Developmental biochemistry of cottonseed embryogenesis and germination XVIII cDNA and amino acid sequences of members of the storage protein families

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Summary

We have sequenced cDNA clones representing each of the three distinct groups of storage proteins of the cotton seed. Characteristics of their mRNAs and derived proteins are given. Dot matrix analysis of the nucleotide and amino acid sequences shows that 2 of these groups of proteins have a great deal of vestigial homology at low stringency and should be considered subfamilies of a single storage protein gene family. The remaining group is quite distinct and should be considered a separate multigene family. It also can be divided into 2 subfamilies based on the presence or absence of glycosyl residues and other sequence differences.

These proteins are processed to smaller species during embryogenesis, and all of the mature storage proteins of cotton can be traced back to these 2 gene families.

In view of these relationships we propose that these 2 families be called the α and β globulins of cotton storage proteins, each comprised of an A and B subfamily.

Introduction

We have shown that the seed storage proteins of the cotton (Gossypium hirsutum) emanate from 2 sets of preproproteins of apparent size of 69 and 60 kD (1, 2). A single cDNA clone prepared from mRNA of embryonic cotyledons will arrest the translation of all the 69 kD preproproteins, whereas 2 different cDNA clones are required to arrest the translation of the 60 kD preproproteins (2). From this it would appear that 3 sets of genes give rise to the storage proteins of these species. However, the 2 sets of cDNA that each partially arrest the translation of the 60 kD preproproteins exhibit weak cross hybridization at very low criteria suggesting some vestigial homology between these cDNAs and thus their genes.

Sequence analysis of cDNA's representing each of the 3 sets and dot matrix and hydropathy ana-

lyses of these sequences reveal that the 2 sets of cDNAs encoding the 60 kD family of preproproteins are members of a single gene family.

Characteristics of the mRNAs derived from the cDNAs, codon usage, amino acid compositions and the putative cleavage sites for producing the mature storage proteins species also are presented here.

Materials and methods

Cotton plants from which mRNA was extracted for cDNA synthesis was *Gossypium hirsutum*, variety Coker 201. The preparation of cDNAs and their identification as storage protein representatives by hybrid selection and arrest have been described (2).

The nucleotides sequences were determined according to the chemical modification protocols of Maxam and Gilbert (3). DNA fragments after sub-

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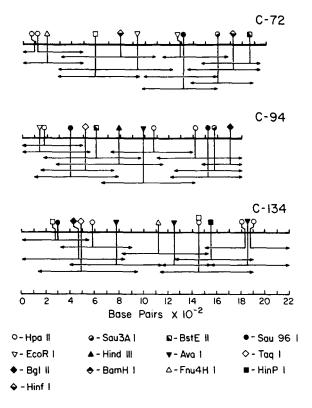


Fig. 1. Sequencing strategy. Clones were cut with the restriction endonucleases shown, labelled by site filling and sequencing in both directions from the cut site.

cloning were digested with restriction enzymes that leave recessed 3' ends. The fragments were labelled by filling in the recessed ends with the Klenow fragment of DNA polymerase I using 32 P-labelled dNTPs. The end-labelled fragments were digested

with a second restriction endonuclease and sequenced. The restriction endonucleases used and the length of sequence obtained from each site are given in Fig. 1. Sequencing was carried out in both directions from the cut sites, which provided the sequence of both DNA strands in over 90% of the cDNA spans.

To increase resolution, one side of the sequencing gels was covalently bound to one of the glass plates by the method of Garoff and Ansorge (4). After electrophoresis the gels were dried and autoradiographed.

The computer program for comparing nucleotide and amino acid sequences by dot matrix analyses was written by D. J. Neigel (UCLA, Los Angeles). This program allows for variation in the span of nucleotides and amino acids covered and in the stringency of matches per span.

The values for amino acid hydrophobicity used in the hydropathy analyses were those of Kyte and Doolittle (5).

Results

Characteristics of the cDNA clones

The notation used for identifying the cDNA clones sequenced is:

Clone C-72 – arrests the translation of the 69 kD preproproteins

Clone C-94 - arrests the translation of some of the 60 kD preproproteins

Clone C-134 – arrests the translation of the 60 kD preproproteins not arrested by C-94

Table 1. Characteristics of cDNA clone sequences and of the derived proteins. Protein sizes given in kDaltons.

	Preproprotein	Proprotein	Mature proteins1	N terminal Met	#AA in leader ²	AATAAA	Poly A	Coding NT	3' NT ³	#AA4
C-72	69725 ⁵	67032	46464 20585	met-val missing ⁵	23	multiple	_	1764	201	586
C-94	58212	56148	24400 20900 10500	met and 1 or 2 AA missing	19	+	+	1521	243	507
C-134	58713		20900 20670 14900	+		multiple overlapping	_	1548	138	516

¹ From gel electrophoresis and sequence assuming cleavages given in text.

² Number of AA in cDNA clones.

³ 3' untranslated nucleotides contained in cDNA clones.

⁴ The number of amino acids of preproprotein encoded by the cDNA clones. For complete preproprotein, add 2 AA to C-72 and 2-3 AA to C-94.

⁵ Sequence of genomic DNA reveals that met-val is missing from the N terminal of C-72 preproprotein (ref. 16). These 2 AA are included in determination of size of this preproprotein.

Significant features of the nucleotide sequence are given in Table 1 and the sequences themselves in Figs. 2-4. Since the cDNAs were cloned with Pst I, the G and C tails are shown as part of the

cDNA inserts in the Figures. Only the non-coding DNA strand is shown to represent the mRNA. All three clones have one or more AATAAA poly (A) addition sites in the 3 untranslated region, although

(met val)ARG ASN LYS SER ALA CYS VAL VAL LEU LEU PHE SER LEU PHE LEU SER PHE GLY LEU CYS SER ALA LYS ASP PHE PRO GLY ARG ARG GLY GGG GGG AGG AAT AAG TCA GCT TGC GTA GTT TTG CTC TTT TCT CTT TCC CTT TCC TTT GGC TTG CTT TGT TCT GCC AAA GAC TTT CCC GGA AGA AGA GGC ASP ASP ASP PRO PRO LYS ARG TYR GLU ASP CYS ARG ARG CAG CGA TGC GAA TGG GAT ACA CGT GGA CAA CAG CAG TGT GAA GAA AGT TGT AAA SER GLN TYR GLY GLU LYS ASP GLN GLN ARG HIS ARG PRO GLU ASP PRO GLN ARG ARG TYR GLU GLU CYS GLN GLU CYS AGG GLN GLU CYS GLN GLN GLU CYS GLN GLN GLU CYS GLN GLN GLN GLN GLN GLU GLU GLU AGT CAG TAT GGG GAG AAA GAT CAA CAA CAA CAA GAA GAA GAA ARG GLN ARG PRO GLN CYS GLN GLN ARG CYS LEU LYS ARG PHE GLU GLN GLU GLN GLN GLN SER GLN ARG GLN PHE GLN GLU CYS GLN GLN HIS CYS HIS AGG CAA CGA CCA CAA TGC CAA AGG TGC TTG AAA CGA TTC GAG CAA GAA CAA TCC CAG AGG CAA TTC CAG GAG TGT CAG CAA CAT TGC CAC ASN PHE ANG VAL LEU GLN ANG PHE ALA SER ANG HIS PRO ILE LEU ANG GLY ILE ASN GLU PHE ANG LEU SER ILE LEU GLU ALA ASN PRO ASN THE PHE AAT TTC ANG GTC CTC CAA ANG TTT GCT TCA ANG CAT CTC ATT CTT ANG GGC ATC AAT GAG TTC CGC TTA TCT ATC TTA GAA GCA AAC CCC AAC ACT TTC VAL LEU PRO HIS HIS CYS ASP ALA GLU LYS ILE TYR LEU VAL THR ASN GLY ARG GLY THR LEU THR PHE LEU THR HIS GLU ASN LYS GLU SER TYR ASN GTT CTC CCA CAC CAT TGT GAC GCT GAG AAA ATT TAC CTT GTC ACC GAA GGA AGG CTT ACG TTC TTG ACA CAT GAA AAC AAA GAG TCT TAC AAT PRO VAL ASH ASH PRO ARG GLN PHE GLU GLU PHE PHE PRO ALA GLY SER GLN ARG PRO GLN SER TYR LEU ARG ALA PHE SER ARG GLU ILE LEU GLU PRO CCT GTT AAT AAC CCT AGA CAC TTT AGT CGT GAA ATT CTT GAG CCT GGA AGC CCT CAG TCA TAT CTC CGA GCC TTT AGT CGT GAA ATT CTT GAG CCT ALA PHE ASN THE ANG SEE GLU GLN LEU ASP GLU LEU PHE GLY GLY ANG GLN SEE HIS ANG ANG GLN GLN GLY GLN GLY MET PHE ANG LYS ALA SEE GLN GCC TTC AAT ACA CBA ABT GAG CAG GTA GAC GAG CTA GAC CTA GAC GAG CTA GAC CTA GAC GAG CTA GAC CTA GAC CTA GAC GAC CTA GAC GAC CTA GAC GAC CTA GAC CTA GAC CTA GAC GAC CTA GA GLU GLN ILE ARG ALA LEU SER GLN GLU ALA THR SER PRO ARG GLU LYS SER GLY GLU ARG PHE ALA PHE ASN LEU LEU TYR ARG THR PRO ARG TYR SER GAA CAA ATA AGG GCA TTG AGC CAA GAA GCC ACT TCT CCA AGG GAG AAA AGT GGC GAG AGA TTC GCC TTC AAC TTA TTG TAC CGG ACC CCT CGC TAC TCC **444 444 446** ASH GLN ASH GLY ARG PHE TYR GLU ALA CYS PRO ARG GLU PHE ARG GLN LEU SER ASP ILE ASH VAL THR VAL SER ALA LEU GLN LEU ASH GLN GLY SER AAC CAA AAC GGC CGC TTC TAT GAG GCT TGC CCA CGT GAA TTC CGG CAA CTC AGT GAC ATC AAT GTC ACC GTT TCA GCC TTG CAA TTA AAC CAA GGA TCA ILE PHE VAL PRO HIS TYR ASN SER LYS ALA THR PHE VAL VAL LEU VAL ASN GLU GLY ASN GLY TYR VAL GLU MET VAL SER PRO HIS LEU PRO ARG GLN ATC TTT GTG CCG CAC TAC AAT TCA AAG GCT ACA TTC GTG GTC CTT GTT AAC GAA GGA AAT GGA TAC GTT GAA ATG GTT TCT CCT CAT CTT CCT AGG CAA GLY ASP ILE PHE YAL VAL PRO ALA ASN PHE PRO VAL THR PHE VAL ALA SER GLN ASN GLN ASN LEU ARG MET THR GLY PHE GLY LEU TYR ASN GLN ASN GGA GAC ATA TIT GTA GTC CCA GCA AAC TTC CCA GTT ACA TTC GTT GCA TCC CAA AAC CAG AAC CTG CGG ATG ACT GGG TTC GGT CTT TAC AAC CAG AAC ILE ASN PRO ASP HIS ASN GIN ARG ILE PHE VAL ALA GLY LYS ILE ASN HIS VAL ARG GIN TRP ASP SER GIN ALA LYS GLU LEU ALA PHE GLY VAL SER ATT AAC CCA GAC CAT AAC CAG AGG ATC TTC GTG GCT GGG AAA ATC AAC CAT GTG AGA CAA TGG GAT AGC CAA GCC AAG GAG TTG GCC TTT GGG GTG TCA SER ARG LEU VAL ASP GLU ILE PHE ASN ASN ASN PRO GLN GLU SER TYR PHE VAL SER ARG GLN ARG GLN ARG ALA SER GLU
TCG AGG TTG GTG GAT GAG ATA TTC AAC AAC CCA CAA GAG TCT TAC TTC GTG TCT CGA CAG AGG CAG CGT GCA TCT GAA TAA AGA GTT AGA GGC AAT AAT AAC CAC ATA GTC TCG TGT TTA CGG ACT AAT GTG AAA TTT TCT AGT TTT AAG CAG GCA TGT AAT GAA ATA AAA TGG TGA CCT ATG TAA AGG AGG AGA CCC TTG TTT TGT AGT GCA ATA ACT TGT ATG AAC TTT TTG AGT GCC TTT TTT GTC TTT CTA TGT TAA GGA ATA AAA CGT TAA GTA CCC CCC CCC CCC

Fig. 2. Nucleotide and amino acid sequence of cDNA C-72. N terminal leader sequence is over-dotted; CysxxxCys sequences are indicated by wavy overlines; clusters of Glu residues are over-dotted; glycosylation site is disignated by v; poly A signal sites are underdotted. Arrows indicate possible processing cleavage sites.

the sequence AATATA may carry out this function in C-94 since it is within 25 nucleotides of the poly (A) tail (marked on the Figures). The other two clones do not have poly (A) tails. Palindromic tracts capable of forming hairpin structures are found in the 3' untranslated regions of C-94 and C-134 (marked on the Figures).

A curious anomaly was found in clone C-134; namely, the coding sequence from nucleotide 1344 to nucleotide 1728 is found in the inverse complement orientation beginning with the 1st 5' cDNA nucleotide (not counting the poly G of the cDNA construct) and ending 2 nucleotides upstream from the initiating Met codon (denoted in Fig. 4). Only

C 94 GLN ASN GLU CYS GLN ILE ASN ARG LEU ARG ALA SER ALA PRO GLN THR ARG ILE ARG SER GLU ALA GLY THR THR GLU TRP TRP ASN PRO ASN CYS GLN CAA AAT GAG TGT CAA ATC AAT AGG CTC CGC GCC TCT GCA CCC CAG ACC CGA ATT CGC TCC GAA GCC GGC ACC ACC GGG TGG TGG AAC CCC AAC TGT CAA GLN LEU ARG CYS ALA GLY VAL SER VAL MET ARG GLN THR ILE GLU PRO ASN GLY LEU VAL LEU PRO SER PHE THR ASN ALA PRO GLN LEU LEU TYR ILE
CAG CTC AGG TET GCT GGT GTA TCT GTA ATG AGG CAG ACT ATT GAA CCT AAT GGC CTT GTC TTG CCT TCC TTC ACC AAT GCT CCT CAG CTT CTC TAC ATT VAL GLN GLY ARG GLY ILE GLN GLY ILE VAL MET PRO GLY CYS ALA GLU THR PHE GLN ASP SER GLN GLN TRP GLN HIS GLN SER ARG GLY ARG PHE GLN GTC CAA GGA AGA GGT ATT CAA GGT ATT GTG ATG CCT GGA TGT GCC GAA ACA TTC CAG GAT TCA CAG CAA TGG CAG CAT CAA AGC CGT GGC AGG TTC CAG ASP GLN HIS GLN LYS VAL ARG ARG PHE ARG GLN GLY ASP ILE ILE ALA LEU PRO GLN GLY VAL VAL HIS TRP SER TYR ASN ASP GLY ASN GLU ARG VAL GAC CAG CAC CAG AAA GTC CGA CGG TTC CGC CAG GGT GAC ATC ATT GCT TTG CCG CAG GGT GTG GTT CAC TGG AGC TAC AAT GAT GGC CAT GAG CGT GTT VAL THR ILE ASN LEU LEU ASP THR GLY ASN SER ALA ASN GLN LEU ASP ASN ILE PRO ARG ARG PHE HIS LEU ALA GLY ASN PRO GLU GLU GLU GLU ARG GTT ACT ATC AAT CTA CTT GAC ACT GGC AAC AGT GCT AAT CAG CTC GAC AAC ATC CCT AGA AGA TTC CAT CTT GCT GGT AAC CCA GAA GAG GAA CAG AGG ALA GLN ALA PHE ASN VAL ASP HIS ASP ILE ILE ARG LYS ILE GLN ARG VAL ARG GLY ASN ARG GLY THR ILE ILE ARG VAL ARG ASP ARG LEU GLN VAL GCT CAA GCT TTC AAC GTT GAC CAC GTC ATC ATC AGA GTC AGG GAC ATC ATC AGA GTC AGG GAC ATC CAA GTC GLU THR PHE CYS SER MET ARG ILE LYS GLU ASN LEU ALA ASP PRO GLU ARG ALA ASP ILE PHE ASN PRO GLN ALA GLY ARG ILE SER THR LEU ASN ARG GAA ACT TTC TGC TCC ATG AGG ATC AAG GAG AAC CTT GCT GAC CCT GAA CGT GCC GAC ATT TTC AAC CCA CAA GCC GGT CGT ATT TCC ACC CTC AAC AGG Phe ASN LEU PRO ILE LEU GLN ARG LEU GLU LEU SER ALA GLU ARG GLY VAL LEU TYR ASN ARG ALA GLY LEU ILE PRO GLN TRP ASN YAL ASN ALA HIS
TTT AAC CTC CCC ATC CTC CAA CGA CTT GAG CTC AGC GCC GAG AGG GGT GTC CTC TAC AAC AGA GCT GGT TTG ATT CCA CAA TGG AAC GCT CAC LYS ILE LEU TYR MET LEU ARG GLY CYS ALA ARG VAL GLN VAL VAL ASN HIS ASN GLY ASP ALA VAL PHE ASP ASP ASN VAL GLU GLN GLY GLN LEU LEU
AMA ATA CTC TAC ATG TTA AGA GGC TGT GCC AGA GTG CTC CTG TAC ACC GGC GAC GCA GTG TTC GAC GAT AAC GTG GAG CAA GGT CAG CTC CTG THR VAL PRO GLA ASN PHE ALA PHE MET LYS GLA ALA GLY ASN GLU GLY ALA GLU TRP ILE SER PHE PHE THR ASN SER GLU ALA THR ASN THR PRO MET AGG GTG CCA CAG AAC TTT GCG TTC ATG AAA CAA GCA GGA GAC GAA GGA GGC GAA TGG ATT TCC TTC TTC ACC AAT AGC GAA GCC ACC ACC ACC ACC CCA ATG ALA GLY SER VAL SER PHE MET ARG ALA LEU PRO GLU GAU VAL VAL ALA ALA SER TYR GLN VAL SER ARG GLU ASP ALA ARG ARG ILE LYS PHE ASN ASN GCT GGA AGT GTC TCC TTC ATG CGA GCA CTG CCG GAG GAA GTG GTG GCA GCG TCG TAT CAG GTG TCA AGA GAA GAT GCA AGG CGG ATT AAG TTC AAC AAC LYS ASN THE PHE PHE PHE PHE THE PRO SEE GLN SEE GLN AGG AGG ALA ASP ALA
AMA AMC ACC TIT TIC TIC ACT CCT TCA CAG TCC GAG AGG AGG GCC GAT GCT TAA ACT CCA GCC GAT GCC AAT GCA ATC AAA ATC ATG GGG ATC TIT GTA DE TAA CET AME TAA GAA ATA ATA AMA AGA ATA CAG AAC TAT ATC CAC CAT GGA TIT TAT TAT TAA TCA TIG AAA TAC TGA AAG ATT TTA ATT TAG AAG ATE AAT AAA AGA AAG AAT CTT ACA AGG CGG CCC TTT CAT TCA TAT AAT CGG TCG AAA TGT TAC AAC TTC AAT ATA TGT AAC TTT GTA TTA TCA AAA

Fig. 3. Nucleotide and amino sequence of cDNA C-94. Wavy overline indicates longest stretch of homology with C-134. Other symbols as in Figure 2.

C 134 GGG GGG GGG GAT GAT TOT GGG GTA CGG TTA TGA CCT GAC CCC TCT CAA CCT GCT CAT CGA ATA TCG CCT CTC CAT TIT CCG ACA CAA TIT GAA TCC TTC CAT THE COE THE TEA TET AMA CAA TEE TET GOS CAT TEA TET TEE ABT BAS GAS COT AGA TAG CAT TAT TET AMA GOA CTC CCC TET COS COE TEA STT GGA GGT ATT GGA GAA TGG GAA GAT TGA AAC TGT TAA CTG TGG TGA TGC GAC CAC CTC GTG GGT TGA AAA CAT CAG CAG AGG AAG CAG GGG TCC TGT GTT MET ALA TYR THR SER LEU LEU SER PHE SER VAL CYS LEU LEU VAL LEU PHE HÎB GLY CYS CYS ALA GLN ILE ASP LEU VAL THR ASN HIS HIS GLH ASP ATE BCT TAC ACT TCT TTG CTT TCT TTT AGC GTT TGC TTG CTT GTC TTC CAT GGC TGC TGT GCT CAG ATA GAT CTC GTC ACT AAC CAT CAG GAT PRO PRO TRP GLY GLN PRO GLN GLN PRO GLN PRO ARG HIS GLN SER GLN CYS GLN LEU GLN ASN LEU ASN ALA LEU GLN PRO LYS HIS ARG PHE ARG SER CCA CCT TGG GGG CAG CCT CAG CAA CCT CAG CCA CGT CAC CAA TCC CAA TCC CAA CTC CAG AAC TTG AAT GCT CTT CAG CCT AAG CAC CGG TTT AGG TCA GLU ALA GLY GLU THR GLU PHE TRP ASP GLN ASN GLU ASP GLN PHE GLN CYS ALA GLY VAL ALA PHE LEU ARG HIS LYS ILE GLN ARG LYS GLY LEU LEU GAG GCT GGT GAA ACT GAG TTC TGG GAC CAA AAT GAG GAT CAA TTC CAG TGT GCT GGT GTT GCT TTC CTA CGT CAT AAG ATC CAG CGC AAA GGA CTT TTA LEU PRO SER PHE THR SER ALA PRO MET LEU PHE TYR VAL GLU GLN GLY GLU GLY GLU GLY ILE HIS GLY ALA VAL PHE PRO GLY CYS PRO GLU THR TYR GLN SER TTG CCT TCA TTT ACC AGT GCT CCT ATG CTT TTC TAT GTT GAA CAA GGG GAG GGT ATT CAT GGG GCG GTC TTC CCA GGT TGT CCC GAG ACA TAT CAA TCA GUN SER GLN GUN ASN ILE GUN ASP ARG PRO GUN ARG ASP GUN HIS GUN LYS LEU ARG ARG LEU LYS GLU GLY ASP VAL VAL ALA LEU PRO ALA GLY VAL CAG TCG CAA CAA AAT ATA CAA GAT AGG CCA CAA AGG GAT CAG CAC CAA AAG CTC AGA CGG TTG AAG GAG GGC GAT GTG GTT GCC TTG CCT GCA GTA ALA HIS TRP ILE PHE ASN ASN GLY ARG SER GLN LEU VAL LEU VAL ALA LEU VAL ASP VAL GLY ASN ASP ALA ASN GLN LEU ASP GLU ASN PHE ARG LYS
GCT CAC TGG ATT TTC AAC AAT GGG CGG TCT CAA CTT GTG TTG GTC GCA CTT GTT GGC CAT GAT GCC CAC CAG CTC GAT GAG AAC TTC AGG AAA . 31 GLU GLU SER GLU GLU SER GLY GLY ASN ASN VAL LEU SER GLY PHE ARG ASP ASN LEU LEU ALA GLN ALA PHE GLY ILE ASP THR ARG LEU ALA ARG GAG GAA GAG TOG CAA GAG AGC GGC GGA AAC AAT GTG CTC AGT GGC TTT CGC GAC AAT CTC CTG GGG CAG GCT TTC GGA ATT GAT ACC AGG CTA GCA AGG LYS LEU GLN ASN GLU ARG ASP ASN ARG GLY ALA ILE YAL ARG MET GLU HIS GLY PHE GLU TRP PRO GLU GLU GLY GLN ARG ARG GLN GLY ARG GLU GLU GLY GAR GAR GGC CAG AGG CA ARG ARG SER GLY ASN GLY LEU GLU GLU THR PHE CYS SER MET ARG LEU LYS HIS ARG THR PRO ALA SER SER ALA ASP VAL PHE ASN PRO ARG GLY GLY AGA AGG TCA GGA AAC GGC TTA GAA GAA ACA TTC TGC TCA ATG AGA CTG AAA CAC AGG ACC CCT GCT TCC TCT GCT GAT GTT TTC AAC CCA CGA GGT GGT ARG ILE THR THR VAL ASN SER PHE ASN LEU PRO ILE LEU GLN TYR LEU GLN LEU SER ALA GLU ARG GLY VAL LEU TYR ASN ASN ALA ILE TYR ALA PRO CGC ATC ACC ACA GTT AAC ACT TIC AAT CTT CCC ATT CTC CAA TAC CTC CAA CTC ASC GCC GAG ASG GGA GTC CTT TAC AAT AAT GCT ATC TAC GCT CCT HIS TRP ASN MET ASN ALA HIS SER ILE VAL TYR ILE THR ARG GLY ASN GLY ARG ILE GLN ILE VAL SER GLU ASN GLY GLU ALA ILE PHE ASP GLU GAN TAG TEG AAC ATG GAT GAT GAT GAG AGG GAT ATT GAT GAG CAG VAL GLU ARG GLY GLN VAL ILE THR VAL PRO GLN ASN HIS ALA VAL VAL LYS LYS ALA GLY ARG ARG GLY PHE GLU TRP ILE ALA PHE LYS THR ASN ALA GTT GAG AGG GGT CAG GTT ATA ACC GTA CCC CAG AAT CAT GCA GTG GTG AAA AAA GCA GGA AGG CGA GGG TTT GAA TGG ATA GCA TTC AAG ACA AAT GCC ASH ALA LYG ILE BER GLN ILE ALA GLY ARG VAL SER ILE MET ARG GLY LEU PRO VAL ASP VAL LEU ALA ASH SER PHE GLY ILE SER ARG GLU GLU ALA AAT GCT AAG ATT AGT CAG ATT GCT GGA CGT GCC AGC TCC TTT GGT ATA TCC CGG GAG GAG GCC MET ANG LEU LYS HIS ASN ANG GLN GLU VAL SER VAL PHE SER PRO ANG GLN GLY SER GLN GLN GEN ANG ANG CAT AAC AGA CAG AGG CAG GTG TCG GTA AGG CAA GGC TCG CAA CAG TAG ATC AAA CAA CTA ATC TAG GTG TAC GTA CGT ACG TAM THE CAT CAM CAM TAM AND COM GAT CCC ANT ATC TGG ATT CGG AAT AMA TAT ATA TAT AMA TAM AND TAM AND ACA TGG CCT TAM CAG GCM TGC AMC TCT AGT CCC CCC CCC CCC CCC C

Fig. 4. Nucleotide and amino acid sequence of cDNA C-134. Under-dotted regions show inverse complement artifact as well as poly A signal sites. Other symbols as in Figure 2.

a single mismatch is present in this 385 nucleotide sector which is 6 nucleotides upstream from the Met codon. Obviously this sequence, which would create a very large hairpin in the mRNA itself, cannot exist as a functional molecule in the cell, and, thus, must be an artifact generated in cDNA synthesis. We mention this because we have observed the same phenomenon in 2 other cDNA prepared from cotton mRNA. Thus it is not an isolated incident. An explanation for how a 3' sequence may end up in the inverse complement orientation at the 5' end of a cDNA is presented in Fig. 5.

This proposed mechanism posits a fortuitous hybridization of the 3' end of a nearly full length cDNA first strand with a short complementary region internal to the 3' end. Second strand synthesis is postulated to add on to this loop structure making the complement of the 1st strand. This is what is considered to happen in normal double stranded cDNA synthesis. What is new in this model is the formation of a second loop causing continued cDNA second strand synthesis to form the complement of the portion of the 2nd strand already

made. Continued 2nd synthesis gives a double stranded cDNA longer than the cognate mRNA and containing at the 5' end (mRNA) in the inverse orientation the complement of the 3' region.

This idea is further strengthened by the fact that the fortuitous complementarity that forms the 1st loop, being fortuitous, may not be perfect. In fact in clone C-134 there is a T/C mismatch 5 nucleotides from the beginning of the inverse complement region. There are no mismatches thereafter (379 nucleotides) since what is formed beyond the hybridized region is the result of 2nd strand synthesis. Others have noted similar cloning errors near the 5' end as well (7, 8).

The sequence downstream from the artifact has been verified by the sequencing of genomic DNA (data not given).

Characteristics of derived proteins

Gross characteristics of the derived proteins are given in Table 1 and their sequences in Figs. 2-4. The N-terminal Met is found on only 1 of the 3

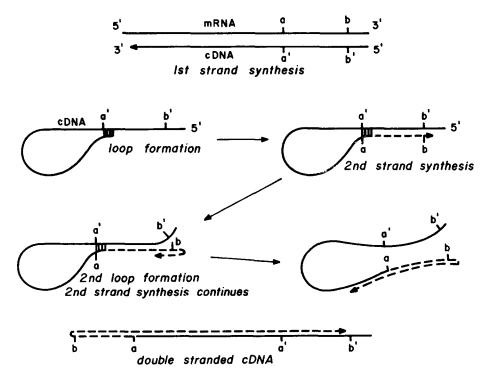


Fig. 5. Model attempting to explain origin of the cloning artifact found in cDNA C-134 (and in other non-storage protein cDNAs). Model is explained in the text.

cDNA clones (C-134). However, the other clones are very close to including the N-terminal coding region. The 5' leader sequence is quite obvious in the sequence of all these proteins (see also Fig. 8, the hydropathy analysis of the proteins). Furthermore, a genomic sequence for the protein family typified by C-72 (16) shows that only a Met-Val is lacking from the coding region in this clone. Although the N terminal amino acids are blocked in the mature proteins, the cleavage point for the leader sequence is also unmistakable in all 3 clones using the formulation of von Heijnes (9). The leader sequences are designated on the Figures.

C-72 protein

The C-72 clone arrests the synthesis of the family of preproproteins of 69 kD which gives rise, after processing, to the mature proteins of 46.5 and 52 (glycosylated) kD and smaller fragments of about 20.5 kD (1). The precise endoproteolytic site has not been determined. However, measurements of molecular weights and calculated isoelectric points

deduced from amino acid sequence, when compared with 2D gel profiles of the mature proteins, allow for a gross placement of this site (1) as does the amino acid composition of the 46.5 and 52 kD proteins determined some years ago (23). Figure 2 designates 2 possible sites (designated by arrows) that follow Arg-Arg pairs. Contiguous pairs of basic residues are common endoproteolytic cleavage sites (10), and, in the case of the specific protein corresponding to C-72, the Arg Arg/Ser site is a possible site since it yields 2 fragments with the molecular weights and pIs observed on 2D gels (1). Since the N terminal amino acid of the 46.5 and 52 kD larger fragment resulting from this cleavage is blocked (11), this cleavage should leave an amino acid suitable for substitution. Either the Glu or Ser residues shown satisfy this requirement. Of course other points of cleavage in this region of the proprotein may be proposed, but we have based the deduced amino acid composition of the mature proteins (Table 2) on the cleavage site given above. The C-72 protein has regions of concentrated Glu

Table 2. Representative amino acid composition of the cotton storage protein families.

	α Globulin fam	ily (cDNA C	C-72)	β Globulin famil		C16 " P /	D.V. 6.44	
	Preproprotein*	Proprotein	Mature protein: large fragment	Cys-rich fragment	Subfamily A (cD Preproprotein**		Subfamily B (contraction Preproprotein	
ASP	16	16	8	8	19	19	17	17
ASN	34	33	31	2	39	37	31	31
GLU	68	68	36	32	49	49	47	47
GLN	69	69	35	34	47	47	50	50
ARG	68	67	40	27	54	54	52	52
LYS	18	17	10	7	7	7	14	14
HIS	17	17	11	6	9	9	15	14
SER	41	37	33	4	32	30	33	30
THR	15	15	13	2	20	20	14	13
CYS	16	14	2	12	9	8	7	4
PRO	29	29	20	9	21	20	22	22
GLY	28	27	22	5	33	32	46	45
ALA	25	23	22	1	33	32	33	31
VAL	33	30	29	1	28	27	32	30
ILE	16	16	16	0	23	23	21	21
LEU	35	29	28	1	38	31	37	32
PHE	37	34	29	5	25	22	24	22
TYR	16	16	12	4	6	6	7	6
TRP	3	3	1	2	6	6	7	7
MET	4	3	3	0	9	9	7	6
	588	563	401	162	507	408	516	494
Mol wt	69725	67032	46464	20585	58212	56148	58713	56352

^{*} The N terminal MetVal, missing from the cDNA sequence but known from a genomic sequence, has been added in this calculation.

^{**} This clone lacks 1-3 amino acids of the N terminal. Thus these values are 1-3 amino acids low.

residues as do the other two storage proteins which are designated on the Figures. These concentrations of negative charge are thought to explain the 'negative staining' characteristics of these storage proteins (12) and help in identifying the cleavage fragments on 2D gels (1). C-72 protein large fragment contains a glycosylation site (designated in Fig. 2) and thus must represent a protein of the 52 kD group of this family of storage proteins. Other sequenced clones of this family lack this site and must represent members of the 46.5 kD group (16).

The most curious feature of the amino acid composition of this family of proteins is the composition of the mature small fragment (~20 kD). This polypeptide is very highly charged and contains 12 Cys residues arranged as 6 CysxxxCys sectors. The amino acid composition is unlike any reported for a plant storage protein and more closely resembles the Cys-rich domains of mammalian cell surface receptor proteins (13, 14, 15). It would seem that this domain has been added to this storage protein family from elsewhere as discussed in ref 16. The sequence of the large fragment of the C-72 protein shares homology with vicilin proteins of legumes (6).

C-94 and C-134 proteins

These proteins superficially do not seem related in sequence. They do have in common regions of concentrated Glu residues and a 13 amino acid region that almost certainly comprises a processing cleavage site for generating some of the mature cotton proteins (marked on Figs. 3, 4). Homologous sequences are found in the legumins of legumes (17, 18, 19) the cruciferins of Brassica (20) and the globulins of Avena (21). This sequence is known to contain the cleavage site Asn/Gly for the processing of the legumins (17). Thus the conservation of this sequence must represent the conservation of an endoprotease recognition site. This feature and others show a common heritage for these cotton proteins, the legumins family of legumes, the cruciferins of Brassica and the oat globulins (6, 22).

Amino acid composition and codon usage

Table 2 gives the amino acid compositions and molecular weights of the preproproteins, proproteins and mature processed proteins encoded in the representive cDNAs. It should be remem-

bered that these cDNAs represent only one of several genes comprising the 2 small multigene families.

The amino acid compositions of the mature protein species are not given for C-94 and C-134, since all the cleavage sites have not been demonstrated. Both proteins are undoubtedly cleaved twice (not counting the removal of the leader sequences). The first cleavage quite likely occurs between the Asn/Gly of the tract of amino acids conserved in many angiosperm storage proteins as pointed out above. Cleavage here generates a small fragment that in each case has a molecular weight and pI of observed mature storage proteins (see ref 1 for other considerations). The large fragment in the case of C-94 has a transient existance in embryogenesis and can be seen on 2D gels (1). Its second cleavage is shown in Fig. 3 to occur in a ArgArg/Ser tract shown in the Figure since this would produce fragments with the characteristics of mature protein species (1). The second cleavage in C-134 can not be speculated at this point, although the mature proteins derived from this cleavage can be identified on 2D gels (1). The identification of the products of the second cleavage of these proteins is borne out by the disulfide linkages observed between mature species (22).

Codon usage in these three sequences is given in Table 3. Noteworthy here is the infrequent use of XCG. The summation of all three sequences shows that in the case of Ser where TCG is one of 6 code words, it is used on by 9/106 times; in the case Pro, Thr and Ala, all having 4 code words, XCG is used only 8/72, 4/49 and 9/91 times respectively.

Relationship among sequences

In order to search for homologous regions of sequence that may suggest evolutionary relations between these proteins, the nucleotide and amino acid sequences were compared by dot matrix analysis. In Fig. 6 the nucleotide sequences are compared at 2 levels of stringencies. In the left panels nucleotides in groups of 4(1-4, 2-5, etc.) are compared and only a perfect match yields a dot. Using this low span of nucleotides, in which the random probability of a match is 1 in 256 sets of 4, considerable background is generated.

In the right panel a span of 12 nucleotides is used and 9 matches in position are required to yield a

Table 3. Codon usage.

		Preproproteins:			Proproteins:			Summation:
		C-72	C-94	C-134	C-72	C-94	C-134	(preproproteins)
Phe	TTT	10	6	8	8	4	7	24
	TTC	27	19	16	26	18	15	62
Leu	TTA	4	1	2	4	1	2	7
	TTG	11	4	9	9	3	7	24
	CTT	11	10	10	8	7	8	31
	CTC	5	17	10	4	15	9	32
	CTA	2	3	3	2	2	3	8
	CTG	2	3	3	2	3	3	8
Ile	ATT	4	12	11	4	12	11	27
	ATC	7	10	4	7	10	4	21
	ATA	5	1	6	5	1	6	12
Val	GTT	10	5	13	9	4	11	28
	GTC	7	9	6	7	9	6	22
	GTA	5	4	3	4	4	3	12
	GTG	10	10	10	10	10	10	30
Ser	TCT	10	5	5	8	4	3	20
	TCC	5	9	5	4	8	5	19
	TCA	9	6	5	8	6	5	20
	TCG	2	2	5	2	2	5	9
	AGT	6	3	6	6	3	6	15
ъ	AGC	9	7	7	9	7	6	23
Pro	CCT	11	8	9	11	7	9	28
	CCC	3	5	4	3	5	4	12
	CCA	11	6	7	11	6	7	24
T)	CCG	4	2	2	4	2	2	8
Thr	ACT	5	5	3	5	.5	2	13
	ACC	3	11	6	3	11	6	20
	ACA	5	2	5	5	2	5	12
A Io	ACG	2	2	0	2	2	0	4
Ala	GCT	8	13	17	7	12	15	38
	GCC GCA	9 8	11	8	8	11	8	28
	GCG	0	7 2	5 3	8	7	5	20
Tyr	TAT	3	1	2	0 3	2 1	3	5
1 91	TAC	13	5	5	13	5	2 4	6
His	CAT	10	3	7	10	3	6	23 20
1113	CAC	7	6	8	7	6	8	20 21
Gln	CAA	46	18	28	46	18	28	92
Om	CAG	23	29	22	23	29	22	74
Asn	AAT	10	12	16	9	11	16	38
	AAC	24	27	15	24	26	15	66
Lys	AAA	10	5	6	10	5	6	21
,-	AAG	8	2	8	7	2	8	18
Asp	GAT	7	6	13	7	6	13	26
F	GAC	9	13	4	9	13	4	26
Glu	GAA	42	26	22	42	26	22	90
	GAG	26	23	25	26	23	25	74
Cys	TGT	7	6	3	6	6	2	16
•	TGC	9	3	4	8	2	2	16
Arg	CGT	6	6	4	6	6	4	16
•	CGC	8	9	3	8	9	3	20
	CGA	9	6	7	9	6	7	22
	CGG	4	3	5	4	3	5	12
	AGA	12	14	14	12	14	14	40
	AGG	29	16	19	28	16	19	64
Gly	GGT	2	10	11	2	10	11	23
	GGC	6	10	7	5	9	6	23
	GCA	14	10	21	14	10	21	45
	GGG	6	3	7	6	3	7	16

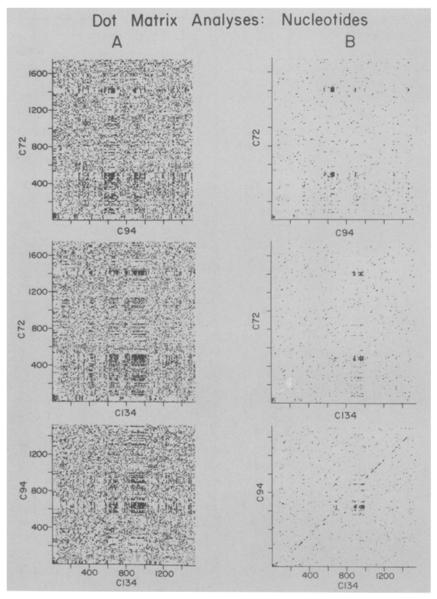


Fig. 6. Comparison of nucleotide sequence of the 3 clones by dot matrix analyses. See text for description of stringencies of comparisons in each panel. Only coding nucleotides are compared.

dot. The increase in span greatly offsets the relaxed stringency and less background is generated, since the random possibility of a 9/12 match is 1 in 2829 sets of 12. Since roughly 1500 sets in one sequence is compared with a similar number in the other sequence, about 800 random dots are anticipated. When C-72 sequence is matched against C-94 or C-134 at either stringency, no regions of homologies are observed, save for the boxes of dots that de-

mark the clusters of Glu found in all these proteins (GAG, GAA clusters). However, when C-94 is compared with C-134 at the low span, a diagonal line from beginning to end of the sequences is discerned. This line of homology becomes more obvious against the low background of dots given by the long span matrix (bottom right panel). Clearly C-94 and C-134 cDNAs are related in their nucleotide sequence throughout and, clearly, sequences in

the middle of the coding regions have moved in time with respect to one another.

Figure 7 gives the same comparisons for the amino acid sequences of the clones. Three comparison stringencies are used here. The first (left panels) compares each amino acid of each sequence and since the random possibility of each comparison is 1 in 20 and since roughly 500 amino acids are being compared, about 12500 random dots are anticipated $(500 \times 500/20)$. In the comparisons of C-72

with C-94 and C-134 only the blocks of poly Glu are noticed against the high background. However, the comparison of C-94 with C-134 gives a diagonal line of homology from N termini to C termini of each sequence.

In the middle panels the span of amino acids has been set at 12 and 7 positions must match to generate a dot. Since the random occurence of a 7/12 match is about about 1 in 2×10^6 sets of 12, no random dots are anticipated ($500 \times 500/2000000$).

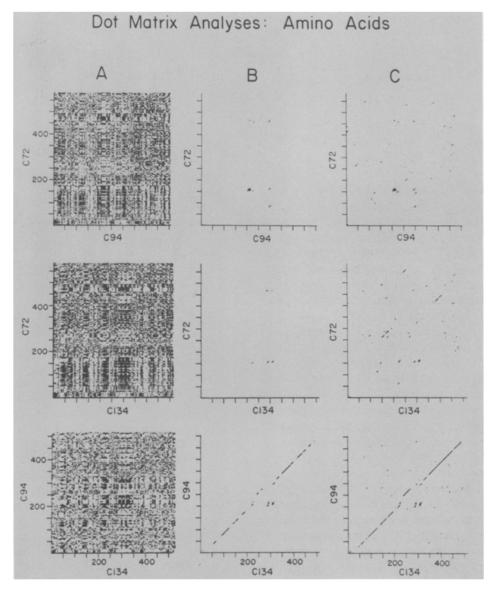


Fig. 7. Comparison of amino acid sequence of the protein represented by the 3 clones by dot matrix analyses. See text for description of the stringencies of comparisons in each panel. The N terminus of each protein is in the bottom left corner of each plot.

This erases all background and leaves only a few dots marking the poly Glu tracts when C-72 is compared with C-94 and C-134. The vestigial homology between C-94 and C-134, however, is clearly shown in this matrix plus the fact that sequences have moved in one of these genes sets relative to the other in the middle of the coding region.

The third comparison again requires that 7 of 12 amino acids match in position but reduces the number of amino acid possibilities to 10 by consolidating what may be considered 'synomyn' amino acids, i.e. grouping as a single entity those amino acids with the R groups that may be functionally similar. These groupings are:

Asp: Glu Asn: Gln Arg: Lys Ser: Thr Phe: Tyr:Trp

Ala: Val:Ile:Leu:Met

This comparison increases the probability of a random 7/12 matched set to about 1 in 21000 sets of 12. This predicts about 12 random dots in each matrix. More than 12 dots appear as background in the comparisons of C-72 with C-94 and C-134. This probably is due to the fact some amino acids occur more frequently in proteins than others which would generate a somewhat greater number of random matches, and/or due to the fact that there are preferred tracts of amino acids in seemingly unrelated storage proteins. The effect of this matching of synonyms in the C-94: C-134 comparison is to increase the number of dots on the horizontal homology line indicating that many of the amino acids changes are between functionally synonymous amino acids. This maximizes the demonstration of relatedness of C-94 and C-134 genes.

The vestigial homology between C-94 and C-134 in both nucleotides and amino acid sequence is not obvious when these sequences are compared by eye. Further, there is obviously greater homology on the amino acid level than between nucleotides indicating that many nucleotide changes have not resulted in amino acid changes.

Hydropathy analysis

Gross structural features of the 3 preproproteins

are shown by their hydropathy profiles given in Fig. 8. Here the relative hydrophilicity/hydrophobicity of amino acid domains is shown by a summation of the hydrophobicity (5) of spans of 11(1-11, 2-12, etc.) amino acids (N terminal on the left). Positive values denote hydrophobicity.

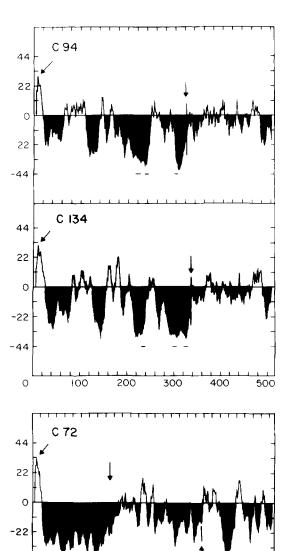


Fig. 8. Hydropathy plots of the amino acid sequences of the proteins represented by the 3 clones. Hydrophilic domains are highlighted in black. N termini on the left. The hydropathic values for amino acid are from ref 5. The span of amino acids is 11. Arrows are explained in the text.

300

-44

100

200

All three preproproteins are extremely hydrophilic. The insolubility of the mature proteins in dilute salts at neutral pH suggest that the proteins are aggregated in protein bodies by salt bridges between charge residues. High salt concentrations (e.g. 0.5 M NaCl) solubilize these proteins (23) presumably by masking these charges.

The N terminal leader sequence is obvious in all 3 instances by their hydrophobicity (shown by left most arrows). The Asn/Gly cleavage sites are shown by downward pointing arrows in C-94 and C-134 as is the putative Arg Arg/Ser cleavage site of C-72, the glycosylation site of C-72 is shown by the upward pointing arrow. Regions of poly Glu are shown by horizontal lines in the hydrophilic portions of the profiles. The profiles of C-94 and C-134 are very similar save in the middle region where a sector containing a clustered Glu tract appears to have moved in one or the other sequence.

Discussion

From the foregoing, it is apparent that the cotton storage proteins represent 2 families of genes one giving rise to the 69 kD preproproteins and the other to the 60 kD preproproteins (actually 58 kD, see Tables 1 and 2). The latter family is composed of 2 subfamilies that superficially do not seem related. However, sequence dot matrix and hydropathy analyses reveal that the 2 subfamilies emanate from a common ancestral gene. The other gene family that is represented here by cDNA C-72 can also be subdivided into subfamilies A and B based on sequence data from genomic DNA that reveals that subfamily B has a glycosylation site, whereas subfamily A does not. Further, the preproproteins of subfamily B are somewhat shorter than those of subfamily A, having a stop codon 19 triplets upstream from the stop codon of subfamily B (16). The genomic DNA sequences and genome organization data, still uncomplete, will be presented elsewhere.

We suggest that the gene family giving rise to the largest preproproteins be considered the α globulins of the cotton seed and the other gene family be considered the β globulins. The following diagram summarizes the characteristics of these gene families and their A and B subfamilies.

Cottonseed storage proteins

α Globulin family

- 15% of seed mRNA
- cleaved once after leader removed to yield Cysrich fragment and large mature protein
- has cryptic sequence homology with vicilin-like storage proteins of other angiosperms
- genes arranged as A-B tandems in genome

A Subfamily

- no glycosylation sites
- yields 46.5 kD mature proteins

B Subfamily

- represented by cDNA C-72
- yields 52 kD glycosylated mature proteins

β Globulin family

- cleaved 2× after leader removed to yield 3 mature proteins of 10-25 kD, 2 of which are disulfide linked
- has cryptic sequence homology with leguminlike storage proteins of other angiosperms
- has no glycosylation sites

A Subfamily

- represented by cDNA C-94
- 15% of seed mRNA

B Subfamily

- represented by cDNA C-134
- 5% of seed mRNA.

Some of these characteristics were published in earlier work (1, 2, 11, 23, 24) while still others (disulfide bonding, genome arrangement and sequence relationships with proteins of other plant families) will be published elsewhere (6, 16, 22).

The fact that the precise cleavage points involved in the processing of the proproteins are not known does not prevent the identification of all the small cottonseed storage proteins as products of the cleavages of the 2 families of proproteins. As pointed out in ref 1, characteristics of the mature proteins such as molecular weight, pI, 'negative staining' of poly Glu tracts, kinetics of processing, transient processing intermediates and more recently disulfide linkages all predict the precursor/prod-

uct relationships between the mature species and the preproproteins. Thus, all the cotton seed storage proteins are seen to be derived from the α and β globulins.

The genome of the commercial cotton used in this work is an amphidiploid tetraploid formed by the combination of the A ('old world') and D ('new world') genomes of the cotton tribe (25). An appealing notion would be that perhaps the α globulin family is derived from one of these ancestral genomes and the β globulins from the other ancestral genome. Another possibility would be that the A and B subfamily of each family are derived from the one or the other of the ancestral genomes. However, 1D electrophoresis of the storage proteins from species carrying exclusively the A genome (G. arboreum) and the B genome (G. thurberi) show that existance of both families and both subfamilies of proteins in each genome, albeit with some small differences in molecular weights (data not given). Thus both families and their subfamilies came into existance in the evolution of cotton before the separation of the A and D genomes.

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