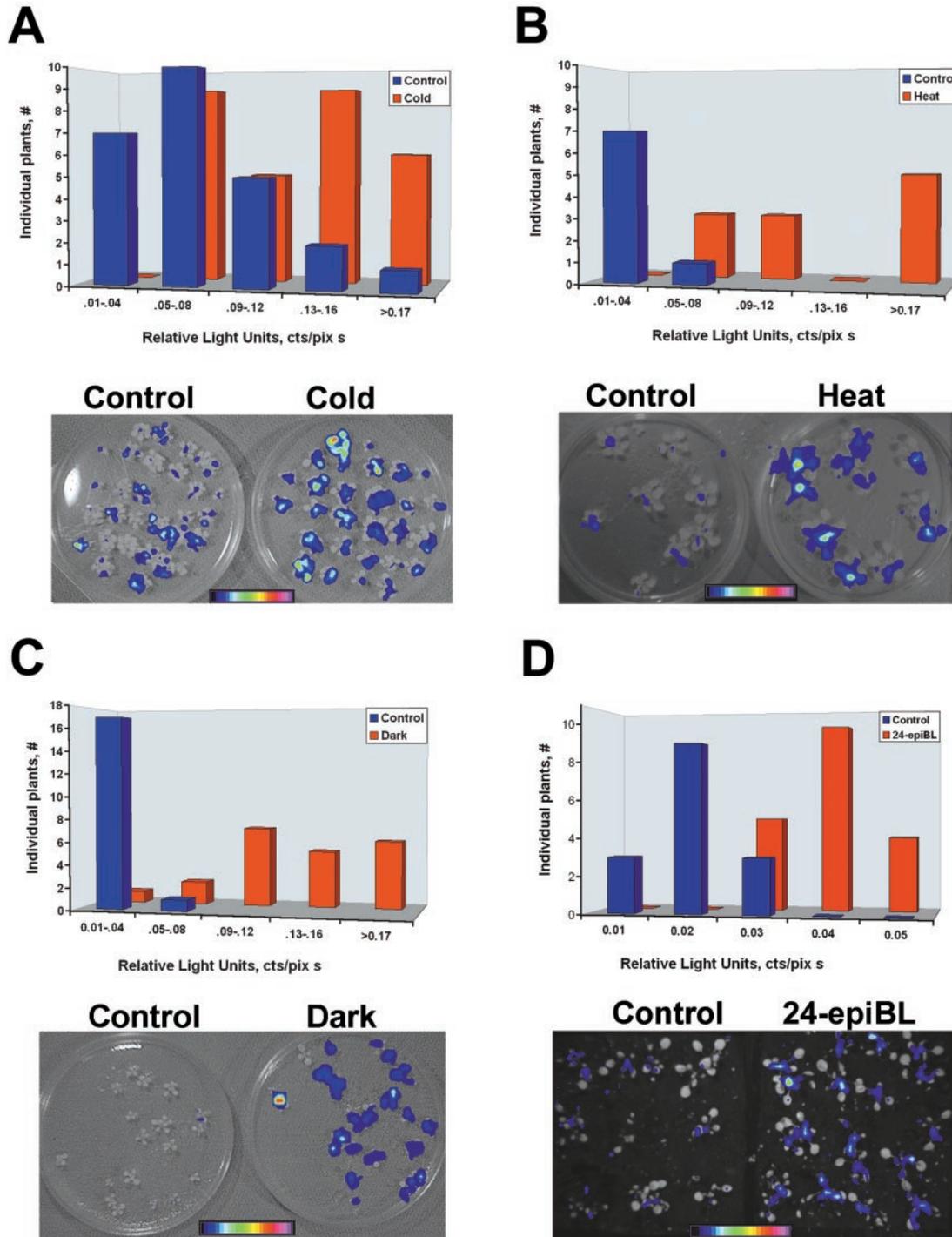
*TCH3* (Sistrunk et al., 1994), and CBF1 and 2 (Gilmour et al., 1998) and found potential sequence similarities with the *TCH4* region  $-143$  to  $-114$ . To test the necessity of these sequences for gene regulation, we generated a  $-958$  to  $-1$  *TCH4::GUS* transgene in which the sequences between  $-143$  and  $-114$  were altered by interchanging purine and pyrimidine residues. In the context of the  $-958$  to  $-1$  region, the sequences between  $-143$  and  $-114$  are nonessential for up-regulation because this transgene still showed touch, darkness, auxin (IAA), and 24-epiBL inducibility of expression (data not shown).

#### Induced Expression of *TCH4* in Response to Cold, Heat, Touch, Darkness, and Auxin Is Conserved in *bri1-2*

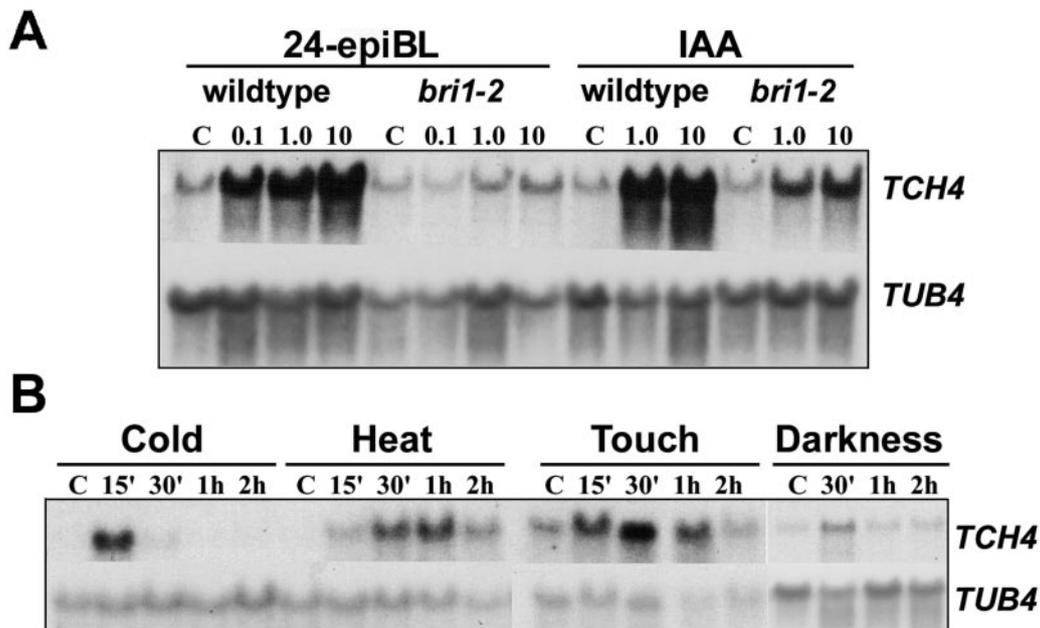
One scenario to explain how diverse stimuli lead to the common response of *TCH4* up-regulation of expression is that all the inducing stimuli lead to an increase in an endogenous hormone. Therefore, we tested the hypothesis that mechanical stimuli, darkness, temperature shifts, and application of exogenous IAA all result in increases in endogenous BR that act to up-regulate *TCH4* expression. *BRI1* encodes a Ser/Thr receptor kinase that perceives BR via its extracellular domain (He et al., 2000). The *bri1-2* mutant is insensitive to BR and develops as a severe dwarf (Clouse et al., 1996; Kauschmann et al., 1996). Figure 6A illustrates that *TCH4* mRNAs accumulate significantly within 2 h after treatment of wild-type plants with 0.1 to  $10\ \mu\text{M}$  24-epiBL, similar to that reported previously (Xu et al., 1995). In contrast, *bri1-2* shows insensitivity to 24-epiBL; *TCH4* expression is not enhanced by application of 24-epiBL, except possibly at high concentrations of 24-epiBL where a weak response is observed (Fig. 6A). The *TCH4* expression response to IAA is maintained in *bri1-2*, although the magnitude of the response is reduced compared with wild type (Fig. 6A). Figure 6B shows that *TCH4* mRNAs also accumulate in *bri1-2* in response to temperature extremes, mechanical perturbation, and darkness. These results indicate that the signal transduction pathways utilized by Arabidopsis to induce *TCH4* expression in response to these exogenous stimuli do not require the perception of BR. Therefore, the signal transduction pathway used for BR activation of *TCH4* expression must be distinct from the signaling pathways employed by other stimuli, or alternatively, the signaling pathways activated by these diverse inducing stimuli converge at some point downstream of BR perception.

#### *TCH4::GUS* Expression in *bri1-2* and *det2-1*

The availability of *bri1-2* and BR biosynthesis mutants such as *det2-1* (Chory et al., 1991; Fujioka et al., 1997) enables an investigation into the potential role



**Figure 5.** *TCH4* sequences between  $-147$  and  $-45$  are sufficient to confer up-regulation of expression in response to cold shock, heat shift, darkness, and 24-epiBL. Plants harboring  $-147$  to  $-45$  *TCH4::LUC* were grown under constant light on agar plates for 10 to 12 d (A–C) or in soil for 14 d (D). A, Control plants remained at room temperature ( $23^{\circ}\text{C}$ ), whereas others were placed for 10 min on ice (cold); then all plants were left to recover for 2 h at room temperature. B, Control plants remained at room temperature ( $23^{\circ}\text{C}$ ), and others were placed in  $37^{\circ}\text{C}$  incubator for 1 h (heat). C, Control plants were left undisturbed, whereas others were exposed to darkness for 2 h. D, Control plants were misted with 0.01% (v/v) Triton X-100, whereas others were misted with  $10\ \mu\text{M}$  24-epiBL and 0.01% (v/v) Triton X-100 and incubated for 3 h. After treatments, plants were sprayed with 1 mM luciferin and 50 mM sodium citrate, pH 5.6, and placed in a low-light imager. The data acquisition was delayed for 5 min to avoid chlorophyll phosphorescence. Fifteen-minute luminescent readings were collected and the data were extracted and analyzed with Microsoft Excel. Top, Relative light units (cts/pix s) of individual control and stimulated plants were binned and the distribution plots. Computer-generated false-color overlays of the light emission at 30 min after substrate addition are shown at the bottom of each panel. Minimum light is indicated with dark blue and maximum light is shown in magenta.



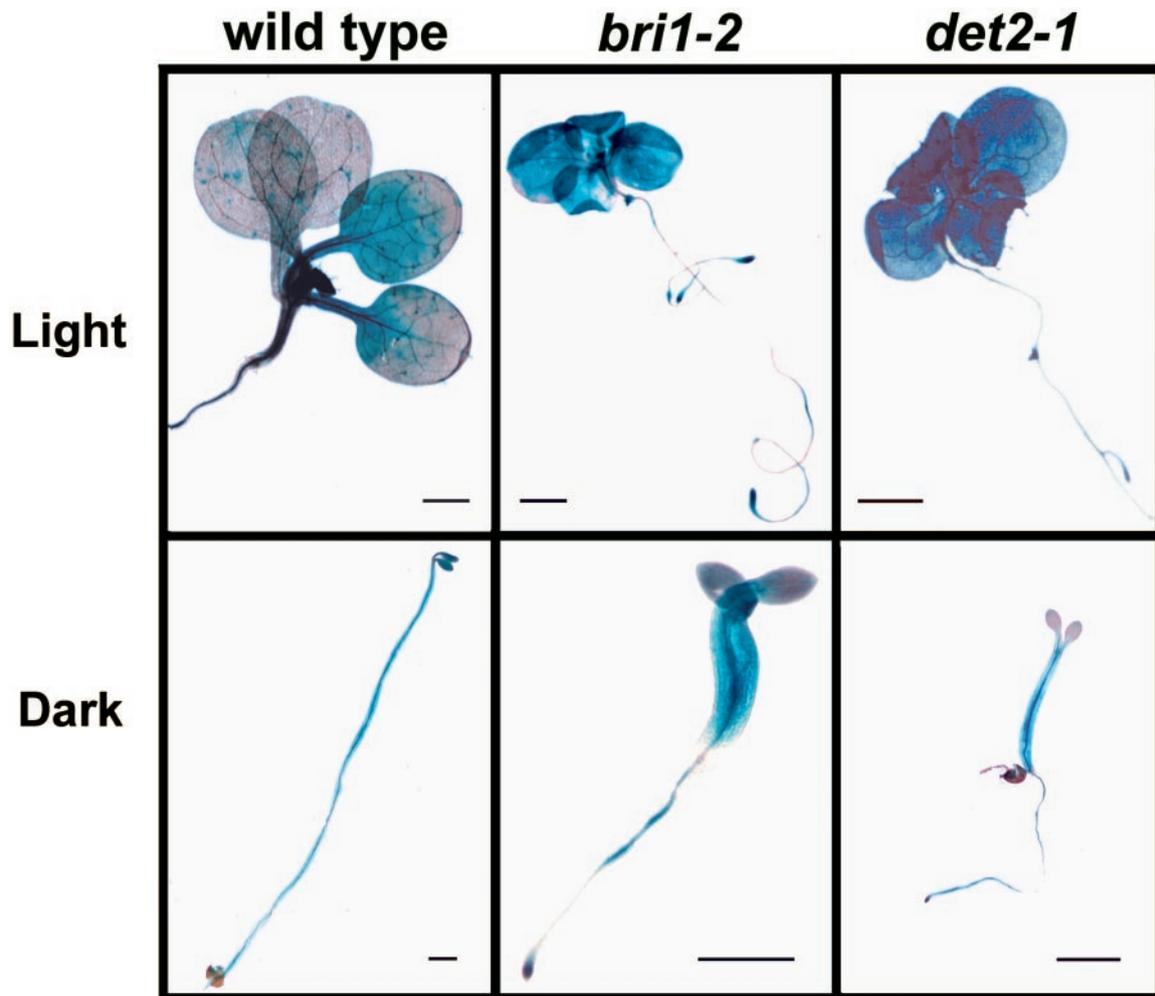
**Figure 6.** *TCH4* up-regulation of expression in *bri1-2* plants in response to environmental and hormonal stimuli. Wild-type and *bri1-2* (A) or *bri1-2* (B) plants were grown in liquid media, shaking gently and continuously at 60 rpm for approximately 2 weeks. A, Control plants (C) were treated with solvent alone (0.001% [v/v] ethanol); others were treated with increasing concentrations of 24-epiBL or auxin (IAA) as indicated in 0.001% (v/v) ethanol. The concentrations of 24-epiBL and IAA shown are in micromolar. Plants were collected after 2 h and immediately frozen in liquid nitrogen. Total RNA was extracted and 4  $\mu$ g of RNA was size fractionated on 1% (w/v) formaldehyde gels, blotted to nylon filters, and hybridized sequentially to probes listed on the right. The prime indicates min; "h" indicates hour(s). *TUB4* is shown as a loading and transfer control. B, "Cold," Control plants (C) were left undisturbed, whereas others were shaken gently in 0°C water bath for 2.5 min, quickly brought back to room temperature by gently shaking in a 23°C water bath, and returned back to room temperature shaker for the indicated times. "Heat," Control plants (C) were left undisturbed, whereas others were transferred to a 35°C water bath and collected at the indicated times. "Touch," Plants were removed from the shaker and left undisturbed a day before the experiment. Control plants (C) were untreated, whereas others were shaken for 10 s and collected at the indicated times. "Darkness," Control plants (C) were left undisturbed, whereas others were covered to minimize light and collected at the indicated times.

of endogenous BR in regulating developmental expression of *TCH4*. *TCH4* expression, assessed by *TCH4::reporter* gene fusions (Xu et al., 1995) and immunolocalization of XTHs (Antosiewicz et al., 1997), correlates with growth and cell expansion and with predicted presence of mechanical stress. One possibility is that *TCH4* expression at these sites may be regulated by endogenous BR. That is, BR may mediate gene regulation during cell expansion or in response to mechanical stress. For example, *TCH4::GUS* expression is high in etiolated hypocotyls, but low in hypocotyls of photomorphogenetic seedlings. Because hypocotyl elongation in the dark requires BR (Azpiroz et al., 1998), *TCH4::GUS* expression detected in etiolated hypocotyls could be a consequence of BR regulation. The components of the *TCH4::GUS* expression pattern that are dependent on BR should be lost or at least reduced in the *bri1-2* and *det2-1* mutants. As shown in Figure 7, *TCH4::GUS* expression is high in young leaves of light-grown plants and the hypocotyl of etiolated seedlings. Surprisingly, in *bri1-2* and *det2-1*, *TCH4::GUS* expression remains strong in the hypocotyls and leaves of light-

grown plants (Fig. 7, Light) and in dark-grown plants (Fig. 7, Dark) even though expansion of these organs is strongly inhibited. The intense blue staining in the mutants may be a consequence of more concentrated accumulation of X-Gluc precipitate because the mutant cells fail to expand normally (Chory et al., 1991; Clouse et al., 1996; Kauschmann et al., 1996). These results indicate that neither the presence nor perception of BR are required for *TCH4::GUS* expression in these organs. Therefore, we conclude that other response pathways most likely function to regulate *TCH4* expression during morphogenesis.

## DISCUSSION

*TCH4* is an unusual gene in that its expression is up-regulated by a variety of seemingly unrelated stimuli, including temperature perturbations, such as touch, temperature extremes, darkness, and the growth-promoting hormones BR and IAA. Induced *TCH4* expression is also remarkably transient. In this report, we investigated which regions of the *TCH4* locus contribute to its regulatory behaviors.



**Figure 7.** *TCH4::GUS* expression in *bri1-2* and *det2-1* mutants. 5-Bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronide (X-Gluc) staining of wild-type, *bri1-2*, and *det2-1* plants harboring the  $-958$  to  $+48$  *TCH4::GUS* transgene. Seedlings were grown for 8 d under 24 h of light (light, top) or in the dark (dark, bottom) for 3 d. The bar in each panel = 1 mm.

### Regulation of Transient Gene Expression Induction

By northern analyses, one can demonstrate that *TCH4* transcripts accumulate very rapidly after stimulation of plants; for example, 10 to 30 min after touch stimulation, *TCH4* mRNA levels peak (Braam and Davis, 1990; Figs. 2A and 3B). Subsequently, there is also a rapid decline in transcripts with an apparent return to basal levels of mRNA by 1 to 3 h after stimulation (Braam and Davis, 1990; Figs. 2A and 3B). Two mechanisms probably account for this rapid disappearance of transcripts. Most likely, transcription initiation is quickly inhibited to halt the production of nascent transcripts. In addition, the *TCH4* mRNAs must be unstable. As yet, we do not know whether *TCH4* mRNA stability is decreased in a regulated manner or, alternatively, the mRNAs are always unstable. In the latter case, the transient accumulation of *TCH4* mRNAs in stimulated plants would be due solely to changes in transcriptional activity. *GUS* transgenes composed of both 5'-

untranscribed *TCH4* sequences and transcribed but untranslated *TCH4* sequences show expression kinetics that resemble those of the endogenous *TCH4* gene (Xu et al., 1995). Because the rapid decay of transcripts after stimulation requires that the mRNAs are unstable, the *TCH4*-derived sequences,  $+1$  to  $+48$  of the UTR, present in the reporter mRNA are most likely responsible for the transcript instability. When the *TCH4* UTR is removed, the *GUS* transcripts remain abundant at least 2 h, a time when the endogenous *TCH4*-derived transcripts are near basal levels (Figs. 2 and 3, A; 3, B, I; and C, I). This prolonged response of transgenes lacking the *TCH4* UTR is seen consistently with transgenes driven by the  $-958/-1$ , the  $-958/-45$ , and the  $-258/-45$  untranscribed regions of the *TCH4* (Figs. 2 and 3A). However, with the latter two constructs, it is also possible that transcription initiation may continue for a longer duration, which could contribute to the prolonged accumulation of transcripts. Sequences from  $-45$  to  $-1$  of

*TCH4* were replaced with a  $-90$  to  $+8$  region of the CaMV 35S regulatory region that has been used as a minimal promoter. However, this  $-90$  region harbors additional regulatory sequences in addition to the TATA and CAT boxes (Benfey et al., 1989; Lam et al., 1989); therefore, there may be sequences that also influence the arrest of transcription initiation. Surprisingly, transgenes driven by the comparable transcriptional regulatory regions of *TCH4* in which the TEV 5'-UTR was added (to enhance translation initiation) show transcript accumulation kinetics nearly indistinguishable from the native *TCH4* (Fig. 3, B, IV; and C, IV), suggesting that the TEV 5'-UTR may also confer RNA instability; such an effect of the TEV 5'-UTR on RNA stability has not been reported previously.

### Transcriptional Regulation of *TCH4* Induction of Expression

Sequences found upstream of the *TCH4* transcribed region are sufficient to confer touch, darkness, 24-epiBL, cold shock, and heat shock inducibility of expression upon reporter genes (Figs. 2–5). This indicates that *TCH4* regulation of expression most likely takes place, at least in part, through regulation of the rate of transcription initiation. *BRU1* is an XTH-encoding gene from soybean (*Glycine max*) that is also regulated in expression by BR; however, in contrast to *TCH4*, *BRU1* is thought to be regulated through a posttranscriptional mechanism based on nuclear run-on assays (Zurek and Clouse, 1994).

### Sequences Sufficient to Confer Sensitivity to Diverse Stimuli

The 5'-upstream region of *TCH4* has numerous motifs that might be predicted to function in the complex regulation of *TCH4* expression. Surprisingly, we found that a single, relatively short 102-bp region between  $-147$  and  $-45$  is sufficient to confer upon a reporter gene the ability to be up-regulated in expression in response to touch, darkness, cold shock, heat shock, and 24-epiBL (Figs. 4 and 5). Because GUS activity could not be detected in transgenics harboring  $-128/-45$  *TCH4::GUS* transgenes (data not shown), sequences between  $-147$  and  $-128$  must be important for *TCH4* expression regulation. However, sequences between  $-143$  and  $-114$ , in the context of approximately 1 kb of *TCH4* upstream sequences, are not necessary for the regulated expression. It is likely, therefore, that there are redundant functional elements within the 1-kb region upstream of the *TCH4* transcriptional start site. Consistent with the idea of redundant functional elements, longer 5' regions generally conferred regulatory behavior that more closely reflected that of the native gene. Sequences between  $-958$  and  $-258$  and  $-258$  and  $-147$  enhance the magnitude of the expression induction

and the kinetics of up-regulation (Figs. 3A and 4; additional data not shown). Whether sequences in these regions can also act alone to confer inducible expression is not yet known. However, within these distal sequences, there are additional E boxes and Myb motifs related to those found between  $-143$  and  $-114$  (Fig. 1A). The finding that inducible expression by multiple, diverse stimuli can be conferred by the same subregion is consistent with the possibility that there is a common cis-element that can serve to control at least most of the complex regulation of *TCH4* expression. The inability to define separable regions able to confer only a subset of inducible properties indicates that the signal transduction pathways activated by the diverse stimuli that lead to inducible *TCH4* expression most likely share at least some common elements. Precise identification of the regulatory sequences that drive *TCH4* expression characteristics will require examination of the effects of combinations of site-specific mutations of the 5'-upstream sequences of *TCH4*.

### The Role of BR in *TCH4* Expression Regulation

BR has been implicated as a hormone that can influence stress responses in plants. For example, BR treatment improves the cold tolerance of maize (*Zea mays*) and cucumber (*Cucumis sativus*) seedlings (Khrupach et al., 1999). Exogenous BR also leads to the up-regulation of *TCH4* expression; expression of *TCH4* is also regulated by diverse abiotic stresses (Xu et al., 1995). Therefore, we tested the possibility that *TCH4* induction of expression is mediated through activation of BR signaling. Inducible expression of *TCH4* by touch, darkness, cold, and heat remains robust in *bri1-2*, a BR-insensitive mutant (Fig. 6B); therefore, BR perception is not required for *TCH4* regulation of expression by these environmental stimuli. Although steady-state *TCH4* mRNA in unstimulated wild-type and *bri1-2* plants is comparable (Fig. 6A), *TCH4* up-regulation of expression in response to 24-epiBL is abolished in *bri1-2* plants, except at very high concentrations. Previous studies (Kauschmann et al., 1996) observed a reduction in steady-state *TCH4* mRNA in *bri1-2* compared with wild type. No up-regulation of *TCH4* mRNA expression in *bri1-2* was observed when grown on media supplemented with  $0.5 \mu\text{M}$  24-epiBL for 13 d (Kauschmann et al., 1996); surprisingly, however, no up-regulation of *TCH4* expression was evident in wild-type plants grown on 24-epiBL-supplemented media as well (Kauschmann et al., 1996). It is possible that 24-epiBL induction of *TCH4* expression occurs transiently; therefore, the differences between the results described by Kauschmann et al. (1996) and our results (Xu et al., 1995; Fig. 6A) could be attributed to different growth conditions and the duration of 24-epiBL exposure.

IAA induction of *TCH4* expression is weaker in *bri1-2* than in wild type (Fig. 6A); this result indicates

that IAA may act in a synergistic manner with BR in some aspects, including the regulation of *TCH4* expression. In addition, we have found that *bri1-2* has reduced expression of *TCH3*, a calmodulin-related gene (D.H. Polisensky and J. Braam, unpublished data). This reduction in *TCH3* expression in *bri1-2* is likely not directly related to BR insensitivity because *TCH3* is not up-regulated in expression by exogenous BR (D.H. Polisensky and J. Braam, unpublished data). *TCH3* is, however, up-regulated in expression by IAA (Antosiewicz et al., 1995), and *TCH3* expression is returned to wild-type levels in the *bri1-2* mutant when supplemented with exogenous IAA (D.H. Polisensky and J. Braam, unpublished data). These results are consistent with the possibility that IAA levels are reduced in plants that are incapable of sensing BR, strengthening the hypothesis that BR and IAA regulation may occur in a synergistic manner. Links between BR and IAA have been reported; for example, the addition of 24-epiBL restores wild-type sensitivity to auxin in *sax1* plants (Ephritikhine et al., 1999). However, BR and auxin have also been shown to act independently in many systems (Clouse and Sasse, 1998).

BR is also not required for the expression of *TCH4* during morphogenesis; there is strong *TCH4::GUS* expression in developing *bri1-2* and *det2-1* seedlings. *TCH4::GUS* expression in wild type strongly correlates with cell growth and expansion (Xu et al., 1995; Fig. 7, wild type). Because *TCH4* expression is not lost in the *bri1-2* and *det2-1* mutants, it is unlikely that *TCH4* developmental regulation is a result of BR signaling or a consequence of the process of cell expansion. However, because *TCH4* encodes a cell wall-modifying activity, one possibility is that properties of the wall and, in the case of *bri1-2* and *det2-1*, deviations from wall homeostasis are sensed and transmitted through a signaling pathway that impacts *TCH4* expression.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Arabidopsis plants were grown at 22°C to 24°C in 65% to 75% humidity under constant 90  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light. For northern analyses, plants were grown for 12 to 14 d in liquid Murashige and Skoog media (Murashige and Skoog, 1962). Plants for LUC analyses were grown in 1-inch pots at a density of one plant per pot or on agar plates containing 0.5 $\times$  Murashige and Skoog media supplemented with 1% (w/v) Suc. Plants for GUS assays were grown on filter paper on agar-containing plates with 0.5 $\times$  Murashige and Skoog with 0.2% (w/v) Suc for 4 to 10 d under constant light or for 3 to 4 d covered (darkness).

### Generation of *TCH4::GUS* and *TCH4::LUC* Reporter Constructs

For construction of  $-958$  to  $-1$  *TCH4::GUS* plasmid, the region from  $-958$  to  $-1$  was isolated by PCR using the forward primer (5'-TCTAGACTTTCGTAAG-3') and reverse primer (5'-TTTGAGGGTTT-ATGGAGG-3') and cloned into pCRII vector (Invitrogen, Carlsbad, CA). The recombinant plasmid was cut with *Xba*I, and ligated into *Xba*I-cut pBI101 vector (CLONTECH, Palo Alto, CA) in front of the *GUS* gene. For

construction of  $-958$  to  $-45$  *TCH4::GUS* and  $-258$  to  $-45$  *TCH4::GUS* with  $-90/+8$  CaMV 35S promoter, the region from  $-958$  to  $-45$  was PCR amplified using the forward primer (5'-TCTAGACTTTCGTAAG-3') and the reverse primer (5'-AAGATTTTAAAGAG-3') and the region from  $-258$  to  $-45$  was PCR amplified using the forward 5'-CCAATTAAT-CTGAAACC-3' and reverse primer 5'-AAGATTTTAAAGAG-3' and cloned into pCRII. The *TCH4* regions between *Hind*III and *Eco*RV were ligated into a pBI221- (CLONTECH) derived plasmid with sequences between *Bam*HI and *Eco*RI deleted; this cloning step resulted in fusion between *TCH4* sequences and the  $-90/+8$  CaMV 35S promoter. The hybrid regulatory regions, flanked by *Hind*III and *Xba*I sites, were inserted at the *Hind*III and *Xba*I sites of pBI101, forming the  $-958$  to  $-45$  *TCH4::GUS* and  $-258$  to  $-45$  *TCH4::GUS* plasmids. The construct containing *TCH4* region from  $-128$  to  $-46$  linked with the  $-90/+8$  CaMV 35S promoter was made by nested deletion from the 5' end of the *TCH4* region in the recombinant plasmid,  $-258/-45$  *TCH4::GUS*. The exact site of the deletion was determined by sequencing analysis.

For construction of fusion genes with the  $-258$  to  $-45$  region and smaller *TCH4::GUS* and *TCH4::LUC* fusions with the  $-46$ -bp CaMV 35S promoter, promoter fragments were PCR amplified as follows:  $-258$  to  $-45$  using the forward primer (5'-CCCAAGCTTCCAATTAATCT-3') and the reverse primer (5'-GAAGATCTAAGATTTTAAAGA-3'), and  $-147$  to  $-45$  using the forward primer (5'-CCCAAGCTTCTTTACTACAA-3') and the reverse primer (5'-GAAGATCTAAGATTTTAAAGA-3'). The primer-introduced *Hind*III and *Bgl*II sites are underlined. The PCR products were cloned into pCRII and sequenced. For *TCH4::LUC* constructs, the *TCH4* regions were excised as *Hind*III-*Bgl*II fragments, then subcloned into pKS 35S-TEV, a pBluescript KSII-based vector that has a  $-46$ -bp CaMV 35S minimal promoter and 143-bp TEV UTR inserted at the *Bam*HI site. The fragments were further subcloned as *Hind*III-*Bam*HI fragments from pKS 35STEV into pCR 35S-TEV, a pCRII-based vector that harbors a PCR-amplified  $-46$ -bp CaMV 35S minimal promoter and 143-bp TEV UTR. Direct cloning of the *Hind*III-*Bgl*II *TCH4* fragments into pCR 35S-TEV was not possible due to the presence of an additional *Bgl*II site. The *TCH4* sequences, along with the  $-46$ -bp CaMV 35S minimal promoter and TEV UTR, were subcloned as *Hind*III-*Xho*I into pKAJ201, a pBI101-based vector into which the *GUS* gene was replaced with *Sal*I-*Sac*I fragment from pJD300 (Luehrsen et al., 1992) containing the *LUC* gene. For *TCH4::GUS* constructs, the PCR-amplified *TCH4* sequences were subcloned from pCRII as *Hind*III-*Bgl*II fragments into pBI 35S-TEV GUS, a pBI 101-based vector in which a PCR-amplified  $-46$ -bp CaMV 35S minimal promoter and 143-bp TEV UTR were inserted in front of *GUS*.

To generate a *TCH4* region with mutated sequence between  $-143$  and  $-114$ , a two-step PCR procedure was followed starting with the plasmid pBITG as the template. pBITG consists of bases  $-958$  to  $-1$  of *TCH4* upstream sequences (nucleotides 40, 185 through 39, and 227 of GenBank accession no. AB011482), the *GUS* gene, and the nopaline synthase terminator of pBI101, all cloned into the binary vector BIN19 (Frisch et al., 1995). First step PCR utilized the 5' primer *Isp*5 (5'-CTCAAAGCTTGCATGCC-TGCAGGTCGAC-3') coupled with a 54-mer (jmb2, 5'-ACCACTAGTTG-TCGGTTTAAACGAGGTGCTGAAAAGCCTGTGTTTATTATTGG-3') consisting of 24 bases complementary to wild-type *TCH4* promoter sequence, and 30 bases mutated by exchange of pyrimidines and purines. The second PCR reaction contained the 3' primer, *Isp*3p (5'-CTCAGGATCCTCTAGATG-CATGCTCGAT-3') and a 34-mer (jmb1, 5'-GACAACTAGTGGTCCTAAA-GACGGCTTCTTC-3') consisting of 13 bases of overlap with jmb2 and 21 bases complementary to wild-type *TCH4* sequence. PCR conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2.5 min. After a final extension of 10 min at 72°C, PCR products were purified by phenol/chloroform extraction and ethanol precipitation and digested with *Spe*I. The two fragments were then ligated and further digested with *Sal*I/*Bam*HI. The mutated *TCH4* was cloned into *Sal*I/*Bam*HI-digested pBITG from which the wild-type *TCH4* region had been removed. The resulting construct was identical to pBITG with the exception of 30 bp within the *TCH4* region, which was verified by sequencing to confirm an exchange of Gs for Ts, Ts for Gs, Cs for As, and As for Cs in the region between  $-143$  and  $-114$ .

The constructs were moved into Arabidopsis via *Agrobacterium tumefaciens*-mediated vacuum infiltration (Bechtold et al., 1993). Independent homozygous  $T_3$  lines were obtained by selfing followed by selection on kanamycin and DNA-blot hybridization analyses. Two to five independent *TCH4::reporter* lines per construct were examined and found to have comparable inducible expression behavior.

## RNA Analysis

For RNA analyses, treated and control plants were harvested at the indicated time points and then immediately frozen in liquid nitrogen. Total RNA was purified (Verwoerd et al., 1989), electrophoresed on formaldehyde gels, blotted overnight onto nylon membranes (Micron Separations, Westborough, MA), and hybridized with hexamer-labeled DNA fragments (Feinberg and Vogelstein, 1983). The probes used were described by Xu et al. (1995).

## LUC Data Acquisition and Analysis

In vivo LUC analyses were performed with a NightOWL low light imager (Perkin-Elmer Applied Biosystems, Foster City, CA). Plants were finely misted from a 15-cm distance with 1 mM luciferin (Biosynth AG, Staad, Switzerland) and 50 mM sodium citrate, pH 5.6, and placed in the NightOWL after 30 min. Multiple images were acquired over 5- to 15-min intervals as noted. Computer-generated representations of luminescence emissions were overlaid with photographs acquired before and/or after the completion of time courses. Data extraction and analysis were performed with WinLight software (Perkin-Elmer Applied Biosystems) and exported into Excel spreadsheets (Microsoft). Control plants were similarly and simultaneously treated (with the exclusion of the stimulus) and concurrently viewed.

## Histochemical Analysis

Plant assays of GUS activity were performed as described by Gallagher (1992). In brief, the plants were fixed in 2% (w/v) paraformaldehyde, 100 mM sodium phosphate, pH 7.0, and 1 mM EDTA for 20 to 25 min on ice. After washing twice with 100 mM sodium phosphate, pH 7.0, the plants were incubated in 2 mM X-Gluc (Molecular Probes, Eugene, OR), 50 mM sodium phosphate (pH 7.0), and 0.01% (v/v) Triton X-100 overnight at 37°C. After the reaction was stopped with a water wash, the tissues were cleared with several washes of 70% (v/v) ethanol. The tissues were mounted on microscope slides in 50% (v/v) glycerol, and the slides were fitted into a Pathscan Enabler (Meyer Instruments, Houston) and scanned with a 35-mm film scanner (Nikon, Tokyo).

## Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial purposes. No restrictions or conditions will be placed on the use of any materials described in this paper that would limit their use in noncommercial research purposes.

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