Expression patterns of Arabidopsis PHYD and PHYE phytochrome genes antisense inhibition of the PHYB gene
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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences
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Abstract:
Phytochrome is the photoreceptor in plants that senses red and far-red light. In Arabidopsis, it is encoded by a small gene family with 5 members: PHYA, PHYB, PHYC, PHYD and PHYE. The regulation of the PHYA and PHYB genes has been characterized to an extent, but as yet little is known about the PHYD and PHYE genes. These two genes are more closely related to PHYB and form a sub-family of PHYB-like genes. So, the spatial, temporal and photoregulation of the PHYD and PHYE gene promoters was studied in comparison with that of the PHYB gene. Using the promoter-reporter fusion system with the gus reporter gene, I show here that, in spite of the amino acid sequence homology between them, there are distinct differences in the expression patterns of the three genes. The PHYB gene is expressed throughout the plant at medium to high intensity and shows no regulation by light. On the other hand, the PHYD gene shows light-dependent regulation in the roots. It is also expressed to a much lower intensity, the level of expression being strongly correlated to the transgene copy number. The PHYE gene also shows a very low intensity of expression. The difference in the pattern of expression of these 3 genes is very apparent in the male gametophyte: PHYB is expressed strongly in the filament, the anther and the pollen, while PHYD is expressed only in the pollen, and PHYE is expressed only in the anther.

In order to determine whether the activity of an individual phytochrome gene can be selectively suppressed with an antisense transgene, inhibition of the PHYB gene was attempted. Multiple copies of the antiB transgene succeeded in decreasing the amount of PHYB protein in the plant by about 50%. The phenotype observed in these plants was intermediate between the wild-type and that shown by the phyB null mutant, hy3, in characteristics of hypocotyl length and chlorophyll levels. However, certain variant phenotypes were also observed that deviated from both wild-type and mutant phenotypes. It is possible that these variant phenotypes might be caused by the antiB transgene interfering with the expression of other members of the phytochrome gene family.

EXPRESSION PATTERNS OF *ARABIDOPSIS PHYD* AND *PHYE* PHYTOCHROME GENES AND ANTISENSE INHIBITION OF THE *PHYB* GENE

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APPROVAL

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Signature Lakshmi. T. A.

Date May 3, 1994.

I dedicate this thesis to my beloved parents,
Mrs. Suseela Ananthakrishnan and Mr. T.V. Ananthakrishnan.

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ABSTRACT

Phytochrome is the photoreceptor in plants that senses red and far-red light. In *Arabidopsis*, it is encoded by a small gene family with 5 members: *PHYA, PHYB, PHYC, PHYD* and *PHYE*. The regulation of the *PHYA* and *PHYB* genes has been characterized to an extent, but as yet little is known about the *PHYD* and *PHYE* genes. These two genes are more closely related to *PHYB* and form a sub-family of *PHYB*-like genes. So, the spatial, temporal and photoregulation of the *PHYD* and *PHYE* gene promoters was studied in comparison with that of the *PHYB* gene. Using the promoter-reporter fusion system with the *gus* reporter gene, I show here that, in spite of the amino acid sequence homology between them, there are distinct differences in the expression patterns of the three genes. The *PHYB* gene is expressed throughout the plant at medium to high intensity and shows no regulation by light. On the other hand, the *PHYD* gene shows light-dependent regulation in the roots. It is also expressed to a much lower intensity, the level of expression being strongly correlated to the transgene copy number. The *PHYE* gene also shows a very low intensity of expression. The difference in the pattern of expression of these 3 genes is very apparent in the male gametophyte: *PHYB* is expressed strongly in the filament, the anther and the pollen, while *PHYD* is expressed only in the pollen, and *PHYE* is expressed only in the anther.

In order to determine whether the activity of an individual phytochrome gene can be selectively suppressed with an antisense transgene, inhibition of the *PHYB* gene was attempted. Multiple copies of the antiB transgene succeeded in decreasing the amount of PHYB protein in the plant by about 50%. The phenotype observed in these plants was intermediate between the wild-type and that shown by the *phyB* null mutant, *hy3*, in characteristics of hypocotyl length and chlorophyll levels. However, certain variant phenotypes were also observed that deviated from both wild-type and mutant phenotypes. It is possible that these variant phenotypes might be caused by the antiB transgene interfering with the expression of other members of the phytochrome gene family.

CHAPTER I

INTRODUCTION

Photomorphogenesis in Plants

Light is the primary source of energy for green plants. In addition to this, however, light also functions as a regulator of plant development. Plants are sedentary organisms that are unable to relocate under unfavourable conditions. Therefore, they have evolved to sense environmental cues and vary their responses accordingly. Light provides a number of cues by way of changes in light intensity, direction, duration and spectral quality. These are sensed by photoreceptors in the plants, and used to regulate the developmental program, a process commonly known as photomorphogenesis. Many diverse plant responses throughout the entire life of the plant have been found to be light-regulated including seed germination, tropic responses, chloroplast development, stem growth, and flowering, to mention just a few (1, 2, 3, 4).

Plant Regulatory Photoreceptors

Response to a light cue occurs via photodetection and subsequent signal transduction. Photoregulation of plant responses depends, therefore, on the ability of the plant photoreceptors to detect accurately the quality and quantity

of light received. Plant photoreceptor molecules are present in a wide variety of cells and not confined to a few specialized cells, as is the case with the animal light-sensory pigment rhodopsin (3). There are three major kinds of photoreceptors in plants: a) phytochrome, sensing red (600-700 nm) and far-red (700-780 nm) light, b) cryptochrome, sensing UV-A /blue light (320-520 nm), and c) a UV-B absorbing pigment (280-320 nm). Of these, phytochrome is the best-characterised (2, 3, 4, 5,) and the subject of this study. The first gene for a blue-light photoreceptor, has recently been isolated in *Arabidopsis thaliana* (6). The protein encoded by this gene shows considerable homology to microbial photolyases, which are flavoproteins that catalyse blue light-dependent reactions. The UV-B photoreceptor has not yet been isolated. The action spectra for a wide variety of plant photoresponses show that the chlorophylls do not play a major part in photomorphogenesis (3).

Biological Activity Of Phytochrome

Phytochrome was first identified in 1959 on the basis of its unique spectral properties (7) and is now the best characterised of the three photoreceptors. It is a regulatory photoreceptor that functions as a binary molecular switch controlling plant gene expression in response to light signals from the environment (2). The phytochrome molecule can assume two spectrally distinct

forms: Pr, which has maximum absorbance at 666 nm, and Pfr, which has maximum absorbance at 730 nm (4). These two forms are photointerconvertible: red light converts the Pr form to the Pfr form and far-red light reverses this reaction. Phytochrome is synthesised as Pr, which is inactive for most phytochrome-mediated responses. When converted to the Pfr form by photons of red light, phytochrome is activated and initiates a transduction process that culminates in altered expression of selected genes and ultimately in altered growth and development appropriate for the prevailing light environment (2). Studies of genes that are regulated by phytochrome show that the alteration of expression may be through transcriptional activation or repression of the genes. Many of the phytochrome-mediated responses can be cancelled by the subsequent reconversion of Pfr back to Pr (4). On repeated irradiation of plant tissue with alternating doses of R and FR, a simple response has been shown to be completely dependant upon the nature of the last stimulus given (3).

Recently, it has been shown that the red-absorbing (Pr) form of phytochrome B is required for normal hypocotyl gravitropism in *Arabidopsis* (9). This demonstrates that distinct biological activities can be associated with each of the two interconvertible forms of phytochrome and raises the possibility that the Pr form may not be completely inactive as has been the concept so far.

Since the interconversion of Pr and Pfr is rapid and reversible, the proportion of phytochrome in either form reflects a photoequilibrium based upon the proportion of red and far-red (R and FR) light entering the cell. This equilibrium is dependent on the ratio of the amount of R to FR light, and can change rapidly in response to changing light conditions. Thus, the phytochrome system is sensitive not merely to the presence or absence of light, but also to the spectral composition of the radiation (10).

The first phytochrome to be studied was the light-labile Type I phytochrome, now called PHYA phytochrome. Much more is known about this phytochrome than any of the other members of this receptor family. In the absence of any activating light signal, PHYA phytochrome accumulates in the red-absorbing Pr form. PHYA Pr is very stable with a half-life of about 100 hours, while the Pfr form is 50 to 100-fold less stable in the cell. Hence, within seconds of Pfr formation following illumination, the majority of this type of phytochrome becomes associated with discrete, amorphous subcellular bodies within the cytoplasm and is degraded (3). PHYA phytochrome photoreceptor is found in almost all plant tissues and organs examined, but varies in abundance in different cell types and in different regions of the plant. Cells that have been newly derived from shoot and root meristematic regions have larger amounts of this phytochrome compared to other cell types. Within

the cell, PHYA phytochrome in the Pr form shows no preferential association with any subcellular compartment (11).

Molecular Properties Of Phytochrome

Most of the biochemical characterization of phytochrome has been done on the Type I PHYA form and it is not yet clear whether other members of the phytochrome family conform to all of the properties listed here. Phytochrome is a soluble homodimeric chromoprotein. Each monomer is an approximately 120 kD protein (1100 amino acids) consisting of two discrete domains: an N-terminal domain of about 74 kD that contains a linear tetrapyrrole chromophore covalently linked via a thiol-ether linkage to a Cys residue in a hydrophobic pocket and a C-terminal domain of about 55 kD that possesses a site(s) responsible for dimerization. These two domains are linked together by a proteolytically vulnerable hinge region (4, 12). On dimerization, the molecule is thought to assume a Y-shaped structure (4). The chromophore is a bile pigment, phytochromobilin (13). Attachment of the chromophore occurs autocatalytically (in vitro), indicating that the protein has an intrinsic chromophore lyase activity. Other posttranslational modifications include the removal of the N-terminal Met, N-acetylation of the penultimate Ser and possible phosphorylation and glycosylation (4). The phototransformation between the Pr and the Pfr forms occurs

in the absence of any additional factors. The chromophore is the site of the R/FR light absorption and sets off a number of changes in the molecule. These include a cis- to trans-isomerization of one of the double bonds within the chromophore, a 31^0 reorientation of the chromophore relative to the polypeptide, and multiple conformational changes within the polypeptide, especially near the N terminus (3, 4).

Efforts made to delineate activities of various domains of the phytochrome through deletion analysis of the carboxy terminus of the oat *PHYA* gene have revealed the presence of separate domains required for spectral and biological activity (14).

The Phytochrome Gene Family

Data from early physiological and biochemical experiments suggested that there were at least two pools of phytochrome in plants, one that predominates in dark-grown, etiolated plants and the other which predominates in light-grown plants. These were referred to as Type I and Type II phytochrome respectively. The first phytochrome gene to be sequenced was the *PHYA* gene, which was found to encode the Type I or etiolated tissue or light-labile phytochrome (17). The Type II or green tissue or light-stable phytochrome was found to be heterogeneous and encoded by more than one gene. In *Arabidopsis*, it was shown that phytochrome is encoded by a small family of genes (8).

This gene family was found to include five members, *PHYA*, *PHYB*, *PHYC*, *PHYD* and *PHYE*, that are at least 50% identical with each other at the amino acid sequence level (15). These genes, though variable in sequence, are conserved in terms of their basic structure. They are all predicted to be soluble proteins located in the cytoplasm. No large non-homologous domains have been found in any one of them. The regions of highest sequence identity are around the portions of the phytochrome required for chromophore attachment and spectral integrity of the molecule. Analysis of amino acid sequence similarity among the five *PHY* genes shows *PHYA, PHYB* and *PHYC* to be equally divergent, and *PHYD* and *PHYE* to be more closely related to *PHYB* than to the other two (15; Table 1). In fact, the PHYD polypeptide shows a striking 80% sequence similarity to PHYB. The evolutionary pattern of the *PHY* genes derived from all these data is that *PHYA*, *PHYB* and *PHYC* presumably arose by duplications of an ancestral *PHY* gene either before or very early in angiosperm evolution and that the *Arabidopsis* PHYB, PHYD and PHYE phytochromes constitute a subfamily of proteins that are more recently derived and more structurally related (15). The high degree of sequence similarity among the five *PHY* genes suggests that they may work via similar signal transduction mechanisms. Characterisation of phytochrome mutants which are deficient in only one phytochrome protein (e.g. the *hy3* mutant that

	PHYA	PHYB	PHYC	PHYD	PHYE
PHYA	--	52	52	52	48
PHYB		--	52	80	56
PHYC			--	50	46
PHYD				--	55
PHYE					--

Table 1. Percent amino acid sequence identity among the *Arabidopsis* phytochrome polypeptides (from Clack et al. (15)).

lacks only PHYB protein (18, 19) and the *hy8* mutant that lacks only PHYA protein (20, 21)) has shown that these genes are not redundant. Nonetheless, it is likely that they interact in synergistic or complementary ways to control plant responses (15). Analysis of the five *PHY* mRNA levels under different light conditions, in different plant organs and in different stages of growth indicates that the phytochrome genes are relatively constitutive in expression, and do not exhibit diurnal cycling under a 12 hour photoperiod (15).

The *PHYA* gene is highly controlled by light and codes for the light-labile protein which is most abundant in dark-grown plants. Since *PHYA* transcription is rapidly repressed by red light and the Pfr form of PHYA is approximately 100-fold less metabolically stable than Pr, the level of the PHYA protein in green plants is as much as 100 times lower than in etiolated plants (4). The *hy8/fre1* mutants of *Arabidopsis* that are deficient in the PHYA protein show a lack of the far-red high irradiance response (FR-HIR) as dark-grown seedlings, but are nearly unaffected as light-grown plants. Thus, PHYA protein appears to be active mainly in etiolated seedlings and primarily responsible for enhancing the photosensitivity of seedlings before they emerge from the soil. In *Arabidopsis*, the *PHYA* gene is located on chromosome 1.

In contrast to *PHYA*, the *PHYB* and *PHYC*, and perhaps

PHYD and *PHYE*, genes encode proteins that are light-stable (15, 18) and can be classified under the Type II or light-stable forms. A mutant in the *PHYB* gene has been identified in *Arabidopsis* (*hy3*), that shows normal FR-HIR but shows alteration of red and white-light-controlled responses including cell elongation, chloroplast differentiation and timing of flowering as light-grown plants (16, 19). In *Arabidopsis*, this gene is located on chromosome 2. The physiological roles of the other phytochrome genes, *PHYC*, *PHYD* and *PHYE*, are yet to be determined.

Regulation Of Plant Genes By Phytochrome

Several plant genes have been found to be positively regulated by phytochrome. Among them are the cab genes that encode chlorophyll a/b binding proteins (23, 26), the *rbcS* genes that encode the small subunit of the ribulose-1,5-bisphosphate carboxylase-oxygenase enzyme (22, 24, 25), and the gene encoding the enzyme NADPH-protochlorophyllide reductase (27). Genes that are negatively regulated by phytochrome have also been identified and include the gene for asparagine synthetase (28, 29) and the NPR (Negatively phytochrome regulated) genes in *Lemna gibba* (30). Short DNA sequences in the promoter regions of these light-responsive genes have been shown to function as cis-acting elements involved in photocontrol (22, 23).

Though the light-responsive nature of phytochrome is

now well understood, little is known about the signal transduction mechanisms that follow the light perception and lead to gene regulation. Calcium/calmodulin and a heterotrimeric G protein have been implicated in several in vivo phytochrome responses (31). In addition, the phytochrome of the moss *Ceratodon* has been found to carry a protein kinase domain (32), and a C-terminal section of higher plant phytochromes has been found to share sequence homologies with the transmitter modules of bacterial sensor proteins (33). However, the actual mechanism of signal transduction by phytochrome molecules still remains to be understood.

Agrobacterium-mediated Plant Transformation

In order to understand the complexities of plant gene expression, plant gene cloning must be coupled with efficient methods of gene transfer into plants. The most important characteristic that differentiates plant gene transfer systems from animal systems is the totipotency that is observed in plant cells (34). This means that a typical plant cell can give rise to a complete, differentiated organism. Thus, gene transfer into plants does not have to use germ-line cells like in animals. Also, isolated plant cells do not necessarily retain a differentiated state determined by the tissue of origin, as is the case with animal cells. Thus, any plant cell in culture is capable of de-differentiating from the tissue of origin and

re-differentiating to give a whole, new plant. Consequently, transgenic plants can be produced in large numbers more easily than transgenic mice or *Drosophila* (35).

The production of morphologically normal plants that contain and express foreign genes is most easily accomplished through the use of the natural gene-transfer capacity of *Agrobacterium tumefaciens*. *Agrobacterium tumefaciens* is a soil bacterium that causes crown gall disease in plants (36). This bacterium is naturally capable of transferring a piece of DNA into the genomes of most dicotyledonous plants. The expression of genes located on this transferred DNA (T-DNA) inside the plants causes an imbalance of endogenous hormones leading to tumour formation. Foreign genes inserted on this T-DNA can be co-transferred, and can integrate into the plant genome (35, 36).

Furthermore, it is possible to 'disarm' the *Agrobacterium* Ti plasmids such that gene transfer can occur without disturbing the endogenous hormone balance in the plants. It has been shown that, for gene transfer to be accomplished, only the T-DNA borders and some flanking sequences need to be present in cis. Thus, the normal T-DNA-encoded genes have been deleted and easily selectable markers, such as those for bacterial antibiotic resistance, have been added to these T-DNA vectors. In this way, transgenic plants that differ from the wild-type only by the

presence of a transferred gene can be generated and studied (34).

Arabidopsis as the Model Plant System

Arabidopsis thaliana or 'Thale cress' as it is commonly called, belongs to the Cruciferaceae or the Mustard family. It is a small and inconspicuous weed, whose unique properties have rendered it a model plant system, ideal for use both in classical and molecular genetics. *Arabidopsis* is important to plant scientists because it has the smallest, simplest known genome of any angiosperm. In its haploid state, it has 5 small chromosomes. It has a haploid genome measuring close to 100,000 kilobases with a very low level of dispersed repetitive DNA (37). This genome is about 15 times that of *E. coli*, 5 times that of *Saccharomyces cerevisiae* and less than half that of *Drosophila melanogaster*, for which it is sometimes referred to as *Drosophila botanica* or a botanical *Drosophila* (38). The recombinational length of the genome is approximately 500 cM. RFLP maps of the genome are already available (37). Thus, this plant is ideal for molecular genetic work.

Other factors that make this plant favourable to plant scientists are its small stature, its ability to grow well indoors, and its short generation time of 5 to 6 weeks. Thus it is possible to grow a large number of these plants in a small area. The plant is self-pollinated and yields up to 100 seeds per seed pod or silique. Seeds are very small

and easy to store. The small size of the seed also makes it easy for mutagenesis on a large scale and ensures that the effects of mutations are carried to seed. This has resulted in a large number of mutants in various genes, both previously known and unknown. Finally, *Arabidopsis* is readily amenable to transformation with the *Agrobacterium tumefaciens*' Ti plasmid (37, 38).

Aims of Our Study

It is now well-known that the PHYA phytochrome is light-labile, whereas the PHYB and PHYC phytochromes are light-stable (18). Furthermore, the *PHYA* gene is transcriptionally light-regulated (40). The structure of the 5' flanking sequence of the oat and *Arabidopsis PHYA* genes has been studied and found to contain the promoter to the gene. A large intron (1.2 kbp) has been found within the 5'untranslated region of *PHYA* genes (39, 40). The photoregulation of the *PHYA* gene promoter has been extensively studied in monocots, notably the expression of the oat promoter in rice using the reporter gene chloramphenicol acetyl transferase (CAT) (40). However, little is known as yet about the photoregulation of the other *PHY* genes. One of the objectives of this study was, therefore, to elucidate the temporal, spatial and photoregulation of the *PHYD* and *PHYE* genes, and to compare them to the expression patterns of the *PHYB* gene.

The other objective was to observe the effects of

reduced PHYB protein levels in *Arabidopsis* plants. The null mutation in the *PHYB* gene in *hy3* strains of *Arabidopsis* causes alterations in hypocotyl elongation, chlorophyll content and flowering time (19). These alterations in the wild-type phenotype can be restored to normal by introduction of a *PHYB* transgene, while more than one copy of the transgene results in exaggeration of many of the same responses (16). *Arabidopsis* plants with reduced PHYB levels were generated using an antisense construct to that gene to look for deviations from both normal receptor functions and null mutant phenotypes. Furthermore, developing the antisense system to the *PHYB* gene would contribute to future work on making antisense to the *PHYD* and *PHYE* genes by determining if the activity of an individual phytochrome gene can be selectively suppressed.

In short, the objectives of our study were:

I. To see if the upstream flanking sequences of the Arabidopsis genes *PHYD* and *PHYE* contain promoter sequences using the *gus* (β-glucuronidase) reporter gene and if so, to characterise the expression of these promoters; also, to compare the expression patterns of these genes with that of the *PHYB* gene promoter.

II. To generate *Arabidopsis* plants that have reduced levels of the *PHYB* gene product using antisense to that gene and observe the effects.

CHAPTER II

PROMOTER ANALYSIS

Introduction to Promoter-reporter Gene Fusion System

One of the ways to elucidate the temporal and spatial regulation of genes is through a promoter-reporter gene fusion system. The concept for this technique is that one gene with a product that is easily detectable/assayable is used to infer the behaviour of another gene that is functionally fused to it (41). The functional fusion could be a transcriptional fusion, in which transcription of the reporter gene (with the easily detectable protein) starts from the +1 nucleotide of the gene whose regulation is to be studied, or a translational fusion in which the start codon for the reporter gene's protein is that of the regulated gene. This system can also be used in deletion analysis, in which truncated promoter sequences fused to the reporter gene can indicate positive and negative cis-acting sequence elements.

In most cases, the transferred genes are expressed under the control of the cotransferred cis-acting regulatory sequences and, at least qualitatively, retain their tissue- and developmental-specific expression patterns. However, occasionally in plants, the evolutionary distance between monocots and dicots appears to prevent such expression,

probably because the host plant does not contain appropriate trans-acting factors (35).

The most common reporter genes used in studying plant genes are:

1. the CAT or Chloramphenicol acetyl transferase gene (42)
2. the NPTII or the Neomycin phospho-transferase gene (43)
3. the LUC or the Luciferase gene (44), and
4. the GUS or the β-glucuronidase gene (45).

CAT and NPTII reporter gene systems have often been used to study light-regulation of plant genes (22, 23). Luciferase (from fireflies) is another reporter gene which is most useful in situations that require transient expression assays (44). Of these reporter genes, the β-glucuronidase or GUS reporter system is the most frequently used tool for the analysis of plant gene expression. The major advantages in this system include fast and non-radioactive analysis which is extremely sensitive, and also the potential to obtain both quantitative and qualitative data with the same reporter gene.

Vectors that Carry the *gus* Gene

The gene encoding GUS or β-glucuronidase is the *gusA* gene (formerly known as *uidA*), originally isolated from *Escherichia coli* (46).

For the purposes of promoter analysis, a set of vectors containing the *gus* gene and based on the binary plasmid pBIN19 (47) has been developed by Jefferson (45). The use

of these vectors is greatly facilitated by the ability of the β-glucuronidase enzyme to tolerate large amino-terminal additions for translational fusions. These allow both transcriptional fusions and translational fusions in all three reading frames. The vectors that were used in this study are pBI101.1 which contains a promoterless *gus* gene to which putative promoters can be fused, and pBI121, a derivative of pBI101 containing the 35S promoter of the cauliflower mosaic virus fused to *gus*.

The enzyme, β-glucuronidase, has a monomeric molecular weight of approximately 68,000, but exists in vivo as a tetrameric species. It has a wide specificity range for β-conjugated glucuronides, but will not cleave other glycosides, such as α- or β-glucoside subtrate types (48). The appropriate substrates all contain the sugar D-glucopyranosiduronic acid attached by glycosidic linkage to a hydroxyl group (usually a phenolic hydroxyl) of a chromogenic, fluorogenic, or other detectable molecule (48).

The *gus* Reporter System in Plants

One of the most attractive features of the *gus* reporter gene system in plant molecular biology is the virtual absence of background activity in a broad spectrum of higher plants (45, 46). There exists, however, a small amount of endogenous GUS activity. Some plants may lack GUS activity in vegetative organs but show activity in reproductive tissue (49). Several solanaceous plants show

activity of the enzyme in the male gametophyte (50). Endogenous GUS activity has also been found in a wide variety of species at pH 5 (51).

In *Arabidopsis thaliana*, native GUS activity was found in leaves, stems, roots and flowers of untransformed plants at pH values below 7. However, no activity was observed at pH 7 and 8. On the other hand, GUS-transformed plants showed *gusA* derived activity at all pH values, but the activity was higher at values equal and above pH 7. Thus, endogenous GUS activity is pH-dependent and can be suppressed by increasing the pH of the staining solution (52). Use of 20% methanol in the staining solution also serves this purpose (52).

Assaying for GUS Activity

The GUS reporter gene system has a number of advantages over other reporter gene systems. First among them is the nonradioactive nature of the analysis. The assay is fast and extremely sensitive, and it is possible to obtain both quantitative and qualitative data with the same reporter gene. Qualitative data, i.e., specificity of expression in tissues and organs, can be obtained using histochemical substrates such as X-gluc, whereas quantitative data, i.e., level of gene expression, can be derived by using fluorigenic substrates such as 4-MUG. It is even possible to use GUS as a selectable marker using certain chemical modifications of the substrate (52). The

most popular substrate for the histochemical assay is X-gluc which is 5-bromo-4-chloro-3-indolyl β-D-glucuronide. The insoluble blue precipitate, dichloro-dibromoindigo, is formed as a result of a two-step process involving production of a colorless intermediate by the GUS enzyme which then undergoes oxidative dimerization. The insoluble nature of the dye ensures its localization in vivo at the sites of enzyme activity. The GUS enzyme is very stable. It is in fact so stable that even very weak expression will eventually lead to significant staining (52).

Fresh tissue is usually fixed before it can be assayed. Low concentrations of wetting agents like Triton ensure that the substrate enters the plant organs. Oxidative catalysts like potassium ferricyanide and ferrocyanide are included in the substrate mix to accelerate the oxidative dimerization of the colorless cleavage intermediate into the colored final product, ClBr-indigo (53).

Quantification of enzyme activity can be done with maximum accuracy using fluorometry. The most widely used fluorigenic substrate for the GUS enzyme is 4-methylumbelliferyl β-D-glucuronide or 4-MUG (48).

GUS assays can be performed with either whole tissue or with homogenized or ground-up plant tissue, since there are protocols for both destructive and nondestructive assays.

Since the β-glucuronidase reporter gene system was first described in 1987, it has been used to study temporal and spatial patterns of gene expression as well as define cis-acting regulatory regions of several gene promoters (55, 56, 57, 58).

Disadvantages of the GUS Reporter System

One of the advantages of using the GUS enzyme is its stability. But this can lead to overinterpretation of data since even very weak expression will eventually lead to significant staining, also making it difficult to differentiate between early onset of transcription and strong promoter activity (52). Cell size and vacuolization can also affect the results of a GUS assay. Assuming that GUS is localized in the cytoplasm, small active cells, like meristematic cells,with few small vacuoles will appear to express the enzyme more than large, highly vacuolated cells. The histochemical staining of GUS transgenics can also show a preferential staining of vascular tissue, which can be attributed to accumulation of the substrate or product, and to the permeability of substrate in the tissue (52).

Another problem encountered is the variable results seen in some GUS transgenic lines. Sometimes, a decrease and even complete cessation of GUS expression is noticed in some lines. This may be due to an increased methylation of the introduced DNA, a phenomenon that is reported in expression of genes in transgenic plants in general (54).

Materials and Methods

Plant Material and Growth Conditions

The plant material used in all the transformations was *Arabidopsis thaliana* ecotype Nossen (No-0 WT).

For all seedling assays, seeds were surface sterilized for 30 min in 15% bleach/0.2% SDS, rinsed at least 5 times with sterile water, and plated in 150x25mm Petri dishes containing GM medium (60) (see Appendix for media content). Plates were treated at 4°C in the dark for 2-5 days and then transferred to continuous white light. For dark-grown seedlings, germination was induced by placing the plates in white light for 10 min prior to transferring them to darkness at 24°C. Experiments requiring continuous white light used white light from a bank of 40 watt fluorescent bulbs (50 μm m^{-1} sec^{-2}). For one experiment involving mature plants, seedlings grown on petri dishes were transferred to GM contained in magenta boxes. For mature plant assays and for generating seed of the next generation, seedlings were moved to pots containing potting soil overlaid with vermiculite, and grown at 24°C under a 16 hour-photoperiod.

Construction of the P_DGUS Translational Fusion

pBI 101.1 is the vector that was used in the translational fusion experiments. This is part of the pBI 101 series. pBI 101 is a 'promoter-less' GUS cassette in

the *Agrobacterium* binary plasmid vector pBIN19 (47). It consists of the GUS gene blunt-end ligated into a filled-in KpnI site of the pBIN19 polylinker upstream of a 260 bp SstI - EcoRI fragment containing the polyadenylation signal from the nopaline synthase gene (*nos*) of the *Agrobacterium tumefaciens* Ti plasmid. Upstream of the ATG of the GUS cassette is a polylinker region containing Hind III, Sph I, Pst I, Sal I, Xba I, Bam HI and Sma I sites. This allows plant promoters to be easily cloned upstream of GUS and transferred to plants with all the advantages of the binary vector systems. This vector has a lowcopy RK2 origin of replication, and confers kanamycin resistance, due to the presence of the Neomycin phosphotransferase II gene (*npt II*) with the *nos* gene promoter and termination sequences. The kanamycin resistance can be used both in bacteria and in plants. The pBI 101.1, 101.2 and 101.3 represent the 3 reading frames in which the GUS gene can be read meaningfully (45).

The *PHYD* upstream sequence, that contains the putative *PHYD* gene promoter, the 5' untranslated region and the first 65 amino acids of the *PHYD* open reading frame was taken from the genomic clone λD 6-1. This 3 kb fragment, flanked by BglII restriction enzyme ends had been cloned into the BamHI site of M13mp18 in the reverse orientation and designated mpTC304. The *PHYD* promoter fragment was excised out of mpTC304 at the flanking SphI and SmaI

restriction sites and ligated into the SphI and EcoRV sites of the pGEM5Z+ vector. The ligated DNA was used to transform competent cells of *E. coli* strain HB 101 through heat-shock method (59). Selection of transformant colonies was done by screening the transformation on LB plates containing ampicillin at 100 μg/ml. DNA from selected colonies was minipreped according to the protocol described by Maniatis et al. 1982 (59). One clone thus obtained proved to contain the *PHYD* promoter fragment in the right orientation and was designated pLT1.

Plasmid pLT1 was then digested with SalI and the *PHYD* promoter fragment was inserted into the Sal I site in the polylinker 5' to the GUS gene of pBI101.1. The ligated DNA was used to transform *E. coli* HB 101 competent cells and selected on LB-kan_{50} plates. Transformants carrying the translational fusion of the *PHYD* promoter and coding sequence to the GUS gene were identified and designated P_DGUS (Fig. 1).

Presence of the P_DGUS construct in the transformed colonies on LB-kan plates was determined by colony hybridization with a radioactive probe containing a 900 bp fragment of the *PHYD* promoter region (59). The hybridization revealed 2 transformant colonies with the P_DGUS construct. The two clones were designated P_DGUS3 and P_DGUS12, and used to transform *Agrobacterium tumefaciens* strain LBA4404 (60). *Agrobacterium tumefaciens* carries the

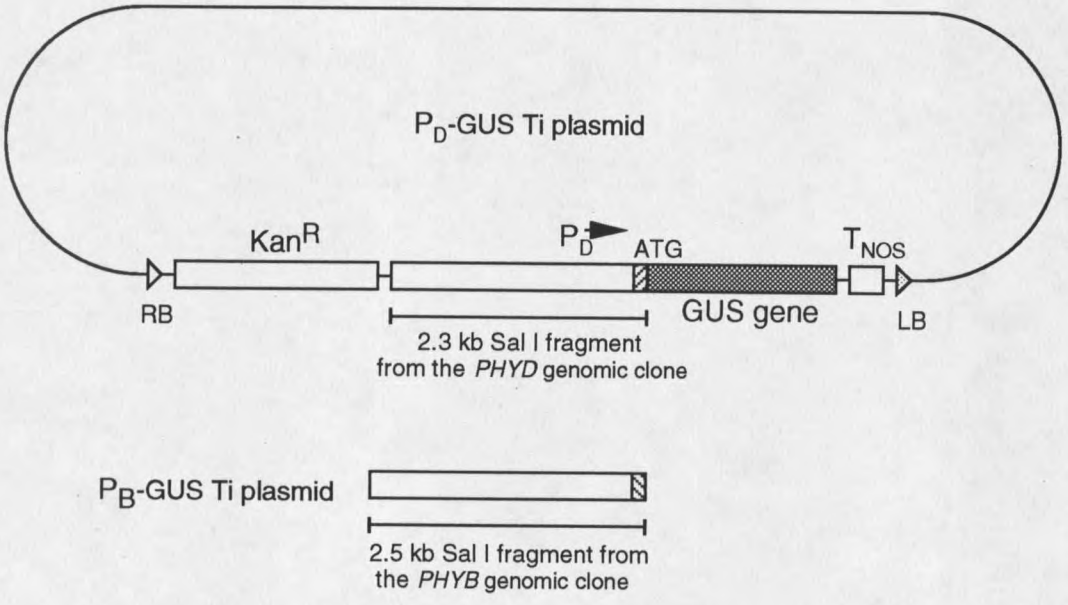


Fig. 1. Construction of the *PHYD*-GUS translational fusion. LB, left border; RB, right border; Kan^R, kanamycin resistance; P_D, *PHYD* gene promoter; T_{NOS}, nopaline synthase terminator. In the *PHYB*-GUS fusion, the 2.5 kb Sal I fragment from the *PHYB* gene was used to make a similar fusion with the GUS gene.

gene for resistance to streptomycin, so selection of transformed *Agrobacteria* was accomplished by plating out the transformation on LB $kan_{50}str_{25}$. The 2 transformed clones of *Agrobacteria*, P_DGUS3 and P_DGUS12, were then used to transform *Arabidopsis thaliana* (see below).

Construction of P_EGUS

The 2.6 kb sequence 5' to the *PHYE* gene (containing the putative promoter to the *PHYE* gene, P_E, and the first 21 amino acids) had originally been cloned into M13mp19 between the Hind III and Sal I sites. This sequence was cut out of M13mp19 and cloned into the pBI101.1 vector using the same restriction sites on the polylinker. This construct was designated P_EGUS (Fig. 2). The ligated DNA was used to transform competent cells of *E. coli* HB 101 by electroporation (59) and selected on LBkan_{50} plates. The insertion of the P_E sequence in the polylinker of the vector in the selected cells was confirmed by restriction digests using the enzyme BamHI.

The DNA from the transformants was used to transform *Agrobacterium tumefaciens* strain LBA 4404 cells by electroporation (59). Screening for transformants was on LB $kan_{50}str_{25}$ and yielded 2 positive clones, designated P_EGUS 1 and P_EGUS 2, which were then used to transform *Arabidopsis thaliana* (see below).

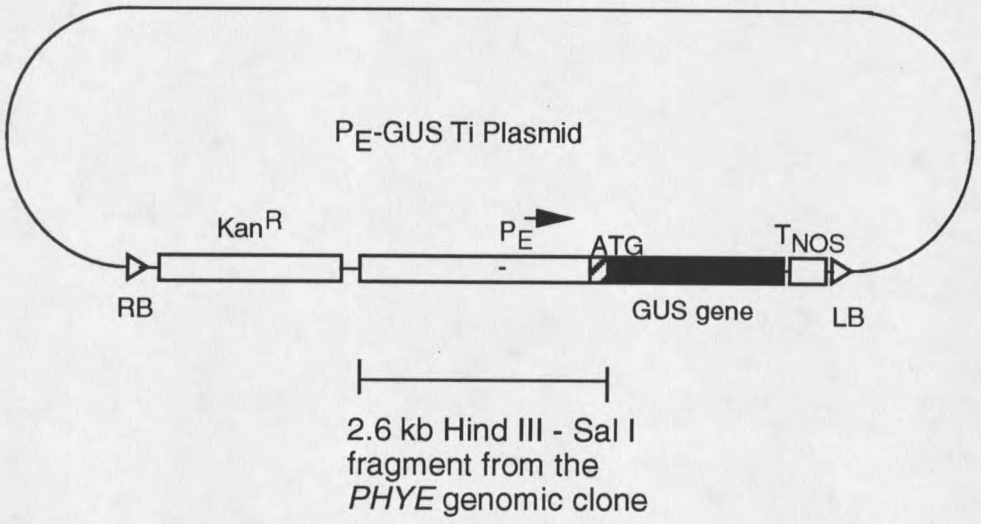


Fig. 2. Construction of the *PHYE*-GUS translational fusion. LB, left border; RB, right border; Kan^R, kanamycin resistance; P_E, *PHYE* gene promoter; T_{NOS}, nopaline synthase terminator.

Transformation of *Arabidopsis thaliana*

Transformation of Arabidopsis can be done using a variety of plant parts such as leaf discs, meristems, root bits, etc. (60). All transformations done for these projects used 2-week-old root bits as explants. Seeds of No-O WT were first subjected to a 70% ethanol wash for about 5 minutes and rinsed with sterile water. Then they were sterilized with 15% clorox/0.2% SDS solution for about 30 minutes with shaking. This was followed by 5 rinses with sterile water. The seeds were then transferred into 50 mls of liquid GM medium contained in 250 ml conical flasks and incubated under low light conditions at 100 rpm for 2 weeks. The medium was changed once a week. At the end of two weeks, the roots from the seedlings were harvested and placed individually on solid CIM plates. These plates were then sealed with air-permissive tape and incubated for 3 days. On the same day as roots were put on CIM (Callus-Inducing Medium) plates, the relevant strain of *Agrobacterium* containing the required construct was streaked out on LB $kan_{50}str_{25}$ plates and incubated at room temperature. After 2 days, a liquid culture of the streaked-out *Agrobacterium* was started in 2xYT $kan_{50}str_{25}$ medium and incubated at room temperature at 100 rpm. Thus, in 3 days' time, the explants and the *Agrobacterium* were both ready for the inoculation process. On the 4th day, the CIM-treated roots were taken out and cut into 0.5 cm

explants and stored in 20 mls of B5 medium. To this, 1 ml of the liquid *Agrobacterium* culture was added and the mixture shaken gently for 2 minutes. Following this, explants were blotted on sterile filter paper and transferred to new (solid) CIM plates in small bundles of about 5 rootbits each, in which they were incubated aerobically for 2 days.

After 2 days, the root segments bundles were lifted out carefully and washed with liquid B5 medium containing sterile carbenicillin (500 μg/ml). This wash was meant to kill all the bacteria adhering to the explants. After the antibiotic treatment, the root segments were once again blotted on sterile filter paper and transferred to solid SIM C_{500} (Shoot-Inducing Medium containing Carbenicillin at 500 μg/ml and kanamycin at 50μg/ml) plates. These plates were incubated aerobically in a plant tissue culture incubation chamber.

Plantlets that arise from transformed tissue began to appear after about a month of incubation. As they appeared, they were transferred to new solid SIM with a lower Carbeniclin content of 200 μg/ml. Healthy plantlets that formed rosettes were then transferred to solid GM media containing kanamycin at 50 μg/ml concentration (GM Kan_{50}) in tall magenta boxes and covered with two layers of Mira cloth held down by the lid of the magenta box. These plantlets were maintained for the rest of the life cycle in this

manner in the tissue culture incubator. In case of contamination appearing in the medium, the plantlets were transferred into rehydrated Jiffy 7 peat pellets (#703 Hummert) along with a plug of agar and watered regularly. Seeds were harvested from ripe siliques that these plantlets produced.

Analysis of Transformants

The T_2 seeds from the original transformants (the T_1 generation) (63) were sterilized with 15% chlorox/0.2% SDS with shaking for about 30 minutes and rinsed 5 times with sterile water. They were plated on solid GM plates containing kanamycin at 50 μg/ml concentration and placed at 4°C for 2 to 5 days. Then they were unwrapped and transferred to continuous light. The ratio of kanamycin-resistant to susceptible seedlings was counted on the 7th day from the date of transfer. After a couple more days, the resistant seedlings were transferred onto peat soil (Sunshine mixture) soaked with nutrient solution and covered with a layer of vermiculite. The same procedure was used for selection in all the transformations carried out and was repeated for the seeds of successive generations. Individual transgenic lines were selfed out to the T_3 or T_4 generation by which point they were no longer segregating kanamycin-sensitive progeny and were likely homozygous for the transgenes. Histochemical GUS assays were performed on the T_3 or T_4 progeny which are identified

in the text. For example, P_D12#1:1-1 is the T_4 progeny of a P_D12 original transformant.

Arabidopsis Transgenic Plants used as Control Lines

The plasmid pBI101.1 was used to transform *Arabidopsis thaliana* and the transformant was designated CTL 4. The plasmid pBI101.1 was the same as the one used to construct the *PHYD*-GUS and the *PHYE*-GUS fusions; in this case, the promoter-less GUS cassette was used as a negative control.

Three independent lines of *Arabidopsis* carrying a translational fusion between the upstream sequences of the *PHYB* gene (containing the first 63 amino acids along with the putative promoter sequences) and the β-glucuronidase gene (designated P_BGUS) had been developed earlier by Sharrock (unpublished) (Fig. 1). These were used to compare the expression of the *PHYB* gene promoter to *PHYD* and *PHYE* gene promoters.

Two independent lines of *Arabidopsis* carrying the plasmid pBI121 (which contains the *gus* gene under the control of the cauliflower mosaic virus 35S promoter) were also used to compare the expression of the *PHY* gene promoters to that of the constitutive CaMV 35S promoter.

Southern Analysis of Transformants

It has been observed in transgenic plants that sometimes more than one copy of the transgene gets inserted into the plant genome. The presence of multiple copies may

then affect the pattern or degree of expression of the transgene. Therefore, the transgenic lines obtained from transformation with the P_DGUS constructs were subjected to analysis by Southern blotting (62). Plant genomic DNA was extracted using the methods described by K. Edwards and co-workers (61). The P_DGUS genomic DNA was digested with EcoRI, fractionated on a gel and probed with a fragment from 5' flanking sequence of the *PHYD* gene (see Fig. 1). This analysis was done with the help of Ted Clack.

Histological GUS Staining

During the course of study, a wide variety of plant tissues from P_DGUS and P_EGUS transgenic plants were assayed for GUS activity. At different points of time, seedlings, rosette leaves, cauline leaves, portions of stem, inflorescence clusters, single flowers, etc. were stained either as whole tissue or as large sections as in the case of large rosette leaves. In all the assays performed, the same histological staining procedure was used, regardless of the type of transgenic (P_BGUS/P_DGUS/P_EGUS) and the plant tissue assayed.

Assay Protocol

After the excision of the plant tissue from the parent plant, it was first fixed in a solution containing 100mM NaH2PO4/Na2HPO4 buffer (pH 7.0), 0.1% formaldehyde, 0.1% Triton X-100 and 0.1% dithiotreitol for 15 minutes under

vacuum, and then rinsed for 5 minutes with shaking, first with 100mM NaH2PO4/Na2HPO4 buffer (pH 7.0), and then with the same buffer at 50mM concentration. The tissue was then infiltrated with the GUS substrate for 6 minutes under vacuum. The GUS substrate was made up with 50 mM NaH2PO4/Na2HPO4 buffer (pH 7.0), 0.5 mM potassium ferri-/ferrocyanide, 1.0 mM Na2EDTA and 1mg/ml X-gluc. The tissue in the X-gluc solution was incubated at 37° C for the required time period. At the end of the incubation, the substrate was removed and the tissue rinsed with the Na2HPO4/NaH2PO4 buffer, after which the tissue was fixed again, this time with 5% formaldehyde, 5% acetic acid and 20% ethanol for at least 2 hours (45). In order to observe the GUS staining patterns in tissues better, the tissue was clarified in increasing concentrations of ethanol, i.e., in successive steps with 50%, 70% and 95% ethanol. Observations were then made of the tissue with the aid of a dissecting microscope. The tissues were then stepped down through the decreasing concentrations of ethanol (95%, 70%, 50% and 25%) and transfered into 25% glycerol and finally into 50% glycerol. This was done to facilitate photography of the tissues.

Results

Construction of *PHYD*-GUS Transgenic Lines

The 5' flanking region of the *PHYD* gene that contains about 2.3 kb of upstream DNA and includes the start codon

was cloned from the λ D-6 genomic clone into the plasmid pBI101.1 in a translational fusion (Fig. 1). This construct was designated P_DGUS. The independent clones found to contain this construct were P_DGUS3 and P_DGUS12. These P_DGUS constructs were used to transform *Arabidopsis thaliana* root-bits, and transgenic plantlets were obtained.

The number of kan-positive original transformants were:

P_DGUS3 =20

P_DGUS12=10

Total =30.

Of these, some turned out to be chimaeric on the basis of their phenotype on kanamycin-containing media, and were neither carried through subsequent generations nor assayed. Thus, 6 P_DGUS3 lines and 3 P_DGUS12 lines were carried on. Of these, even in the T_3 and T_4 generations, some of the transgenic lines like P_DGUS3#12:1-1 were neither homozygous, nor showed the normal 3:1 segregation pattern on GMkan, expected when the transgene is inserted at a single locus (Table 2). Moreover, even the lines that appear to have only a single locus could be carrying more than one copy of the transgene at that locus because of tandem insertion. The copy numbers of all the transgenic lines of both P_BGUS and P_DGUS transgenics were therefore examined through Southern analysis.

Transgenic Line	Generation	Ratio (Kan^R/Kan^S)
P_BGUS#6	T3	35:0
P_DGUS3#1	T4	33:0
P_DGUS3#2	T3	30:17
P_DGUS3#5	T4	35:0
P_DGUS3#6	T4	29:0
P_DGUS3#10	T3	49:9
P_DGUS3#12	T4	30:1
P_DGUS12#1	T4	40:0
P_DGUS12#2	T4	35:1
P_DGUS12#8	T4	28:0
CTL4#2	T3	43:0

Table 2. Segregation ratios (kan^r/kan^s) of progeny of the 9 P_DGUS lines that were studied. It can be seen that while some of the lines appear to be homozygous, others show ratios that vary from the normal heterozygotic ratio of 3:1, indicating multicopy insertions in the genome. The ratios of the P_BGUS#6 line and the CTL4#2 line which were used as controls are also presented. The ratios give the actual number of seedlings evaluated.

Southern Analysis of the P_BGUS and P_DGUS Transgenic Lines

Southern blot analysis of genomic DNA from transgenic P_DGUS lines cut with EcoRI was performed. This enzyme cuts within the T-DNA so the copy number of the transgene can be estimated from the number of bands on the blot, using the endogenous *PHYD* promoter fragments as a single copy control (Fig. 3).

P_DGUS lines: Of the nine P_DGUS lines, P_DGUS3#5 and P_DGUS12#8 proved to have single copy insertions. P_DGUS3#1 contained 2 copies, and the lines P_DGUS3#2, P_DGUS3#6 and P_DGUS3#12 had 3 to 4 copies of the transgene. P_DGUS3#10 and P_DGUS12#1 had multiple copies. However, the line P_DGUS12#2 was found to lack detectable transgene sequences even though it was GUS positive. When the blot shown in Fig. 3 was stripped and rehybridized with a probe for the GUS gene, the single bands appeared for all lines as expected, but again no hybridization was observed for line P_DGUS12#2. The origin of the GUS activity in this strain is not known.

P_BGUS lines: The copy number of the P_BGUS transgene in the three lines used in these experiments were also determined by similar Southern analysis. Line P_BGUS#3:1 was found to carry a single copy of the transgene, while P_BGUS#6:1 and P_BGUS#7:5 had multiple copies (data not shown).

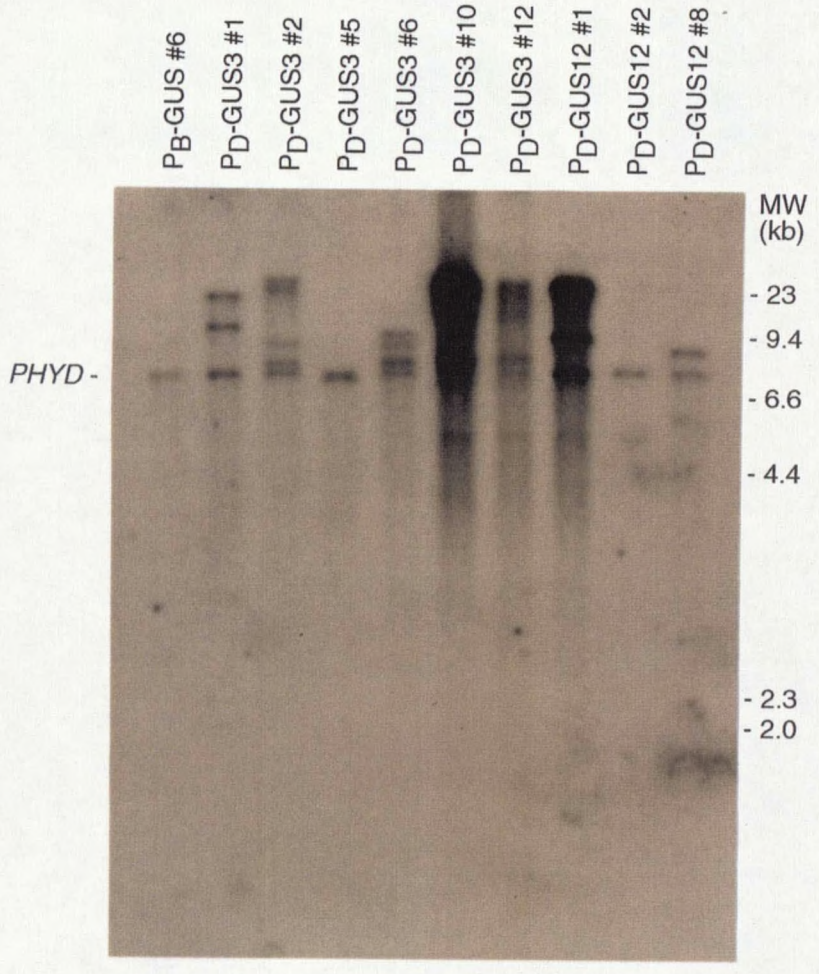


Figure 3. Southern blot analysis of P_D-GUS transgenic lines showing the transgene copy number. Total DNA was extracted from the indicated lines, cut with EcoRI, and probed with a fragment from the 5' flanking sequence of the *PHYD* gene. The single band hybridizing to the probe at approximately8 kb is due to the endogenous *PHYD* gene.

Histochemical Assays

Assays of Control Lines

The pBI121 plasmid has the GUS gene constitutively turned on by the CaMV35S promoter. Plants carrying this plasmid were chosen to be the positive control for histochemical GUS staining. 7-day-old seedlings of these two lines, pBI121#1A and pBI121#4 were assayed for histochemical GUS expression. Both lines showed a high intensity of staining in all parts of the seedlings, i.e., the cotyledons, hypocotyl and root. This level of staining was scored as +++, the highest attainable score (Fig. 4C). No further assays were done with these lines.

The negative controls for the histochemical assays were the wild type line, No-0 WT, and the control line generated by transforming Arabidopsis with the plasmid pBI101.1, CTL4 (see Materials and Methods). When seedlings of both these lines were assayed for GUS, no visible activity could be detected. This was scored as '-', denoting no visible GUS expression (Fig. 4A, B). These controls were important because they showed that there was no intrinsic B-glucuronidase activity under the assay procedures followed, and also that the promoter-less GUS construct was inactive.

Assay of P_BGUS Lines: When the seedlings of the 3 transgenic lines of P_BGUS were assayed for histochemical GUS activity, all of them showed the same pattern: a high

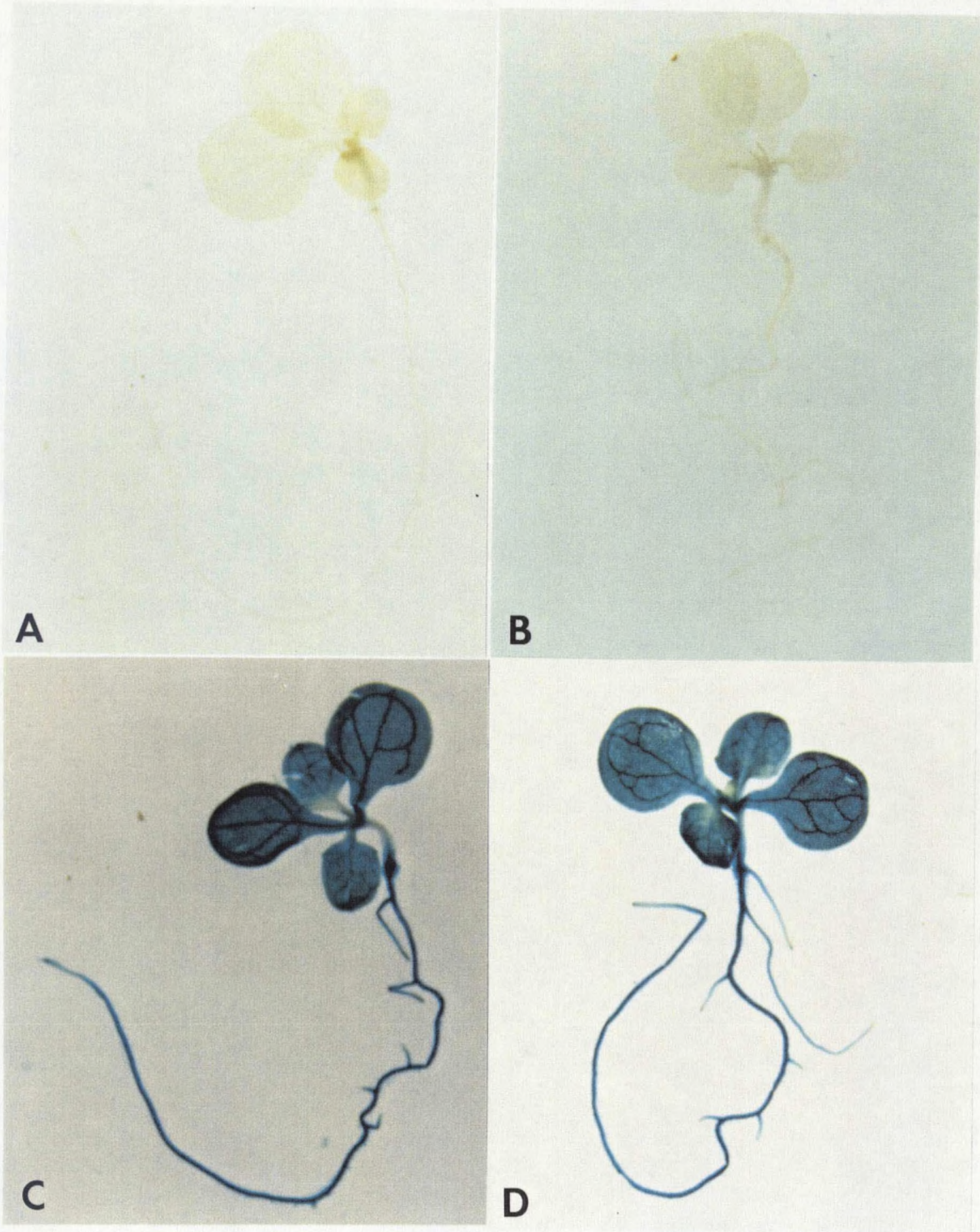


Fig. 4. Histochemical GUS assays of 7-day-old light-grown seedlings. A. Untransformed seedling of the wild-type *Arabidopsis* line No-0. B. Seedling from the CTL4#2 line which carries the plasmid pBI101.1 (promoter-less GUS gene). C. Seedling from the pBI121 line carrying the GUS gene driven by the CaMV35S promoter. D. P_BGUS#6 seedling in which the promoter for GUS gene is the *PHYB* gene promoter.

intensity of staining in cotyledons, hypocotyl and roots (score: +++), comparable to that observed in the pBI121 lines (Fig. 4D). The copy number of the transgene did not seem to affect the staining pattern in this case. Of these lines, the line P_BGUS#6:1 was selected for comparison with P_DGUS and P_EGUS transgenics and was included in all experiments involving P_D- and P_E-GUS.

Assay of P_DGUS Lines

Unlike the P_BGUS transgenic lines, the histochemical activity of GUS in the P_DGUS lines were strongly affected by the copy number of the transgene. Therefore, these lines have been divided into three categories based on the histochemical staining patterns they exhibit.

Category 1: 2 lines of P_DGUS 3 (P_DGUS 3#10:3 & P_DGUS 3#12:1-1) and 1 line of P_DGUS 12 (P_DGUS 12#1:1-1) showed a higher intensity of staining. They were also found to have multiple copies (Fig. 3) and so were designated as multicopy lines.

Category 2: 4 lines of P_DGUS 3 (P_DGUS 3#1:1-3, P_DGUS3#2:1, P_DGUS 3#5:4 & P_DGUS 3#6:1) and 1 line of P_DGUS 12 (P_DGUS 12#8:1) showed essentially the same pattern of staining in seedlings as the multicopy lines, but at a much lower intensity. These lines had a lower copy number (between 1 and 4 (Fig. 3)) and may be more representive of the true nature of the promoter, in terms of level of expression. Therefore, they were designated as standard lines.

Category 3: One line of P_DGUS 12 (P_DGUS 12#2:1-1) did not carry the transgene (see Southern Analysis) but still showed histochemical staining with the X-gluc substrate. This line stands alone in the third category and will be referred to as the variant line.

The P_DGUS transgenic lines showed a strong correlation between intensity of expression and the transgene copy number, indicating that the expression of the *PHYD* promoter varies in proportion to the copy number of the transgene. As for the difference in expression levels between P_DGUS3#12, P_DGUS3#2 and P_DGUS3#6, all of which apparently carry an identical number of copies of the transgene (Fig. 3; Table 3), this is most likely to be due to position effect (35).

8-hour Assay

Most of the histochemical GUS assays were allowed to run for about 16 to 18 hours (overnight), since it was observed from the outset that the P_DGUS transgenics did not stain over short assay periods. Once the general staining patterns had been established, an 8-hour histochemical assay was conducted to observe the effect of time on the staining pattern. Twenty five-day-old seedlings of the P_BGUS line and the P_DGUS multicopy lines, all grown on GM medium in magenta boxes, were assayed and the staining observed at various times. The progression of histochemical staining of the roots could be observed by periodical monitoring during

Line	# of trans-genes	seedling			mature plant						
		cotyledon	hypocotyl	root	leaf	stem	sepal	petal	stamen	pollen	pistil
P_BGUS#6	>4	+++	+++	+++	+++	+++	+++	(+)	+++	++	+++
P_BGUS#3	1	+++	+++	+++							
P_BGUS#7	>4	+++	+++	+++							
P_DGUS3#1	2	+	-	+	++	+	(+)	-	-	(+)	-
P_DGUS3#2	3-4	+	-	(+)	-	-	-	-	-	(+)	-
P_DGUS3#5	1	+	-	+	-	-	-	-	-	(+)	-
P_DGUS3#6	3-4	+	-	+	-	-	-	-	-	(+)	-
P_DGUS3#10	>4	++(+)	-	+	++(+)	(+)	++	-	-	++	-
P_DGUS3#12	3-4	++	-	+	++(+)	(+)	+	-	-	++	-
P_DGUS12#1	>4	++(+)	-	+	++(+)	(+)	++	-	-	++	-
P_DGUS12#2	?	++	-	-							
P_DGUS12#8	1	+	-	(+)	-	-	-	-	-	(+)	-

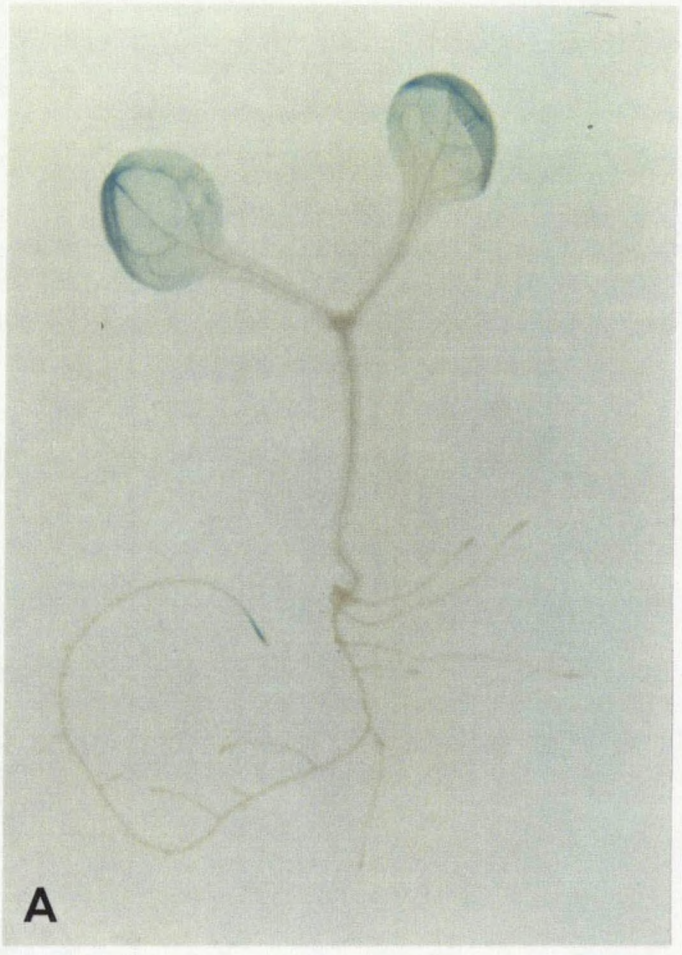
Table 3. Patterns of histochemical GUS expression in transgenic lines carrying the P_BGUS and P_DGUS fusions. - indicates the absence of visible GUS expression and is the lowest score obtainable; (+) indicates barely discernable staining; +, ++ and +++ indicate low, medium and high intensity of histochemical GUS staining. No assays were conducted on mature plant parts of P_BGUS lines #3 and #7.

the eight-hour period. P_BGUS showed staining in roots within half an hour. The P_DGUS standard lines started to show staining in roots in about 2 hours' time. The assay was terminated after 8 hours and the plants destained, whereupon the staining pattern was found to be the same as observed in the overnight seedling assays (data not shown). However, flowers of P_DGUS transgenics required overnight assaying before staining could be detected in any appreciable way. In this way, an effort was made to determine the time of onset of GUS expression in the P_B- and P_DGUS transgenic plants.

Seedling Assays

Seven-day-old GM- and GMkan_{50}-grown seedlings were assayed for histochemical GUS activity. P_BGUS invariably stained very deeply in all parts of the seedlings (score: +++, the maximum observed), namely cotyledons, hypocotyl and roots. This was observed irrespective of whether the medium contained kanamycin or not. The control line CTL 4#1 did not stain at all, again irrespective of the medium, as also did the No-0 WT (Fig. 4).

P_DGUS seedlings stained with low to medium intensity in the cotyledons (score: + to ++ or ++(+)), with no staining observable in the hypocotyl (score: -) and low staining in roots (score:+) (Table 3). Fig. 5A shows a stained seedling from a standard line, P_DGUS3#5:4, which contains a single copy of the P_DGUS transgene. This staining pattern in



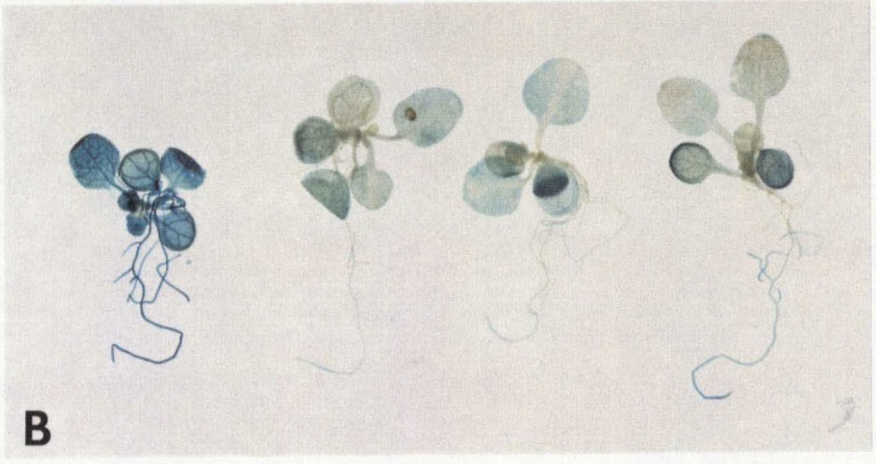


Fig. 5. A. Histochemical GUS activity exhibited by a 5-day-old light-grown seedling of the standard line, P_DGUS3#5, which carries a single copy of the transgene. B) Histochemical staining in 10-day-old seedlings of P_BGUS#6 and the P_DGUS multicopy lines, P_DGUS3#10, P_DGUS3#12 and P_DGUS12#1.

seedlings was reproducable in all P_DGUS lines (Fig. 5B). Surprisingly, when grown on GMkan$_{50}$ medium, expression of GUS in roots in all lines was increased (data not shown). The presence of kanamycin in the medium apparently induced expression from the *PHYD* promoter or increased the activity of the GUS enzyme or the access of the substrate, though it is unclear how.

P_DGUS transgenics were grown in GM liquid medium and assayed for GUS activity in comparison with GM solid medium-grown seedlings. For this study, the sterilized seeds were cultured in liquid GM medium under continuous light with constant shaking. These liquid-grown seedlings showed a slight increase in staining intensity in the roots (score:++). However, the staining intensity in the cotyledons and the staining pattern remained unchanged (data not shown).

Overall, the seedlings of all the P_DGUS transgenic lines showed essentially the same pattern of staining: present in cotyledons and near the root tip region and absent in the hypocotyl. Only the staining intensity differed. Therefore, it can be concluded that the copy number of the transgene does not affect the staining pattern, while it does affect the intensity. This justified the decision to use the more highly staining multicopy lines to analyze the light-dependent nature of the *PHYD* promoter.

Seedling Assays: Light versus Dark Experiments

In order to find out if the *PHYD* promoter acts in a light-dependant manner, light versus dark experiments were conducted. In these, seeds of line P_BGUS#6:1 and the 3 P_DGUS multicopy lines were plated on 3 GM plates and 2 of these plates were placed in continuous dark and 1 plate in continuous light. After 7 days, one plate of dark-grown seedlings was transferred to continuous light conditions, and a time-course was followed, with histochemical assays of the seedlings conducted at 0 hours, 24 hours, 48 hours and 72 hours of light exposure.

In these experiments, 7 day-old dark-grown seedlings of the P_BGUS transgenic showed high intensity staining (score: +++) of the cotyledons, the hypocotyl and the root, the same as in light-grown seedlings (Fig. 6A). Seven day-old dark-grown seedlings of the P_DGUS multicopy lines showed strong staining only in the cotyledons (score: +++), and, unlike the light-grown seedlings, no staining was observed in the roots (Fig. 6B). When 7 day-old dark-grown seedlings of the P_DGUS lines were transferred to continuous white light and assayed at 24-hour intervals, they showed progressive staining in the root tips. 24 hours after dark-grown seedlings were transferred to white light, P_DGUS 3#10:3 and P_DGUS 12#1:1-1 showed low intensity staining in the root (score: +), while no staining was observed in the root of P_DGUS 3#12:1-1 (data not shown). Very faint

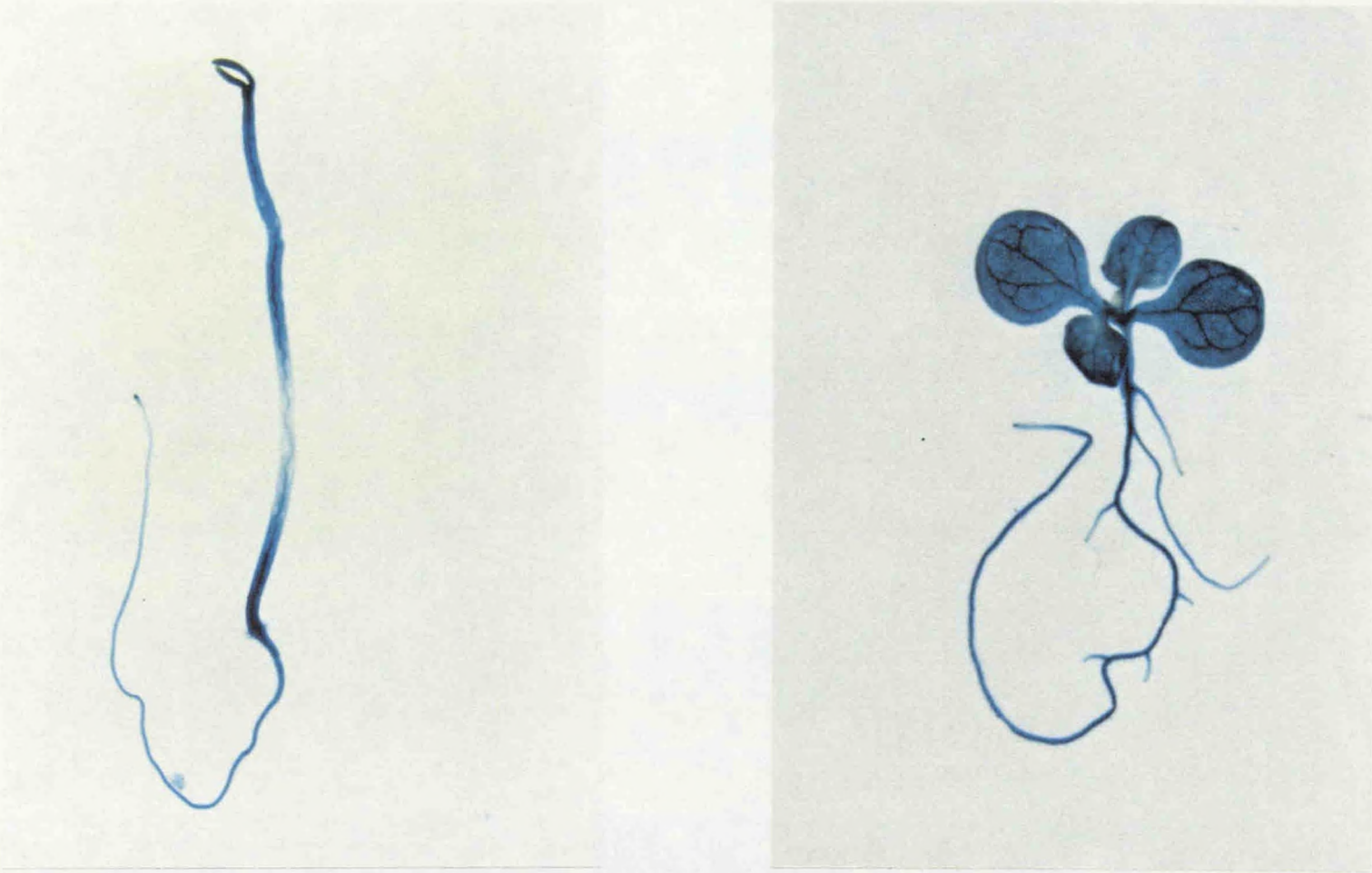


Fig. 6A. Histochemical GUS assays of dark-grown (left) and light-grown (right) 7 day-old seedlings of line P_BGUS#6.

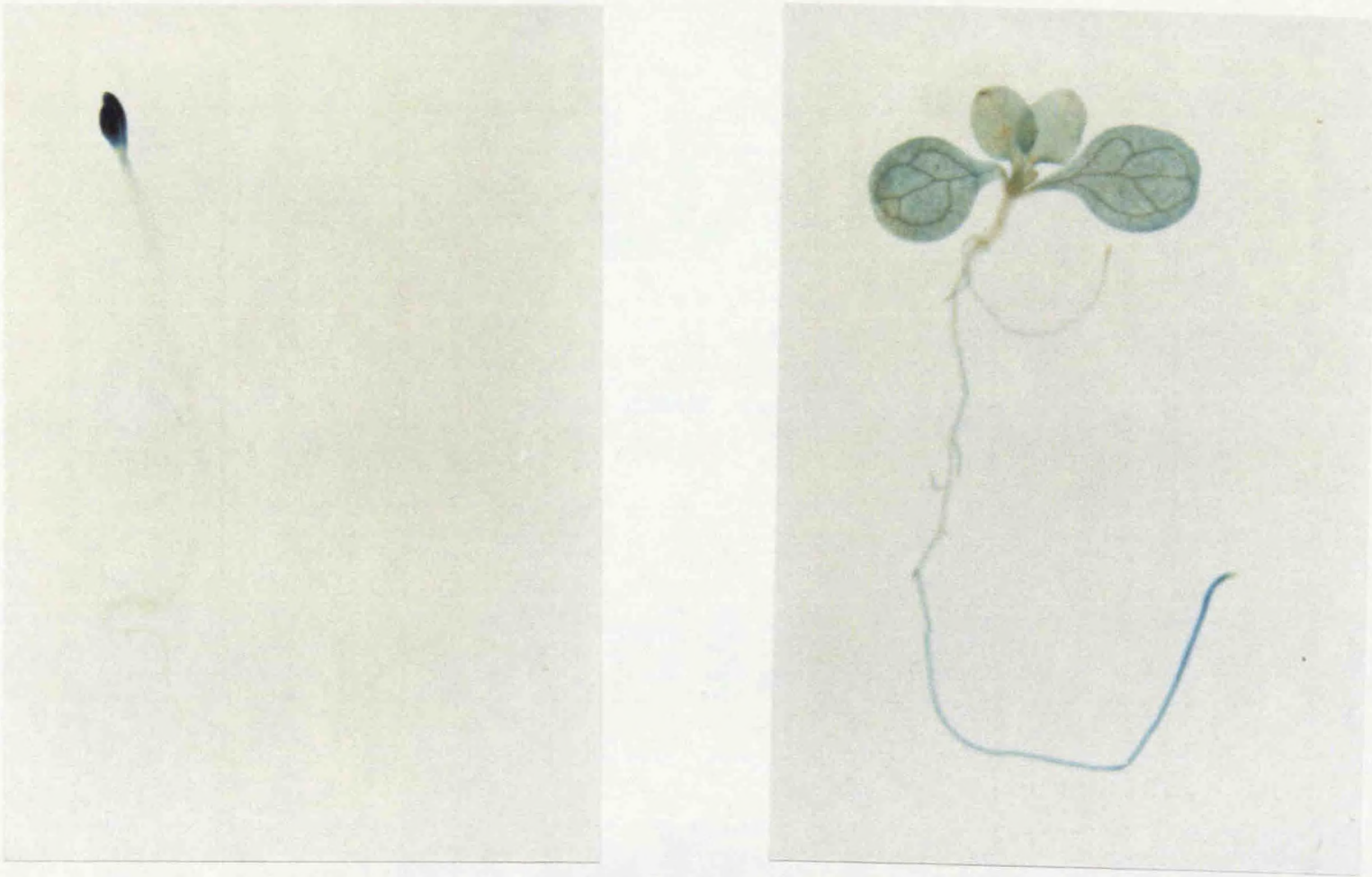


Fig. 6B. Histochemical GUS assays of dark-grown (left) and light-grown (right) 7 day-old seedlings of line P_DGUS12#1.

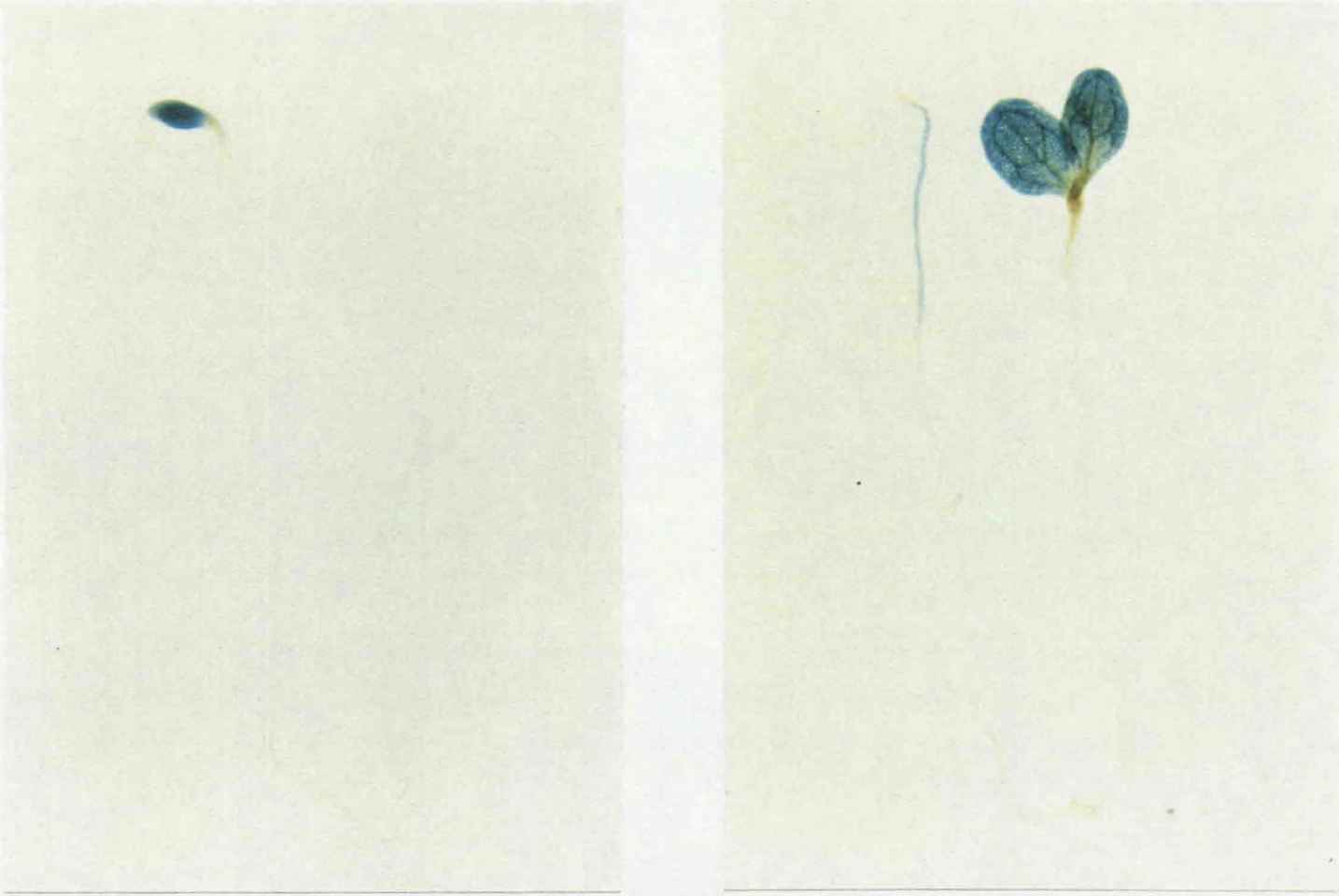


Fig. 6C. Ten-day-old seedlings of line P_DGUS3#12. The seedling on the left was grown in continuous darkness. The seedling on the right was grown for 7 days in continuous darkness followed by 72 hours in continuous white light.

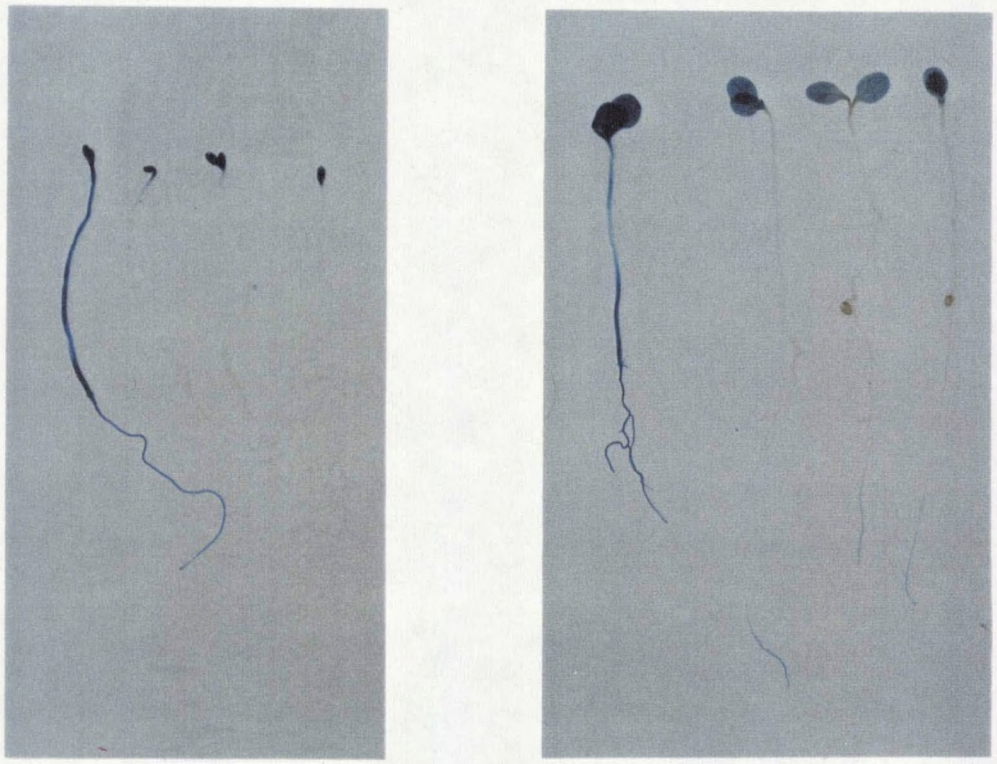


Fig. 6D. Histochemical GUS staining of seedlings of lines P_BGUS#6, P_DGUS3#10, P_DGUS3#12 and P_DGUS12#1. Seedlings on the left were grown for 7 days in continuous darkness. The seedlings on the right were grown for 7 days in continuous dark followed by 72 hours in continuous white light.

staining was then observed in this line after 48 hours under continuous light. When assayed after 72 hours of exposure to white light, seedlings of all 3 P_DGUS lines showed staining in the root tips (Fig. 6C, D). This indicates that, in the leaf, the *PHYD* promoter is independent of light conditions, while in the root, the *PHYD* promoter is off in the dark, but is turned on by exposure to continuous white light. The *PHYB* promoter, however, is on constitutively throughout the entire seedling, irrespective of the presence or absence of light.

Assay of Mature Plant Parts

Roots: Roots harvested from 14 day-old soil-grown P_BGUS plants stained for GUS activity while roots from the 3 P_DGUS multicopy lines did not (data not shown). When grown on GM in magenta boxes, roots of the P_DGUS multicopy lines showed low intensity staining (score: +), while no staining was observed in the P_DGUS standard lines. P_BGUS, on the other hand, showed a score of ++ or medium staining. GM medium is translucent and may conduct an amount of light, which would not happen in the case of soil. Thus, the observation that expression of the *PHYD* promoter in roots is dependent on light gains further evidence.

Leaves: Leaves of Arabidopsis are basically of 2 types: 1) rosette leaves, found at the base of the plant, and 2) cauline leaves, found on the stalks of the bolt. Inflorescences arise from the axils of cauline leaves.

Large rosette leaves were sometimes assayed in approximately 5 mm sections. Small rosette leaves and cauline leaves were assayed whole. P_BGUS transgenics showed a high intensity of staining in both kinds of leaves (score: +++). The P_DGUS multicopy lines showed medium intensity of staining, in some cases tending towards high intensity (score: ++ to ++(+)) (Fig. 7A). The P_DGUS standard lines showed no visible staining in leaves (score:-) indicating both that the *PHYB* promoter is stronger than the *PHYD* promoter, and that the P_DGUS transgene copy number strongly influences the intensity of expression. The example of a stained rosette leaf from P_DGUS3#12 shown in Fig. 7A shows even staining throughout the leaf, suggesting that many leaf cell types may express this gene.

Assays of the Stem

Sections from the lower (older) part of the stem and from the upper (younger) part of the inflorescence stem were assayed. The upper stem sections included side-branches. Lower stem sections stained with medium intensity in the P_BGUS transgenic (score: ++). Except for one multicopy P_DGUS line, P_D3#10:3, which showed a low intensity of staining (score: +(+)), all P_DGUS lines showed no staining of lower stem sections. The upper stem sections of P_BGUS showed high intensity of staining (score: +++), while in the P_DGUS multicopy lines, the side-shoots stained with

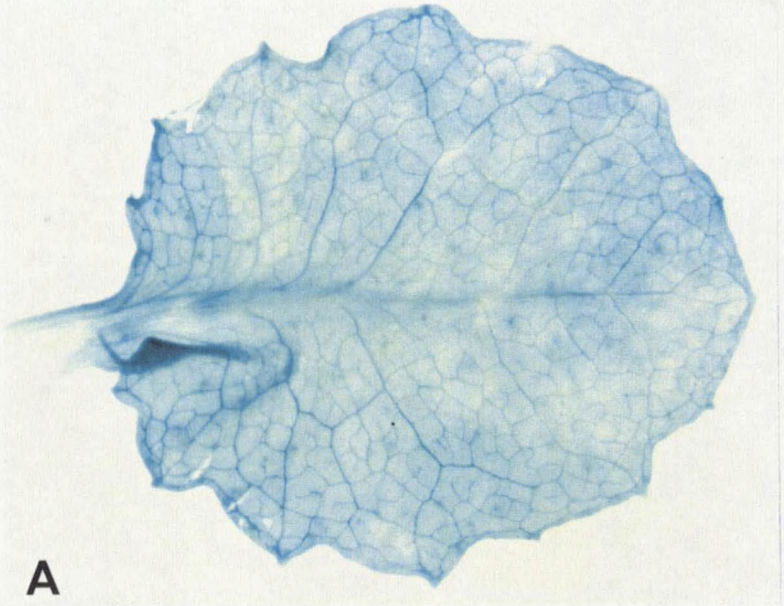


Fig. 7A. Histochemical GUS activity exhibited by a rosette leaf from a light-grown P_DGUS10#3 plant, and B. an inflorescence from P_DGUS12#1.

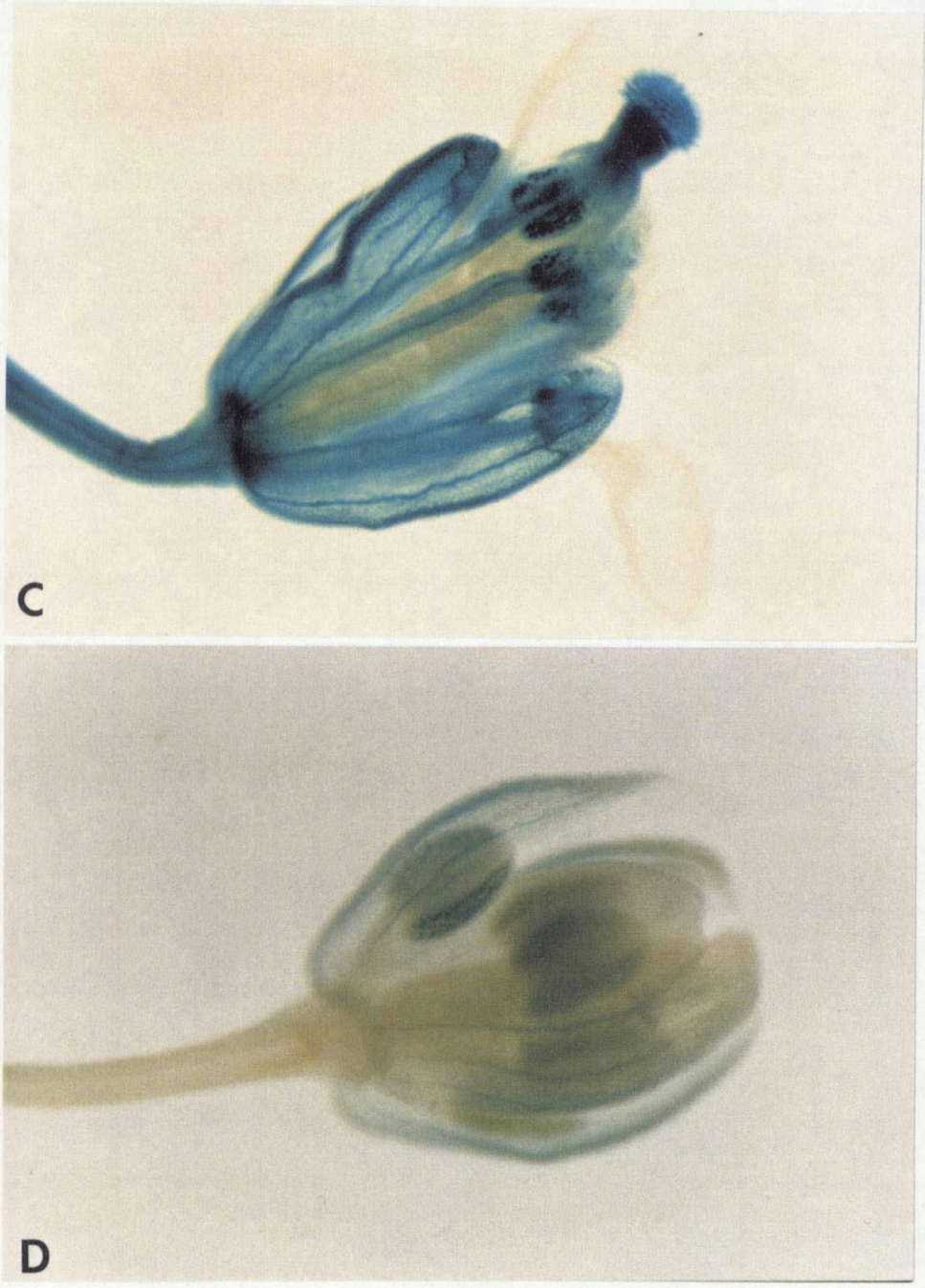


Fig. 7C. Histochemical GUS activity exhibited by a single flower from the P_BGUS#6 line, and D. single flower from P_DGUS3#10.

low intensity (score: +) and the main stem did not stain at all (data not shown).

Assays with Flowers

Flowers of the P_B- and P_DGUS transgenics showed distinct differences in the pattern of staining (Fig. 7B,C,D). The P_BGUS transgenic showed intense staining in sepals (score:+++). There was some variability among the P_DGUS standard lines with regards to sepal staining. The multicopy lines P_DGUS 3#10:3 and P_DGUS 12#1:1-1 showed medium staining (score: ++), while P_DGUS 3#12:1-1 showed barely discernable staining. This pattern of GUS expression in the sepals is for most part consistent with that seen in leaves, which is understandable since sepals are a form of leaf.

In the histochemical GUS assays conducted for 16 to 18 hours, a very faint staining (score: (+)) was seen in the petals of the P_BGUS line in a couple of instances. No staining was observed in the petals in any of the P_DGUS strains even though all the assays were conducted for the same period of time.

In the stamens, the P_BGUS line showed high intensity of staining of the filament (score: +++), medium intensity in the septum or anther wall (score: ++) and medium intensity in pollen (score: ++). All the multicopy P_DGUS lines showed no detectable staining of both the septum and the filament, but the pollen stained with medium

intensity (score: ++). Pollen staining was also observed in young buds in the standard lines.

In the carpel, the P_BGUS line showed strong staining of the ovary, style and stigma (score: +++). Detectable staining was absent in the carpels of all the P_DGUS lines assayed. Thus, while the *PHYD* promoter seems to be expressed in the male gametes of the flower along with *PHYB*, it is not on to an appreciable extent in other fertile flower parts. *PHYB*, on the other hand, is constitutive in its expression.

Siliques responded poorly to the assays and were not investigated to a great extent.

Construction of P_EGUS Fusion

The 2.6 kb 5' flanking sequence of the *PHYE* gene was cloned as a translational fusion with the B-glucuronidase gene in the plasmid pBI101.1 and designated P_EGUS (Fig. 2). On transformation of *Agrobacterium tumefaciens* with this construct, 2 clones carrying the construct were identified and designated P_EGUS1 aand P_EGUS2. Both these constructs were used to transform *Arabidopsis* root-bits and transformants were regenerated.

Characterization of P_EGUS Transgenic Lines

A total of 12 putative transgenic lines were obtained from transformation using the 2 constructs.

P_EGUS1 = 5

P_EGUS2 = 7

Total =12.

Again, several of these lines appeared to be chimeric and six lines were chosen for further analysis (Table 4). The ratios for kanamycin resistance of some of the lines showed deviation from the standard 3:1 ratio observed on insertion of the transgene into a single locus. This was similar to the ratios observed in the P_DGUS transgenics.

Histochemical Assay of Seedlings

Seven day-old light-grown and dark-grown seedlings of 5 P_EGUS lines (P_EGUS2#7 was not included in this assay) were assayed for histochemical GUS activity. Dark-grown seedlings showed a low intensity of staining in the cotyledons (score:+) while no GUS activity was observed in the hypocotyl and the root (score:-). The light-grown seedlings of these lines also showed no staining of the hypocotyl or the root, though they showed an apparent increase in the intensity of staining in the cotyledons, scoring ++ for medium intensity staining (Table 5, Fig. 8). These results indicate that the *PHYE* gene promoter is on in the cotyledons and off in other seedling tissues irrespective of the presence or absence of light. The photographs in Fig. 8 suggest that there may be an induction of *PHYE* promoter activity in leaves by light, a possibility which could be investigated with fluorometric GUS assays.

Transgenic line	Generation	Ratio (Kan_r/Kan_s)
P_EGUS1#2	T3	25:9
P_EGUS1#4	T3	24:0
P_EGUS2#3	T3	13:9
P_EGUS2#4	T3	25:5
P_EGUS2#5	T2	1:3
P_EGUS2#7	T2	12:5

Table 4. Segregation ratios for P_EGUS transgenic lines. As in Table 2, some of the transgenic lines show ratios that vary from the normal heterozygotic ratio of 3:1. The ratios give the actual number of seedlings evaluated.

	seedling			mature plant					
Line	cotyledon	hypocotyl	root	leaf	sepal	petal	anther	pollen	pistil
P_EGUS1#2	++	-	-						
P_EGUS1#4	-	-	-						
P_EGUS2#3	++	-	-	++	+	-	+	-	-
P_EGUS2#4	-	-	-						
P_EGUS2#5	++	-	-	++	+	-	+	-	-
P_EGUS2#7	+	-	-						

Table 5. Expression pattern of P_EGUS transgenic plants. The method of scoring histochemical GUS expression used here is the same as in Table 3.

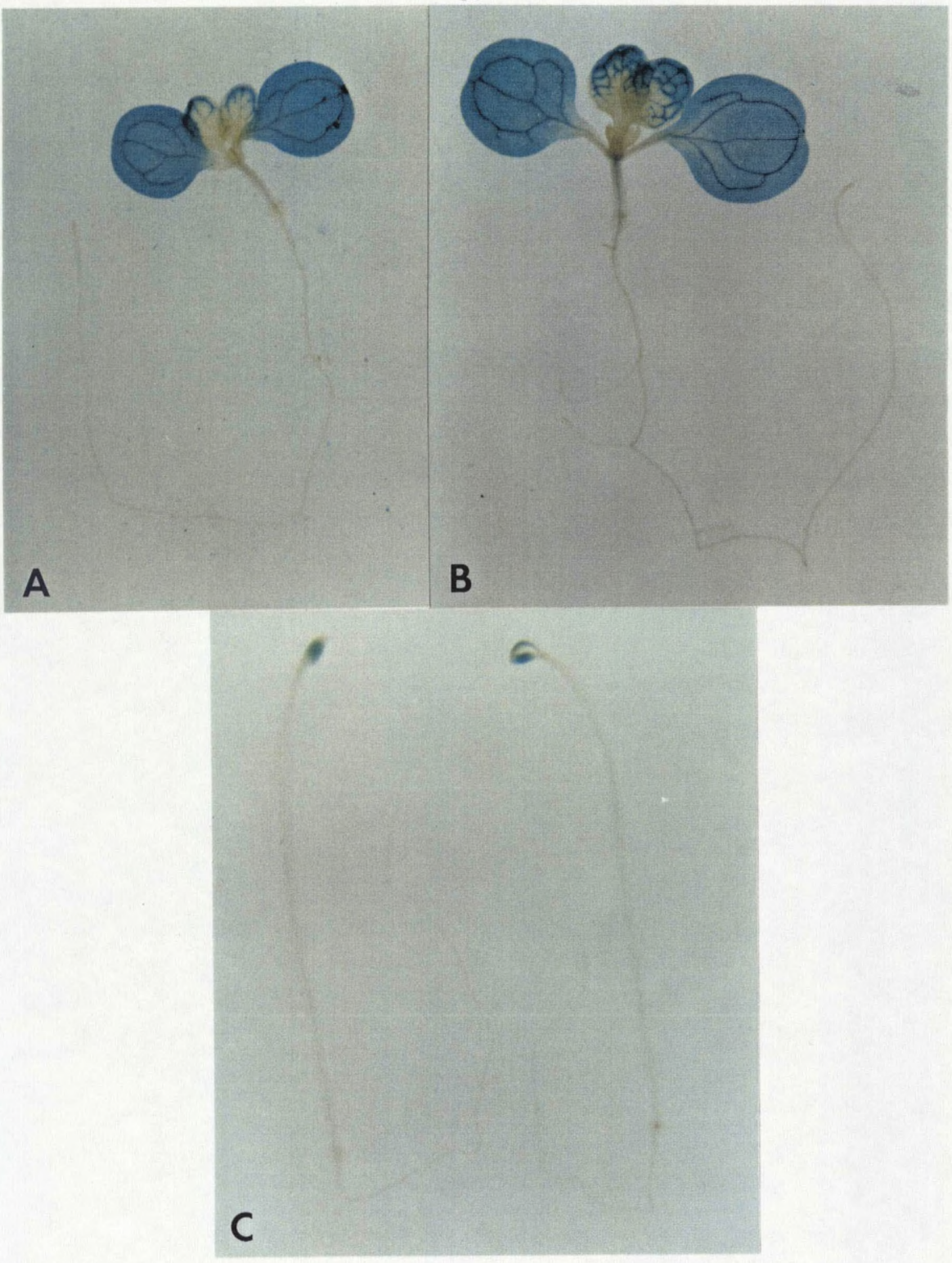


Fig. 8. Histochemical GUS assays of: A. 5 day-old light-grown seedling from line P_EGUS1#2, B. 7 day-old light-grown seedling from line P_EGUS2#5, C. two 7 day-old dark-grown seedlings from line P_EGUS1#2.

Unlike the *PHYD* promoter, there appears to be no activity of the *PHYE* promoter in the root region.

Among the 6 lines selected for kanamycin resistance, 2 (P_EGUS1#4 and P_EGUS2#4) showed no visible histochemical activity. The kanamycin resistance indicates the presence of the transgene in these lines (Table 4), so it is likely that the fusion has been silenced in these plants.

Mature Plant Assays

Cauline leaves and flowers of 2 P_EGUS lines, P_EGUS2#3 and P_EGUS2#5, were assayed for histochemical GUS activity. In both these lines, the same pattern of staining was observed. Cauline leaves in both lines stained with medium intensity (score:++) (Fig. 9A). Staining in leaves in general was not uniform, very low at the base but increasing towards the leaf tip, as can be seen in the first true leaves in seedlings (Fig. 8A,B), cauline leaves (Fig. 9A) and in the sepals (Fig. 9B,C). In the flowers, sepals showed a low intensity of staining (score:+), whereas in the stamens, GUS staining was seen in the anther walls (Fig. 9B,C). Here, too, the staining was of low intensity (score:+). No staining was observed in the petals, pollen, or the carpels (Fig. 9B,C).

Discussion

Using the GUS reporter gene system, it has been possible to show temporal and spatial regulation of the

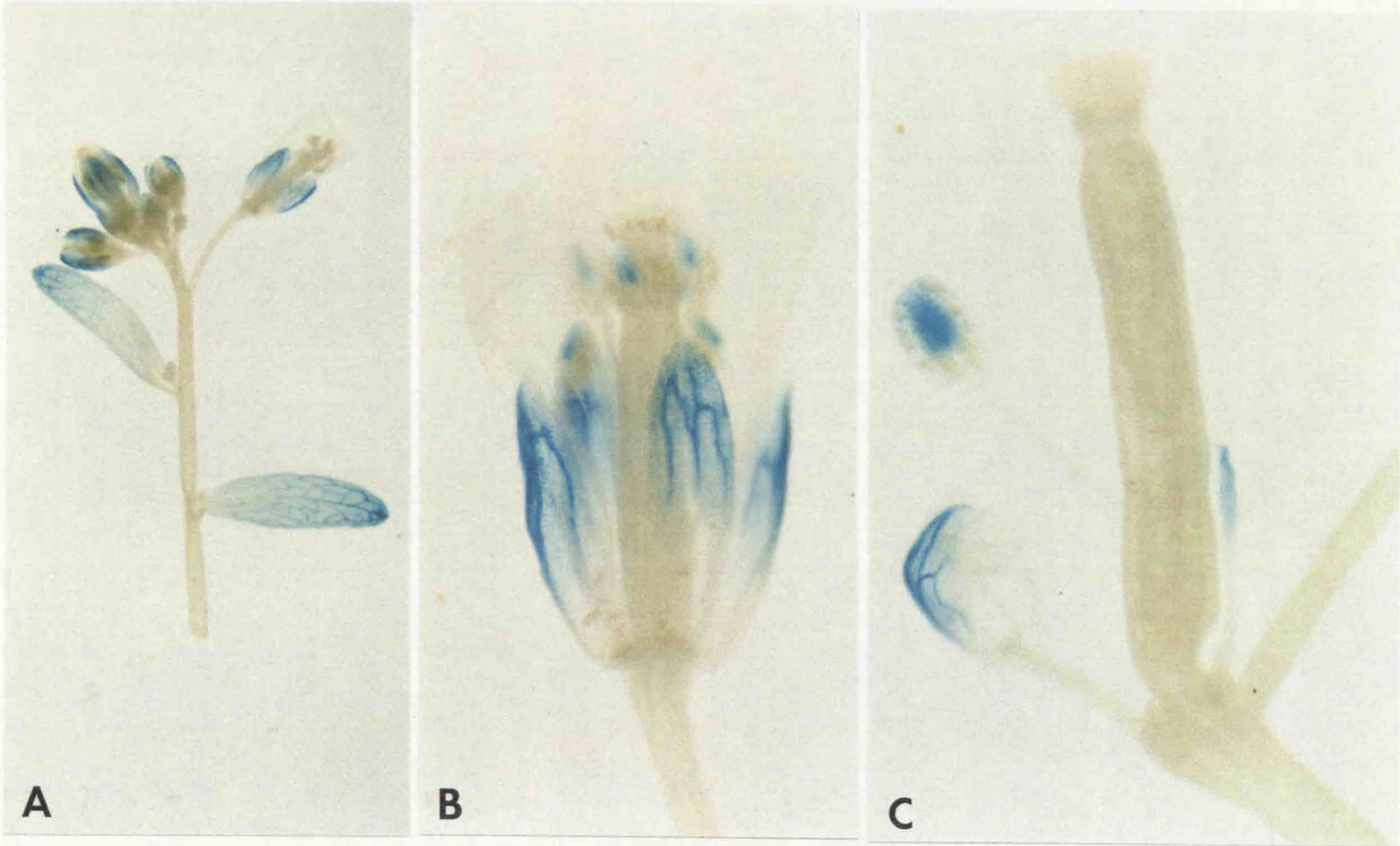


Fig. 9. Histochemical GUS activity shown by: A. an inflorescence with a part of the stem and cauline leaves attached, from line P_EGUS2#3, B. single flower from line P_EGUS2#5, and C. another view of a single flower from line P_EGUS2#5.

PHYB-PHYD-PHYE subfamily of phytochrome genes. It has been shown here that 2.3kb of the 5' flanking sequence of *PHYD* gene and 2.6kb of the 5' flanking sequence of the *PHYE* gene, including the translation start sites for both reading frames, contain the information necessary for the expression and regulation of the genes. These regions presumably contain the transcription activating regions or promoters, though comparison of the DNA sequences shows no significant homology (Sharrock, unpublished data). Expression of the β-glucuronidase enzyme in the translational fusions has also been shown to be due solely to these promoter regions since the promoter-less GUS construct did not show any visible GUS expression.

Levels of Expression. Preliminary studies on the levels of mRNAs encoded by the phytochrome genes had indicated that the *PHYB* transcript predominates in 14 day-old light-grown plants while *PHYD* and *PHYE* are the least abundant (15). The GUS fusions produced in this study show a similar pattern in that the P_DGUS lines with a single copy of the fusion constructs show a very low level of GUS expression compared to the P_BGUS line. The P_BGUS construct shows a level of expression comparable to that of the CaMV35S constitutive promoter, while the P_DGUS lines with a single copy of the fusion constructs show a very low level of GUS expression. However, the histochemical GUS assays cannot be relied on to give the exact levels of expression of the genes, due to the

stability of the bacterial β-glucuronidase enzyme (52). This becomes a problem when comparing the expression of P_BGUS fusion to the constitutive CaMV35S promoter, since the strong staining noticed in P_BGUS plants may be due more to the duration of the assay than to early onset of transcription and strong promoter activity. The quantification of GUS activity by fluorometry would be the best way to resolve this issue. Another way would be to conduct histochemical assays of shorter duration on the P_BGUS- and pBI121-transgenic plants. The *PHYD*-GUS multicopy lines show a distinct increase in GUS activity over the standard lines which have a lower copy number of the transgene. The *PHYE* promoter appears to have a very low level of expression. In both these cases, GUS assays using fluorometric substrates will help confirm the strength of the promoter as indicated by the histochemical assays.

There appears to be no effect of the transgene copy number on the staining pattern in the case of the P_BGUS transgenics. This may be due to the saturation of expression due to strong *PHYB* gene promoter activity.

Pattern of expression. The *PHYA* gene encodes a light-labile, Type I phytochrome and shows significant light regulation patterns. The level of the *PHYA* transcript is high in dark-grown seedlings and drops markedly within a few hours following transfer to white light (8). On the other hand, the *PHYB*, *PHYD* and *PHYE* transcript levels are

unaffected by light, indicating that they may encode light-stable, Type II phytochromes (15). The PHYB protein has been shown to be light-stable (18). The expression of these *PHY* genes as revealed by the reporter fusions is consistent with the earlier findings on mRNA levels. Overall, the GUS expression in the P_BGUS, P_DGUS and P_EGUS fusions was found to be similar in seedlings grown in the dark and in the light. An exception to this is that the *PHYD* gene promoter is off in dark-grown roots and on in the roots in the presence of light. It is not known whether this reflects transcriptional regulation of *PHYD* in roots since the mRNA levels in light- and dark-grown roots has never been analyzed. There has been no previous account of light-induced regulation of a phytochrome gene in the root before.

Clack et al. (15) also found that all five *Arabidopsis PHY* mRNAs were present at relatively uniform levels in roots, leaves, stems and flowers of mature *Arabidopsis* plants. The expression patterns of the *PHYB*-GUS transgenics fit this finding, but the P_DGUS and P_EGUS transgenics did not. In both P_D- and P_E-GUS lines, leaves showed higher levels of expression than the other organs as judged by histochemistry. Notably, in P_EGUS lines, no visible GUS expression could be detected in roots, even though the mRNA level in roots is not much lower than in the other organs (15). These discrepancies might be due to the regulation at the mRNA level, either preventing or

decreasing translation of the genes in these organs. Since the GUS fusions used in these studies contain the 5′ untranslated regions and the beginning of the coding regions for both *PHYD* and *PHYE*, translational regulation mechanisms would likely be operative in these lines.

In flowers, each of these promoters showed a difference in expression. The *PHYB* promoter is expressed at a fairly high level in all parts of the flower except the petals, while *PHYD* and *PHYE* are on only in the sepals and the anthers. In the anthers, *PHYD* appears to be turned on only in the pollen grains, while *PHYE* is turned on only in the anther walls. It would be interesting to discover what function these three *PHY* genes, which form a part of the light sensing apparatus of the plant, could be performing in the male reproductive organs.

All the P_DGUS transgenic lines with the exception of the variant P_DGUS12#2:1 showed more or less the same pattern of expression, though they varied in strength. The P_EGUS transgenics also show a distinct pattern of expression. While studying these patterns, however, it must be remembered that cell size and vacuolization affect the results of GUS assays. GUS has also been known to accumulate preferentially in the vascular tissue (52). All these factors must be taken into consideration while viewing the results obtained.

Studies on the amino acid sequence similarity of the 5 *Arabidopsis* phytochrome genes have shown that *PHYD* and *PHYE* show the most similarity to *PHYB* gene and so could be considered to form a subgroup of phytochrome genes containing *PHYB*-like forms (15). PHYD shows 80% sequence similarity with PHYB, yet the phenotypes of the *PHYB* null mutants indicate that the two genes are not functionally redundant. The experiments described here show that though the *PHYB* and *PHYD* promoters are both fairly general in expression, there are significant differences between them. This indicates that the two genes have distinct spatial and regulatory patterns in the plant and adds further evidence to the functional heterogenity of the *PHYB* and *PHYD* genes. The *PHYE* gene also appears to have a distinct pattern of expression in the plant. However, it is still not known how the pattern of expression of these genes relate to their physiological function. The *PHYA* gene is strongly photoregulated and is known to mediate the FR-HIR in etiolated plants (20, 21). The phenotypic effects of the PHYB protein have been understood to an extent through characterisation of the null mutant for that gene (19) and through complementation of the null mutation with the *PHYB* minigene (16). But so far, nothing is known yet about the *PHYD* and *PHYE* genes. They appear to be transcribed in practically all parts of the plant body, suggesting that they are likely to play an important role in different

responses at all times of development. The presence of these genes in the male gametophytic tissue may be linked to viability of pollen also. It is also likely that *PHYB* controls many more plant responses than are currently understood.

CHAPTER III

ANTISENSE TO THE *PHYB* GENE

Introduction to Antisense

A key strategy for studying gene function is to observe how the absence of expression of that gene affects the normal phenotype. This is done either by isolating mutants in which the particular gene has been rendered nonfunctional or by blocking the expression of the gene with its antisense construct. In higher plants, unlike in yeast or in animal transgenic systems, it is not currently possible to knock out specific genes, so the most common approach to blocking gene expression is using antisense RNA.

During normal transcription, the antisense strand of DNA in the gene is the template for the single-stranded messenger RNA, which is then translated into the protein by ribosomes. The single-stranded mRNA is the only transcription product of most genes. However, in prokaryotes, some genes are regulated by the additional transcription of an antisense RNA from the sense DNA strand (64). The regulatory role of the antisense RNA molecules was first discovered in *E. coli* by Tomizawa and Itoh in 1981 (66). Following this, antisense RNA has been identified in the regulation of diverse phenomena in prokaryotes (67). The regulation of genes results when the antisense RNAs bind

specifically to sense RNAs by Watson and Crick base-pairing to form RNA duplexes, that are either rapidly degraded, or impair mRNA processing in the nucleus, or block mRNA for translation (68).

Although several RNAs from eukaryotic cells are known to be complementary to known genes, none have been shown to have an in vivo regulatory role. However, strategies have been evolved to artificially regulate genes using antisense RNA. Direct microinjection of artificially synthesised RNA can result in specific inhibition of gene expression (65). Another strategy for introducing antisense nucleic acids into cells is to use short synthetic single-stranded DNA oligonucleotides that are complementary to the sequence around the translation initiation site. These prevent translation by hybridizing to the mRNAs and thus prevent intiation of translation (69). The third and the most-often used method is to use expression vectors to make antisense RNA in vivo (70). The antisense constructs carry the inverted cDNA of the gene to be studied, either whole or in part, driven by a strong promoter with an appropriate transcription terminating sequence. The first work on artificial antisense regulation of gene expression in plants was done by Ecker and Davis (71), who showed effective transient inhibition of chloramphenicol acetyl transferase activity in carrot cell cultures by its antisense gene. Subsequently, several plant genes have been artificially

reduced in expression using this technique (72, 73, 74, 75, 76, 77, 79), some with commercial value (72). Most successful antisense experiments have used the strong constitutive Cauliflower mosaic virus 35S promoter. The constitutive expression derived from this promoter provides a pool of antisense RNA already present at the onset of activity of a developmentally regulated gene. Thus, a large excess of antisense to sense template is created which increases the efficiency of duplex formation between the antisense and the sense template (68). Even the most abundant protein in plants, ribulose 1,5-bisphosphate carboxylase, could be inhibited with the antisense construct of this gene driven by the CaMV35S promoter (73).

One possible disadvantage to this system is that, in general, antisense systems are leaky, perhaps due to the transient dissociation of the antimessenger/mRNA hybrid (69). Therefore, even though reduction in gene expression is achieved, the gene might not be completely silenced in all cases. It has also been observed that only a small fraction, less than 20% of the transgenics obtained, show the phenotype (77, 79).

Our aim in using the antisense technique was to reduce the expression of the *PHYB* gene and observe the phenotype it produced. If the antisense construct to the *PHYB* gene succeeded in silencing the gene completely, then the expected phenotype would be the same as that of the null

mutant in *PHYB* gene, *hy3* (19). If it did not knock out the gene completely but succeeded in reducing the expression of the *PHYB* gene, a partial phenotype could be expected. Using this *PHYB* gene as a control when the expected phenotype is known, it will be possible to test whether antisense technology can be used to determine the roles of the *PHYC*, *PHYD* and *PHYE* genes in *Arabidopsis*.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Nossen, No-0 WT was used in transformation with the antisense construct. The Bo64 allele of the *hy3 (PHYB)* mutation, a nonsense mutation in the *PHYB* gene (19), was used as the *PHYB*-null control strain. Later, *A. thaliana* land race Landsberg erecta and No-0 were used as female parents in crosses with lines carrying the antisense construct. The light source for the hypocotyl length experiments was white light from a bank of 40 watt fluorescent bulbs (50 μm m^{-1} sec^{-2}). Growth conditions for hypocotyl experiments were the same as for the promoter-analysis experiments (refer Materials and Methods, Chap.II). For experiments on rosette leaf morphology and flowering time, seeds were sown directly on pots containing potting soil overlayed with vermiculite, treated at 4°C for 4 days, and grown thereafter at 24°C under an 8-hour photoperiod.

The antiB Construct used

The antisense *PHYB* gene was constructed by Sharrock and Clack (unpublished). For the construction of this plasmid, the GUS gene cassette was first excised from pBI121 using XbaI and SstI restriction sites, and the polylinker from pGEM7Zf+ (Promega) was inserted in its place. This gave the vector pBI127. The *PHYB* cDNA from λB2-1 was cloned into the SmaI and XbaI sites of the pBI127 polylinker in the reverse orientation. This construct, pBI127-antiB thus reads the *PHYB* message in the antisense direction, driven by the CaMV35S constitutive promoter and terminating at the *nos* termination sequence (Fig. 10). The pBI127-antiB construct was transformed into *Agrobacterium* LBA4404 and the antiB transgene was transformed into wild-type *Arabidopsis* as described in Materials and Methods to Chapter II of this thesis. The transgenic lines derived from this will be referred to as WT(antiB) lines.

Southern Analysis and Western Blotting

Southern analysis was conducted using the genomic DNA from 2 WT(antiB) lines in order to confirm the presence of the transgene in them. Genomic DNA was extracted and the Southern analysis was performed using the protocol described by Wester et al (16). The probe used was a 855 bp fragment of the *PHYB* cDNA (8), extending from the HindIII site at position 2875 to the AatII site at position 3730. The levels of the PHYB and PHYA protein in the transgenic plants

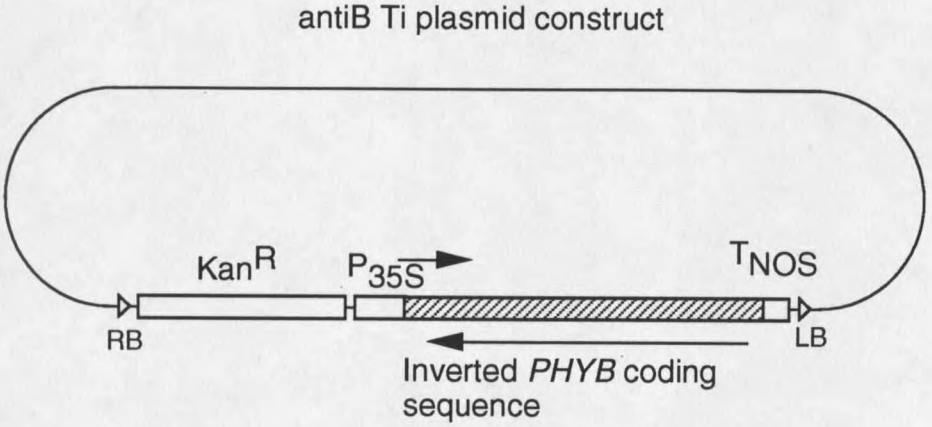


Fig. 10. The anti-B plasmid construction. RB, right border; LB, left border; Kan^R, kanamycin resistance; P_{35S}, the Cauliflower Mosaic Virus 35S promoter; T_{NOS}, nopaline synthase terminator.

were examined by Western analysis using the type-selective monoclonal antibodies described by Somers et al. (18). This assay was performed by Dr. Richard Stout.

Hypocotyl Measurement Experiments

Seeds of the wild-type line, the *hy3* Bo64 mutant line, and the WT(antiB) transgenic lines were sterilized and plated on GM plates. The plates were incubated at 4°C in the dark for 2 to 3 days after which they were transferred to continuous light conditions (described in Plant materials and growth conditions). After 7 days, the seedlings were picked gently from the plates and the hypocotyl length measured individually.

Flowering under 8-hour Photoperiod

Seeds of the wild-type line, the *hy3* Bo64 line, WT(antiB)#2 and WT(antiB)#4 were sown directly on pots filled with soil and overlaid with vermiculite. They were incubated at 4°C in the dark for 4 to 5 days and then transferred to an 8-hour photoperiod. The plants were observed periodically for the appearance of bolts or flowering stalks. Once the bolt appeared, the plant was lifted and the number of leaves were counted. The days to flowering were also noted.

Chlorophyll Assay

36 day-old seedlings of all the above lines were harvested and stored at -80°C. They were then assayed for

chlorophyll content using the protocol described by Chory (78).

Genetic Analysis by Crossing

In order to ensure that the phenotype exhibited by the antiB transgenic plants was heritable, they were crossed into the *Arabidopsis* No-0 and Landsberg erecta wild-type ecotypes. Since selfing is the natural mode of pollination in *Arabidopsis*, the crossing was done by hand.

Results

Selection and Characterization of WT(antiB) Transgenics

Seven original transformants were selected on GMkan medium. Of these, the T_2 seed of 5 lines were kan-positive: WT(antiB)#2, WT(antiB)#4, WT(antiB)#5, WT(antiB)#6 and WT(antiB)#7. The progeny (T3 seed) of these five lines exhibited two levels of kanamycin resistance. Three lines (WT(antiB)#5, #6 and #7) segregated seedlings that were completely resistant to 50ug/ml kanamycin, but lines WT(antiB)#2 and #4 segregated seedlings that were weakly kanamycin resistant. Fig. 11 shows growth of WT(antiB)#2 and #4 on GMkan plates compared to control kanamycin-resistant transgenic *Arabidopsis* lines. Alongside selection on GMkan medium, these lines were also grown on GM medium to observe their phenotype. Seedlings were observed for long hypocotyls, since the expected phenotype was that of the *hy3* mutant, i.e., long hypocotyl. Of the 5 transgenic lines,

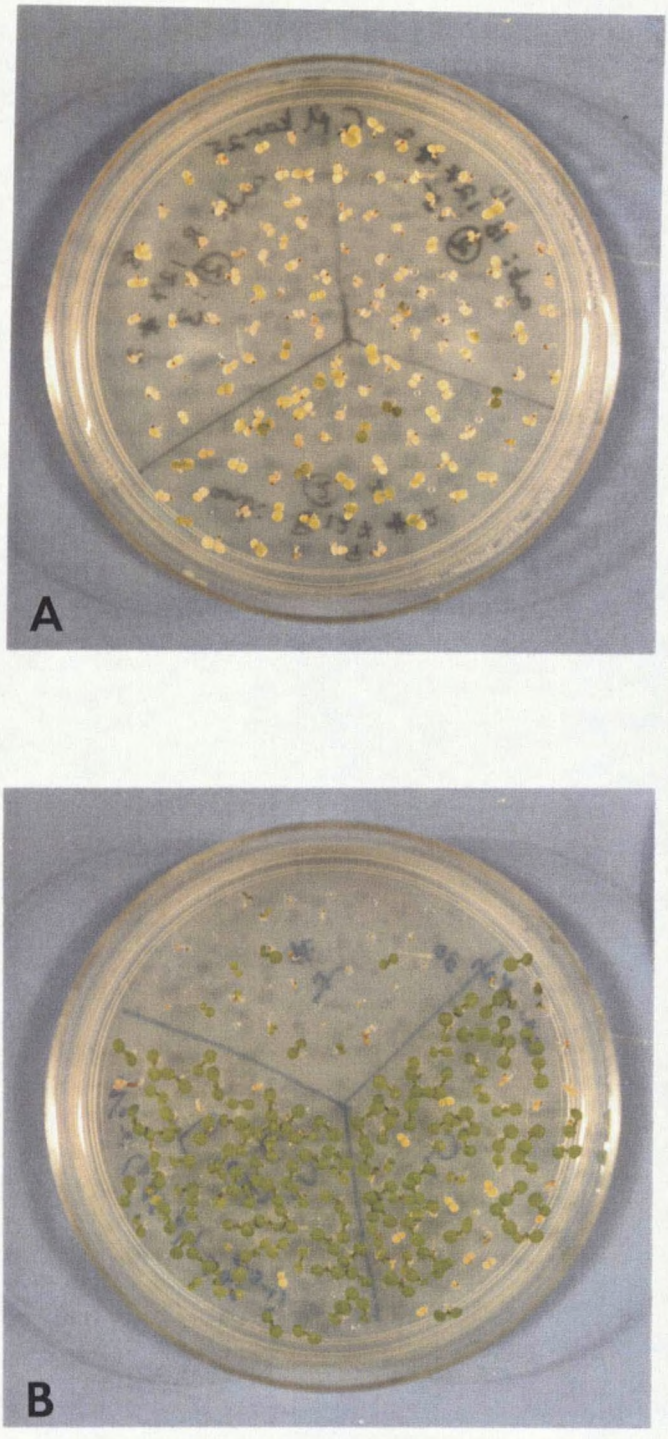


Fig. 11. A. Weak kanamycin resistance shown by WT(antiB)#4 seed lots (T_3 generation) on GMkan medium. B. Segregation normal kanamycin resistant and sensitive seedlings of control transgenic *Arabidopsis* lines.

the 3 lines that were kanamycin-positive were observed to have hypocotyls of wild-type length, while lines WT(antiB)#2 and #4 that were only weakly kanamycin-resistant showed longer hypocotyls. Fig. 12A shows the distribution of hypocotyl lengths in 7-day-old seedlings of wild-type (WT), *hy3* Bo64 and the WT(antiB)#2 and #4 lines. It can been seen that the WT(antiB) lines show an increase in hypocotyl length over the wild-type seedlings. These results indicate that 2 out of 5 *PHYB* antisense transgenic plants show the expected long hypocotyl phenotype and that this phenotype correlates with weak kanamycin resistance. This response to kanamycin in the medium was tested for different concentrations (50ug, 25ug and 15 ug kanamycin/ml) and found to be identical. Presumably, the kanamycin resistance level of these strains was sufficient to allow them to come through under selection in tissue culture and the reason for this correlation of reduced kanamycin resistance and antiB transgene effectiveness is not currently known.

Heritability of Antisense Phenotype

In order to show that the long-hypocotyl phenotype was heritable, the (No-0) WT(antiB)#2 and (No-0) WT(antiB)#4 lines were crossed with the No-0 wild-type. As expected, the F2 progeny from these crosses showed weak kanamycin resistance (data not shown). In the F_2 progeny the long-hypocotyl characteristic was also observed, and the distribution of the hypocotyl lengths observed among the F_2

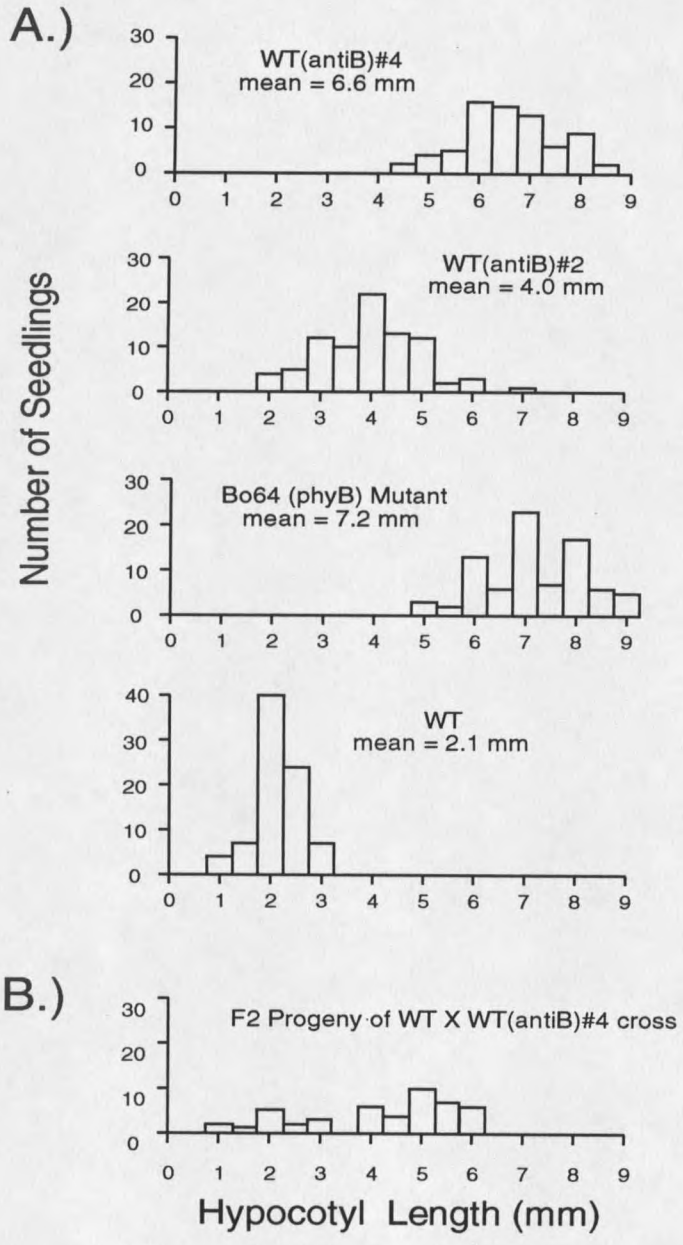


Figure 12 A. Hypocotyl length distributions of the wild-type, the *hy3* Bo64 (phyB) mutant, and WT(antiB) lines #2 and #4.
B. Hypocotyl length distribution of F2 progeny of a No-O wild-type X WT(antiB)#4 cross.

progeny can be seen in Fig. 12B. Though it was not possible to follow kanamycin resistance as an indication of the presence of the transgene, the F_2 progeny show an approximately 1:3 (10:36) segregation of wild-type height to antisense height (Fig. 12B). These results indicate that the presence of the antisense construct results in an increase in hypocotyl elongation in seedlings, a phenotype governed by the *PHYB* gene (16, 19), and also that this characteristic is heritable.

Southern Analysis of the antiB Lines

That the lines WT(antiB)#5, WT(antiB)#6 and WT(antiB)#7 carried the transgene in some form was concluded from their kan-positive nature. However, the lines WT(antiB)#2 and WT(antiB)#4 had to be examined for the transgene, since they showed partial kanamycin-sensitivity. Genomic DNA from these two lines was digested with SstI, fractionated on a gel and probed with a fragment of the *PHYB* cDNA. SstI cuts only once within the transgene so insertion of this gene at different loci should yield various sized hybridizing fragments. A single band due to the chromosomal *PHYB* gene is seen in both strains and the wild-type, and this band represents a single-copy gene. Fig. 13 shows that both antiB#2 and antiB#4 carry multiple copies of the transgene.

Hence, the antisense construct is present in lines WT(antiB)#2 and WT(antiB)#4 as well as in the three

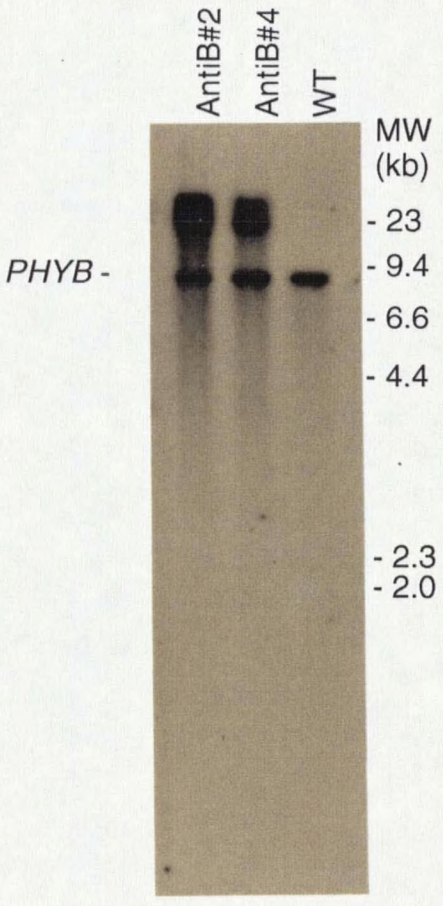


Figure 13. Southern blot analysis of WT(antiB) transgenic lines showing the presence of multiple copies of the transgene. Southern analysis was conducted on the T7 generation of two WT(antiB)lines and the wild-type No-O line as control. Total DNA was extracted and cut with SstI. The probe used was an 855 bp fragment of the *PHYB* cDNA. The single band hybridizing to the probe at approximately 8 kb is due to the endogenous *PHYB* gene.

kanamycin-resistant lines but it is functional only in lines WT(antiB)#2 and WT(antiB)#4, in terms of generating the expected antisense phenotype.

Analysis of PHYB Protein Levels

In order to assess if the antisense construct incorporated in the wild-type genome in lines WT(antiB)#2 and WT(antiB)#4 was actually reducing the quantity of the PHYB protein, Western blot analysis of total protein extracts from the wild-type, the *hy3* Bo64 mutant and WT(antiB)#4 was conducted. The protein extracts were also probed with monoclonal antibodies to PHYA protein to normalize the PHYB levels. Fig. 14 shows that, as expected, the *hy3* mutant line contains no detectable PHYB protein. The two WT(antiB)#4 seed lots assayed showed reduced levels of the PHYB protein relative to the wild-type (Fig. 14). The PHYB protein level was reduced by about one-half to a third (Fig. 14), suggesting that the antiB gene has succeeded in reducing the amount of PHYB protein, but has not knocked the expression completely. Therefore, the phenotypes manifested by the antisense lines are caused by low levels of PHYB protein. This is consistent with the observation that the *phyB* null mutation is incompletely recessive and the long-hypocotyl phenotype is *PHYB* gene copy number-dependent (16). The possibility that the antiB transgene is also lowering the protein levels of other phytochromes like *PHYD* and *PHYE* cannot be ignored, given the

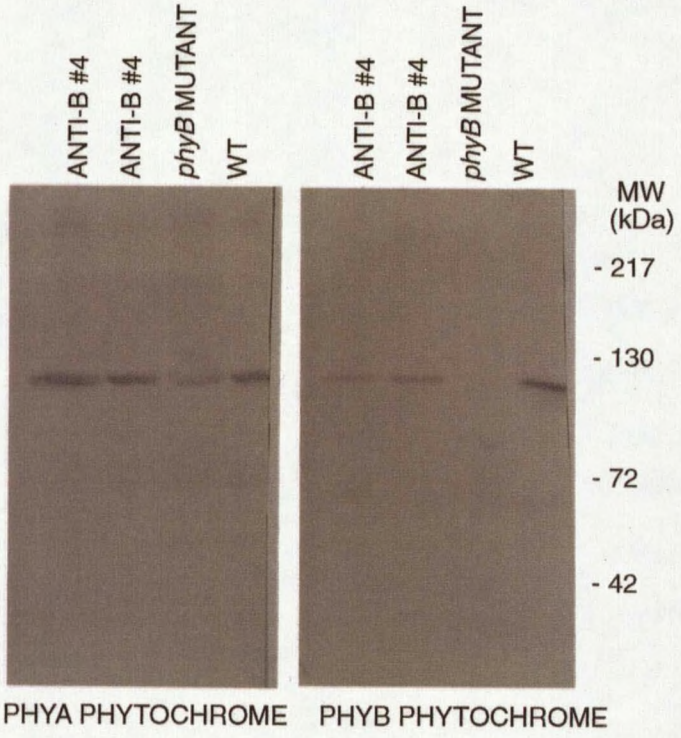


Figure 14. Western blot analysis of the PHYA and PHYB phytochrome levels in the WT(antiB)#4 line, the *hy3* Bo64 (*phyB* null) mutant, and wild-type No-O. Protein was extracted from two week-old dark-grown seedlings. Blots were probed with a monoclonal antibody specific for PHYA (left) or a pool of three monoclonal antibodies that selectively detect PHYB (right). The PHYA phytochrome band in the *phyB* mutant lane is low due to a gel loading error.

sequence similarity between the 3 genes (15). This could not be tested since there are no available monoclonal antibodies to PHYD and PHYE proteins at the present time.

Effect of Antisense Construct on *PHYB* Controlled Phenotypes:

In addition to elongated hypocotyls in seedlings, it has been observed that *hy3* mutants show distinct morphological and developmental changes as adult plants including elongated petioles and extended rosette leaves, reduced chlorophyll content, and reduced time to flowering under a short day photoperiod (16, 19). All of these phenotypes can be complemented with a *PHYB* transgene (16) so they are definitely controlled by the *PHYB* gene. Therefore, to see if the antisense construct affected the *PHYB*-controlled phenotypes in adult *Arabidopsis*, the WT(antiB) lines #2 and #4 were grown under an 8-hour photoperiod and compared to wild-type and mutant plants grown under the same conditions.

Compared to the rosette morphology of the wild-type, the WT(antiB) lines showed an elongation in the petiole length (Fig. 15). This elongation, however, was not as pronounced as that observed in the *hy3* mutant plants. Moreover, the leaf margins in the WT(antiB) plants showed a tendency to curl slightly at the edges, a phenotype not seen in either the wild-type or the *hy3* plants.

The chlorophyll assay on plants grown under the 8-hour photoperiod showed that the levels of chlorophyll in the

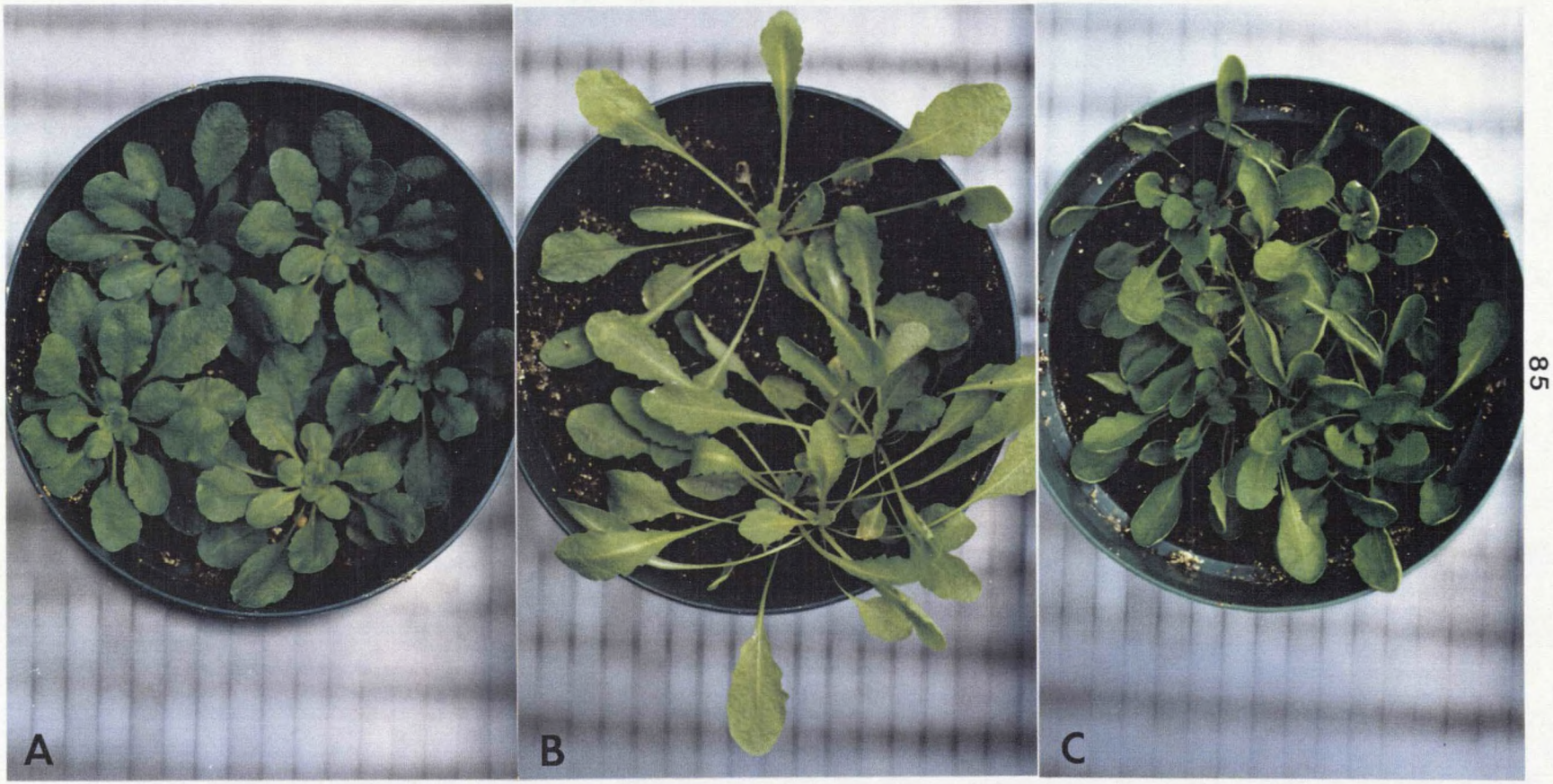


Fig. 15. Rosette leaf morphology exhibited by: A. wild-type *Arabidopsis* No-0 line, B. *hy3* Bo64 (*phyB*) mutant line, and C. WT(antiB)#4 line. All the plants were grown for 43 days under an 8 hour photoperiod.

WT(antiB) plants fell between those of wild-type plants and *phyB* Bo64 plants (Table 6). Hence, for both leaf morphology and chlorophyll content, the antiB lines show effects less extreme than the *phyB* null mutant but consistent with antisense inhibition of *PHYB* expression. The *phyB* mutant flowers considerably earlier than the wild-type allele (16, 19; Table 6) and, along the same lines, it would be expected that the antiB lines would be intermediate in flowering time. The observed results were, however, contrary to this expectation, in that the WT(antiB) lines actually took more time to flower than the wild-type (Table 6). The average number of rosette leaves produced before flowering, a second measure of flowering time, also indicated that the *PHYB* antisense was not producing the expected phenotype (Table 6). The reason for this is not known, but it is possible that some variant phenotypes shown by WT(antiB) plants, like the curved leaf margins and the delay in onset of flowering, may be due to the effect of the antisense construct interfering with other phytochromes.

Discussion

Antisense inhibition of *PHYB* gene expression was attempted in order to determine whether the activity of an individual phytochrome gene can be selectively reduced in *Arabidopsis* to an extent that will yield a recognizable phenotype. There exists a well-characterised null mutant in the *PHYB* gene, *hy3* (19), and it was of interest to see if

Strain	Chlorophyll a+b content (mg/gm fr. wt.)[a]	Ratio of chl a/b[a]	Days to flower[b]	Number of rosette leaves at flowering[b]
Wild-type	1.3	2.2	61.0 (0.5)	44.3 (0.6)
Bo64	0.8	2.1	52.8 (0.6)	24.8 (0.7)
antiB#2	1.1	2.1	74.5 (0.6)	49.2 (0.6)
antiB#4	1.0	2.1	64.5 (0.6)	42.4 (0.5)

Table 6. Effect of the antiB transgene on chlorophyll content and flowering. About 85 plants of each line were grown under an 8 h light/16 dark photoperiod.
[a] A few plants were harvested at 36 days for the chlorophyll assays.
[b] Days to flower and number of rosette leaves were determined for about 75 plants of each line. The figures in parenthesis indicate the standard error.

antisense RNA to this gene produced the same phenotype as this mutant. There is no prior report of antisense inhibition of phytochrome genes.

In addition, though the physiological roles of the phytochrome genes *PHYA* and *PHYB* have been elucidated primarily by studying null mutants in those genes, there are no known mutants in the two latest *PHY* genes, *PHYD* and *PHYE*, whose functions in the plant are as yet unknown. One way to study their functions would be to specifically block their activities with antisense DNA constructs. The antisense to *PHYB* gene would, therefore, be useful to see the extent to which the antisense to a phytochrome gene can block gene activity.

Introduction of an antiB transgene into wild-type *Arabidopsis* resulted in two lines which exhibit partial phenocopies of the *hy3 (phyB)* null mutant. The long-hypocotyl phenotype of these antiB lines was shown to be genetically transmissable and Southern blot analysis showed that they contained several copies of the transgene. But, Western blot analysis showed that the level of the *PHYB* gene product had been reduced by only about 50% of wild-type levels. However, it is common occurrence that antisense is leaky and does not always silence the sense gene completely (69). The *PHYB* antisense could also be interfering with other phytochrome genes. Given the similarity between the *PHYB* and *PHYD* sequences, the *PHYD* mRNA would be the best

match for the *PHYB* antisense. At the present time, there is no available monoclonal antibody for the PHYD protein and so probing the WT(antiB) lines for PHYD protein is not possible. But Northern analysis could be done to probe for the *PHYD* mRNA. This could resolve if the incomplete reduction of the PHYB protein is because the antiB construct is blocking other phytochromes, or if the antisense is not efficient enough to ensure complete silencing of the gene.

Phenotypically, some of the characteristics shown by the WT(antiB) plants were midway between those of the wild-type and the null mutant phenotypes. In this way, characteristics that show the partial phenotype like the hypocotyl length and petiolar elongation (cell size), and also chlorophyll levels, appear to be dependent on PHYB protein levels. However, the tendency of the WT(antiB) plants to delayed flowering is interesting and may be caused by the antiB construct interfering with other phytochromes. All the results that were obtained in this study should be considered with the view that they were all obtained using only two transgenic lines and will have to be further tested.

This experiment with antisense to *PHYB* gene gives preliminary evidence of the efficiency of the antisense technique in the case of phytochrome genes. The data obtained from the antiB experiment shows that the resultant plants were not identical to the null mutant, but similar to

it, and that the antisense to *PHYB* gene was very leaky. Nonetheless, this indicates that antisense inhibition of phytochrome genes is possible and may be used as an approach to determining the physiological roles of the *PHYC*, *PHYD* and *PHYE* genes and, perhaps, in the generation of transgenic plants with useful alterations in light-controlled traits such as plant height and flowering time.

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APPENDIX

Media Contents

GM Liquid Medium:
900 mls deionized water
4.4 g M-S Basal Salts (Sigma)
20 g Sucrose
0.5 g MES
Final volume: 1 litre.

B5 Basal liquid medium
900 mls deionized water
3.2 g Gamborgs B5 salts plus organics (Sigma)
0.5 g MES
20 g Glucose
Final volume: 1 litre.

CIM: Callus-inducing medium
900 mls deionized water
3.2 g B5 salts plus organics
0.5 g MES
20 g Glucose
8 g Agar
0.5 ml of 1 mg/ml 2,4-D
0.5 ml of 0.1 mg/ml kinetin
Final volume: 1 litre.

SIM C_{500}: Shoot-inducing medium
900 mls deionized water
3.2 g B5 salts plus organics
0.5 g MES
20 g Glucose
8 g Agar
5 ml 2ip-adenine (1 mg/ml)
1.5 ml IAA (0.1 mg/ml in 1N KOH)
5 ml Carbenicillin (100 mg/ml)
5 ml Kanamycin (10 mg/ml)
Final volume: 1 litre.

SIM C_{200}: Shoot-inducing medium
900 mls deionized water
3.2 g B5 salts plus organics
0.5 g MES
20 g Glucose
8 g Agar
5 ml 2ip-adenine (1 mg/ml)
1.5 ml IAA (0.1 mg/ml in 1N KOH)
2 ml Carbenicillin (100 mg/ml)
5 ml Kanamycin (10 mg/ml)
Final volume: 1 litre.

GM kan_{50} solid medium:
900 mls deionized water
4.4 g M-S basal salts
20 g Sucrose
0.5 g MES
8 g Agar
Final volume: 1 litre.