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Novel evolutionary variation in transcription and location of two chloroplast genes

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ABSTRACT

We have found major evolutionary changes in the types of transcripts produced by specific chloroplast genes, in particular those encoding the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase and a photosystem II polypeptide (PII). Two distinct patterns of LS gene transcripts are revealed by hybridizing an LS gene probe to electrophoretically separated RNA from 19 angiosperms. Most species, including pea, contain the single transcript of approximately 1.6 kb previously observed in corn, spinach and mustard. However, in mung bean and other members of the legume genera Vigna and Phaseolus, the 1.6 kb transcript represents only a minor fraction of LS transcripts, and instead, two larger LS transcripts of approximately 2.4 and 2.6 kb predominate.

The PII gene produces a single transcript in pea and most other species examined, while members of the related legume genera Vigna, Phaseolus and Glycine contain two additional transcripts which are smaller in size and probably represent specific RNA breakdown products. A single species, sweet pea (Lathyrus odoratus), contains a second PII transcript which is 0.2 kb larger than the approximately 1.2 kb transcript found in all species.

The LS and PII genes map to the same 5 kb region in both pea and mung bean and are transcribed off the same DNA strand. In contrast, published studies indicate that the two genes are approximately 50 kb apart and are transcribed off opposite DNA strands in five other chloroplast genomes. These differences are probably the consequence of an approximately 50 kb inversion which distinguishes the pea and mung bean genomes from those of most other angiosperms (1).

INTRODUCTION

Recent studies have led to the identification of approximately 30 chloroplast translation products (2-4), yet detailed information concerning gene structure, sequence and transcription is presently available for only two protein-encoding chloroplast genes. The gene for the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase has been sequenced in corn (5) and spinach (6), and mapped onto four other chloroplast genomes (7-9). A single LS transcript, approximately 1.6 kb in size and colinear with the LS gene, has been observed in corn, spinach and mustard (5-7). The second well-characterized chloroplast gene is that encoding a rapidly metabolized 32,000 dalton polypeptide (PII) which regulates photosystem II electron transport and is the receptor protein for triazine

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herbicides (10-12). The PII gene has been mapped onto five chloroplast genomes (7,9,13,14) and found to encode a single transcript of 1.2 kb, colinear with the gene, in mustard (7) and corn (15). The positions of the LS and PII genes are highly conserved in all five species examined; the PII gene is located at the margin of the large single copy region, adjacent to one end of the large inverted repeat sequence, while the LS gene is located near the middle of the large single copy region, approximately 50 kb apart from the PII gene (5,7,9,13,14,16,17).

This paper explores the question of evolutionary variation in transcription and location of these two chloroplast genes among flowering plants. A general approach is presented for detecting evolutionary change in the types of transcripts produced by specific genes. We show that among leguminous plants, at least three different mutations have occurred which result in evolutionarily and developmentally stable changes in the size and number of LS and PII transcripts, and discuss the phylogenetic significance of these RNA changes. The LS gene is located only a few kb from the PII gene in pea and mung bean, contrary to all published studies (5,7,9,13,14,16,17), but consistent with our findings that a 50 kb inversion, with its endpoints in the vicinity of the LS and PII genes, has occurred during the evolution of a group of angiosperms which includes all legumes examined (1).

MATERIALS AND METHODS

Chloroplast DNA was purified from spinach and pea as described (18) and from mustard and mung bean according to Palmer (9). *E. coli* plasmid DNA was isolated as described (19). Total cell RNA was extracted from leaves by a modification of the method of Glisin et al. (20). One gm of leaves was frozen in liquid nitrogen and homogenized for 30 sec in a Polytron (Brinkman) at setting 6 in buffer containing 0.1 M Tris-HCl (pH 8.0) and 4% Sarkosyl, with one gm solid CsCl per ml buffer. The homogenate was layered over a pad of 5.7 M CsCl, 0.1 M EDTA and centrifuged overnight in the SW-27.1 rotor at 24,000 RPM. The pellet of precipitated RNA was dissolved in the Tris-Sarkosyl buffer, solid CsCl was added to one gm/ml, and the RNA was again pelleted through a pad of 5.7 M CsCl. The pellet was dissolved in 10 mM Tris-HCl (pH 7.5) and 2.5 mM EDTA, ethanol precipitated overnight, and dissolved in sterile dH_2O.

Restriction endonuclease digestions, agarose gel electrophoresis and nitrocellulose filter transfers of DNA, labeling of isolated restriction fragments by nick translation, and filter hybridizations were performed exactly as described (9). RNA electrophoresis and transfer to nitrocellulose filters

were as described (21) except that the RNA was denatured by heating at 60° for 30 minutes in buffer containing 3% formaldehyde, 25% formamide, 2.5 mM EDTA and 20 mM phosphate buffer.

RESULTS

Transcripts from the LS Gene

Our approach in this study is to utilize cloned chloroplast DNA restriction fragments which contain 3', 5' or internal regions of the LS and PII genes as hybridization probes to map these genes and to study their transcription in a number of different plants. Table 1 lists the nature and source of the LS and PII probes used. The use of mustard and spinach probes for the study of the LS and PII gene transcripts allows quantitative comparisons of transcript abundance in species within the legume family since each legume is expected to be the same evolutionary distance from the reference species. LS gene transcripts

Table 1. Sources of LS and PII Gene Probes

Species	Gene Portion	Clone	Fragment	Reference
corn	5' internal LS	pZmc461	567 bp PstI	(5)
spinach	5' internal LS	S25.5	821 bp PstI-KpnI	(6,22)
Chlamydomonas reinhardtii	3' internal LS	pCR34.1	887 bp HindIII	(23)
spinach	5' terminal PII	S17.3	480 bp SmaI-PstI	(14,24,22)
spinach	internal PII	S8.9	300 bp PstI-HpaII	"
spinach	3' terminal PII	S8.9	920 bp HpaII-SalI	"
mustard	3' terminal PII	--	2.2 kb Pst I	(7)

Fragments were prepared from agarose gels according to methods described previously for the isolation of fragments from acrylamide gels (25; J. Dodd, pers. comm.). The corn and Chlamydomonas probe fragments were carried as the sole chloroplast DNA inserts on the plasmids listed, the spinach probe fragments as part of larger cloned spinach PstI fragments of 25.5, 17.3, and 8.9 kb (22), and the mustard fragment was prepared from a PstI digest of total mustard chloroplast DNA. "Internal" fragments are contained entirely within the translated portions of the LS and PII genes, while "terminal" fragments contain sequences at the 3' or 5' end of the gene plus flanking sequences outside the gene. The 5' and 3' terminal spinach PII fragments each carry approximately 50% gene sequences and 50% flanking sequences, while the mustard 3' terminal PII fragment carries approximately 800 bp of gene sequence and 1400 bp of flanking sequence.

Table 2. Sources of Chloroplast DNAs and Leaf RNAs

	Genus and Species	Common Name	Family
1.	*Geraea canescens*	desert sunflower	Compositae
2.	*Cucumis sativa*	cucumber	Cucurbitaceae
3.	*Brassica juncea*	mustard	Cruciferae
4.	*Spinacia oleracea*	spinach	Chenopodiaceae
5.	*Zea mays*	corn	Gramineae
6.	*Vigna radiata*	mung bean	Leguminosae
7.	*Pisum sativum*	pea	"
8.	*Vicia benghalensis*	purple vetch	"
9.	*Vicia sativa*	spring vetch	"
10.	*Lens culinaris*	lentil	"
11.	*Lathyrus odoratus*	sweet pea	"
12.	*Cicer arietinum*	chick pea	"
13.	*Vigna unguiculata*	black-eyed pea	"
14.	*Medicago sativa*	alfalfa	"
15.	*Trifolium repens*	white clover	"
16.	*Glycine max*	soybean	"
17.	*Phaseolus vulgaris*	pinto bean	"
18.	*Vigna angularis*	azuki bean	"
19.	*Phaseolus vulgaris*	snap bean	"

were visualized by hybridizing an LS gene fragment from spinach to RNA preparations from 19 different angiosperms (Table 2) which had been separated by agarose gel electrophoresis and transferred to nitrocellulose filter paper (Fig. 1). Most species, including corn (5) and spinach (6), contain a single LS RNA of 1.6 kb. However, in samples 6, 13 and 17-20 the 1.6 kb band is reduced in intensity and two larger RNAs of approximately 2.4 kb and 2.6 kb predominate. These qualitative differences in the number and size of LS transcripts overlay quantitative variation in the abundance of LS RNA from species to species among the legumes surveyed. In spite of these absolute differences in LS RNA abundance, the relative proportions of the multiple LS transcripts are constant in samples 6, 13 and 17-20.

It is possible that the two distinct patterns of LS transcripts observed might in part result from developmental variation in the expression of the LS gene among different species. Therefore, we have examined LS RNAs from mung bean leaves grown in either darkness or light, or various intermediate stages of light-induced greening (Fig. 2). Despite changes in the absolute amount of LS RNA, the relative amounts of the three major LS transcripts remain constant throughout light-regulated leaf development (Fig. 2). In addition, minor low molecular weight RNAs are visible. These vary in proportion to the apparent degradation of the various RNA preparations, and are probably *in vitro* degradation

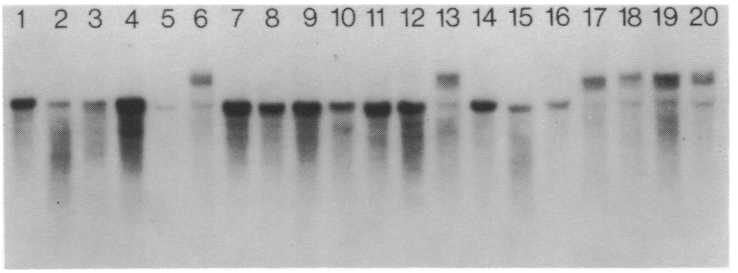

Figure 1. Evolutionary variation in LS transcripts among angiosperms. The spinach LS fragment was hybridized to a nitrocellulose filter containing 5 micrograms of electrophoretically separated RNA from (lanes 1-19) samples number 1-19 (Table 2) and (lane 20) a duplicate lane of mung bean RNA.

products similar to those observed in spinach (6).

Transcripts from the PII Gene

Hybridization of a mustard PII probe to the same 19 RNA preparations reveals three distinct patterns of transcripts (Fig. 3). The major PII transcript in all species, including mustard (7) and corn (15), is an RNA 1.2 kb in length. Two additional, smaller transcripts of approximately 1.0 kb and 0.2 kb are also observed in species 13 and 16-19, while a larger transcript of approximately 1.4 kb is observed in species 11, sweet pea. After longer exposures of the same filter the two small RNAs are also observed in mung bean (lanes 6 and 20; also see Fig. 4). These qualitative changes again overlay quantitative variation

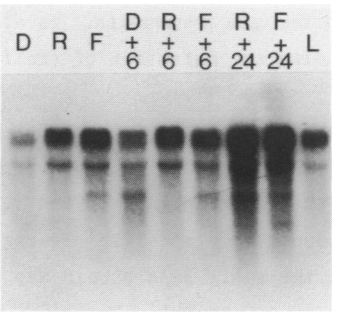

Figure 2. Developmental variation in LS transcripts from mung bean. The corn LS fragment was hybridized to a nitrocellulose filter containing 5 micrograms of RNA from the following mung bean leaf tissues: (D) dark-grown mung bean leaves; dark-grown leaves which were treated with 5 min of red (R) and far-red (F) light every 24 hr for three days before harvesting; (D+6) dark-grown, (R+6) red and (F+6) far-red light treated leaves which were subsequently grown for 6 hr in white light; (R+24) red and (F+24) far-red light treated leaves grown for 24 hr in white light; and (L) light-grown leaves.

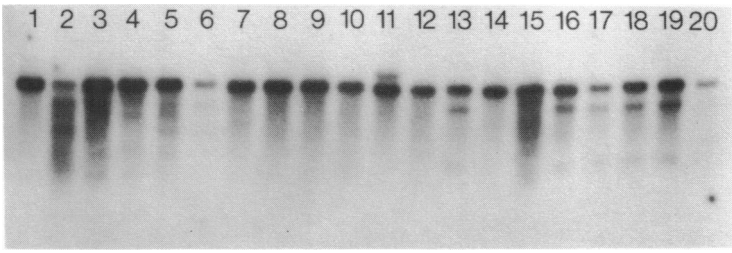

Figure 3. Evolutionary variation in PII transcripts among angiosperms. The mustard PII fragment was hybridized to a nitrocellulose filter containing 5 micrograms of RNA from the samples shown in figure 1.

in the abundance of PII RNA. However, it should be emphasized that despite significant variation in the abundance of PII RNA in lanes 16-20, the relative concentrations of all three transcripts within each of these preparations are quite constant.

We again examined RNAs from a series of mung bean leaves to determine whether there is a developmental component to the observed PII RNA variation. While significant developmental variation in the abundance of PII RNAs is observed, the additional transcript of 1.0 kb is clearly visible, in a constant ratio to the major 1.2 kb transcript, in all stages of leaf and chloroplast development (Fig. 4). The smallest PII RNA, of 0.2 kb, is only barely visible in the preparations which contain the highest levels of PII RNA. We have observed significant filter-to-filter variation in the apparent relative abundance of this RNA within the same preparation. We attribute these differences to variability in the binding and retention of small RNAs on nitrocellulose (21).

Since the approximate sizes of the two smaller PII RNAs sum to the size of the major transcript (1.2 kb), and since the 0.2 kb RNA signal always appears at least five times less intense than that of the 1.0 kb RNA, we suggest that these two RNAs may be produced by a specific cleavage of the 1.2 kb PII RNA. If this is the case, then the small RNAs should map adjacent to one another as component parts of the major 1.2 kb RNA. The mustard probe used in Fig. 3 contains the middle and 3' end of the PII gene [Table 1; (7)]; if our hypothesis is correct then the 0.2 kb RNA must lie at the 3' end of the gene. Analysis with separate 3' and internal PII probes from spinach confirms this interpretation. The 3' probe hybridizes to all three RNAs, while the internal probe hybridizes only to the 1.0 kb and 1.2 kb RNAs (Fig. 5).

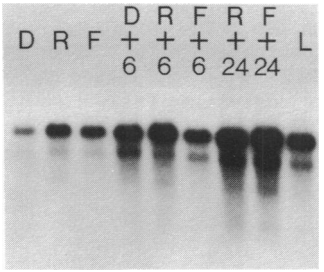

Figure 4. Developmental variation in PII transcripts from mung bean. A combination of all three spinach PII fragments was hybridized to a nitrocellulose filter containing 5 micrograms of RNA from the mung bean leaf samples shown in figure 2.

Location and Orientation of the LS and PII Genes in Mung Bean and Pea

As a first step towards determining the molecular basis for some of the observed LS and PII transcript changes, we have compared the location and arrangement of these two genes in mung bean and pea. Given the extensive series of sequence rearrangements which distinguish the mung bean and pea genomes (1,26), it is not unreasonable to expect that analysis of the arrangement of the LS and PII genes might reveal positional differences which could be related to the observed variation in their transcripts. Hybridization of LS and PII gene probes to restriction digests of total mung bean and pea chloroplast DNA reveals that the two genes are located within 5 to 7 kb of each other on both chromosomes (Figs. 6 and 7). Thus the extensive series of sequence rearrangements which distinguish the mung bean and pea genomes have not altered the relative positions of the two genes.

The direction of LS and PII transcription was determined by hybridizing 3' and 5' gene probes to particular chloroplast DNA restriction fragment patterns. This determination was simplified by the use of cloned segments of the chloroplast genome (22) which carry the genes as shown in Fig. 6. It must be noted that the 5' internal LS probe from corn and the 3' internal LS probe from Chlamydomonas have a common internal region of approximately 350 bp (23). It is therefore possible that the only sequences in the probes that retain sufficient homology to hybridize to the pea or mung bean genes lie within the

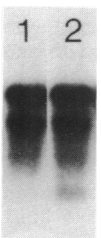

Figure 5. Mapping PII transcripts in Phaseolus. The internal (1) and 3' (2) spinach PII fragments were hybridized to a nitrocellulose filter containing 5 micrograms of RNA from snap bean (Phaseolus vulgaris).

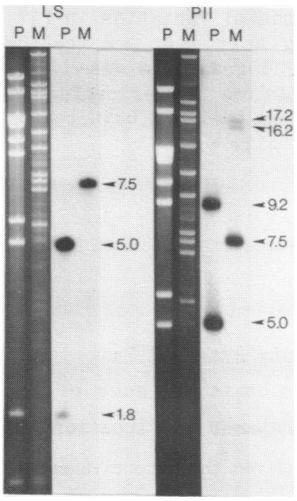

Figure 6. Localization of the LS and PII genes in mung bean and pea. The corn LS fragment (LS) and a combination of all three spinach fragments (PII) were hybridized to nitrocellulose filters containing PstI restriction fragments from pea (P) and mung bean (M) chloroplast DNA.

common region shared by both probes. However, this is unlikely in light of the mung bean hybridization results (Fig. 8), where, although both probes do hybridize to the same two mung bean restriction fragments, the 3' probe hybridizes 10-20 times more strongly to the 6.0 kb fragment than to the 1.5 kb fragment, while the 5' probe hybridizes at least 20 times more strongly to the 1.5 kb fragment than to the 6.0 kb fragment. The simplest interpretation is that the

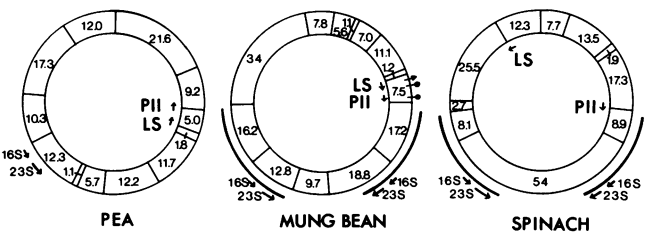

Figure 7. Diagrams of three of the four major angiosperm chloroplast genome types (1). The long, heavy black lines represent the extent of the inverted repeat sequences present in mung bean and spinach, but absent from pea (1,26). Pea and mung bean share a 50 kb inversion relative to spinach (1); a consequence of this inversion is that the LS and PII genes map to the same 5 kb region and are transcribed off the same DNA strand in pea and mung bean (figs. 6 and 8), while in spinach the two genes are approximately 50 kb apart and transcribed in opposite directions (14,17). The fourth major genome type is that found in corn, where a second, smaller inversion has occurred within the 50 kb inversion described above (1). PstI cleavage sites and fragment sizes (in kb), and positions of ribosomal RNA genes, are shown for chloroplast DNA from mung bean and pea (26), and spinach (24). The two HindIII (closed circles) and one KpnI (closed triangle) sites within MB7.5 are also shown.

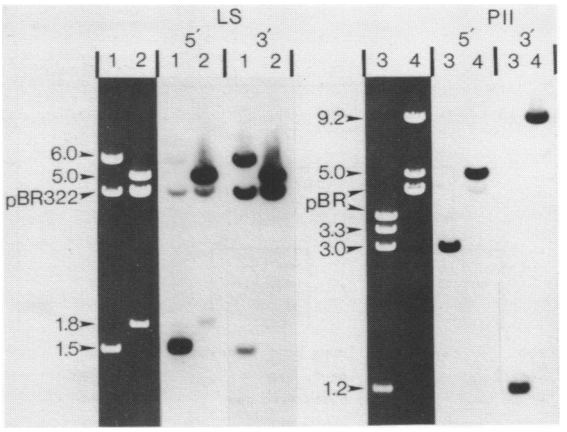

Figure 8. Orientation of the LS and PII genes in mung bean and pea. The corn 5' internal LS fragment (LS 5') and Chlamydomonas 3' internal LS fragment (LS 3') were each hybridized to nitrocellulose filters containing (1) Pst-KpnI digested MB7.5 (22), generating chloroplast DNA fragments of 6.0 and 1.5 kb and a pBR322 vector fragment of 4.36 kb, and (2) PstI digested P1.8 and P5.0 (22), generating chloroplast DNA fragments of 5.0 and 1.8 kb and a pBR322 vector fragment of 4.36 kb. The spinach 5' terminal fragment (PII 5') and spinach 3' terminal fragment (PII 3') were each hybridized to nitrocellulose filters containing (3) PstI-HindIII digested MB7.5 (22), generating chloroplast DNA fragments of 3.3, 3.0 and 1.2 kb and pBR322 vector fragments of 3.58 and 0.78 kb, and (4) PstI digested P5.0 and P9.2 (22), generating chloroplast DNA fragments of 9.2 and 5.0 kb and a pBR322 vector fragment of 4.36 kb.

5' end of the LS gene lies on the 1.5 kb fragment fnd the 3' end on the 6.0 kb fragment. The pea data are not as clear as the mung bean data since both LS probes hybridize primarily to the 5.0 kb pea fragment. However, the 5' corn probe also hybridizes to the 1.8 kb pea fragment, suggesting that the 5' end of the pea LS gene lies within the 1.8 kb fragment and the 3' end within the 5.0 fragment. Accepting this, we conclude that the LS and PII genes are transcribed in the same direction in both the pea and mung bean genomes. Thus we find no readily observable differences in the general organization of these two genes in pea and mung bean.

DISCUSSION

Evolutionary Variation in LS Transcription

The most significant and novel observation in this study is that dramatic evolutionary changes can occur in the structure of RNAs transcribed from two important chloroplast genes. The limited published data indicate that a single RNA of relatively constant size is transcribed from the LS and PII genes in

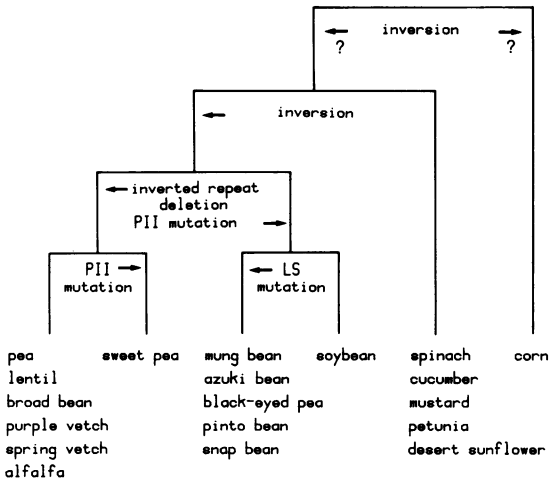

Figure 9. Chloroplast DNA and RNA phylogeny of angiosperms. The inverted repeat deletion and two inversions represent the three major rearrangements known among angiosperms [(1); see Fig. 7], while the LS and two different PII mutations are those described in this paper. The inverted repeat status of the chloroplast genomes of clover and chick pea has not been determined and thus these two species have been omitted from the phylogenetic tree. The inverted repeat status of the species shown are from Palmer and Thompson (1982) and also from unpublished data. The LS and PII transcript status of broad bean (Vicia faba) and petunia (Petunia hybrida) has not been determined; their positions have been assigned based on DNA rearrangements (1) and also, in the case of broad bean, on general taxonomic criteria (28).

corn, spinach and mustard (5-7,14,16). In this report we confirm these results, but find that a more extensive survey, concentrating on a number of leguminous species, reveals hitherto unexpected variation in the number, size and quantitative proportions of LS and PII transcripts.

Perhaps the most interesting RNA variation is the pattern of LS RNAs, which we shall term the mung bean pattern, observed in all members of Vigna and Phaseolus examined. In these species the predominant LS transcripts are two RNAs almost one kb longer than the sole LS transcript observed in all other species; the third and least abundant LS transcript in the mung bean pattern has the same apparent molecular weight as the usual LS transcript. Sequence comparisons of the corn (5) and spinach (6) LS genes indicate that a number of small deletions and insertions have occurred within the 3' and 5' untranslated regions of the gene, resulting in a spinach LS RNA which is 55 bp larger than that from corn. The mung bean LS changes observed in the present study stand out from those found between corn and spinach in two respects - first, in the

magnitude of the LS RNA size changes observed in mung bean, and second, and perhaps most significantly, because three stable LS RNAs accumulate in mung bean compared to the single RNA found in corn, spinach and most other species.

The complicated set of mung bean LS RNAs might be the result of one or a combination of many types of mutational events, including DNA rearrangements (particularly insertions) and 5' and 3' base pair substitutions creating promoter or terminator mutations. New promoters upstream from those described in corn (5) and spinach (6), or terminators downstream from that in spinach (6), could have been created by base pair substitutions in these regions. Since most of mung bean LS RNA is contained in the two additional large RNA species, any new promoters or terminators would have to be strong relative to the old ones, and additional changes weakening the already existing signals must be postulated.

A simple hypothesis explaining the mung bean pattern might call for the insertion of a DNA segment containing promoters into the intergenic region or the 5' untranslated region. In this hypothesis, the relationship between the position of insertion and the location of promoters in the new segment is constrained by the sizes of the three observed LS transcripts. Insertion of a terminator-carrying segment at or near the 3' end of the LS gene has similar explanatory possibilities.

A more involved explanation is that an intron or additional coding sequences have been inserted into the mung bean LS gene. Stiegler et al. (23) have recently provided evidence suggesting that the LS gene from Euglena may contain a large intron 0.5-1.1 kb in size. In Euglena however, RNA processing would appear to be efficient and rapid, as only a single RNA species of approximately 1.5-2.0 kb was detected.

Two additional questions raised by these findings are whether the two large RNAs are translated, and if so, whether LS polypeptides of normal size are produced. We are not aware of any information which bears on the first question. However, the LS polypeptide from mung bean does appear to be of normal molecular weight (27; J. R. Seemann, unpublished data).

Evolutionary Variation in PII Transcription

The extra PII RNA found in sweet pea is larger than the normal PII RNA. It is therefore expected that this RNA could have arisen through any of the changes discussed in the previous section.

We have already discussed data which indicate that the two additional, small PII RNAs observed in Vigna, Phaseolus and Glycine are probably specific breakdown products from the major PII RNA. Whether or not this breakdown is primarily an in vivo or in vitro process is a moot point in the present context.

What is clear is that this quantitative increase in PII RNA lability at a specific site represents a change which has occurred in an evolutionarily and developmentally stable manner and has relatively deep roots within the legume family (Fig. 9). Evolutionary variation in RNA lability has already been noted for the chloroplast 23S ribosomal RNA (29), although no strong conclusions could be made regarding the phylogenetic direction of change in rRNA breakdown products.

Evolutionary Variation in LS and PII Gene Locations

We have previously demonstrated that a 50 kb inversion differentiates the chloroplast genomes of mung bean, and probably pea and broad bean too, from those of spinach, cucumber, petunia and corn (1). Our gene mapping results with mung bean and pea confirm and considerably extend those experiments in which large, undefined restriction fragments were used to locate regions of homology (1). The PII gene is in the same position with respect to the inverted repeat in both mung bean and spinach (Fig. 7). However, the spinach LS and PII genes are 50 kb apart and transcribed in opposite directions, while in mung bean the LS and PII genes are only 5 kb apart and transcribed in the same direction. These data are quite consistent with previously observed discontinuities in hybridization patterns between mung bean and spinach (1). Both sets of data are most easily explained by a large inversion with one endpoint between the mung bean LS and PII genes. We are currently examining this region in greater detail.

In pea, owing to the loss of the inverted repeat structure (26), such comparisons cannot be made directly. However, it seems clear that the LS gene is located and oriented in similar fashion with respect to the PII gene in both pea and mung bean. Thus, the LS-PII gene linkage observed in mung bean appears to be unaltered by the extensive series of rearrangements which have occurred in the pea lineage since its divergence from the mung bean lineage (1,26).

Phylogenetic Implications

The changes in LS and PII transcription observed in this study appear to be the consequence of rare, evolutionarily and developmentally stable mutations which can be used to infer phylogenetic relationships among different angiosperms. In combination with three major chloroplast DNA rearrangements previously described (1), two of which are diagrammed in Fig. 7, these changes can be incorporated into a chloroplast-based molecular phylogeny for angiosperms (Fig. 9). We note that this molecular scheme is both broadly and specifically consistent with notions of angiosperm and legume phylogeny (1,28,30,31). No internal inconsistencies are apparent. In particular, two quite distinct events

- the inverted repeat deletion (1,26) and the mung bean PII mutation - give the same branching order among legumes. These two mutations are almost certainly not related, and in fact they occur in opposite phylogenetic directions. We expect that further analysis of additional legume species will reveal intermediates which either retain the inverted repeat and possess a single PII RNA, or vice versa.

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