The Plant Cell, Vol. 3, 573–582, June 1991

Phytochrome Control of the *tms2* Gene in Transgenic *Arabidopsis*: A Strategy for Selecting Mutants in the Signal Transduction Pathway

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Introduction of the *tms2* gene from *Agrobacterium tumefaciens* into *Arabidopsis thaliana* yields transgenic seedlings with a new selectable phenotype: the seedlings are strongly growth inhibited on micromolar concentrations of auxin amide substrates that do not significantly affect wild-type seedlings. The *tms2* gene encodes an amidohydrolase that catalyzes the conversion of biologically inactive auxin amides into active auxins, which are toxic to plants at elevated concentrations. In the absence of exogenous substrate, *tms2*$^+$ transgenic seedlings grow normally and are fertile. When grown on auxin amides, both etiolated and green *tms2*$^+$ seedlings exhibit a variety of dose-dependent auxin toxicity effects. *tms2* mRNA and the encoded amidohydrolase activity are both detectable in transgenic but not in wild-type seedlings, demonstrating that a cognate activity is lacking in wild-type *Arabidopsis*. Furthermore, when the introduced *tms2* gene is fused to the *Arabidopsis cab140* promoter, the *tms2* RNA and its encoded amidohydrolase activity and, thus, the conditional lethal phenotype can be modulated by phytochrome action. The *tms2* gene can, therefore, serve as a regulatable selectable marker in *Arabidopsis* that should be useful in isolation of *trans*-regulatory mutants that have lost the imposed regulation of *tms2* gene activity.

INTRODUCTION

Phytochrome is involved in the regulation by light of many processes important in the growth and development of higher plants, including the regulation of gene expression. Although the chromophore of this photoreceptor is known to undergo conformational changes in response to irradiation, how the light signal is transduced and relayed to cellular targets, such as the nucleus, remains an unsolved problem. Phytochrome action has been shown to mediate specific changes in nuclear gene transcription (reviewed by Tobin and Silverthorne, 1985; Nagy et al. 1988; Thompson, 1988). Efforts to delineate the signaling pathway(s) involved have focused primarily on characterizing the phytochrome molecule(s) (reviewed by Lagarias, 1985; Furuya, 1989) and on defining the regulatory DNA elements and DNA-binding factors that mediate transcriptional changes (reviewed by Gilmartin et al., 1990; Schindler and Cashmore, 1990).

Genetic approaches to understanding phytochrome-mediated signaling processes have also been attempted, and mutants in which the phytochrome-mediated suppression of hypocotyl growth has been altered have been isolated in *Arabidopsis thaliana* (Koornneef, 1980; Chory et al., 1989) and other species (Koornneef et al., 1985; Adamse et al., 1988). Three of the *Arabidopsis* mutants (*hy1, hy2,* and *hy6*) have greatly reduced levels of spectrophotometrically detectable phytochrome and expression of several phytochrome-regulated genes is reduced, but brief illumination with red light can still result in increases in the RNA levels for these genes (Chory et al., 1989). Two others, *hy3* and *hy5*, have normal levels of phytochrome and do not show any alteration in the phytochrome regulation of *cab* RNA (Sun and Tobin, 1990). This example demonstrates that mutations that cause a visible phenotype may not be in genes whose product is an intermediate component of the signal transduction pathway leading to transcription changes. Conversely, mutations in a biochemically defined process such as transcriptional regulation may not give predictable or unique visible phenotypes. For this reason, we have developed an alternative, more directed genetic approach. This approach involves the fusion of a phytochrome-regulated promoter from an *Arabidopsis cab* gene (Karlin-Neumann et al., 1988) to a selectable reporter gene and the stable transformation of *Arabidopsis* with the construct. The reporter gene confers a phenotype such that mutants in which the phytochrome-regulated transcriptional response is crippled can survive the selection. Although infrequently used,

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such a promoter fusion approach has been successful in higher eukaryotes (Hofstetter et al., 1987; Pellegrini et al., 1989; Shirras and Bownes, 1989) as well as in yeast and bacteria (Guarente et al., 1982; Struhl, 1983).

A major factor that has limited this particular genetic approach in plants is the lack of markers shown to be capable of conferring a regulatable phenotype for negative selection. The immediate aims of this study were to determine whether the *tms2* gene from the *Agrobacterium tumefaciens* T-DNA could function as such a marker in *Arabidopsis* and whether the imparted phenotype could be modulated by phytochrome action.

The *tms2* gene encodes an enzyme that catalyzes the second of two steps in the bacterial pathway of auxin biosynthesis from tryptophan (Schroder et al., 1984; Thomashow et al., 1984). The auxin biosynthesis pathway is different in higher plants (Sembdner et al., 1980); thus, wild-type plants would not be expected to have either the endogenous substrate or the homologous enzyme for the *tms2* gene product. Recently, in a survey of 27 species, only rice showed a significant amount of such an activity (Kawaguchi et al., 1991). The indole-3-acetamide (IAM) hydrolase encoded by the *tms2* gene converts IAM to indole-3-acetic acid (IAA), the natural auxin found in plants. Although auxins are normally present at low concentrations in plants and act as regulators of growth and development, at elevated concentrations they are lethal (Moore, 1979). Thus, the *tms2* gene product converts a relatively nontoxic substrate (e.g., IAM) into a toxic product (e.g., IAA) and so should be suitable for negative selection of seedlings expressing it at a high enough level. Indeed, Budar et al. (1986) showed that tobacco seedlings expressing the *tms2* gene grew normally in the absence of added substrate, but were strongly growth inhibited on concentrations of a synthetic auxin amide that had no effect on wild-type seedlings.

The experiments presented here show that the *tms2* gene fused to a *cab* promoter can be regulated by phytochrome action and can behave as a conditional lethal marker for selection of mutants in *Arabidopsis*. Furthermore, the results suggest that this approach may be useful for selection of mutants in many different kinds of signal transduction pathways.

RESULTS

Creation of Homozygous Lines with *tms2* Constructs

Transgenic *Arabidopsis* lines were created with either a construct containing a 1.3-kb fragment from the *Arabidopsis cab140* promoter fused to the *tms2* gene (*cab140*::*tms2*) or a construct containing a 1.6-kb cauliflower mosaic virus (CaMV) 35S promoter fragment fused to the *tms2* gene (*35S*::*tms2*). The *35S*::*tms2* construct served as a nonphytochrome-regulated control. Homozygous plants were obtained from representative lines transformed with each construct, and their progeny were confirmed to be diploid by analyses of mitotic figures in root tip squashes (data not shown). Results utilizing two such clonal lines—cab(+14)A, containing the *cab140*::*tms2* fusion, and 35S(+1)A, containing the *35S*::*tms2* fusion—are presented here.

Genomic DNA gel blot analysis with probes of various regions established that the *tms2* fusion constructs were intact in these transgenic lines (data not shown). By comparison of amounts of a probe hybridizing both to the endogenous *cab* promoter and to the *cab140*::*tms2* fusion, we estimated that the cab(+14)A plants contained approximately 10 tightly linked copies of the insert per haploid genome. Similar estimates for the 35S(+1)A line suggested that a single copy of the construct was present (data not shown). Thus, these transgenic lines were suitable for use in the experiments reported here, as well as for later mutagenesis and analysis.

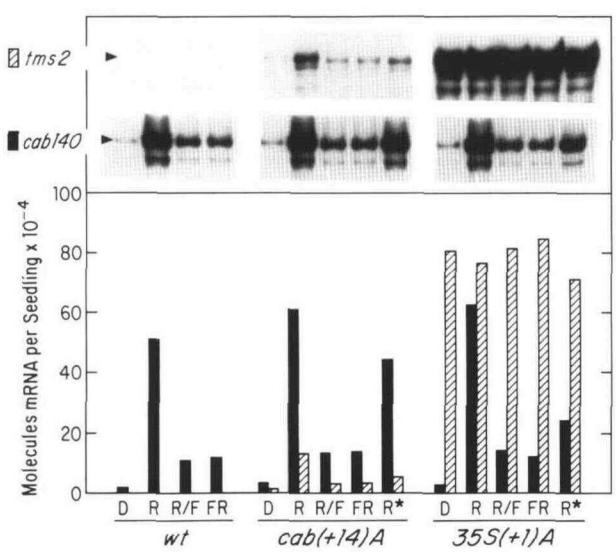

Figure 1. RNase Protection Analysis of Phytochrome Effects on *tms2* and *cab140* mRNA Levels.

Total RNAs analyzed were from 5-day-old etiolated seedlings from wild type (wt) and two transgenic lines, cab(+14)A and 35S(+1)A. Seedlings were grown either in darkness and given designated light treatments 2 hr before harvest (no treatment, D; 1′R only, R; 1′R followed immediately by 10′FR, R/F; or 10′FR only, FR) or grown in darkness supplemented with 1′R every 2 hr (R*) and harvested 2 hr after the last R irradiation. Arrowheads indicate the major protected bands for *tms2* RNA (153 nucleotides) and *cab140* RNA (130 nucleotides). Solid bars, *cab* RNA; hatched bars, *tms2* RNA.

Phytochrome Action Regulates the Expression of the *cab140::tms2* Gene

Etiolated seedlings were tested for phytochrome regulation of the introduced *cab140::tms2* and *35S::tms2* constructs. Transcript levels of both the *tms2* and the endogenous *cab140* genes were determined by RNase protection assays. Data from a representative analysis are shown in Figure 1. As expected, no RNA homologous to the *tms2* gene was present in the wild-type plants. The increases in the RNA level of the endogenous *cab140* gene in response to a single brief red illumination (R) ranged from 19-fold to 25-fold in the three lines shown. The effect of R was reversed substantially by far red illumination (R/FR), as expected for a phytochrome-regulated response. In the cab(+14)A line, the level of the *tms2* RNA was regulated similarly; however, the relative increase in response to a single brief R was routinely lower (11-fold in the experiment shown here). The increases in both RNAs were not as great when the plants were grown with intermittent red (1 min/2 hr) (R*). In the 35S(+1)A line, the *tms2* RNA was expressed constitutively at high levels, and it was not affected detectably by any light treatment used. This experiment demonstrated that the 1326-bp *cab140* promoter fragment was sufficient to confer sensitivity to phytochrome action on the *tms2* gene.

Phytochrome Regulates Amidohydrolase Activity in the *cab140::tms2* Transgenic Plants

We had demonstrated previously that the increase in *cab140* mRNA in response to brief R was transient, peaking after several hours and returning to the basal level by 8 hr (Karlin-Neumann et al., 1988). Because we wanted to induce the maximum sustained difference in seedlings growing for 5 days, we compared the amidohydrolase activity in *cab140::tms2* transgenic seedlings that were grown in darkness with those grown in intermittent red (1 min/2 hr). Figure 2 shows that a fourfold elevation in this enzyme activity could be induced in the cab(+14)A line by this treatment compared to the level in seedlings grown in complete darkness. A similar threefold elevation was found in a second homozygous *cab140::tms2* line, and increases could be seen in seedlings as early as 2 days after sowing (data not shown). Such increases are comparable to the increase seen for the *tms2* RNA (Figure 1, lanes D and R*). By contrast, in the 35S(+1)A line, the enzyme activity was high in the dark-grown seedlings and lower (on a protein basis) in the seedlings grown under the intermittent R conditions (Figure 2). Wild-type seedlings never were found to have detectable amidohydrolase activity under any growth conditions. Thus, Tms2 hydrolase activity levels are positively regulated by phytochrome action in etiolated *cab140::tms2* seedlings.

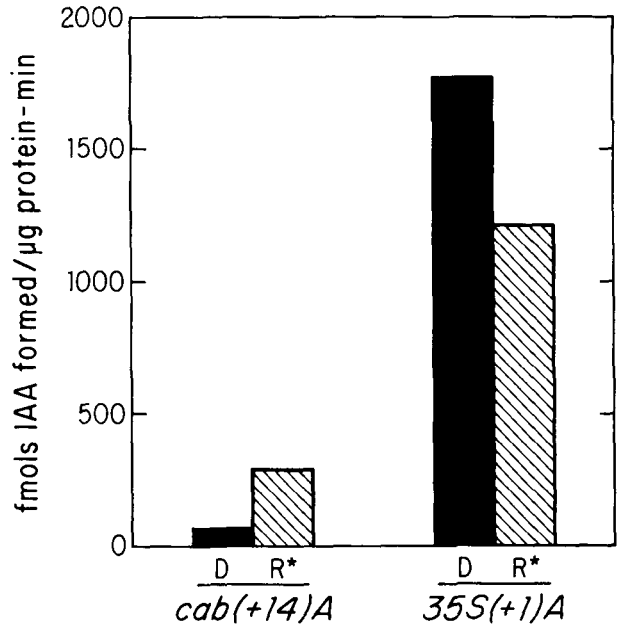

Figure 2. Red Light Inducibility of Tms2 Amidohydrolase Activity in *cab140::tms2* Seedlings.

Amidohydrolase activity in cab(+14)A and 35S(+1)A seedlings was measured as described in Methods. Seedlings were analyzed after 5 days' growth in either darkness (D) or darkness supplemented with 1′R every 2 hr (R*). Note that R*-grown seedlings contain about 50% more total protein per seedling than those that are D-grown; thus, on a per seedling basis, R* values would be 50% higher than shown.

The *tms2* Gene Confers Auxin Amide Sensitivity on Transgenic Seedlings

As a first step in developing conditions that would give a phenotype suitable for selection of mutants, we characterized the growth of transgenic and wild-type seedlings on various auxin amide substrates. Figure 3 demonstrates that, in the absence of substrate, the cab(+14)A seedlings were indistinguishable phenotypically from wild type when grown for either 8 days in continuous white light (8d W) or 5 days under intermittent R (5d R*). Furthermore, they showed no morphological differences at maturity and exhibited normal fertility (data not shown). However, when grown on 5 μM α-naphthalene acetamide (NAM), the growth of the transgenic seedlings, but not wild-type seedlings, was inhibited severely under both conditions of illumination, as expected.

The phenotypes of such growth-inhibited etiolated seedlings are shown in detail in Figure 4. Both root and hypocotyl growth were severely inhibited (Figure 4A; Figure 4B, panel iii), and callus occasionally developed either on or

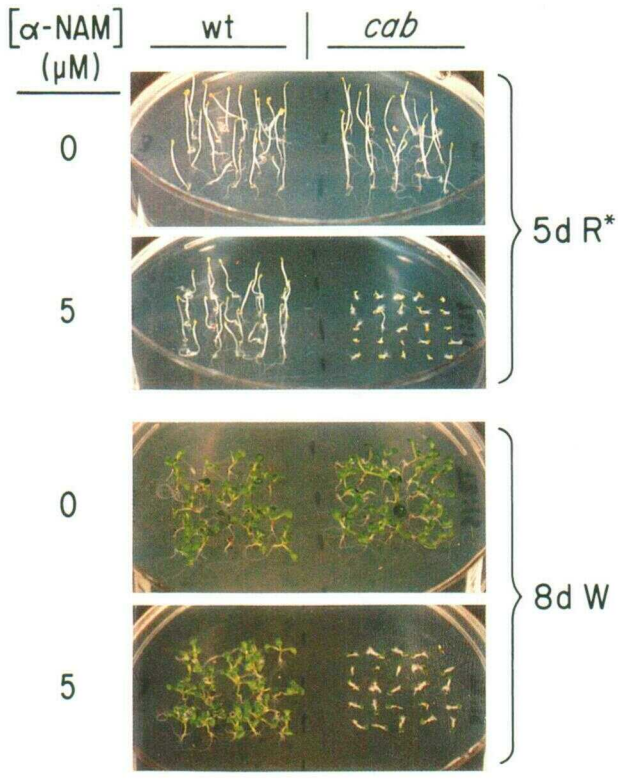

Figure 3. Growth Inhibition of Both Etiolated and Green *tms2*$^+$ Seedlings by an Auxin Amide Substrate.

Seedlings from the wild type (wt) or cab(+14)A (*cab*) lines were grown for the designated times under either intermittent red, 1′R every 2 hr (R*), or continuous white light (W) ± 5 μM α-NAM.

immediately below the cotyledons (Figure 4B, panel iv). By comparison, in green seedlings (in which hypocotyl growth is inhibited by light), the growth of roots and primary leaves was inhibited (Figure 3, 8d W plants). At a lower α-NAM concentration (3 μM), the growth of the hypocotyl is somewhat less inhibited, the tissue shows some disintegration (Figure 4B, panel i), and adventitious roots eventually form on the hypocotyl (Figure 4B, panel ii). Such effects on growth are known to occur as a result of high auxin levels (Key, 1969).

The effects of three different auxin amide substrates on the growth of the hypocotyls are illustrated by the dose-response curves in Figure 5. Two of the substrates, IAM and α-NAM, inhibited growth of the transgenic seedlings by 50% at concentrations of 1 μM to 2 μM. The third, β-NAM, which was presumably converted by the amidohydrolase to the less active auxin, β-naphthaleneacetic acid (NAA), was considerably less toxic, showing 50% inhibition at 50 μM to 75 μM. At high concentrations, growth of the wild-type seedlings eventually was inhibited by both α-NAM and β-NAM.

The results of these experiments demonstrated that various auxin amide compounds can be used to inhibit the growth of the transgenic seedlings expressing the *tms2* gene. The relative efficacy of the compounds seems to correlate with the auxin activity of the converted product. α-NAA is more toxic than IAA, and both are more toxic than β-NAA (Hertel et al., 1969).

Phytochrome Action Can Regulate the Growth Inhibition by Auxin Amides in the *cab140*::*tms2* Transgenic Seedlings

Because we had demonstrated that phytochrome action could regulate the *tms2* RNA levels (Figure 1) and the amidohydrolase levels (Figure 2) in the transgenic seedlings, we expected to see such regulation of the growth-inhibited phenotype on any of the three substrates examined (Figure 5). Unexpectedly, however, dose-response curve determinations with IAM and α-NAM showed no differences between seedlings grown in darkness and in intermittent R (data not shown). Only on the less toxic substrate, β-NAM, could the expected red induction of growth inhibition be observed. Figure 6 shows that there is little difference in growth of either wild type or the 35S(+1)A line under either illumination condition. However, the cab(+14)A line shows greater inhibition at all tested concentrations of β-NAM if the seedlings are grown with intermittent R illumination than if they are grown in complete darkness.

These results suggested to us that the level of the amidohydrolase in the dark-grown plants was sufficiently high so that this enzyme activity was not the limiting factor in the plants' response to inhibitory levels of α-NAM and IAM, the substrates converted to the more toxic products. We thought that by using β-NAM, a substrate that would be converted to a less toxic product, we would be able to partially saturate the amidohydrolase level found in the dark-grown seedlings. Because the highest concentration of β-NAM used gave the greatest difference in sensitivity between dark and intermittent red-grown seedlings but was also beginning to inhibit growth of wild-type seedlings, we considered whether combinations of α-NAM and β-NAM might produce a still greater difference. We determined empirically that a mixture of 3 μM α-NAM and 100 μM β-NAM gave the greatest growth differential between induced and uninduced seedlings, being slightly better than β-NAM alone (data not shown). Figure 7 illustrates the differential growth inhibition of the *cab140*::*tms2* line between intermittent R and dark-grown seedlings. Neither wild type nor the *35S*::*tms2* line was differentially inhibited; the wild type showed no significant effect, and the

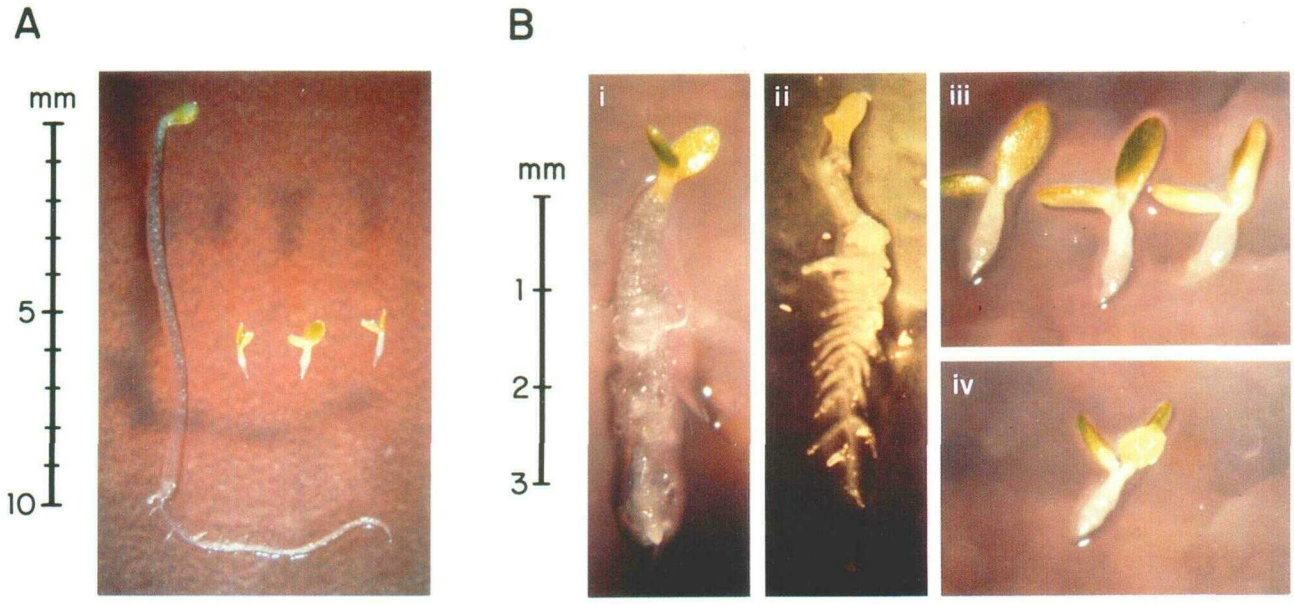

Figure 4. Photomicrographs Showing Auxin Toxicity in *tms2*$^+$ Seedlings Grown on α-NAM under Intermittent R.

(A) Growth inhibition of shoot and root axes in three cab(+14)A seedlings (right) contrasted to an unaffected wild-type seedling (left), all grown for 5 days on 5 μM α-NAM.
(B) Panels (i) and (ii), seedlings grown on 3 μM α-NAM for either 5 days (i) or 10 days (ii). Panels (iii) and (iv), seedlings grown on 5 μM α-NAM for 5 days.

35S::*tms2* line, which expresses high levels of the enzyme (Figure 2), was severely inhibited under both illumination conditions.

Shoot Height Can Be Used as the Parameter To *Distinguish Induced from Noninduced Transgenic* Seedlings

It was important to demonstrate that we would be able to distinguish putative mutants in the phytochrome transduction pathway using the selection system described. We would expect such mutants to be unable to respond to red light and, thus, to grow as well as dark-grown seedlings on the α-NAM + β-NAM mixture, even in the presence of intermittent R illumination. Furthermore, we would need to be able to select such seedlings against a background of growth-inhibited seedlings. Thus, we measured the height distribution of a population of *cab*::*tms2* seedlings grown in darkness or intermittent R light and compared this distribution to wild-type seedlings grown in intermittent R. Figure 8 shows that although there is some overlap, a particular minimal height can be chosen that would exclude all the induced (Figure 8A) from the uninduced (Figure 8B) transgenic plants in a mixed population. Thus, the selection for mutants using this procedure should be feasible.

DISCUSSION

The experiments presented here demonstrate the feasibility of using the *Agrobacterium tms2* gene as a selectable reporter gene for negative selection in *Arabidopsis*. This gene encodes an enzyme activity that was not present in the wild-type plants, and further evidence indicated that *tms2*$^+$, but not wild-type seedlings, converted ^{14}C-IAM to ^{14}C-IAA in vivo (data not shown). Furthermore, the substrate for the enzyme is not present at significant levels in *Arabidopsis*, as evidenced by the lack of any change in phenotype in the transgenic seedlings growing under normal conditions. Thus, the timing and conditions for selection can be controlled easily.

We have characterized the phenotype of *Arabidopsis* seedlings expressing the *tms2* gene under the control of a phytochrome-regulated promoter, derived from the *cab140* gene of *Arabidopsis* (Leutwiler et al., 1986; Karlin-Neumann et al., 1988), or under the control of a CaMV 35S promoter. The expression of the gene conferred greatly increased sensitivity to auxin amide substrates,

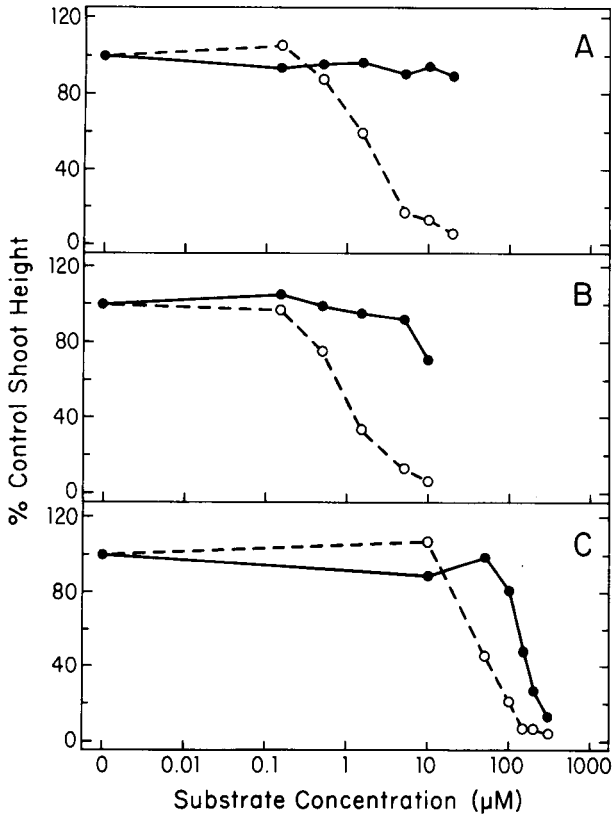

Figure 5. Dose-Dependent Inhibition of Shoot Growth on Auxin Amide Substrates.

(A) cab(+14)A (○) and wild-type (●) seedlings were grown for 5 days with 1 min red/2 hr on IAM.
(B) cab(+14)A (○) and wild-type (●) seedlings were grown for 5 days with 1 min red/2 hr on α-NAM.
(C) cab(+14)A (○) and wild-type (●) seedlings were grown for 5 days with 1 min red/2 hr on β-NAM.
The mean hypocotyl lengths of 25 seedlings per sample are expressed as a percentage of the mean hypocotyl length of control seedlings grown in the absence of substrate. Control hypocotyl lengths for the two lines did not differ.

leading to pleiotropic effects characteristic of auxin toxicity. At substrate concentrations that have little or no effect on wild-type *Arabidopsis* seedlings, *tms2*$^+$ seedlings showed nearly complete inhibition of hypocotyl and root growth when grown in darkness (Figures 3 to 5), and of primary leaf and root growth when grown in white light (Figure 3). Growth-inhibitory effects of IAM and α-NAM have also been seen in transformed tobacco seedlings (Inzé et al., 1984; Budar et al., 1986), tobacco protoplast-derived cells (Depicker et al., 1988), and petunia leaf disc explants (Klee et al., 1987) expressing the *tms2* gene. Therefore, the *tms2* gene may be useful as a selectable marker in many different plant species.

We have also shown that we can place the *tms2* gene under the regulation of the phytochrome system by using a 1.3-kb fragment of the *Arabidopsis cab140* promoter. The red light-induced increase in the mRNA observed for this fusion construct, but not for a construct using a CaMV 35S promoter fragment (Figure 1), demonstrated that this regulation was at the level of transcription. We also note that the *cab140*::*tms2* construct included the first 14 nucleotides of the *cab140* transcript, but we do not think this 14-nucleotide fragment is involved in post-transcriptional regulation because *cis*-RNA elements that are known to affect RNA stability are longer and capable of forming secondary structures (e.g., Casey et al., 1989; Malim et al., 1989). Furthermore, evidence from a study with the wheat *cab1* promoter suggests that this region would not be capable of mediating the phytochrome response because the authentic *cab* transcript produced from a constitutive promoter was unaffected by phytochrome action (Nagy et al., 1987). The fact that the relative increase in response to brief R for the *tms2* RNA was less than for the endogenous *cab140* RNA may be due to additional enhancer elements present elsewhere in the gene and/or to constitutive differences in the half-lives of the *tms2* and *cab140* transcripts.

It is noteworthy that the plants could respond to red light as early as 2 days after sowing, about 12 hr after germination is evident. The early development of responsiveness of mRNA levels to phytochrome action has also been seen in mustard and radish seedlings (Schmidt et al., 1987; Fourcroy et al., 1989).

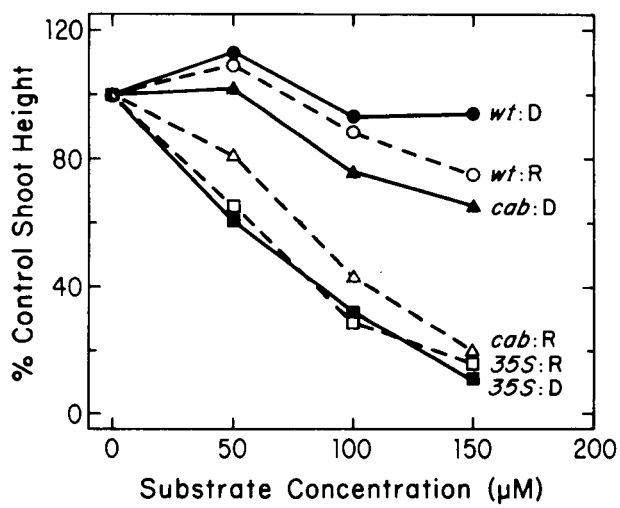

Figure 6. Red Light Increases Sensitivity of *cab140*::*tms2* Seedlings to β-NAM.

Seedlings from three lines (wild type [wt], cab(+14)A [*cab*], and 35S(+1)A [35S]) were grown in darkness (D) or 1 min red/2 hr (R) for 5 days on the concentrations of β-NAM shown.

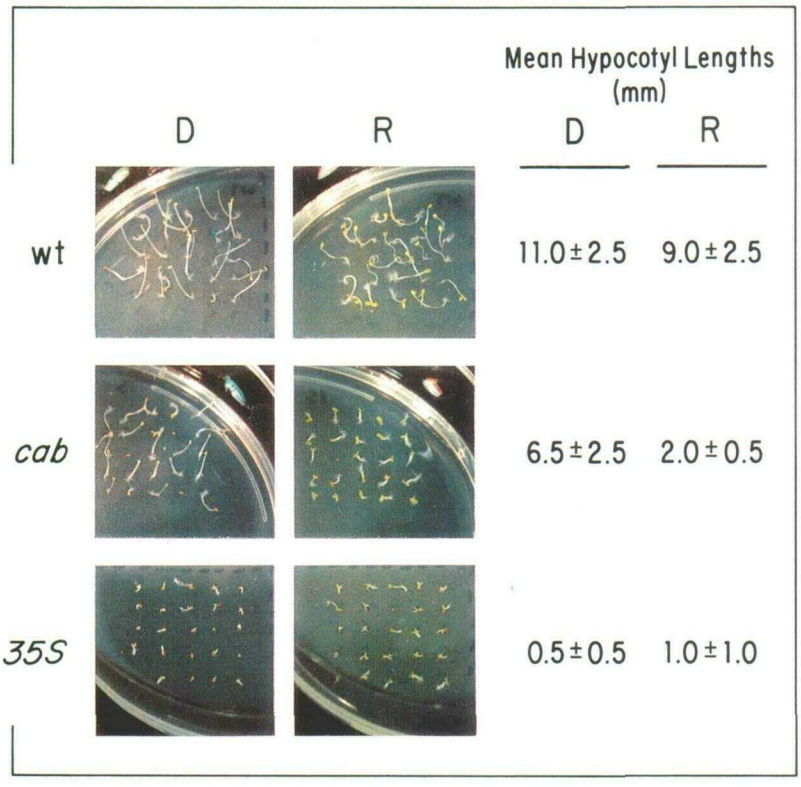

Figure 7. Phytochrome Regulates Growth Inhibition of *cab140*::*tms2* (*cab*) but Not of *35S*::*tms2* (*35S*) Seedlings on a Mixture of α-NAM and β-NAM.

Seedlings were grown on 3 μM α-NAM and 100 μM β-NAM for 5 days under darkness (D) or 1 min red/2 hr (R). Means and SD of hypocotyl lengths are given for each of the seedling populations pictured. wt, wild type.

The relative increase in the *tms2* RNA was less in plants given the intermittent red light treatment than in those given a single red treatment (Figure 1, R and R*). However, because the increase in *cab140* RNA had already been shown to be a transient one (Karlin-Neumann et al., 1988), it was necessary to give repeated stimulation with red light to maintain the increased level of the RNA and, thus, an increase in the amidohydrolase activity over the whole time of seedling growth. Under these growth conditions, the phytochrome regulation of the amidohydrolase level mirrored the increase in the RNA (Figure 2) and increased the enzyme activity about fourfold. Because the enzyme activity in the dark-grown plants was not negligible, and, in fact, apparently was able to convert available substrate to significant amounts of product, we used β-NAM, a substrate that was converted to a less toxic auxin, to try to decrease the effective toxicity of the dark level of the enzyme. We found empirically an appropriate level of mixed substrates that resulted in more substantial growth inhibition of seedlings grown in intermittent red light than of those grown in darkness (Figures 6 to 8). The comparison to the wild-type seedlings suggests that if the dark levels of the enzyme had been lower in the transgenic *cab140*::*tms2* seedlings, the growth inhibition induced by the red light treatments would have been even easier to distinguish.

We are currently using these conditions to screen mutagenized transgenic *cab140*::*tms2* lines as the first step in isolation of mutants in the phytochrome signal transduction pathway leading to transcriptional changes. Reconstruction experiments, in which a small number of wild-type seedlings were grown on 5 μM α-NAM in the midst of about 1000 *cab140*::*tms2* seedlings, have demonstrated that possible cross-feeding effects are not observed (data not shown). Other kinds of mutations that might be selected in this screen include auxin resistance mutants and photoreceptor mutants, but these should be easily distinguishable. The strategy of using the *tms2* gene fused to a regulated promoter should also be applicable to the isolation of signal transduction mutants in other responses.

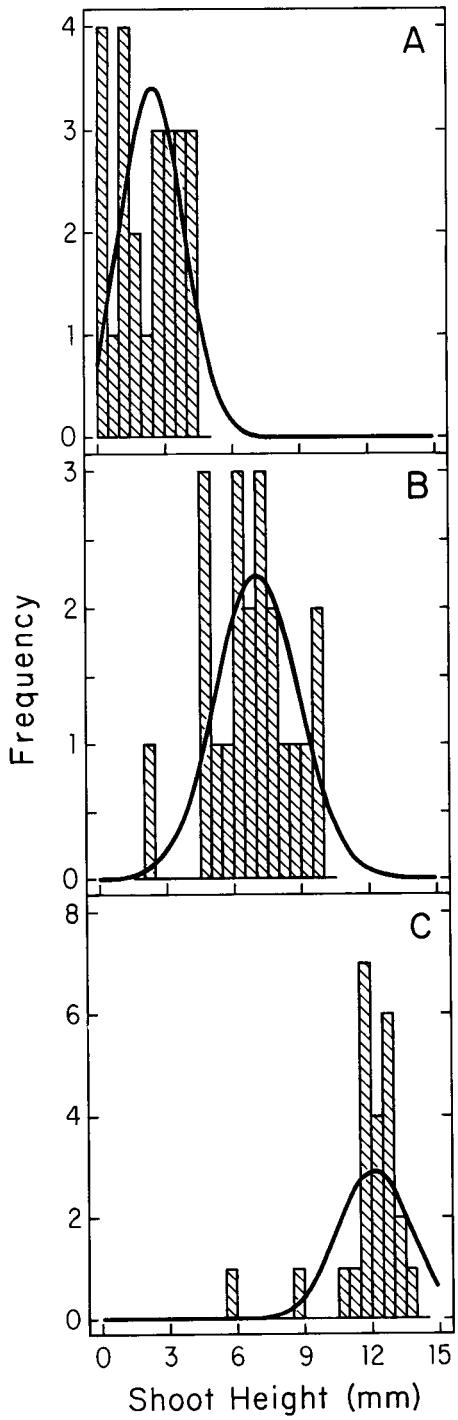

Figure 8. Uninduced *cab140::tms2* Seedlings Can Be Distinguished from Induced Seedlings on a Mixed Substrate.

The distribution of hypocotyl lengths from the seedlings in Figure 7 is shown.
(A) cab(+14)A grown with 1 min red/2 hr.
(B) cab(+14)A grown in darkness.
(C) Wild type grown with 1 min red/2 hr.

METHODS

Plant Material, Growth Conditions, and Chemicals

Arabidopsis thaliana (var Columbia) was used for all experiments. Seeds were surface sterilized in 20% bleach/0.2% Ivory soap, then sown onto Murashige and Skoog medium (MS salts [GIBCO]/3% sucrose/B-5 vitamins/0.7% Phytagar [GIBCO], pH 5.5) ± auxin amide as indicated; or for RNA analysis, onto Whatman No. 1 filters saturated with nutrient solution (Karlin-Neumann et al., 1988). Dishes were placed in darkness at 4°C for 2 days, followed by a 15-min exposure to white light. They were then incubated in darkness or under other light regimes at 24°C. The red and far red light sources used were described previously (Karlin-Neumann et al., 1988).

β-NAM and 1-^{14}C-IAM were synthesized by Dr. Jerry Cohen (U.S. Department of Agriculture Plant Hormone Laboratory, Beltsville, MD). Other auxins and auxin amides were purchased from Sigma.

Construction of Promoter Fusions and Probes

To construct the *cab140::tms2* gene fusion, a 1.34-kb *cab140* promoter fragment (Leutwiler et al., 1986), extending from the EcoRI site at −1326 to +14, relative to the start of transcription, was ligated to a 2-kb BamHI fragment from pMON544 (Klee et al., 1987), containing a modified *Agrobacterium tumefaciens tms2* gene with a BamHI site introduced 10 nucleotides upstream of the translation start. The *cab140::tms2* gene fusion was transferred as a 3.4-kb HindIII fragment to the unique HindIII site in the polylinker of the Ti plasmid-based binary vector pCIT20 (kindly provided by M. Yanofsky and E. Meyerowitz).

To construct the *35S::tms2* gene fusion, the 2-kb BamHI fragment containing the modified *tms2* gene was joined in the sense orientation to a 1.6-kb HindIII-BamHI fragment from pDO396 containing the CaMV 35S promoter (Ow et al., 1986). The resulting *35S::tms2* gene fusion was transferred as a 3.6-kb HindIII fragment to the HindIII site of pCIT20.

Clones for synthesizing antisense probes for RNase protection analyses were constructed in pBluescript SK+ and SK− phagemids (Stratagene). The probe for the *cab* RNA, pSK(+)/cab140RH, contained an EcoRI-HindIII (−1326 to +130) fragment from λbAt1005 (Leutwiler et al., 1986), and the probe for the *tms2* RNA, pSK(−)/α-tms2RV, contained a BamHI-EcoRV fragment from the *tms2* open reading frame of pMON544 (Klee et al., 1987). Both clones were linearized with BglII and transcribed with T7 RNA polymerase.

Complete details of all constructs are given by Karlin-Neumann (1991).

Plant Transformation

pCIT20 recombinants containing *cab140::tms2* and *35S::tms2* promoter fusion constructs were mobilized from *Escherichia coli* MM294 cells into *Agrobacterium* strain A208-SE using the simplified triparental mating system for binary vectors described by Rogers et al. (1987). Sterile leaf and stem explants from 4-week-old to 5-week-old *Arabidopsis* plants, grown under a 14-hr light/10-hr dark photoperiod, were transformed with resulting *Agrobacterium* transconjugants by the procedure of Lloyd et al. (1986).

In Vitro Amidohydrolase Assay

Enzyme extracts were prepared and assayed by modifications of the procedures of Kemper et al. (1985). Samples of 25 to 35 sterile seedlings were powdered in liquid N_2 in 1.5-mL microcentrifuge tubes using a Kontes pestle. Further homogenization was done on ice after addition of 50 μL of ice-cold homogenization buffer (0.1 M Tris-HCl, pH 8.5, 10% sucrose, 5 mM EDTA, 10 mM $MgCl_2$, 2 mM DTT, 50 mM sodium dithionite [added in solid form just before use]). After centrifugation of tissue homogenates at 12,000*g* for 2 min at 4°C, supernatant fractions were assayed for protein (Bradford, 1976) and amidohydrolase activity. Assay samples consisted of 25 μL of extract (either undiluted or diluted 1:1 with homogenization buffer) and 25 μL of assay buffer (0.2 M Tris-HCl, pH 8.5, 20 mM $MgCl_2$, 3 mM EDTA, 80 mM KCl, 2 mM DTT, 10 mM sodium dithionite), in which 60,000 dpm of ^{14}C-IAM per 25 μL had been previously resuspended. Final concentration of the substrate in each reaction was 8 μM. Reactions were initiated by a 5-sec centrifugation followed by gently mixing and incubation at 30°C for 10 min. Reactions were stopped by transfer of samples to ice. The amount of substrate converted to ^{14}C-IAA was determined by scintillation counting after first removing unconverted ^{14}C-IAM with three ethylacetate extractions at pH 11. ^{14}C-IAA produced in the reaction was then extracted at pH 3 with three additional ethylacetate extractions. The assay was linear with respect to time, and conversion of substrate was proportional to the protein concentration (above 10 μg of protein/reaction). Values given for hydrolase activity levels are the average of duplicates from a representative experiment. The experiment was repeated three times.

RNase Protection Assay

Total RNA was prepared from etiolated *Arabidopsis* seedlings following the method of Sharrock and Quail (1989). High specific activity probes (4×10^8 cpm/μg template) were synthesized with T7 polymerase (Promega) as described by the manufacturer, except that 200 μCi of α-^{32}P-UTP (800 Ci/mmol, 40 mCi/mL; Amersham), and no unlabeled UTP were used. The *cab140* antisense probe was 426 nucleotides long, of which 130 nucleotides were protected by *cab140* mRNA, whereas the *tms2* antisense probe was 205 nucleotides long, of which 153 nucleotides were protected by *tms2* mRNA. RNase protection analyses were carried out by the procedures of Zinn et al. (1983) using 10 μg of total *Arabidopsis* RNA and 2 to 4×10^6 cpm of each labeled probe. RNase protection results shown are from a representative experiment and have been repeated at least twice. Radioactivity in protected bands was used to calculate the number of molecules of each mRNA per seedling based on an estimate of 0.1 mg of total RNA per seedling.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Lu Huang. We thank Alan Lloyd and Rob Horsch for advice on *Arabidopsis* transformation and Steve Rogers and Harry Klee for providing us with the *Agrobacterium* host strain A208-SE and pMON544, Marty Yanofsky and Elliot Meyerowitz for pCIT20, and David Ow for pDO396. We are deeply indebted to Jerry Cohen for his advice and for syntheses of various auxin amide substrates. Finally, we wish to thank Sharlene Weatherwax, Steve Rolfe, and Lin Sun for critical comments on the manuscript. This work was supported by National Institutes of Health Grant GM-23167 to E.M.T. G.A.K.-N. was supported by the McKnight Foundation and by United States Public Health Service Cell and Molecular Biology Training Grant GM-07185 to University of California, Los Angeles. J.A.B. is a recipient of a postdoctoral fellowship from the American Cancer Society.

Received February 5, 1991; accepted April 2, 1991.

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