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Expression Analysis of a Pseudogene in Transgenic Tobacco: A Frameshift Mutation Prevents mRNA Accumulation

Toni A. Voelker,[1] Joaquin Moreno,[2] and Maarten J. Chrispeels[3]

Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0116

Seeds of the Pinto cultivar of the common bean, *Phaseolus vulgaris*, are deficient in phytohemagglutinin (PHA), a lectin normally composed of two different polypeptides (PHA-E and PHA-L). In Pinto seeds, there is no PHA-E and only small amounts of PHA-L. The gene coding for the Pinto PHA-E, *Pdlec1*, is a pseudogene as a result of a single base pair deletion in codon 11, causing a frameshift and premature termination of translation. This mutation explains the absence of the PHA-E polypeptide but not the several-hundredfold reduction of the cytoplasmic *Pdlec1* mRNA in developing seeds when compared with a normal PHA-E gene. To find the cause for this reduction in mRNA levels, we swapped gene fragments of *Pdlec1* with the homologous parts of a normal PHA gene from the cultivar Greensleeves and introduced these fusions into tobacco. Analysis of the transgenic seeds showed that the *Pdlec1* promoter is fully functional. We also repaired the *Pdlec1* coding frame in vitro and inserted the repaired and unrepaired versions into a PHA gene expression cassette. In transgenic tobacco, both constructs showed *Pdlec1* transcript accumulation in the second half of seed maturation. The single-base frame repair boosted the peak transcript levels by a factor of 40 and resulted in the synthesis of PHA-E at normal levels. We propose that the premature translational stop caused by the frameshift leads to a faster breakdown of the *Pdlec1* mRNA, thereby preventing this transcript from accumulating to high levels.

INTRODUCTION

Seed development is characterized by the accumulation to high levels of a small number of seed proteins, such as storage proteins, lectins, and enzyme inhibitors (Higgins, 1984). In several species, a number of cultivars have been found to be defective in the expression of specific seed protein genes, and the molecular lesions of some of these have been identified (Goldberg, Barker, and Perez-Grau, 1989). To understand how the expression of seed protein genes is regulated, we have characterized the genes for phytohemagglutinin (PHA), the lectin of the common bean, and studied their expression (Staswick and Chrispeels, 1984; Chappell and Chrispeels, 1986; Voelker, Staswick, and Chrispeels, 1986; Voelker, Sturm, and Chrispeels, 1987; Riggs, Voelker, and Chrispeels, 1989). PHA is a tetrameric glycoprotein made up of two polypeptides, PHA-E and PHA-L, with M_r 34,000 and 36,000, respectively; these polypeptides are encoded by two tandemly linked genes, *dlec1* and *dlec2*, respectively, that show a high degree of sequence identity (Hoffman and Donaldson, 1985).

A number of bean cultivars have been identified that are deficient in PHA accumulation (Brücher, 1968). For example, the Pinto cultivar contains very low levels of PHA-L (about 5% the normal level) and no PHA-E (Pusztai, Grant, and Stewart, 1981; Vitale, Ceriotti, and Bollini, 1985). We have previously analyzed the Pinto PHA genes, called *Pdlec1* and *Pdlec2*, and studied their expression during seed development (Voelker et al., 1986). In the *Pdlec2* gene, which encodes a PHA-L polypeptide, a quantitative element has been deleted from the promoter, resulting in a down-regulation of the mRNA and protein levels, compared with a normal cultivar such as Greensleeves (Voelker et al., 1987; Riggs et al., 1989). The *Pdlec1* gene that encodes a PHA-E polypeptide is a pseudogene that has a single-base pair deletion in codon 11. This deletion results in the translational shift into another frame leading into a stop at codon 53. Only a truncated, aberrant, 52-amino acid polypeptide could be synthesized instead of the 275-amino acid full-length PHA molecule. This explains the lack of PHA-E in Pinto seeds. Besides this frameshift, the nucleotide sequence of *Pdlec1* is more than 90% identical to a normal PHA-E allele, including the 5′ promoter region and the poly(A) signal 3′ of the coding frame. No other obvious lesions could be found in the pseudogene. With gene-specific probes, we found in Pinto cotyledons a 600-

[1] Current address: Calgene, 1920 Fifth St., Davis, CA 95616.
[2] On leave from Departament Bioquimica i Biologia Molecular, Universitat de Valencia, Burjassot, 46100, Spain.
[3] To whom correspondence should be addressed.

fold reduction in cytoplasmic poly(A) RNA for PHA-E, compared with the normal cultivar Greensleeves (Voelker et al., 1986). According to this description, *Pdlec1* is comparable with the human ζ-globin pseudogene, which is near identical to its functional neighbor, but has its sixth codon mutated to a chain termination signal (Proudfoot, Gill, and Maniatis, 1982). In this case, after a conversion to a functional frame, no transcripts could be detected in vivo, i.e., this pseudogene must carry additional regulatory mutations besides the nonsense mutation (Hill et al., 1985). Recently, Jofuku, Schipper, and Goldberg (1989) published a detailed study of a soybean trypsin inhibitor pseudogene and found that greatly reduced levels of poly(A) mRNA in vivo were accompanied by near-normal rates of transcription in isolated nuclei. They interpret their findings as indicating that the frameshift mutation results in mRNA destabilization and leads to reduced accumulation levels.

Previously, we have shown that a PHA gene from the normal cultivar Greensleeves is expressed in a conserved manner in the seeds of transgenic tobacco (Voelker et al., 1987). This allowed us to take the approach of functionally testing the expression of the *Pdlec1* pseudogene in this heterologous system. We find its promoter to function normally. The repair of the PHA-E coding frame of the *Pdlec1* pseudogene results in the accumulation of much higher levels of mRNA in the seeds of transgenic tobacco.

RESULTS

The observations (Jofuku et al., 1989) that a pseudogene may be transcribed at a high level and yet have a low level of transcript accumulation in the cytoplasm led us to determine whether the significant reduction of transcript levels of the pseudogene *Pdlec1* was caused solely by the frameshift mutation or whether yet undetected promoter mutations or a genomic position effect was responsible. By using RNase protection assays, we compared the gene-specific levels of *Pdlec1* and *Pdlec2* transcripts in isolated nuclei and total tissue extracts of developing Pinto cotyledons (50-mg stage; see Methods for details). At this stage, PHA gene expression is close to its maximum (T. A. Voelker, unpublished data). We found that, in 10 μg of total RNA, the transcript level of *Pdlec1* (700 cpm of protected RNA) was threefold less than that of *Pdlec2* (2100 cpm protected RNA). As for isolated nuclei, in 10 μg of RNA we obtained a *Pdlec1* signal twice as large (900 cpm) as the *Pdlec2* signal (500 cpm). Thus, the relative level of *Pdlec1* (the pseudogene) transcripts drops sixfold when one compares a nuclear extract with a total cell extract and normalizes with respect to *Pdlec2* transcripts. This indicates that the *Pdlec1* pseudogene is transcribed more rapidly than the accumulation of the poly(A) mRNA transcripts in the cytoplasm would indicate. That result is consistent with the observation of Jofuku et al. (1989) on the soybean trypsin inhibitor pseudogene.

Strategy of PHA Gene Swap

To dissect the *Pdlec1* pseudogene and analyze the influence of each part on gene expression, we swapped gene fragments between *Pdlec1* and the fully functional PHA-L gene *dlec2* obtained from the cultivar Greensleeves. The hybrid genes were introduced into tobacco and gene expression was measured in seeds. Our strategy of swapping DNA segments was facilitated by the fact that the intron-free PHA coding regions of all four known genes are flanked by stretches of completely conserved sequences, which contain sites for the restriction enzymes BsmI (5′) and XbaI (3′), as shown in Figure 1. This situation allowed us to swap fragments at strategic points between PHA genes without altering the sequences at the splicing points, thereby eliminating the possibility of side effects due to recombination in vitro. Using these enzyme restriction sites, we created seven hybrid genes, five of which are shown in Figure 1, together with the two genes, *dlec2* and

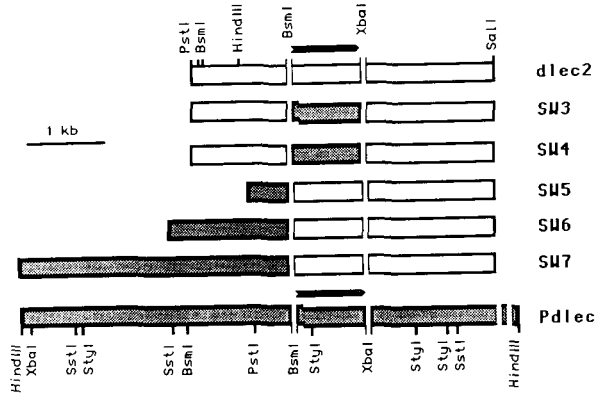

Figure 1. Scheme of the Swap Constructs.

The top bar (open) represents the restriction enzyme map of a 3.4-kb PstI/SalI fragment carrying the PHA-L gene *dlec2* of *Phaseolus vulgaris* cv Greensleeves (Hoffman and Donaldson, 1985; Voelker et al., 1987). The extent of coding sequence (816 bp, no introns) is shown by the rightward-pointing solid arrow-bar. The bottom bar (shaded) represents the major portion of the 7.5-kb HindIII fragment (Voelker et al., 1986) that carries the pseudogene *Pdlec1* from *P. vulgaris* cv Pinto. This gene carries a coding region (rightward-pointing arrow-bar) of 824 bp that has no introns and would be a PHA coding sequence if it were not interrupted by a single-base pair deletion in the 11th codon (shown by the break in the bar). The coding regions of *dlec2* and *Pdlec1* are delimited by BsmI and XbaI sites, which are shown as interruptions. BsmI is located at −12 relative to the translation start codon and XbaI covers the translation stop at the end of the coding region. The structure of all chimeric genes, SW3 to SW7, is depicted as assemblies of portions of the two parent genes. Repair of the coding sequence of *Pdlec1*, as shown in SW4, was achieved by swapping the otherwise identical region between the BsmI and StyI restriction sites of the two Pinto PHA genes (see Methods).

Pdlec1, from which the fragments were obtained. The hybrids SW3 and SW4 were created to study the effect of the frameshift mutation. SW3 consists of the coding sequence of *Pdlec1* with the 5′ and 3′ sequences of *dlec2* as an expression cassette. SW4 is identical to SW3 except that the frameshift mutation has been repaired. This was accomplished by exchanging the 119-bp BsmI/StyI fragment at the 5′ end of the coding region of *Pdlec1* and replacing it with the homologous fragment from *Pdlec2*, the PHA-L allele of Pinto. These two fragments differ only by the single base pair responsible for the frameshift in *Pdlec1* (Voelker et al., 1986). This replacement repairs the PHA reading frame of *Pdlec1* and allows the translation of a polypeptide of 275 amino acids closely related to PHA-E. The constructs SW5, SW6, and SW7 were designed to test the 5′ upstream region of *Pdlec1* and its ability to drive the expression of the intact coding region of *dlec2*. The reading frame and 3′ region of *dlec2* serve as the reporter gene in these constructs.

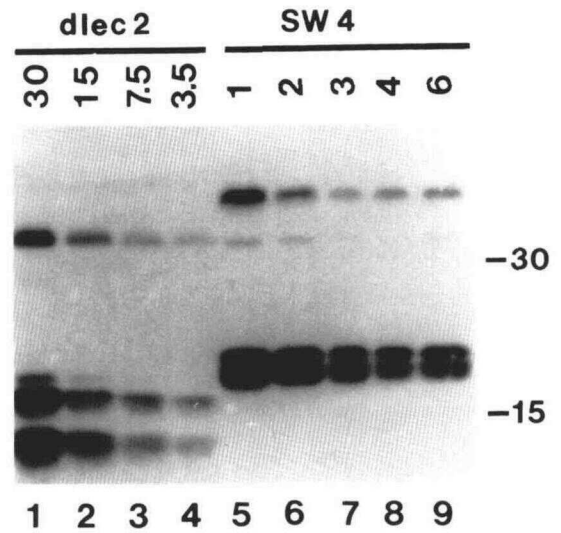

Figure 2. Immunoblot Analysis of the SW4 Transformants.

Mature tobacco seeds were extracted with a low-salt, nondenaturing buffer and the proteins separated by SDS-PAGE, electroblotted to nitrocellulose, and developed with a rabbit PHA antiserum as described (Voelker, Herman, and Chrispeels, 1989). Lanes 1 to 4, dilution series of seed extracts of *dlec2*-transformed tobacco plants from a previous study (Voelker et al., 1987); the numbers at the top indicate the micrograms of protein loaded per lane. Lanes 5 to 9, seed extracts of five independent plants transformed with the SW4 constructs (30 μg of protein per lane). Molecular size markers on the right are in kilodaltons.

Pdlec1 Codes for a PHA-E-Like Polypeptide

All the genes were transferred into tobacco by co-cultivation of leaf discs with transconjugant *Agrobacterium tumefaciens* using the Bin19 vector (Voelker et al., 1987). We generated five or more independently transformed plants per construct, and the insertion of the PHA gene into the tobacco genome was monitored for each plant by DNA gel blot analysis of total leaf DNA using a PHA coding region probe (not shown). Only plants with at least one hybrid gene copy without obvious rearrangements were selected for further analysis. The flowers were selfed, and protein extracts of mature seeds were assayed for PHA by immunoblotting with a polyclonal rabbit antiserum after separating the proteins by SDS-PAGE. The results from all independent transformants containing the repaired *Pdlec1* coding sequence (SW4) are shown in Figure 2. The lanes marked *dlec2* serve as a control and show a dilution series of PHA-L present in the seeds of tobacco transformed with an unmodified *dlec2* gene (Voelker et al., 1987). Besides the full-length polypeptide of M_r 34,000, representing twice-glycosylated PHA-L, several polypeptides of lower molecular weight are present. This material represents processing products caused by cleavage of PHA-L polypeptides after arrival in the tobacco protein storage vacuoles (Voelker et al., 1989). All five independent SW4 transformants shown in Figure 2 showed a similar polypeptide pattern with a full-length polypeptide and two processing products. The repair of the coding sequence and elimination of the frameshift caused the synthesis of a PHA polypeptide that is slightly larger (M_r = 36,000) than PHA-L and similar in size to PHA-E. This confirms our previous conclusion that *Pdlec1* encodes a PHA-E-like polypeptide (Voelker et al., 1986). As with PHA-L, a major proportion of the SW4 product is proteolytically processed in tobacco. We observed a fourfold plant-to-plant variation in PHA levels in transgenic seeds. To compensate for this moderate plant-to-plant variation when measuring expression levels, we pooled equal amounts of seeds from four different plants transformed with the same construct, and the results of this analysis for the different constructs are shown in Figure 3. Comparing the signal strengths of the pooled extracts from the cognate *dlec2* gene (lane 1) with the construct SW4 (lane 3) shows that the repaired *Pdlec1* coding frame behaves completely normally in quantitative terms. This means that the prepared *Pdlec1* coding region does not have any sequences that render its mRNA unstable or cause inefficient translation. As expected, no PHA protein was produced with *Pdlec1* (lane 7) or with SW3 (lane 3); both constructs have the frameshift mutation in the coding region.

The Promoter of *Pdlec1* Is Fully Functional

The constructs SW5, SW6, and SW7 were designed to test the promoter of *Pdlec1*. Progressive deletion of the 5′ end of the *Pdlec1* fragment did not result in an attenuation of expression (Figure 3, lanes 4 to 6), indicating that

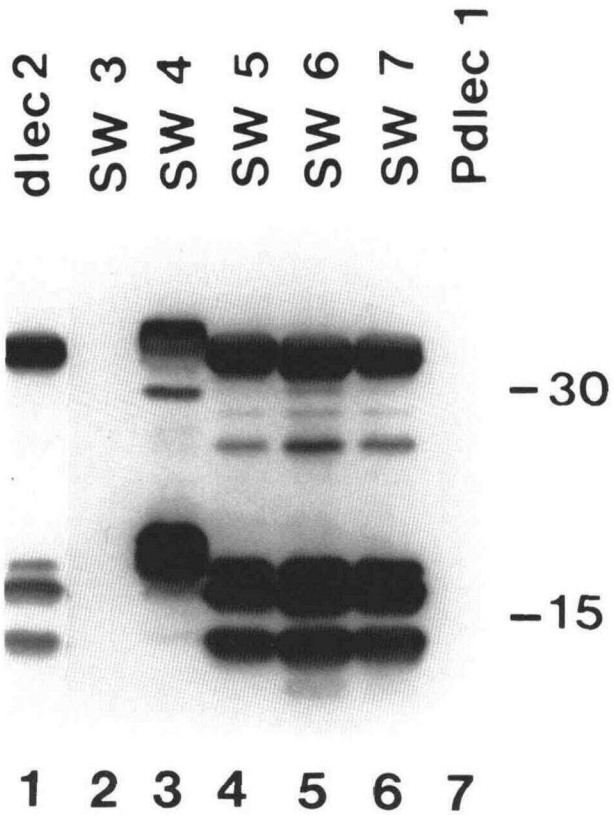

Figure 3. Immunoblot Analysis of PHA Accumulation in the Seeds of Transgenic Tobacco.

Proteins of fully mature seeds were extracted with a low-salt/Triton X-100-containing buffer (Voelker et al., 1989) and equal proportions of protein from four to five independently transformed plants mixed before SDS/PAGE (30 μg of total protein per sample). Immunodetection with a rabbit PHA antiserum was as described (Voelker et al., 1989). The seed pool for lane 1 (*dlec2*) was obtained from a previous transformation study (Voelker et al., 1989). The positions of marker proteins are indicated on the right (in kilodaltons).

a 400-bp segment of DNA at the 5′ end of the *Pdlec1* gene is sufficient to obtain a level of expression similar to the one observed with *dlec2*, a highly expressed allele of PHA (lane 1). An analysis of the time course of the expression of PHA in SW7 plants showed that PHA polypeptides accumulate in the second half of seed development (15 days to 30 days after pollination). Furthermore, very little protein accumulated in the vegetative organs of the plant (data not shown). In conclusion, the analysis of the promoter of *Pdlec1* shows that the 5′ upstream fragment of *Pdlec1*, ligated to the coding region of *dlec2*, contained all the necessary elements for the correct temporal and tissue-specific expression of the gene. In seeds, it causes the same level of PHA accumulation as a normal PHA promoter, indicating that all quantitative elements must be functional.

A Single-Base Deletion Lowers mRNA Accumulation

The single-base deletion in the *Pdlec1* coding sequence clearly abolishes the accumulation of PHA protein (Figure 3, lanes 3 and 7). To assess the effect of the single-base deletion on mRNA accumulation, we measured the levels of PHA mRNA during seed development in transgenic tobacco plants containing the constructs SW3 and SW4. These two constructs are identical except for the single-base pair repair in SW4. We used an antisense RNA transcript probe that hybridizes to 700 bases of the *Pdlec1* coding region (from StyI to XbaI, identical in both constructs; see Figure 1, bottom). After hybridization of the probe with total tobacco seed RNA isolated at different stages of development, the samples were digested with RNase and subjected to gel electrophoresis. The resulting autoradiograms (Figure 4) show that, in SW4 plants, PHA message started to accumulate 15 days to 17 days after pollination (dap) with large amounts of mRNA still being present at 25 dap. (For comparison, the intact 600-base probe is shown in lane P.) In seeds of SW3 plants, PHA mRNA appeared with a similar time course, but accumulated to a much lower level. To quantitate the signals, the gel areas containing the undigested probe were cut out and the radioactivity was determined (Figure 4, bottom panel). The data show a twofold difference of mRNA levels at the earliest levels of expression (15 dap) and a 30-fold to 40-fold difference late in seed maturation (23 dap). Both constructs have identical promoters, but, in the SW4 plants, there is a 50-fold increase in mRNA levels during the second half of seed development and, in SW3 plants, only a threefold increase. We interpret these data to indicate that the mRNA is much less stable in the SW3 plants and that this is caused by the one-base deletion and resulting frameshift in the coding sequence present in the SW3 construct.

DISCUSSION

Pseudogenes are present in the genomes of many eukaryotes and probably arise as by-products of gene duplication events that occur when gene families are formed (Loomis and Gilpin, 1986). Depending on the degree of sequence degeneration as defined by the number of changes from the functional gene, a pseudogene can be classified as evolutionary, old, or of recent origin (see Wilde, 1986, for review). We showed by gene expression analysis in transgenic tobacco that the single-base insertion repair of the coding sequence of *Pdlec1* restores this pseudogene to full function. This small degeneracy of *Pdlec1* indicates that its ancestor gene may have been

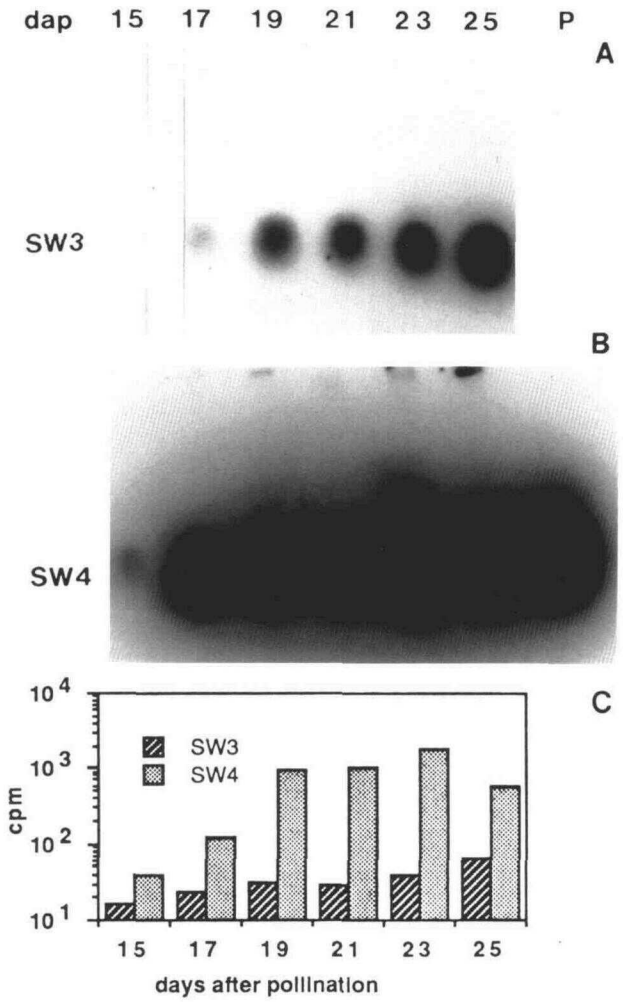

Figure 4. Quantitation of *Pdlec1* mRNA Levels in the Developing Seeds of Transgenic Tobacco.

Total RNA was isolated from developing seeds at 2-day intervals after pollinating (dap). Equal numbers of capsules from four independently transformed plants from the same stage were pooled before extraction to integrate for plant-to-plant variation. For RNase protection, we used 10 μg of total RNA pools. The *Pdlec1* mRNA was detected with a 700-base antisense RNA probe (1 μCi) derived from an in vitro run-off transcription with bacteriophage T7 RNA polymerase using ^{32}P-UTP at 200 Ci/mM. This probe is protected by the StyI/XbaI 3′ reading frame sequence of *Pdlec1* (Figure 1). After RNase digestion, the samples were electrophoresed in a 1% agarose/formaldehyde gel. Autoradiographs (same experiment and exposure time) are shown. All protected probe migrates as a 700-base piece in the same position as the original probe (lane P).

(A) SW3 pool RNA.

(B) SW4 pool RNA.

(C) Quantitation of the signals in **(A)** and **(B)**. Appropriate pieces of the original gel were cut out, and the ^{32}P activity was measured in a scintillation counter. (Baseline, 20 cpm, obtained with RNA from seeds of untransformed tobacco as a negative control, is already subtracted from all values in the plot.)

fully functional until recently, because pseudogenes continue to accumulate mutations because of the absence of selection pressure after the first inactivating mutation (Wilde, 1986). Brücher (1968) suggested, on the basis of his surveys of wild and cultivated bean varieties, that different PHA-deficient cultivars are not phylogenetically related and that mutations leading to the loss of PHA must have occurred sporadically. It is not known whether the repaired *Pdlec1* gene encodes a protein that agglutinates red blood cells. We did not detect agglutinating activity against rabbit erythrocytes in the seeds of SW4 tobacco plants.

Although the single-base pair frameshift in *Pdlec1* easily explains the absence of the protein from the seeds, it is not immediately obvious why the level of transcript should be low in developing cotyledons. The evidence presented here points toward instability of the mRNA. Our results obtained in transgenic tobacco (SW3 and SW4) are in agreement with our other results in which the transcriptional activity of PHA genes in Greensleeves and Pinto were compared (Chappell and Chrispeels, 1986) and with the analyses of PHA transcripts in nuclei and total tissue extracts. Taken together, all observations support the interpretation that transcription from *Pdlec1* is about normal, but that the RNA has a short half-life in the cytoplasm, leading to a dramatic reduction in the steady-state levels of *Pdlec1* mRNA in the cytoplasm. Furthermore, the frameshift mutation is apparently the only significant functional lesion influencing the expression of the *Pdlec1* pseudogene. It has been shown that transcripts of a null allele of a soybean glycinin gene with a mutation in the translation initiation codon are present in the total RNA extract but not in the polysomal RNA (Scallon, Dickinson, and Nielsen, 1987). Untranslatable or only partially translatable mRNA probably does not form polysomes or the polysomes are very unstable. In the examination of a soybean trypsin inhibitor pseudogene, Jofuku et al. (1989) observed near-normal transcription rates but significantly reduced poly(A) mRNA levels in vivo. Nucleotide sequencing shows that the pseudogene differs from the normal gene by only three nucleotide changes in the coding region. This alteration results in a frameshift leading to premature termination during translation. These findings further support our functional analysis in transgenic tobacco.

Studies of nonsense mutations in *Escherichia coli* show that genetic polarity is caused by instability of mRNA (Morse and Yanofsky, 1969), and it has been proposed that the 3′ ends of truncated transcripts are exposed to attack by ribonucleases because they are not physically protected by ribosomes (Schneider, Blundell, and Kennell, 1978). This phenomenon, first observed in prokaryotic cells, where translation and transcription are coupled, has also been found in yeast (Losson and Lacroute, 1979). Nonsense mutations toward the 5′ end of a coding region reduce poly(A) RNA half-life without changing the rate of transcription. From a mouse hybridoma line that secretes

IgM, Baumann, Potash, and Köhler (1985) isolated several frameshift mutations causing in-frame termination codons in the active IgM gene. Most of the mutations reduced steady-state mRNA levels, especially when the reading frame was severely truncated. More recently, Daar and Maquat (1988) constructed a series of nonsense and frameshift mutations in the gene for triosephosphate isomerase and checked their effect on transcription rate and mRNA levels in nuclei and cytoplasm of tissue culture cells. They demonstrated that premature termination of translation does not affect transcription rate or stability of the mRNA in the nucleus. However, there was an increased rate of turnover of the mutant mRNAs in the cytoplasm. Although we lack this direct evidence, we suggest that the *Pdlec1* mRNA becomes unstable in the cytoplasm because of the termination of translation. The precise mechanism of this phenomenon is not known.

METHODS

RNA Isolation and RNase Protection

Total plant RNA was isolated from frozen, powdered cotyledons (*Phaseolus vulgaris* cv Pinto UI111) using a phenol/SDS lysis protocol (Gilman, 1987). Nuclei from developing bean cotyledons (50 mg/cotyledon) were isolated according to Walling, Drews, and Goldberg (1986), and RNA was extracted (Gilman, 1987). PHA transcripts were detected by RNase protection (Gilman, 1987). To probe for the *Pdlec1* and *Pdlec2* transcripts, we used an in vitro 700-bp antisense (T7 polymerase) transcript spanning the homologous StyI/XbaI fragment of the respective PHA genes *Pdlec1* and *Pdlec2* of the cultivar Pinto. These probes hybridize to 80% of the reading frames of these genes, which have more than 90% sequence identity (Voelker et al., 1986).

Cloning, Plant Transformation

The plasmid pTV781, which contains the complete *dlec2* 3.4-kb PstI/SalI fragment from the *dlec2* gene from *P. vulgaris* cv Greensleeves (Voelker et al., 1987), was opened with BsmI (partial) and XbaI. For the creation of SW3, the fragment containing the vector and the 5′ *dlec2* upstream region was ligated to the BsmI/XbaI PHA frame of *Pdlec1* from the plasmid pTV481 (= pHI1; see Voelker et al., 1986). In a subsequent step, the *dlec2* 3′ XbaI/SalI fragment was added. The assembly of SW4 was essentially the same except for the first cloning step. Instead of the *Pdlec1* frame fragment, we added the 200-bp BsmI/StyI fragment of *Pdlec2* (Voelker et al., 1986) together with the StyI/XbaI fragment from *Pdlec1*. In this three-fragment ligation, we repaired the frameshift in *Pdlec1* without introducing any other changes. For the construction of SW5, SW6, and SW7, we opened pTV781 with HindIII (vector sequence next to the PstI site) and BsmI partial. Into the fragment containing the vector and the *dlec2* frame plus downstream DNA, we cloned the HindIII/BsmI fragment spanning the complete *Pdlec1* 5′ region of pTV481 to arrive at SW7. SW6 and SW5 are deletion derivatives of SW7 using, respectively, an internal SalI site and an internal PstI site in the upstream portion of *Pdlec1* (see Figure 1). All gene assemblies were cloned into the plant binary vector Bin19 (Bevan, 1984) and transferred into the tobacco genome (Horsch et al., 1985).

Protein, Protein Extraction, and Immunoblotting

For protein extraction of tobacco seeds, fresh material was ground at 0°C with a buffer containing 50 mM Tris, pH 8, 30 mM NaCl, 0.1% Triton X-100, and 1% β-mercaptoethanol. The supernatant of the subsequent centrifugation (14,000*g* for 5 min) was termed the low-salt extract. For immunoblotting, appropriate quantities of protein (determined according to Lowry et al., 1951) were fractionated by SDS-PAGE, the proteins transferred to nitrocellulose, and PHA detected using a rabbit anti-PHA-L antibody generated as described in Voelker et al. (1987). We used goat anti-rabbit horseradish peroxidase-coupled IgGs (Bio-Rad, Hercules, CA) as secondary antibody and 4-chloro-1-napthol as a peroxidase substrate.

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