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The Salk Institute

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Genetic Analysis of Photoreceptor Action Pathways in *Arabidopsis thaliana*

The specific strategies and long-term goals of this proposal remain intact relative to the original proposal. We continue to isolate and characterize photomorphogenic mutants of *Arabidopsis thaliana*. The molecular and biochemical characterization of one of these mutants, *det*1, has led to one publication of original data and to one Society for Experimental Biology Symposium paper (see below). The phenotype of a second mutant, *det*2, has also been studied during this funding period. In addition, we have continued work on a general strategy to isolate mutations in trans-acting regulatory factors that mediate light-regulated gene expression, and have identified several potentially interesting regulatory mutants. In the third funding period, we will concentrate on the genetical, biochemical, and molecular characterization of these new mutants. Construction of double mutants between the new mutants and the previously characterized morphological mutants should allow us to construct a pathway for light-regulated seedling development in *Arabidopsis*.

Progress Report:

A. Characterization of Morphological Mutants. When the original proposal was submitted, I had isolated and partially characterized photomorphogenic mutants which fell into two phenotypic classes: *det* or de-etiolated mutants which had some of the phenotypes of light-grown plants when grown in complete darkness and long hypocotyl (*hy*) mutants which had some of the phenotypes of etiolated plants when grown in the light.

(1). Further Characterization of the Phenotype of *det*1 Mutants. Our original analysis of *det*1 mutants revealed that these plants display many phenotypic characteristics of light-grown plants when grown in darkness (Chory et al., 1989, Cell, 58:991-999). When grown in the dark, these mutants have the gross morphology of light-grown plants, including the development of chloroplasts and leaf mesophyll tissue. In dark-grown mutant seedlings, mRNA levels for several nuclear and chloroplast photogenes are similar to the levels found in light-grown wild-type plants, and are 20-100 fold higher than the levels found in dark-grown wild-type seedlings. Since the *det*1 mutations are recessive, we proposed that DET1 is a master regulatory molecule exerting negative control over the light response.

In addition to this phenotype in the dark, *det*1 mutants, grown in the light, are small, pale-green, and lack apical dominance. These observations imply that DET1 has a function in light-grown plants, as well as in dark-grown plants. During this year, we showed by histology and RNA analysis, that the role of *DET*1 in light-grown plants is likely to be in regulating the cell-type specific expression of light-regulated genes and chloroplast development. Using several light-regulated promoters (*cab*, *chs*) fused to screenable marker genes that were introduced into *det*1 and wild-type plants, we showed that in light-grown *det*1 plants, both *cab* and *chs* promoters are active in cell-types where they are normally silent or expressed at very low levels in wild-type plants. We extended this analysis to show that the aberrant dark expression of light-regulated nuclear genes in the *det*1 mutants is correlated with increased transcription from the *cab* and *chs* promoters. Taken together, these results suggest the *DET*1 gene product is a negatively acting regulatory molecule that is used in common by both the light stimulus transduction pathway and by temporal or spatial regulatory signals in plants. The data from these studies were recently accepted for publication in the Proceedings of the National Academy of Sciences. I have included a preprint with this progress report.

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(2). Characterization of *det*2 Mutants. During year 2, we have been characterizing the phenotypes of *det*2 mutants as well. In general, *det*2 mutations are less pleiotropic than *det*1 mutations. Both *det*1 and *det*2 mutants show cotyledon expansion and an inhibition of the rate of hypocotyl elongation in the absence of light. *det*2 mutants differ from *det*1 mutants in that *det*2 fails to develop true leaves in the dark. By ultrastructural analysis, we have shown that even though the cotyledons are expanded in *det*2 mutants, the chloroplast developmental program has not been initiated. Though *det*2 mutants fail to undergo the chloroplast differentiation program in the dark, a number of genes whose products function within chloroplasts are expressed at high levels in dark-grown *det*2 mutants. This suggests that the expression of these genes does not require functional chloroplasts. Further, unlike *det*1 mutants, the cell-type specificity of the chloroplast developmental program, as well as gene expression in light-grown *det*2 plants does not appear to be affected. Instead, *det*2 mutants appear to have defects in photoperiodic timing, characterized by a prolonged juvenile phase and failure to repress the accumulation of light-regulated RNAs during dark periods.

A summary of the phenotypes of *det*1, *det*2 and wild-type plants is shown on page 6. It is clear from our characterization that *det*1 and *det*2 mutations have some important similarities, as well as some notable differences. Both DET1 and DET2 appear to be involved in light signal transmission because mutations at either locus uncouple the light signal from a number of light-dependent processes. Mutations in the *DET*1 gene result in derepression of the whole light light developmental program, including leaf and chloroplast development and gene expression. Further, *det*1 mutants are also defective in the proper integration of temporal or spatial regulatory signals since we observed a loss of tissue-specific expression of the chloroplast developmental program, and the light-regulated gene expression program, including aberrant expression of *cab* genes in roots, and *chs* in leaf mesophyll cells and flowers. Mutations in the *DET*2 gene affect two downstream light-regulated processes, morphology and gene expression, but do not affect the etioplast to chloroplast transition. These results are important because they show that chloroplast differentiation and photogene expression can be uncoupled from each other. This should help us to analyze the downstream light responses.

(3). Summary of Phenotypes of *hy* Mutants and New Studies with *hy* Mutants. In addition to the *det* mutants, we have been studying a second class of mutants that have a long hypocotyl (*hy*) when grown in the light. Sixty-five mutant alleles have been analyzed by M. Koornneef and by our laboratory. These efforts have identified 7 complementation groups; 3 of these (*hy*1, *hy*2, and *hy*6) have deficiencies in photoreversible phytochrome activity, and are presumably signal perception mutants. During the first two funding periods, we have analyzed *hy*1, *hy*2, and *hy*6 mutant alleles biochemically. Our data (see last year's summary), indicate that the *HY*1, *HY*2, and *HY*6 genes may encode enzymes in the phytochrome chromophore biosynthetic pathway, rather than in the apoprotein itself. These mutations are therefore of particular interest because they are likely to affect all phytochrome forms within the plant, including the light-labile and light-stable forms.

It is unlikely that all the genes acting in a phytochrome signalling pathway have been identified, therefore we undertook a search for others by screening for suppressor mutations of the long hypocotyl phenotype of *hy*2 mutants. A *hy*2 *gl*1 line was mutagenized with ethane methyl sulfonate (EMS) and M2 seeds collected. From among these M2 seeds, 3 revertants with a short hypocotyl were identified. In conjunction with Lee Pratt at the U. of Georgia, we have shown that one of these revertants still has no photoreversible phytochrome activity. Thus, by biochemical analysis, this mutation is probably at a second site from the original mutation. Crosses to the *hy*2 parent and to wild-type have been performed to confirm this, as well as to determine the phenotype of plants carrying the suppressing mutation in the absence of the original *hy*2 mutation. We expect to find several types of mutations in these suppressor screens. First, we hope to find mutations in other genes acting in the same pathway of red-light signal transduction as HY2. In addition, mutations in pathways that could act in parallel to the phytochrome sensing pathway might also

have the potential to suppress *hy*2 mutations. For example, the suppressing mutations might increase sensitivity to light of another color, or activate a second red-light sensing pathway that uses a chromophore different from that of phytochrome. For these reasons, we are very excited about the feasibility of obtaining such mutants, and plan to continue the mutant screens and biochemical analysis during year 3 of funding.

(4). Double Mutant Studies. We have constructed double mutants between the phytochrome-deficient *hy* mutants and the *det* mutants. The phenotype of the *hy*1-*det*1, *hy*2-*det*1, or *hy*6-*det*1 double mutant is *det*1, indicating that *det*1 is epistatic to *hy*1, *hy*2, and *hy*6. Likewise, *det*2 is epistatic to *hy*1, *hy*2, and *hy*6. These results are consistent with a model where formation of the active form of phytochrome results in a decrease in DET1 or DET2 activity, which in turn leads to the de-etiolation response. The phenotype of *det*1-*det*2 double mutants is additive. This additive effect suggests that the DET1 and DET2 gene products do not interact. These experiments indicate that at least two distinct pathways are involved in the de-etiolation response in *Arabidopsis*.

B. Isolation of New Mutations that Affect the Expression of the *Arabidopsis cab*3 Promoter.

(1). Strategy. Our long-term goals are to saturate the photoreceptor pathways with mutations so that we can eventually reconstruct the number and order of events associated with chloroplast biogenesis. One difficulty to saturating the pathway with mutations is that all the possible phenotypes will be difficult or impossible to predict. Therefore, we have pursued a parallel molecular genetic approach in which we identify mutants by aberrant gene expression patterns, rather than by phenotype. We chose the nuclear *cab*3 gene of *Arabidopsis* as an indicator of light-regulated, developmental gene expression. The *cab* promoter-marker gene chimeras needed for these studies have been introduced into wild-type plants at a single site in each transgenic line. The transcriptional chimeras (see Fig 1, page 7) contain a fully regulated *cab*3 promoter sequence fused to either of two selectable marker genes: (a) the *hph* (hygromycin phosphotransferase) gene, which confers hygromycin resistance; or (b) the *Arabidopsis adh* (alcohol dehydrogenase) gene which allows either positive or negative selection. Both constructs show low basal expression from the *cab* promoter and carry a screenable marker GUS gene (*E. coli* B-glucuronidase), also under control of the *cab* promoter. Our strategy is to mutagenize and select for plants which aberrantly express the marker transgenes from the *cab*? promoter under a variety of conditions. Following selection, we will also screen for GUS activity. This step is important so that true signal transduction mutants can be distinguished from cis-acting promoter mutations.

At the end of the first funding period, we had transformed wild-type *Arabidopsis* with both the pOCA107 and pOCA108 constructions and had obtained several independent transgenic lines that were (a) diploid and (b) showed proper light/dark and tissue-specific expression of the *cab*3 promoter. During year 2, we bulked up seeds of one of these lines (pOCA107-2) that was homozygous for the insertion. We mapped the transgene to chromosome 2, position 15.0 on Koornneef's linkage map. We have mapped the transcription start site of the chimeric *cab-hph* fusion gene using primer extension to show that the mRNA was initiated at the proper start site (Fig 2, page 8). 50,000 seeds were mutagenized with EMS, the plants were selfed, and the M2 seeds collected in pools of 200 families. We have been screening for trans-acting regulatory mutations that affect the expression of the two *cab* promoters in the pOCA107-2 line. The rationale for the screens follows.

The expression of *cab* genes is tightly regulated. These genes are expressed only in the light in green plants, with little or no detectable levels in etiolated seedlings. In addition, *cab* genes are expressed in a tissue-specific manner, transcripts being most abundant in leaves and lower or undetectable in other organs. Finally, the developmental stage of the chloroplast itself appears to regulate the expression of *cab* genes. For example, in photooxidative mutants of maize or in a variety of plants where chloroplast development is arrested with an inhibitor, *cab* genes are not expressed. Thus, *cab* gene expression is regulated by light, intrinsic developmental signals, and

must also be sensitive to signals originating from the chloroplast itself. We have looked for and obtained mutants where the *cab*3 promoter is aberrantly expressed in each of these 3 situations. Specifically, we searched for mutants where the *cab* promoter was expresed either: (1) in the dark; (2) in the wrong organs (e.g., roots); or (3) in the presence of inhibitors that eliminate chloroplast function. We designed our experiments such that all three classes of mutants would represent gain-of-expression of the *cab* promoter, which is important because the greening process is essential. The results from each screen are described separately.

(2). Trans-acting Mutations that Affect the Etiolated Expression Levels of *cab*3. 100,000 M2 seeds from 200 families were screened for hygromycin-resistance in the dark. 500 seeds from each family were germinated on a 100 mm Petri dish containing synthetic medium plus hygromycin (40 μg/ml) for 9 days in the dark. After 9 days, the plates were removed from the dark growth chamber, and were scored for growth. Seedlings from the unmutagenized pOCA107-2 parent had an average hypocotyl length of 1-4 mm in these conditions. 285 putative mutants were picked that had a hypocotyl longer than 6 mm. Of these 285, approximately 90 survived and set seeds (M3). M3 seeds from 47 putative mutants were assayed for GUS activity in the light and dark. An increased amount of GUS activity in the dark would distinguish a trans-acting mutation from a *cab*3 promoter mutation. Of the 47 M3 seedlings examined, 7 also had a greater than 3-fold increase in GUS activity in the dark. All seven are from independent M2 pools, indicating that they are independent mutations. The data are summarized in the following Table:

Summary of Phenotypes of Mutants Affected in Etiolated Expression Levels of the *cab*3 Promoter

Mutant Designation	Hypocotyl Length (mm)	Cotyledon Phenotype	GUS Activity[1] Light	Dark	Ratio L/D
pOCA107-2	1-4	open	42,800	365	117
80-473	10-12	open	53,840	1287	42
115-299	7-10	open	70,233	1335	53
92-345	6-9	open	22,548	1487	15
134-635	9-12	closed	48,095	1049	46
184-714	6-9	open	56,714	1057	54
145-659	2-7	open	40,299	1067	38
182-709	<10	open	26,785	2035	13

[1]GUS activity is defined as pmol of 4-methylumbelliferone per min per mg of protein.
Hypocotyl length is defined for etiolated seedlings grown on 40 μg hygromycin for 9 days in total darkness.

We are currently continuing genetic analysis to determine if these are single gene mutations and performing complementation analysis to determine how many genes these mutations define. The mutants will be further characterized with respect to other aspects of light-regulated seedling development, as we have previously done with the *det* and *hy* mutants.

(3). Trans-acting Mutations that Affect the Tissue-Specific Expression of *cab*. M2 seeds (100,000) were screened for root elongation after growth for 14 days in the light on synthetic medium containing 20 μg/ml hygromycin. Under these conditions, the pOCA107-2 control plants had short roots many of which did not contact the agar surface. 182 putative mutants were picked that had long, branched root growth into the hygromycin-containing medium. A small piece of root from these 182 putative mutants was excised and placed into a solution containing X-glucuronide (X-gluc), which allows for histochemical staining of GUS activity. Of the 182 M2 seedlings, 17 also showed X-Gluc staining in the roots, indicating a possible trans-acting

mutation. Eight of these 17 M2 plants yielded M3 seeds. Of these 8, 4 gave rise to M3 seedlings with a hygromycin-resistant root phenotype (i.e., branching roots that grow into the hygromycin medium). M3 seedlings derived from all 4 of these M2 plants had weak X-gluc staining in the roots (see Fig 3, page 10). In summary, from this screen, we have 4 potential mutants that show tissue-inappropriate expression of *cab*3. Genetic and biochemical characterization of these mutants is currently underway.

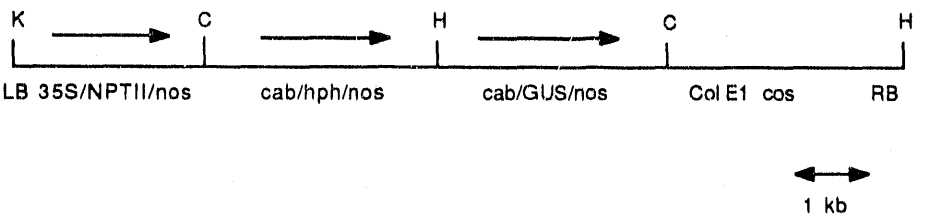
(4). Mutations that Affect Signalling Between the Nucleus and Chloroplast. The herbicide, Norflurazon, is known to block carotenoid accumulation, which in bright light results in photobleaching and the inhibition of chloroplast development. In a variety of plants that have been treated with Norflurazon, the *cab* genes are not expressed. This has led to the hypothesis that a signal originating from the chloroplast is necessary for optimal transcription or mRNA accumulation for nuclear genes encoding proteins destined for the chloroplast. Nothing is known about the nature of the signal, or the signal transduction pathway(s) involved in coupling gene expression in the chloroplast and nucleus during photosynthesis. We have used the mutagenized pOCA107-2 transgenic line to select for mutations where the *cab*3 promoter is expressed at high levels in the presence of Norflurazon. 100,000 M2 seeds, from 200 families, were grown for 10 days in bright light conditions in the presence of 5×10^{-6}M Norflurazon and 20 μg/ml hygromycin. The visual screen is for photobleached plants that look larger and healthier (i.e., hygromycin-resistant) than the pOCA107-2 control seedlings. A cotyledon was "snipped" off each seedling and stained in X-gluc to test for expression of the second *cab* promoter. Of the original 664 plants selected, 283 had high levels of GUS activity, and 92 of these survived and set seeds (M3). M3 seeds from each individual M2 putative mutant were re-screened. Progeny from thirty-six M2 plants are both hygromycin-resistant and GUS+, indicating possible trans-acting mutations. A photograph of four such GUS+ cotyledons is shown in Fig 4, page 11. Interestingly, 15 of the 36 potential mutants are pale when grown in the absence of Norflurazon, and we plan to concentrate on these during our initial genetic studies.

In summary, we have isolated mutants that have aberrant *cab* promoter expression under one of a variety of conditions, and have demonstrated the feasibility of a strategy that should be applicable to a variety of regulated promoters. We are currently analyzing these new mutants, both genetically and biochemically. First, we will establish how many complementation groups define each class of mutant; then we will determine if we have identified some of the same genes using the different strategies. Finally, we will construct double mutants between these new mutants and the previously identified *det* and *hy* mutants to determine the number and order of events involved in the early gene expression events associated with light-regulated seedling development.

Comparison of *det*1 and *det*2 Mutants

	wild-type	*det*1	*det*2
A. DARK PHENOTYPES			
Morphology:			
Leaves	Unexpanded cotyledons	Expanded cotyledons and leaves	Expanded cotyledons (I° leaf buds)
Hypocotyl	Long	Short	Short
Pigments	Absent	Anthocyanins	Anthocyanins
Chloroplasts	Undifferentiated	Some differentiation	Undifferentiated
Gene Expression:	**(% of wild-type levels in the light)**		
nuclear	1-2	25-100	10-30
chloroplast	1-2	100	10
B. LIGHT PHENOTYPES:			
Tissue-Specific Gene Expression Affected:			
	no	yes	no
Light-Dark Regulation Affected:			
	no	no	yes
Morphology:			
Leaf Number	9 ± 2	10 ± 2	19 ± 3
Bolt #	2 - 4	5 - 7	5 - 7
Days to Flower:			
	21	21	35

pOCA107



pOCA108

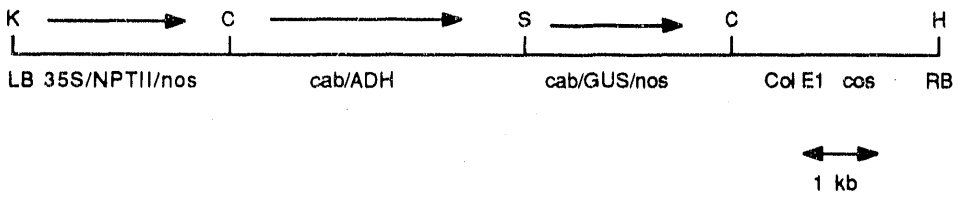


Figure 1: In pOCA107, selection will be for hygromycin resistance in the dark and subsequent screening for GUS activity. GUS stands for the gene for the B-glucuronidase enzyme from E. coli. Many colorimetric and fluorometric substrates are available which make GUS screening feasible on small amounts of tissue. In pOCA108, either a positive or negative selection can be performed for the presence or absence of the alcohol dehydrogenase (ADH) gene. As for pOCA107, screening for GUS activity subsequent to selection should help delineate promoter mutations from trans-acting mutations. In both cases, the selection for the transformation event is by selection for G418 or kanamycin resistance, which is expressed from a highly and constitutively expressed plant promoter from cauliflower mosaic virus.

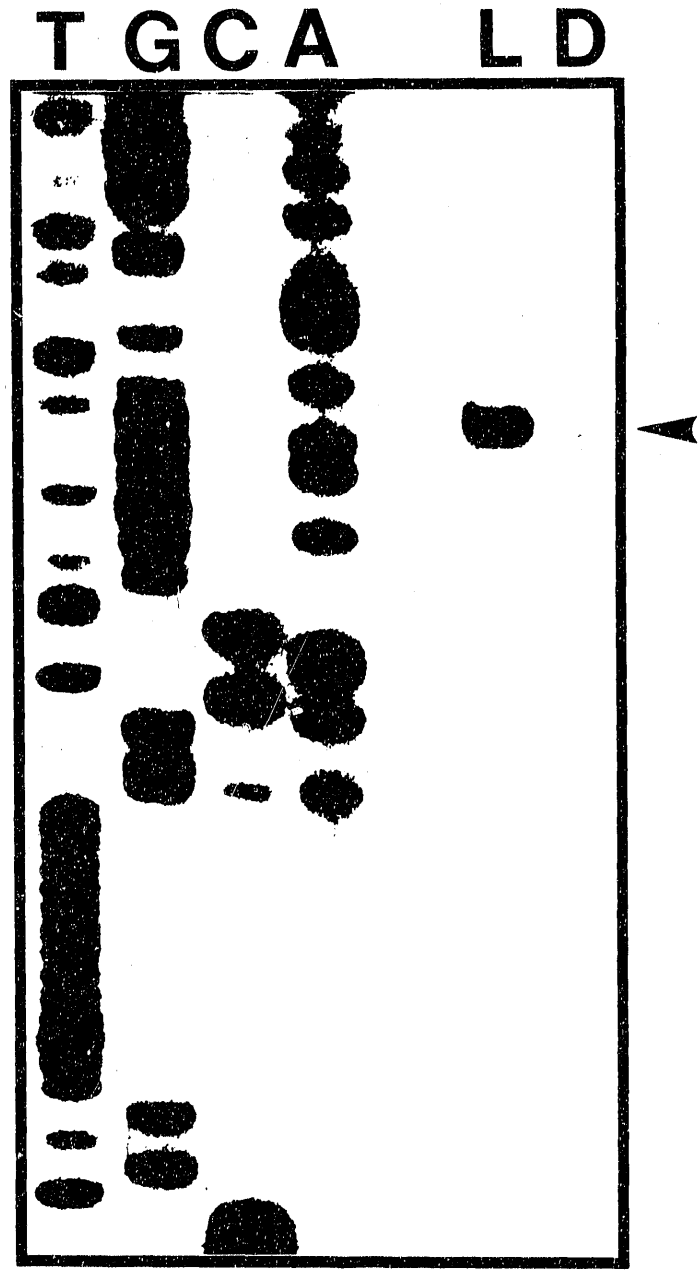


Figure 2: Light/dark regulation of the *cab*3-*hph* fusion in the pOCA107-2 transformant analyzed by primer extension analysis. The arrow indicates the transcription start site, which is the same in this fusion construction as was previously published for the intact gene.

Figure 3. Screen for Expression of *cab*-GUS in Mutant Roots. Unmutagenized control seeds were germinated and grown (at 22°C and 1-2 x 10^{16} quanta/sec/cm^2 white light) for 14 days on synthetic medium (MS salts supplemented with 2% sucrose and B5 vitamins) containing 20 μg hygromycin B/ml. Wild-type Col-O (A); pOCA107 transgenic (B); pHG1-4 transgenic (C); pHG1-4 construct is identical to pOCA107 except the *A. thaliana pal*1 promoter, which is strongly expressed in leaves and roots, replaces the *cab*3 promoter). Note the pOCA107 transgenic plant is slightly more resistant to hygromycin than the wild-type control but not nearly as resistant as the pHG1-4 transgenic plant. Seedlings then were stained for GUS activity using 1mg X-GLUC/ml (Jefferson, 1987, Plant Mol. Biol. Reporter, 5:387). Wild-type Col-O (D), pOCA107 transgenic (not shown) and pHG1-4 transgenic (F) plants were stained on day 14. Note the *pal*1-GUS transgene of pHG1-4 is strongly expressed in both leaves and roots. 14 day old plants were allowed to recover on fresh medium lacking hygromycin for several weeks, then leaf and root cuttings stained. Leaf and root cuttings from pOCA107 transgenic (E) and root cutting from one M2 mutant plant (G) are shown. Note the mutant but not the unmutagenized pOCA107 transgenic parent stains for GUS activity in the roots.

Figure 4. Mutant screen for *cab*-GUS expression in the absence of the Chloroplast Signal. Control seedlings were germinated and grown for 10 days on synthetic medium (described in figure 3 legend) containing 5 x 10^{-6}M Norflurazon (Sandoz Chemical Co.) and 20 μg hygromycin B/ml. Wild-type Col-O (A); pOCA107 transgenic (B); pHG1-4 transgenic (C). Note the complete photobleaching of all plants and the hygromycin resistance of the pHG1-4 transgenic plant. Photobleached seedlings (wild-type Col-O [D], pOCA107 transgenic [E]; pHG1-4 transgenic [F]) were then stained for GUS activity (as described in figure 3 legend). Cotyledons from M2 mutant seedlings were snipped off and stained (G). Note the staining of photobleached mutant cotyledons but not of the photobleached leaves from the unmutagenized pOCA107 transgenic parent. Expression of the *pal*1-GUS transgene of pHG1-4 is unaffected by photobleaching (compare to figure 3)

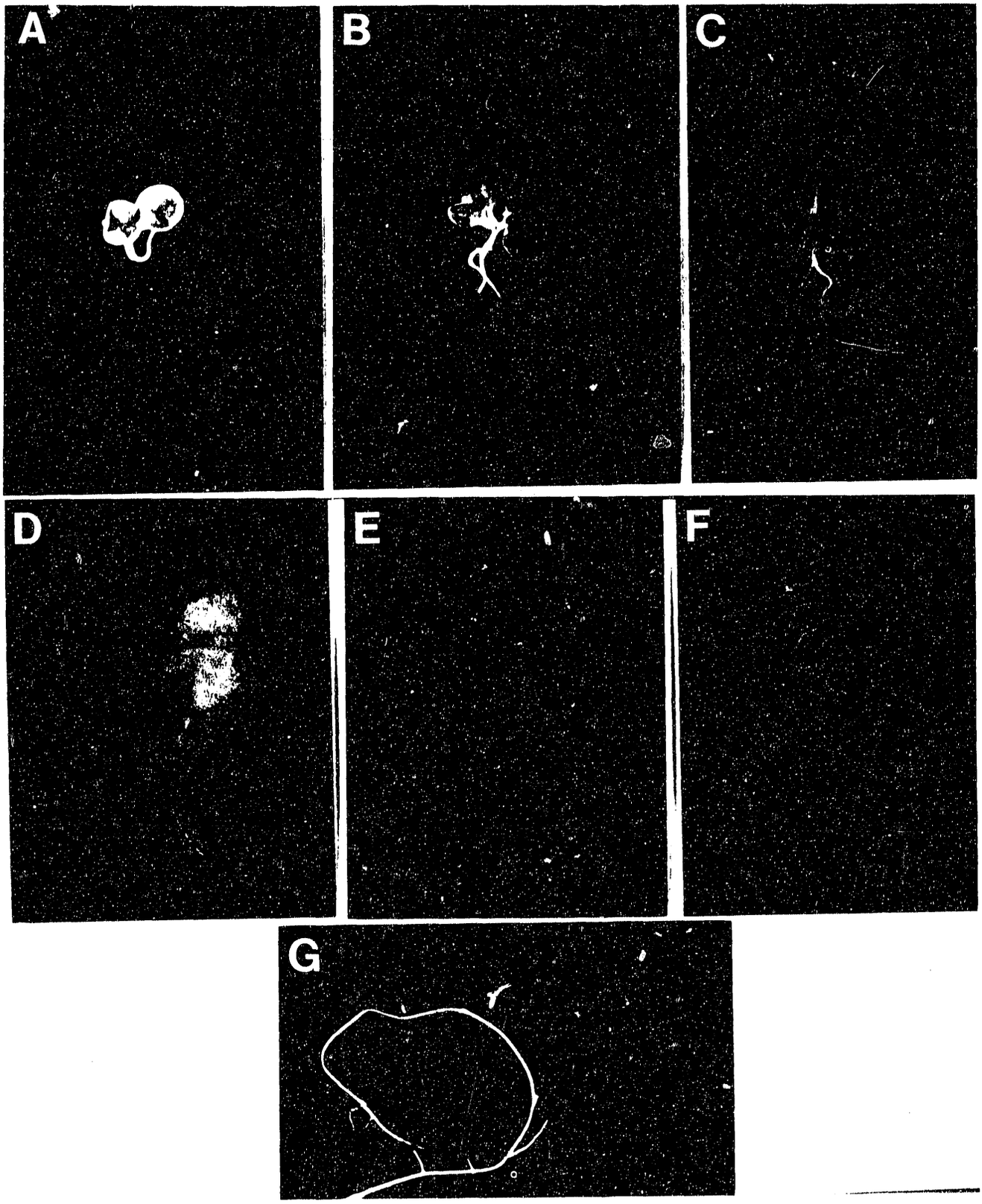


Figure 3

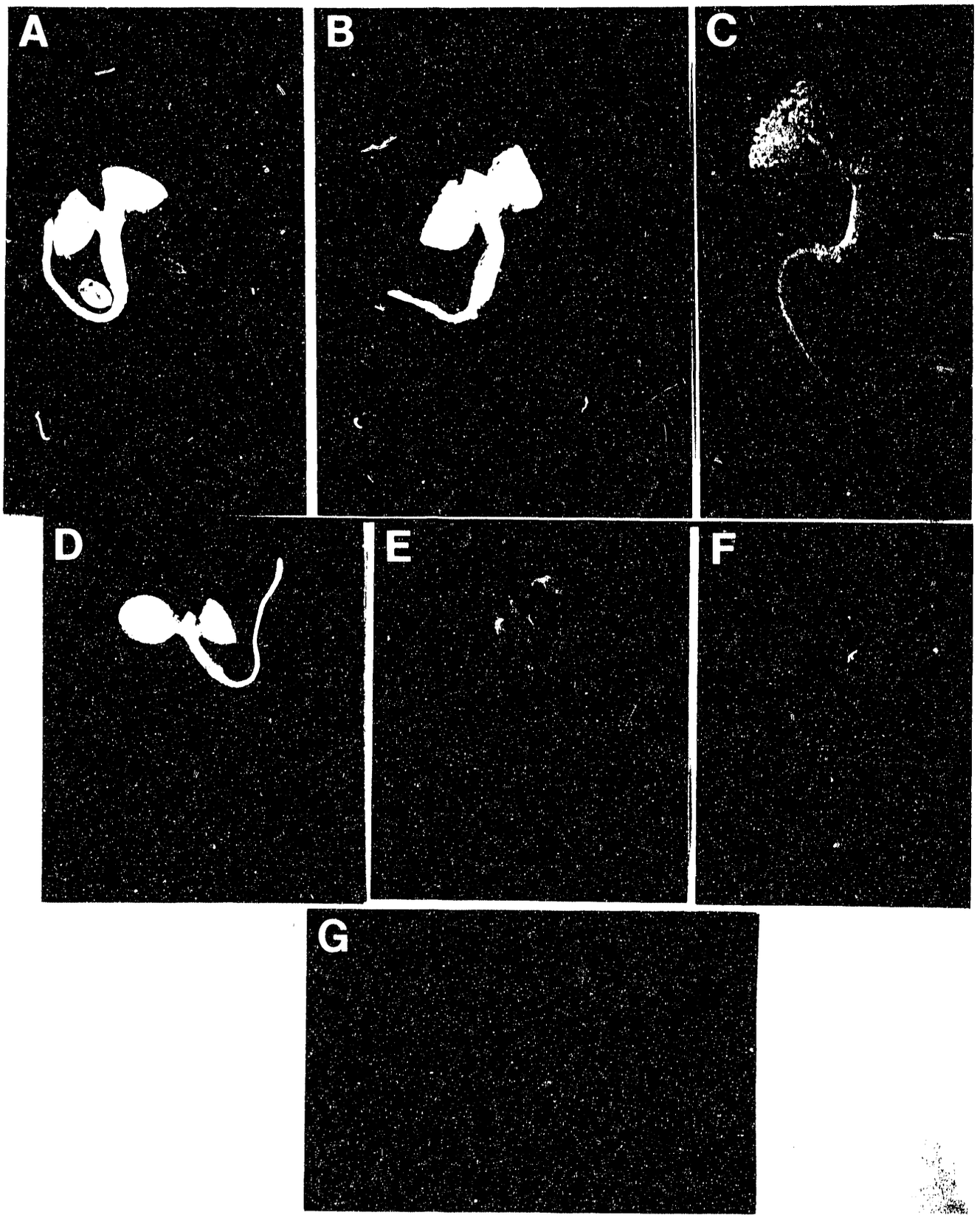


Figure 4

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