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Transcriptional and Posttranscriptional Control of Phaseolin and Phytohemagglutinin Gene Expression in Developing Cotyledons of *Phaseolus vulgaris*[1]

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ABSTRACT

The expression of phaseolin and phytohemagglutinin (PHA) in the developing cotyledons of a normal (Greensleeves) and a PHA-deficient (Pinto 111) cultivar of *Phaseolus vulgaris* was investigated. Phaseolin mRNA translational activity and abundance were present at similar levels in both cultivars. In contrast, PHA mRNA translational activity and abundance in Pinto 111 were less than 1% of the levels measured in Greensleeves. Using nuclear runoff assays, the transcription rate of phaseolin gene sequences was similar in both cultivars. The transcription rate of PHA gene sequences in Pinto 111 was only 20% of that measured in Greensleeves. Comparison of the transcription rates with the relative mRNA amounts measured in RNA blot hybridizations indicated that the normally expressed storage protein gene mRNAs were very stable with half-lives greater than several days. Because a low level of PHA gene transcription in Pinto 111 was measurable but no PHA mRNA accumulated, these results suggest that the PHA deficiency in Pinto 111 is due to a reduced transcription rate and possibly an instability of the mRNA.

The development of legume cotyledons is accompanied by the synthesis and deposition of large amounts of storage protein and lectin in the protein bodies of the storage parenchyma cells (15). In the common bean, *Phaseolus vulgaris*, the major storage protein is the 7S globulin phaseolin. This trimeric protein which accounts for 50% of the total seed protein has polypeptides with M_r as 47,000 to 50,000 (2) and is encoded by a small gene family (22). The lectin of the common bean, called phytohemagglutinin, makes up 5 to 10% of the total seed protein; it is a tetrameric protein that has polypeptides with M_r 32,000 to 34,000 (2) encoded by several genes (12).

The regulation of storage protein synthesis in developing cotyledons has recently been investigated with the aid of cloned DNA fragments (cDNA or genomic) containing the coding sequences for these proteins (20, 21). RNA blot hybridizations have been used to show that the abundance of storage protein mRNA increases soon after cotyledon development begins and decreases when the seeds enter the drying-off stage. The mRNAs are readily detectable throughout cotyledon development because they represent a considerable proportion of the total poly-A$^+$ mRNA. These mRNAs are known to be associated with polysomes which are translationally active (3).

Recently, the transcription rate of the globulin gene sequences in nuclei isolated from developing pea cotyledons has been studied (1, 9). These reports correlate the level of the globulin mRNAs with the transcription rate of the respective genes, and present evidence for posttranscriptional as well as transcriptional control of the mRNA levels.

Another dimension in the regulation of seed protein accumulation can be studied by examining cultivars which lack specific proteins. For example, approximately 10% of the cultivars of *P. vulgaris* examined have no or very low levels of hemagglutinin activity and equally low levels of PHA[3] (5, 6, 18). Explanations for these deficiences include loss of the structural gene(s), or transcriptional or translational inactivation of gene expression. In this regard, a soybean lectin minus cultivar was shown to have a reduced transcription rate (10) apparently due to the insertion of a putative transposable element (23).

In our efforts to characterize a lectin-minus cultivar of *P. vulgaris* (Pinto 111), we have demonstrated that little or no *in vivo* synthesized PHA is detected when developing cotyledons of Pinto 111 are supplied with radioactive amino acids (20). Using a cDNA clone for the PHA mRNA from a lectin containing cultivar (Greensleeves), the PHA mRNA in Pinto 111 cotyledons was shown to be less than 1% that found in Greensleeves (20). Subsequent genomic cloning and sequencing indicates that Pinto 111 does contain PHA genes without any major structural rearrangements (P Staswick, T Voelker, MJ Chrispeels, unpublished data).

We now provide further evidence that the PHA mRNA translational activity and abundance are at very low levels at all times during the development of Pinto 111 cotyledons relative to Greensleeves cotyledons. In contrast, the phaseolin polypeptides and mRNAs are present in both cultivars at similar levels. Using nuclear runoff transcription assays, the transcription rate of phaseolin gene sequences is similar in both cultivars; however, the transcription rate of the PHA gene sequences in Pinto 111 is less than one-fifth of that measured in Greensleeves. Finally, a comparison of the changes in mRNA amounts with changes in the transcription rate of the respective genes during cotyledon development indicates that storage protein and lectin gene expression is under transcriptional and posttranscriptional regulation.

MATERIALS AND METHODS

Plant Material. The two cultivars of *Phaseolus vulgaris*, Greensleeves and Pinto, were grown in a greenhouse with natural lighting. Bean pod development in both cultivars took approxi-

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[3] Abbreviation: PHA, phytohemagglutinin.

mately 5 weeks from the time of flowering to the mature, dry bean. In the present study, we were interested in the midstage of development from the start of storage protein accumulation to the period of rapid water loss. We arbitrarily choose six stages of development based on cotyledon fresh weight to represent the sequence of events. Stage 1 is just before protein accumulation begins, 10 to 25 mg/cotyledon. Stages 2, 3, 4, and 5 represent various times in the rapid accumulation phase, respectively, 50 to 100, 150 to 225, 250 to 350, and 400 to 550 mg/cotyledon. Stage 6 is just as water loss begins, approximately 4 to 4.5 weeks after flowering. These cotyledons typically weigh more than 550 mg. The Pinto 111 seeds used were 20% smaller than Greensleeves. The developmental sequence of both cultivars was slower in winter than in summer.

RNA Isolation, *in Vitro* Translation and Immunoprecipitation. Total RNA was isolated from 1 to 3 g of cotyledons (20) and 10 to 40 μg RNA used in a micrococcal nuclease-treated rabbit reticulocyte *in vitro* translation system (BRL). Equal amounts of *in vitro* synthesized radioactive products were analyzed directly by SDS-PAGE or challenged with anti-PHA (3) or antiphaseolin antisera (3). Immunoprecipitates were absorbed to protein A-sepharose, washed, released, and analyzed after SDS-PAGE by fluorography (4, 14).

RNA Blot Hybridization. Total RNA (3–15 μg) was separated on 1.2% agarose-formaldehyde gels and blot transferred to nitrocellulose according to Maniatis *et al.* (17). Filters were hybridized with nick translated probes (17) at 0.5×10^6 cpm/ml in 50% formamide, 5 × SSPE, 0.5% SDS, 10 μg/ml tRNA 10 μg/ml herring sperm DNA, and 0.02% each of Ficoll (polyvinylpyrrolidone), and BSA. 20 × SSPE contains 3 M NaCl, 0.2 M NaH_2PO_4 (pH 7.4), 20 mM EDTA. The specific activities of the hybridization probes were greater than 1×10^8 cpm/μg DNA. Hybridizations were for 12 h at 37°C. Hybridization probes included a PHA-specific cDNA (pSC-1) (20), a phaseolin genomic clone (p 3.8.177.4) (19) provided by S. Slightom of Agrigenetics Corp., and a Dictyostelium ribosomal genomic cluster (8) provided by R. Firtel of the University of California, San Diego. Filters were then washed and hybridizations visualized by autoradiography as previously described (20). Autoradiograms were scanned with a Joyce-Loeb densitometer, peak areas integrated by a cut-and-weigh procedure, and the data normalized with the heaviest band being 1.

Nuclei Isolation and Runoff Transcription Assay. Cotyledons (5–20 g fresh weight) were powdered in liquid N_2. All the following procedures were performed at 4°C. The powdered tissue was homogenized for 40 to 60 s in a Brinkman Polytron in 25 mM Tris (pH 8.0), 10 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 10mM 2-mercaptoethanol, 0.5% (w/v) Ficoll-400, 2% (w/v) Dextran-70, 0.6% (w/v) Triton X-100, and 0.44 M sucrose. The homogenate was filtered through a 70/40 μm nylon mesh sandwich and the starch grains and nuclei collected by centrifugation at 1000 *g* for 10 min. The resulting pellet was resuspended with 25 mM Tris (pH 8.0), 10 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 10 mM 2-mercaptoethanol, 0.134 M sucrose, 85% (v/v) Percoll, transferred to a clean centrifuge tube, overlayered with homogenization buffer and centrifuged at 8000*g* for 30 min. The nuclei floated to the homogenization buffer-Percoll interface. The collected nuclei were diluted with homogenization buffer, concentrated by centrifugation at 1000*g* for 5 min, and the pellet washed twice with 50 mM Tris (pH 7.8), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 20% (v/v) glycerol. The final pellets were resuspended with the same buffer at 5 to 50 μg DNA/ml. As determined by microscopic examination, this procedure does not differentiate between nuclei and chromatin and, depending on the cotyledons' developmental stage, the proportion of intact nuclei varied.

The runoff transcription assays (7) were performed in 100 to 250 μl reaction volumes containing 75 mM $(NH_4)_2SO_4$, 8 mM $MgCl_2$, 0.5 mM each GTP, CTP, and ATP, nuclei equivalent to 10 to 50 μg DNA and 100 to 200 μCi:[^{32}P]UTP (400 Ci/mmol). After incubation for 10 min at room temperature, 2 μg DNase (DPFF, Worthington) plus 100 μg tRNA were added and incubated for 5 min more. The reaction mixture was made to 0.5 mM EDTA, 1% SDS, and 20 μg proteinase K added for a further 15 min. The mixture was then extracted two times with phenol:$CHCl_3$ (1:1), two times with $CHCl_3$, and the RNA precipitated with ethanol. The pelleted RNA was washed two times with 3 M sodium acetate, two times with 70% ethanol, and finally resuspended in hybridization buffer. The [^{32}P]RNA was hybridized to DBM filters containing probes specific for PHA, phaseolin, and ribosomal RNA as previously described (7).

RESULTS

Changes in Phaseolin and PHA mRNA Translational Activities during Cotyledon Development. During the midmaturation phase of cotyledon development there is a greater than 100-fold accumulation of protein per cotyledon. Upwards of 50% of the total protein at the later stages of development can be attributed to the phaseolin storage polypeptides and approximately 10% to the lectin PHA (2). To investigate the mechanisms controlling the accumulation of these proteins and to identify the lesion(s) in expression of the PHA genes in Pinto 111, we first analyzed the translational activity of RNA isolated from various stages of cotyledon development. Figure 1 shows fluorographs of the total *in vitro* synthesized proteins (A) as well as those *in vitro* synthesized proteins adsorbed by phaseolin (B) or PHA (C) antisera.

The total *in vitro* synthesized protein pattern for Greensleeves (Fig. 1, upper panel) shows prominent changes for proteins of M_r 42,000 to 46,000 (putative phaseolin) and M_r 30,000 to 32,000 (putative PHA). These polypeptides are indeed phaseolin and PHA as demonstrated by their adsorption to the respective antibodies (Fig. 1, B and C). Also notable is the increase in both phaseolin and PHA mRNA translational activities up to stage 3 after which the translational activities remain constant.

The pattern of total *in vitro* synthesized proteins for Pinto 111 (Fig. 1, lower panel) is similar to that of Greensleeves. The most striking exception is the absence of polypeptides of M_r 30,000 to 32,000, although there is an abundant polypeptide with M_r 29,000, just as in Greensleeves. The reaction of the *in vitro* synthesized proteins with antisera against the phaseolin and PHA protein (Fig. 1, B and C) shows that Pinto 111 contains very little mRNA which can be translated into polypeptides which bind to antibodies against PHA.

The phaseolin polypeptides of Pinto 111 are of similar size and show a similar pattern of accumulation to those of Greensleeves. However, the double band migrates closer together indicating less mol wt difference. In quantitative evaluations, the amount of phaseolin encoded by RNA from cotyledons of Greensleeves and Pinto was comparable as determined by immunoprecipitation of radioactivity. The *in vitro* synthesized phaseolin accounted for more than 20% of the total *in vitro* translational activity in both cultivars at stage 3 of cotyledon development and later.

Very little *in vitro* synthesized protein encoded for by Pinto 111 RNA was precipitated with antibodies against PHA (Fig. 1C, lower panel), while significant amounts of PHA were encoded by Greensleeves mRNA (Fig. 1C, upper panel). As determined by immunoprecipitation of radioactivity, upwards of 15% of the total *in vitro* synthesized proteins encoded for by Greensleeves RNA can be attributed to PHA. In the case of Pinto 111, less than 0.5% of the total translational activity was devoted to PHA synthesis.

Phaseolin and PHA mRNA Levels in Developing Cotyledons. The level of mRNAs coding for storage proteins in several

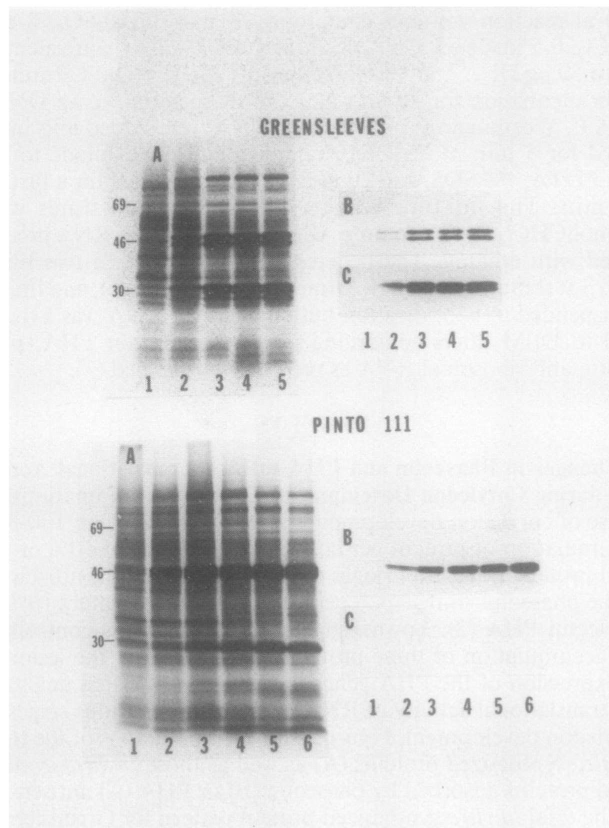

FIG. 1. Comparison of the *in vitro* translation products of RNA isolated from Greensleeves and Pinto 111 cotyledons. RNAs from various stages of cotyledon development of Greensleeves (upper panel) or Pinto 111 (lower panel) were translated *in vitro* in the presence of [^{3}H]leucine. Aliquots of equal radioactivity were challenged with phaseolin-specific or PHA-specific antisera. Total *in vitro* translation products (A), and antibody-precipitable phaseolin (B) and PHA (C) were separated by SDS-PAGE and visualized by fluorography. Panels B and C are portions of gels positioned relative to panel A to the correct molecular mobility of the precipitated antigens. Numbers underneath the panels refer to the stages of cotyledon development and those along the side refer to mol wt markers, $M_r \times 10^{-3}$.

legumes has previously been shown to increase during cotyledon development. The low level of Pinto 111 RNA translational activity for PHA therefore indicated that the level of PHA mRNA may be reduced in these cotyledons. Hybridization probes specific for PHA (20) and phaseolin (19) mRNAs were then used to determine the respective mRNA levels. Figure 2 shows the RNA blot analysis of RNA isolated from different stages of cotyledon development for Greensleeves and Pinto 111 and hybridized with the phaseolin and PHA probes.

The phaseolin mRNA levels in both cultivars increased during the early stages of development after which the phaseolin mRNA level was rather constant. RNA samples of 3 μg were analyzed for both cultivars and the blots where hybridized together with nick-translated probe. Assuming equal hybridization efficiency of the probe to the phaseolin mRNAs, the relative abundance of phaseolin mRNA in the two cultivars appears similar.

The patterns of hybridization with the PHA probe varied between the cultivars. In Greensleeves, the PHA mRNA accumulated in a similar manner as the phaseolin mRNA. In contrast, the hybridization pattern of Pinto 111 RNA was much weaker and showed a transient induction of hybridizing material with a maximum between stages 3 and 4 of development. To obtain the weak signal shown in Figure 2, 5 times as much Pinto 111

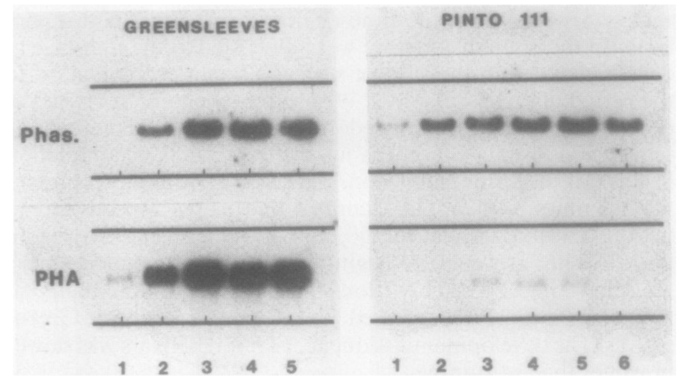

FIG. 2. Comparison of phaseolin and PHA mRNA levels in developing cotyledons of Greensleeves and Pinto 111. RNA isolated from different developmental stages (1–6) was size-separated on formaldehyde-agarose gels, transferred to nitrocellulose, and probed with either a phaseolin genomic clone or a PHA cDNA clone. All RNA samples were 3 μg except for 15 μg samples for Pinto 111 RNA hybridized with the PHA probe.

RNA (15 μg samples) was used as for Greensleeves (3 μg samples).

The Pinto 111 RNA hybridizing to the PHA probe is not likely to be PHA mRNA, but a closely related mRNA coding for a lectin-like protein (11). Upon rehybridization of the Pinto blot with the lectin-like specific probe, no additional bands appeared, only the intensity of the present one increased. Previously, *in vitro* translation of Pinto 111 mRNA hybrid-selected with the lectin-like cDNA clone was shown to encode a protein of lower mol wt than authentic PHA, and which had very low cross-reactivity with the anti-PHA antibodies (20). Additionally, the lectin-like mRNA is smaller than the true PHA mRNA and they separate from one another under the conditions used here. The Pinto 111 RNA hybridizing to the PHA and lectin-like cDNA probes is not the correct size for authentic PHA mRNA.

***In Vitro* Transcription Rate of Phaseolin and PHA Gene Sequences.** We performed nuclear runoff experiments to determine the transcription rate of phaseolin and PHA gene sequences. We were not successful with previously published techniques for the isolation of nuclei from cotyledons (1, 9, 16) and found it important to separate nuclei from starch grains; starch grains apparently inhibited *in vitro* transcriptional activity. Also, the procedure described is more general for the isolation of nuclei and chromatin. The nuclear preparations incorporated [^{32}P]UTP continuously into TCA precipitable products for 20 min at rates of 100 to 200 pmol UMP/10 min·10μg DNA. Incorporation was inhibited 57% by 10 μg/ml of α-amanitin.

Table I compares the transcription rates of rDNA, phaseolin, and PHA gene sequences in nuclear preparation from Greensleeves and Pinto 111 cotyledons. Ribosomal DNA sequences were transcribed at a much higher rate (greater than 45-fold) than either phaseolin or PHA gene sequences. Between the two cultivars, ribosomal RNA and phaseolin RNA synthesis rates were comparable. However, the transcription rate of PHA gene sequences in nuclear preparations from Pinto 111 cotyledons was only one-fifth of that measured in Greensleeves' nuclear preparations.

In further experiments, the transcription rates of phaseolin and PHA gene sequences were measured in nuclear preparation from various stages of cotyledon development of both cultivars. Figure 3, B and D, shows the results of those experiments and, for comparison, the changes in the respective mRNA amounts determined in Figure 2 are also shown (Fig. 3, A and C). The transcription rate of phaseolin and PHA gene sequences in nuclear preparations from Greensleeves cotyledons showed a similar pattern of change. The transcription rates were high at

Table I. *Comparison of the In Vitro Transcription of Ribosomal, Phaseolin, and PHA Gene Sequences in Nuclear Preparations from Developing Cotyledons of the Pinto 111 and Greensleeves Cultivars*

Cultivar[a]	*In Vitro* Transcripts Hybridizing (ppm) to Cloned DNA for		
	rDNA	Phaseolin	PHA
Greensleeves	2760	59	36
Pinto	2041	75	7

[a] Cotyledons of similar developmental stage were used for nuclear preparations. Nascent chains of RNA were completed *in vitro* in the presence of [^{32}P]UTP for 10 min, the radiolabeled RNA purified and hybridized to DBM filters containing either cloned rDNA, phaseolin, or PHA sequences. A control filter containing λ and pBR322 DNA was included for determination of background hybridization. Specific hybridization is presented as ppm of input RNA and has been corrected for background hybridization. Independent hybridizations were with 5×10^6 and 1×10^6 cpm and background hybridizations were 180 and 100 cpm, respectively. The amount of specific hybridization was proportional and linear with respect to input RNA. No corrections for hybridization efficiency or probe size were made.

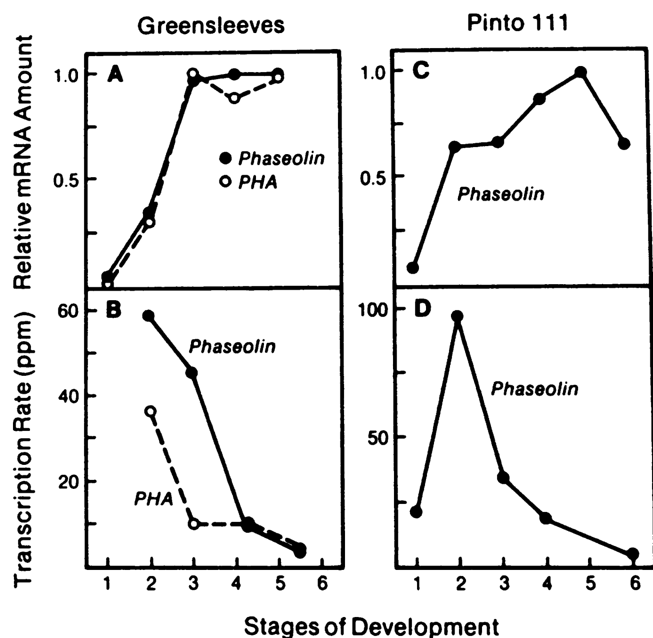

FIG. 3. Comparison of the mRNA levels and transcription rate of phaseolin and PHA gene sequences in developing cotyledons of Greensleeves and Pinto 111. Relative changes in the mRNA amounts were measured by densitometry of the autoradiograms shown in Figure 2. The transcription rate of phaseolin and PHA gene sequences in nuclear preparations from the indicated stages of cotyledon development were determined as described in Table I. Specific hybridization is presented as ppm of input RNA (1 and 5×10^6 cpm) and has been corrected for background hybridization (50–200 cpm). The amount of specific hybridization was proportional and linear with respect to input RNA. No corrections for hybridization efficiency or probe size were made.

the early stages of development and declined to very low rates by the latter stages.

Changes in the transcription rate of phaseolin gene sequences in nuclear preparations of Pinto 111 cotyledons were similar to those of Greensleeves. However, the transcription rate of PHA gene sequences was very low at all stages of Pinto 111 cotyledon development. These runoff assays were dependent on a RNA to DNA probe hybridization step which was routinely performed at two concentrations of *in vitro* synthesized RNA. The data for the transcription rate of PHA gene sequences in Pinto 111 were barely above background and difficult to determine with accuracy; therefore, they are not plotted in Figure 3D. Nevertheless, the maximum rate of PHA gene transcription measured was 10 to 14 ppm at stage 2 of cotyledon development.

The transcription rate of ribosomal DNA was also measured for both cultivars in the experiment depicted in Figure 3. Ribosomal gene transcription showed an overall pattern similar to phaseolin with maximum rates of 4,000 ppm at stage 2, declining to 900 ppm by stage 5.

DISCUSSION

The developing seed constitutes an excellent system to study the temporal regulation of gene activity and gene product accumulation because a few proteins accumulate to very high levels. In addition, specific cultivars may lack certain polypeptides or proteins. We report here data on the accumulation of mRNA translational activity, mRNA abundance, and the transcription rate of specific genes for the two major proteins phaseolin and PHA in a normal and a PHA-deficient cultivar of the common bean.

Both cultivars accumulate similar amounts of phaseolin protein, and have similar amounts of phaseolin mRNA and transcription rates of the phaseolin genes. Cotyledon development is characterized by a rapid increase and subsequent decline in the transcription rates. The decline is accompanied by the maintenance of the mRNA levels, indicating that mRNA abundance is not only regulated at the level of transcription (the initial increase) but also by posttranscriptional events. Alternatively, the measured decline in the transcription rates could be a reflection of an increasing, nuclear localized, RNA processing (turnover) activity at the later stages of development. No matter, the cytoplasmic levels of the storage protein gene mRNAs remain relatively constant at the later stages of cotyledon development, and have apparent half-lives greater than several days.

The Pinto 111 cultivar has very low levels of PHA protein (2% of normal), and equally low levels of mRNA as measured by RNA blot hybridization or *in vitro* translation of mRNA. Nuclear runoff experiments show, however, that the transcription of PHA genes in Pinto 111 may be as high as 20% of the transcription rate measured in Greensleeves at the time when the transcription rates are highest (stage 2). This may be an overestimation of the true transcription rate of the PHA genes in Pinto 111. Because both cultivars contain other lectin-like genes with sequence homology to the PHA genes (12), we are not able to exclude the possibility that some of the nuclear runoff transcripts measured for Pinto 111 are derived from the other lectin-like genes. We did however use hybridization conditions which were previously shown to minimize cross-hybridization (20). Nevertheless, the present results indicate that the failure of Pinto 111 to accumulate PHA is due to a much lowered transcription rate of the PHA gene(s), and possibly an instability of the Pinto 111 PHA mRNA.

Recent DNA sequence analysis of the Pinto 111 PHA genes has shown that these genes are structurally very similar to the genes from normal cultivars (P Staswick, T Voelker, MJ Chrispeels, unpublished data). Hence, nucleotide sequence analysis and comparison may not be sufficient to identify the mechanism(s) responsible for low levels of PHA expression in Pinto 111. However, a transformation analysis comparing the expression of the Greensleeves and Pinto 111 PHA genes in yeast and higher plants may help in defining those regions of the PHA gene responsible for transcriptional control and mRNA stability.

During preparation of this manuscript, Horowitz (13) published results on the PHA protein level in Pinto 111 consistent with our results. Using a lectin-like cDNA probe Horowitz also

noted a decreased level of the corresponding mRNA in Pinto 111, inconsistent with our previous results (20).

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