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Biochemistry

Linkage and homology analysis divides the eight genes for the small subunit of petunia ribulose 1,5-bisphosphate carboxylase into three gene families

(multigene families/photosynthetic genes/nuclear DNA/λ phage libraries)

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ABSTRACT **Twenty-six λ phage clones with homology to coding sequences of the small subunit (SSU) of ribulose 1,5-bisphosphate carboxylase have been isolated from an EMBL3 λ phage bank of *Petunia* (Mitchell) DNA. Restriction mapping of the phage inserts shows that the clones were obtained from five nonoverlapping regions of petunia DNA that carry seven *SSU* genes. Comparison of the *Hin*dIII genomic fragments of petunia DNA with the *Hin*dIII restriction fragments of the isolated phage indicates that petunia nuclear DNA encodes eight *SSU* genes, seven of which are present in the phage clones. Two incomplete genes, which contain only the 3′ end of an *SSU* gene, were also found in the phage clones. We demonstrate that the eight *SSU* genes of petunia can be divided into three gene families based on homology to three petunia cDNA clones. Two gene families contain single *SSU* genes and the third contains six genes, four of which are closely linked within petunia nuclear DNA.**

Ribulose 1,5-bisphosphate carboxylase is the primary enzyme of the carbon fixation pathway in the chloroplast. It is composed of two subunits; the large subunit, which is encoded by chloroplast DNA (1) and synthesized inside the organelle, and the small subunit (SSU) which is encoded in the nucleus (2), synthesized as a higher molecular weight precursor in the cytoplasm, and imported by the chloroplasts (3). *In vitro* experiments have demonstrated that the uptake and processing of the SSU precursor protein are post-translational events (4). Many studies have demonstrated that the expression of the SSU polypeptide is regulated by light (5, 6) via the light receptor, phytochrome (7). Recent studies suggest that this *SSU* gene expression is transcriptionally regulated (8).

There are multiple copies of the structural gene for the SSU protein in the nuclear DNA of petunia, pea, wheat, and soybean (9–12). In *Petunia* (Mitchell), Dunsmuir *et al.* (9) have isolated three SSU cDNA clones with differing sequence (pSSU51, pSSU71, and pSSU117, illustrated in Fig. 1), indicating that at least three *SSU* genes are transcribed in leaf tissue. When the overlapping regions of these three cDNA clones are compared, there is one predicted amino acid difference in the encoded SSU proteins. This difference occurs at the COOH-terminal amino acid (residue 123) and changes a tyrosine residue in pSSU51 to a phenylalanine residue in pSSU71 (13).

Dunsmuir *et al.* (9) have proposed that the multiple nuclear *SSU* genes of petunia could be classified into distinct gene families based on their homology with the cDNA clones. The division of multiple nuclear genes into gene families has been reported for several plant genes, including storage protein genes for maize (14–16) and pea (17), petunia chlorophyll *a/b* binding-protein genes (18), and soybean leghemoglobin genes (19). Although there has been extensive characterization of multigene families in animal systems (20–22), the multigene families in plants have been only partially characterized. This has been due in part to the high copy number of these genes [for example, the zein genes (16) and the chlorophyll *a/b* protein genes (18)]. We decided to investigate the organization of the multiple *SSU* genes (present in 4–12 copies; ref. 9) in the nuclear DNA of *Petunia* (Mitchell) in order to fully characterize the organization of this plant multigene family. We are also interested in whether the multiple *SSU* genes are differentially expressed, which DNA sequences are necessary for the regulation of their expression, and whether the different genes encode polypeptides that are functionally distinct.

We describe here the isolation of seven of the eight *SSU* genes of *Petunia* (Mitchell) and demonstrate that there are three families of genes. Two of these families contain single genes and one contains six genes, four of which are closely linked in petunia nuclear DNA. We have also found two gene fragments in petunia nuclear DNA that contain only the 3′ end of a *SSU* gene.

MATERIALS AND METHODS

Plant Material. The *Petunia* (Mitchell) strain is a doubled haploid produced by another culture from a hybrid between *Petunia hybrida* var Rose of Heaven and *Petunia axillaris* (23). The plants were grown under greenhouse conditions.

Genomic Blots and Preparation of Probes. Petunia DNA was isolated as described (18). Ten micrograms of DNA was cut to completion with *Hin*dIII and electrophoresed on a 0.7% agarose gel containing 40 mM Tris acetate, pH 7.8/5 mM Na acetate/1 mM EDTA. DNA was transferred to Genescreen (New England Nuclear) for 12 hr by the Southern procedure (24). The filters were baked under vacuum for 2 hr at 80°C, prehybridized for 4 hr at 42°C in 4× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate)/5× Denhardt's solution [0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidine (25)]/20 mM Na phosphate, pH 6.5/0.5% $NaDodSO_4$. Hybridization was at 42°C in 50% formamide/4× NaCl/Cit/0.5% $NaDodSO_4$/20 mM Na phosphate, pH 6.5/5× Denhardt's solution/denatured salmon sperm DNA (100 μg/ml). After 16 hr, the filters were washed in 2× NaCl/Cit/0.1% $NaDodSO_4$ two times for 5 min at room

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Abbreviations: SSU, small subunit; kb, kilobase(s).

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temperature followed by two washes in 0.1× NaCl/Cit/0.1% $NaDodSO_4$ at 60°C for 1 hr. For high stringency washing, the filters were washed twice in 0.1× NaCl/Cit/0.1% $NaDodSO_4$ at 70°C for 1 hr.

The three cDNA clones diagrammed in Fig. 1, pSSU51, pSSU71, and pSSU117 (9), are *Bam*HI inserts in the vector pBR322. These were labeled with [^{32}P]dCTP (400 Ci/mmol; 1 Ci = 37 GBq) using the T4 polymerase procedure of O'Farrell (26) after cutting the cDNA clones with *Bam*HI. The labeled inserts were purified through one cycle on a 5% polyacrylamide gel.

Preparation of a *Petunia* (Mitchell) Genomic Bank. Petunia DNA was partially digested with *Sau*3A to give a mean fragment size of 15–20 kilobases (kb). The DNA was then fractionated on a 10–40% sucrose gradient with 1 M NaCl/20 mM Tris·HCl, pH 8/1 mM EDTA. The gradient fractions containing DNA fragments between 15 and 20 kb were concentrated with butanol and then by ethanol precipitation. λ phage EMBL3 (27) DNA was completely digested with *Bam*HI and then digested with a 10-fold excess of *Eco*RI. This step allows a biochemical selection against the reinsertion of the phage stuffer fragment during the ligation reaction. The small linker fragment was removed by isopropanol precipitation (27). The double-cut EMBL3 DNA was ligated to the fractionated petunia DNA in a 2:1 molar excess of EMBL3. Ligated DNA was packaged into λ phage heads in 0.75 μg (EMBL3) aliquots using the Hohn and Murray procedure (28). Two phage banks (200,000 recombinant phage) were screened by hybridization by the method of Benton and Davis (29).

SSU Phage DNA Isolation. The plaque-purified phage were stored at 4°C as single phage plug stocks in 1 ml of 5 mM Tris·HCl, pH 7.5/100 mM NaCl/10 mM $MgSO_4$/0.01% gelatin. Ten microliters of this stock was adsorbed at 37°C for 20 min to 150 μl of a saturated culture of *Escherichia coli* LE392. The adsorbed cells were grown at 37°C in 5 ml of Sinsheimer's broth (30) until cell lysis began to occur. The culture was treated with chloroform and centrifuged at 5000 × *g* for 5 min to remove cell debris. The phage were pelleted by centrifugation at 100,000 × *g* for 4 hr. The phage pellet was resuspended in 300 μl of 10 mM Tris·HCl, pH 7.5/10 mM $MgSO_4$ and incubated with 0.25 μg of DNase I (Calbiochem) for 2 hr at 4°C. The phage were extracted with an equal volume of chloroform, followed by an equal volume of phenol/chloroform/0.3 M Na acetate. The final aqueous phase was passed through a 1-ml Sephadex-G50 (Pharmacia) spin column. This procedure yielded ≈20 μg of DNA.

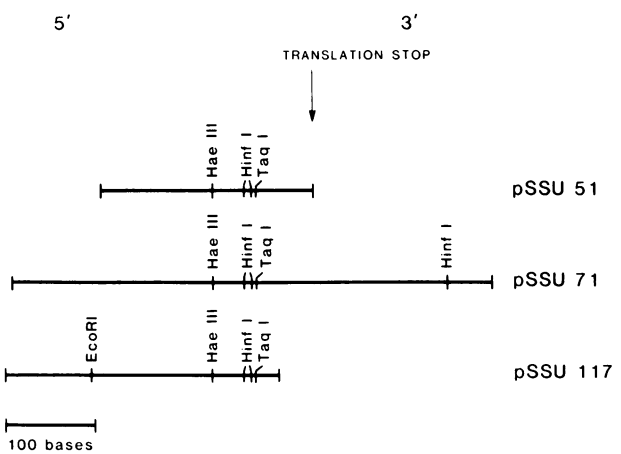

FIG. 1. Restriction endonuclease maps of petunia SSU cDNA clones. The cDNA clones isolated by Dunsmuir *et al.* (9) are all *Bam*HI inserts in the vector pBR322. Restriction site maps were determined with single and multiple enzyme digests. Position of the translation termination signal was determined by sequence analysis (9, 13).

RESULTS AND DISCUSSION

***SSU* Gene Copy Number.** After hybridization of the petunia SSU cDNA clones to genomic blots of petunia DNA, Dunsmuir *et al.* (9) found at least 12 discrete *Eco*RI fragments of petunia DNA ranging from 2 to 12 kb with homology to the cDNA clones. The presence of *Eco*RI restriction sites in one of the cDNA clones and at least three of the *SSU* genes (9, 13) made the estimate of the copy number of the *SSU* genes extremely difficult. We have therefore repeated the hybridization of the petunia SSU cDNA clones to genomic blots of *Hin*dIII-restricted petunia DNA. We chose to use *Hin*dIII for this experiment because sequence analysis of the three cDNA clones (9, 13) and the two SSU genomic clones isolated by Dunsmuir *et al.* (9) (unpublished results) has shown that *Hin*dIII does not cut in the coding region of these five SSU clones. After hybridization of the petunia cDNA clone pSSU117 (which contains only SSU protein coding sequences) to a genomic blot of *Hin*dIII restricted petunia DNA (42°C, 50% formamide) and washing of the genomic blot at normal stringency (60°C, 0.1× NaCl/Cit) 10 *Hin*dIII fragments of petunia DNA hybridized to the cDNA clone (Fig. 2, fragments A–J). The same hybridization profile is found for the other two cDNA clones pSSU71 and pSSU51 (data not shown). The *Hin*dIII fragments range in size from 10.5 to 1.2 kb. The weak band of hybridization between fragments I and J does not appear reproducibly in different genomic blots. We therefore do not consider this to be hybridization to SSU coding sequences. The results from the genomic blot suggest that petunia DNA contains up to 10 copies of the *SSU* gene.

***SSU* Gene Isolation.** To isolate all of the *SSU* genes from petunia, we rescreened the petunia *Eco*RI genomic bank constructed in the λ vector Charon 28 (18). This screen gave only the SSU clones that were previously isolated by

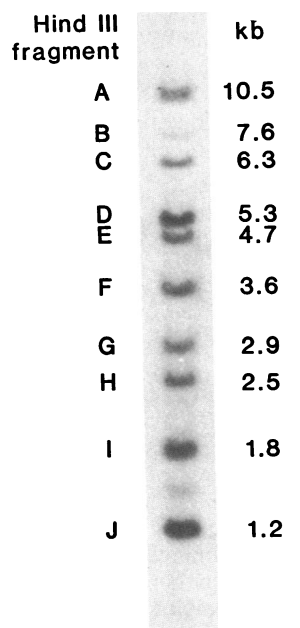

FIG. 2. Hybridization of SSU cDNA clone pSSU117 to *Hin*dIII-digested petunia DNA. Petunia DNA (10 μg) was digested to completion with *Hin*dIII, electrophoresed on a 0.7% agarose gel, and transferred to Genescreen. The filter was hybridized with the *Bam*HI insert of the cDNA clone pSSU117. The filter was washed at 60°C in 0.1× NaCl/Cit.

Dunsmuir *et al.* (9). We therefore cloned petunia DNA partially digested with *Sau*3A into the *Bam*HI sites of the λ vector EMBL3, and 200,000 recombinant phage were screened and plaque-purified by hybridization to the petunia cDNA clone pSSU117. Twenty-six phage containing SSU coding sequences were recovered. Restriction endonuclease site maps of the SSU phage were determined by hybridizing purified and labeled restriction fragments from each λ phage DNA to Southern blots of single and multiple restriction enzyme digests of the same λ phage DNA. Twelve of the 26 phage containing SSU coding sequences contain different inserts, and these are shown in Fig. 3. The other 14 phage were found to be duplicates of the 12 phage shown in Fig. 3 (determined from a comparison of their DNA fragments). Surprisingly, 10 of these 14 phage contain approximately the same region of genomic DNA. Phage clones 1, 3, and 9 shown in Fig. 3 are those previously isolated by Dunsmuir *et al.* (9). Comparison of the DNA fragments of the SSU phage generated by using several different restriction enzymes shows that many of the inserts of the SSU phage contain overlapping regions of petunia DNA. This was confirmed by hybridization of the overlapping restriction fragments to Southern blots of the appropriate restriction digests of the different phage. Chromosomal maps of the five regions of petunia genomic DNA generated when the overlapping inserts of the λ phage clones are linked together are shown in Fig. 3.

Location of SSU Coding Sequences and Orientation of the Genes. SSU coding sequences in the λ phage clones were determined by using hybridization of the cDNA clone pSSU117 to Southern blots of multiple digests of the λ phage DNA. The orientation of the *SSU* genes within the λ clones was established by using 3′ and 5′ specific *SSU* gene probes. These probes were made by using a conserved *Eco*RI site located in the coding region of petunia *SSU* genes (determined during sequence analysis; unpublished observations). This *Eco*RI site is also located centrally in the cDNA clone pSSU117 (Fig. 1). The sequences of pSSU117 5′ to the *Eco*RI site and those 3′ to the *Eco*RI site were separately hybridized to Southern blots of restriction enzyme digests of the λ phage clones. The location and orientation of the *SSU* genes on the five regions of petunia genomic DNA are shown in Fig. 3. Subsequent analysis of the *SSU* gene carried on phage 30 has shown that the *Eco*RI site in this gene is not the conserved *Eco*RI site present in all the other isolated petunia *SSU* genes. However, hybridization of the 5′ and 3′ coding sequences of pSSU117 still allows correct orientation of this gene (confirmed by sequence analysis; unpublished).

The information illustrated in Fig. 3 indicates that one region of petunia genomic DNA contains four *SSU* genes (confirmed to be complete genes by sequence analysis; unpublished) that are located within ≈20 kb and organized in a tandem array. The three other regions of petunia DNA contain single *SSU* genes and one other contains an SSU 3′ gene fragment.

SSU 3′ Gene Fragments. During the analysis to determine the orientation of the *SSU* genes in the genomic clones, we found that the sequences homologous to the cDNA clone pSSU117 carried on phage 44 did not contain the sequences of pSSU117 5′ to the *Eco*RI site. Sequence analysis of several of the petunia *SSU* genes (unpublished) allowed us to construct a probe containing sequences encoding the transit peptide of the SSU precursor protein (these sequences are not present on the incomplete cDNA clone pSSU117). This probe also showed no hybridization to any of the restriction fragments of the petunia DNA carried on phage 44 (data not shown). From this evidence, we conclude that the genomic DNA cloned in phage 44 contains an SSU 3′ gene fragment and not an internal deletion of coding sequences 5′ to the *Eco*RI site. Sequence analysis (unpublished) has shown that this 3′ gene fragment contains homology to DNA sequences that encode the COOH-terminal 76 amino acids of the mature SSU protein (encoded in the third exon).

A second *SSU* gene fragment was also located on the overlapping inserts of the phage 2, 7, and 23. This was also shown to contain homology to sequences in pSSU117 that lie 3′ but not 5′ to the *Eco*RI site.

Isolation and restriction mapping of 15 different λ phage clones from genomic banks of *Petunia* (Mitchell) has thus enabled us to isolate seven complete *SSU* genes and two *SSU* gene fragments. These genes are located on five regions of petunia nuclear DNA with one region containing four complete *SSU* genes and one gene fragment within 20 kb. The four linked genes are arranged in a tandem array. The three genes for which no evidence of linkage has been obtained must be, respectively, at least 3 kb (phage 49, 31, and 56), 11.5 kb (phage 4, 6, and 33), and 11 kb (phage 30 and 22) from any other *SSU* gene.

We can also now relate the *Hin*dIII genomic fragments that hybridize to the SSU cDNA clones (Fig. 1) to the restriction fragments on the isolated λ phage by a comparison of their size. The labels in Fig. 3 (A–J) relate the *Hin*dIII restriction fragments of the phage to the *Hin*dIII genomic fragments shown in Fig. 2. Only one of the *Hin*dIII fragments (G) cannot be accounted for in the phage clones. This fragment (2.9 kb) shows the same level of hybridization to the cDNA clones as the other *Hin*dIII genomic fragments that carry one *SSU* gene. We therefore assume this fragment carries one *SSU* gene. Since only one *SSU* gene is cut by *Hin*dIII, we can estimate that the nuclear genome of *Petunia* (Mitchell) contains eight *SSU* genes and two *SSU* gene fragments, of which we have cloned all but one.

Relationship of the *SSU* Genes to Isolated cDNA Clones. Dunsmuir *et al.* (9) have shown that the three petunia SSU cDNA clones remain hybridized after stringent washing conditions to nonoverlapping subsets of *Eco*RI fragments of petunia nuclear DNA. On the basis of these data, they proposed that the *SSU* genes could be classified into distinct gene families based on their homology to the cDNA clones. We have tested this hypothesis by hybridizing the petunia cDNA clones to genomic blots of *Hin*dIII-restricted petunia DNA. After washing the blots at normal stringency, 10 *Hin*dIII fragments remain hybridized to the cDNA clone pSSU117 (Fig. 2). After washing at high stringency (70°C, 0.1× NaCl/Cit), each of the three cDNA clones remains hybridized to nonoverlapping subsets of the *Hin*dIII fragments (Fig. 4). The cDNA clone pSSU51 shows strong homology at high stringency to six *Hin*dIII fragments (C, D, E, F, G, and H). The cDNA clone pSSU117 shows strong homology to a 1.2-kb *Hin*dIII fragment (J) and weaker homology to a 7.6-kb *Hin*dIII fragment (B), whereas pSSU71 shows strong homology to only one *Hin*dIII fragment of 1.8 kb (I). The *Hin*dIII fragment A (10.5 kb), which corresponds to the fragment of phage 44 carrying the incomplete gene, has been found to comigrate on the genomic blots with a DNA fragment containing pBR322 homology (data not shown). After washing at high stringency, all three cDNA clone probes remain hybridized to this fragment because of contamination of the gel-purified probes with low levels of vector DNA (pBR322).

All the *Hin*dIII fragments with SSU homology can be accounted for in the blots after high-stringency washing. We conclude from this that the *SSU* genes of *Petunia* (Mitchell) can be divided into three gene families based on their sequence homology to the three isolated cDNA clones. The cDNA clone pSSU51 defines a gene family that includes six *SSU* genes, which all show approximately equal hybridization to the cDNA clone pSSU51 after high-stringency washing of the genomic blots. Four of these genes are linked in a tandem array within ≈20 kb of genomic DNA. The remaining isolated member of this gene family lies on a separate piece

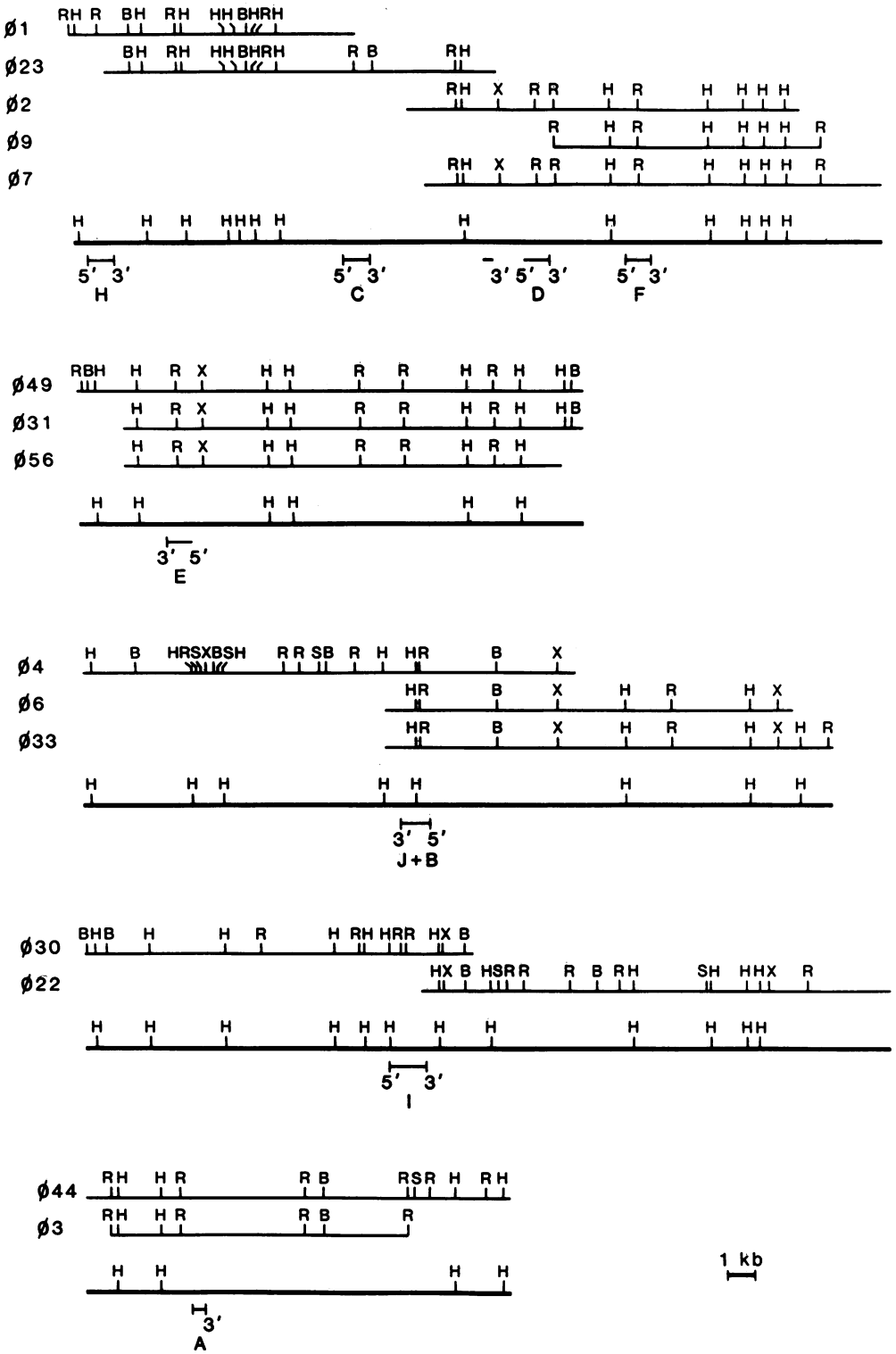

FIG. 3. Restriction endonuclease maps of petunia λ phage clones with homology to the SSU cDNA clones. Overlapping regions of these clones have been linked to give five regions of petunia nuclear DNA containing *SSU* genes. These are diagrammed underneath the overlapping phage with just the *Hin*dIII restriction sites indicated. The phage inserts (except for that in phage 33) are oriented so that the sequence adjacent to the long arm of the phage DNA is to the left. Restriction sites are abbreviated as follows: H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; S, *Sal* I; X, *Xho* I. The location and orientation of the SSU coding sequences were determined by hybridization (see text). Bars on lines depicting SSU coding sequence indicate the limits of the coding sequence, as determined by sequence analysis. Where bars are missing, the limits of the coding sequence have been inferred from conserved restriction endonuclease sites in the coding region of the transit peptide (unpublished observations). The letters A–J relate the *Hin*dIII restriction fragments on the phage clones to the *Hin*dIII fragments carrying SSU homology seen in the genomic blot (Fig. 2).

of genomic DNA (phage 49, 31, and 56) and must be at least 3 kb 3′ and 14 kb 5′ away from any other *SSU* gene. The sixth member of this gene family is represented by *Hin*dIII genomic fragment G and is not represented in the λ phage clones.

The gene family defined by cDNA clone pSSU117 contains one *SSU* gene carried on two *Hin*dIII fragments J and B. This is the only *SSU* gene of petunia containing an internal *Hin*dIII site. The cDNA clone pSSU117 is an incomplete cDNA clone lacking the sequences encoding the transit peptide and the

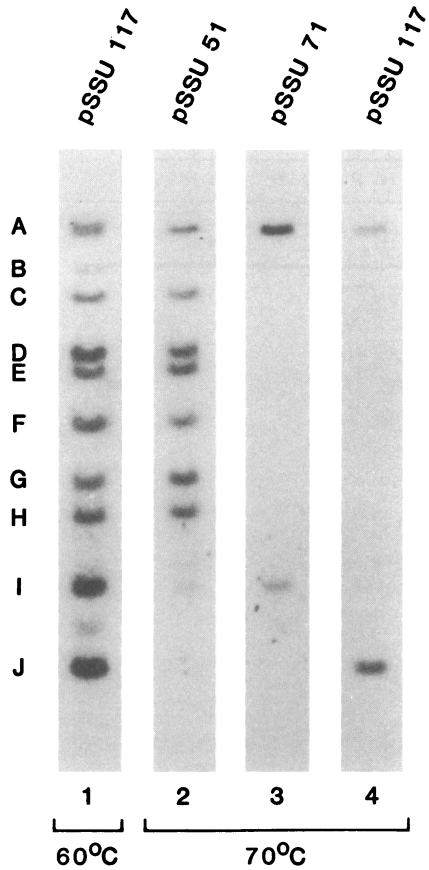

FIG. 4. Hybridization and thermal elution of SSU cDNA clones to *Hin*dIII-restricted petunia DNA. Petunia DNA (10 μg) was digested to completion with *Hin*dIII, electrophoresed on a 0.7% agarose gel, and transferred to Genescreen. Different filters were hybridized with gel-purified inserts of the three cDNA clones. The filter in lane 1 was washed at normal stringency (60°C, 0.1× NaCl/Cit) for 2 hr. The filters in lanes 2–4 were washed at high stringency (70°C, 0.1× NaCl/Cit) for 2 hr.

first 11 amino acids of the mature protein. This accounts for the weaker hybridization to the *Hin*dIII fragment B, which carries the 5′ region of the *SSU* gene. The third gene family defined by cDNA clone pSSU71 also contains one *SSU* gene, which is carried on a 1.8-kb *Hin*dIII fragment (I).

Hybridization of the three cDNA clones to Southern blots of the isolated phage DNA after normal and high-stringency washing confirms the results from the melts of the genomic blots (data not shown). In addition, these data showed that the gene fragment located on phage 44 did not remain hybridized to any of the cDNA clones at high stringency, although sequence analysis (unpublished) has shown that this gene fragment is more closely related to the *SSU* gene carried on phage 4, 6, and 33 (117 gene family) than to any of the other *SSU* genes examined.

The *SSU* genes fall into three gene families based on their homology to the three cDNA clones (pSSU51, pSSU71, and pSSU117). This infers that the genes in the pSSU71 and pSSU117 gene families and at least one gene of the pSSU51 gene family are expressed in light-grown petunia leaf tissue. It will be interesting to examine if the linkage of the genes of the pSSU51 gene family influences their expression. It will also be interesting to investigate whether there is any functional significance to the division of these *SSU* genes into gene families. We have completed a sequence analysis (unpublished) on representatives from each of the three gene families.

R. Fraley and D. Shah (personal communication) have recently isolated a cosmid clone containing two *SSU* genes from *Petunia* (Mitchell). We have compared the restriction endonuclease map of this clone with the restriction site maps of the λ phage clones reported here. One of the *SSU* genes on the cosmid clone is the *SSU* gene carried on *Hin*dIII fragment H (Fig. 3), the other is carried on a 2.9-kb *Hin*dIII fragment and represents the one *SSU* gene not cloned in our λ phage bank. This *SSU* gene is located 2 kb 5′ and in the same orientation as the *SSU* gene carried on *Hin*dIII fragment H. Five *SSU* genes of the pSSU51 gene family therefore lie in a tandem array within 23 kb of petunia nuclear DNA.

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