The Pennsylvania State University

The Graduate School Program in Genetics

THE MOLECULAR GENETICS OF FRUIT CHROMOPLASTS FROM *CUCURBITA PEPO* L.: VARIATION IN STEADY-STATE QUANTITIES OF *RBCL*, *PSBA*, AND ORF 2280 TRANSCRIPTS, CHANGES IN LEVELS OF RUBISCO LARGE SUBUNIT AND RUBISCO ACTIVITY DURING FRUIT DEVELOPMENT

A Thesis in

Genetics

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 1992

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ABSTRACT

The psbA gene encodes the D1 protein involved in electron transport of photosynthesis leading to production of NAD(P)H and ATP. The rbcL gene encodes the large subunit of the ribulose,-1,5-bisphosphate carboxylase (Rubisco), an enzyme involved in the initial step of CO_2 fixation. A large open reading frame (ORF) of about 2131-2280 amino acids has been located on several plastid genomes. The objectives of this study were to determine the patterns of the expression of the psbA, rbcL genes and to examine whether the ORF 2280 was expressed in the plastid of three Cucurbita pepo L. varieties, namely, Early Prolific (EP), Fordhook Zucchini and Bicolor gourds. These objectives were achieved by isolation of total RNA from the skins of squash fruits and probing with gene-specific and ORF 2280-specific probes. Changes in rbcL mRNAs were also compared with variations in Rubisco LSU and Rubisco activity.

In the three varieties of squash the expression of the rbcL and psbA genes was developmentally regulated. For example in EP fruits, both transcripts increased steadily with fruit development up to 21 days postpollination. Evidence of post-transcriptional regulation of rbcL gene was observed in Zucchini and Bicolor fruits. In Zucchini, the amounts of rbcL mRNAs increased to a maximum at 3 days and slightly declined at day 14 and 21. However, the Rubisco

activity at 14 and 23 days significantly exceeded activities in young fruits.

The expression of the rbcL and psbA genes did not correlate with the plastid type in EP fruits but correlated with plastid type in Bicolor fruits. In Bicolor fruits, the levels of both transcripts were slightly greater in the chromoplast tissue than in the chloroplast tissue. Rubisco activity was significantly higher in the chromoplasts than in the chloroplasts of the same fruit.

Differential gene expression was observed in Bicolor and Zucchini fruits, where the steady-state amounts of psbA mRNAs exceeded the expression of the rbcL mRNAs. For example, the steady-state amounts of psbA mRNAs exceeded rbcL transcripts in Fordhook Zucchini by 2.5- to 3.9-fold, while in Bicolor fruits the amounts of psbA mRNAs were greater than rbcL transcripts by 3.3- to 4.2-fold. Regulation of gene expression at the tissue specific level was also observed in Zucchini and Bicolor fruits and leaves. For example, in Bicolor and Fordhook Zucchini fruits the amounts of psbA mRNAs in the leaf exceeded the levels in the fruits by a range of 3.3- to 8-fold, and 2.8- to 59-fold, respectively.

ORF 2280 transcripts of 2.6 kb were detected in EP and Bicolor fruits, while a 1.4 transcript was also detected in Bicolor fruits.

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ACKNOWLEDGEMENTS

I would like to convey my special thanks to my advisor, Dr. Charles Boyer, for his innovative ideas and encouragement which contributed to the modeling of the information in this thesis. I further wish to express my gratitude to members of the thesis committee, Drs. Paul Grun, Ross Hardison and Jack Shannon, for their constructive criticisms and input.

The completion of this thesis would not have been possible without the financial support of the University of Nairobi/Penn State Student Exchange Program, the Department of Horticulture at Penn State and the Genetics Program at Penn State.

Sincere thanks go to Christina Richards for her constant guidance in the technical aspects of the research. I also wish to extend my appreciation to other members of the laboratory, Marceline Egnin and Dane Fisher, for being good workmates.

Special thanks go to members of my family, Enoch and Rose Obwogo, Jaredi and Rhoda Ochiengi, Harry Werre Silas, and my brothers and sisters. This thesis is dedicated to the memory of my late mother, Agnes Napunyi Obwogo.

Finally, I thank God for giving me the strength and life to endure the training program.

Chapter 1

INTRODUCTION

Plant cells contain a unique group of cellular inclusions collectively called plastids. These organelles, which include proplastids, etioplasts, chromoplasts, chloroplasts and amyloplasts, are the sites of many agricultural processes. For example, photosynthesis is located in the chloroplasts, making them the most intensively studied plastids. Chromoplasts contain vitamin A precursors of fruits and vegetables. Amyloplasts are the site of starch synthesis in all major cereals and hence the major site of agricultural productivity. Other important biological pathways, including lipid and amino acid synthesis, are partially compartmentalized in the plastids. Understanding plastid physiology, biochemistry and molecular biology is therefore pivotal to future improvement of agricultural productivity by classical techniques as well as new biotechnologies.

Although plastids are functionally and structurally different, they are interconvertible. For example, chromoplasts can develop from proplastids, amyloplasts or chloroplasts (Tilney-Basset, 1989). Amyloplasts are transformed into chloroplasts during light-induced greening of lemon roots (Casadoro et al., 1990) and carrot roots (Gronegress,

1971). Chromoplasts are converted into chloroplasts when oranges are exposed to sodium gibberellate (Thomson et al., 1967). In ripening tomato and pepper fruits, the chloroplasts are converted into chromoplasts (Camara et al., 1983; Rhodes, 1980). Contrary to previous thought, fruit ripening is not a degenerative process, but an active one involving several synthetic processes. For example, during tomato fruit ripening, the chloroplasts break down (Rosso, 1968; Harris and Spurr, 1969), starch is converted into sugars (Davies and Cocking, 1965) and lycopene is synthesized (Kushwaha et al., 1970). Similarly in ripening pepper, chlorophyll is degraded, the thylakoids are lost and replaced by a dilated membranous, filamentous structure and new kinds of xanthophylls are synthesized (Spurr and Harris, 1968, Camara and Brandgeon, 1981). Plastid differentiation is therefore a dynamic process involving not only degradative processes but also new synthetic activities. The study of plastid transformation would provide an understanding of the control of fruit ripening and photo-synthetic processes, and the regulatory mechanisms of plastid differentiation.

Plastids contain their own functional genomes which range in size from 71 kb in the parasitic plant, beechdrops (dePamphilis and Palmer, 1990), to 217 kb in geranium (Palmer, 1991). The plastid genomes (ptDNA) have been sequenced in tobacco (Shinozaki et al., 1986), in rice (Hiratsuka et al., 1989) and in liverwort (Ohyama et al.,

1986). Genes have been located on these ptDNAs. For example, genes encoding four different rRNAs, 30 different tRNAs, 44 proteins and nine predicted open reading frames (ORFs) have been localized by complete sequencing of the plastid DNA of tobacco (Shinozaki *et al.*, 1986). Out of 120 genes encoded by the plastid genome (Mullet, 1988), *rbcL* and *psbA* are the two most intensively studied.

The *rbcL* gene in conjunction with the nuclear encoded *rbcS* gene, code for the multi-subunit enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a bifunctional enzyme catalyzing the initial step in CO_2 fixation and a competing oxidation reaction (photorespiration). Photorespiration is a wasteful process in which fixed CO_2 is liberated. Therefore, much effort has been geared towards engineering a Rubisco enzyme with less photorespiration.

The regulation of *rbcL* gene expression is complex and control mechanisms have been reported to be at transcription, post-transcription, translation and post-translation levels. No simple regulatory mechanism has been documented and regulation seems to vary from species to species, within cultivars in a species (Richards *et al.*, 1991), in a tissue specific manner, with stage of development (Nikolau and Klessig, 1987; Piechulla *et al.*, 1986) and with the level of illumination (Klein, 1991).

The *psbA* gene encodes the D1 protein (also called the herbicide binding protein) (Roichaix and Erickson, 1988)

involved in electron transport during the light cycle of photosynthesis. Point mutations in the psbA gene, resulting in a specific amino acid substitution, have been observed to induce herbicide resistance in Amaranthus hybridus (Hirschberg and McIntosh, 1983), Solanum (Hirschberg et al., 1984) and in cyanobacteria (Gingrich et al., 1988). The ability of a mutation in the psbA gene to confer herbicide resistance in plants and the role of D1 protein in electron transport makes this gene a potential candidate for plant improvement. Due to the vital importance of the psbA gene, several previous studies focused on understanding its structure and mechanisms of regulation. These goals have been pursued through several strategies including analysis of transcript abundance and DNA sequencing. Notably, the psbA gene has been reported to have a strong promoter (Gruissem and Zurawski, 1985a), the transcript is highly stable (Mullet and Klein, 1987) and the D1 protein is highly unstable under darkness (Edelman et al., 1985). This is partly because the nascent D1 protein is stabilized by chlorophyll during its synthesis (Mullet et al., 1990). Other studies reported post-translational regulation of the psbA gene through processing of the 33-36 kDa to the precursor D1 protein (Vermaas et al., 1983; Herrin and Michaelis, 1985), by removal of approximately 1.5-2.0 kDa fragment from the C-terminus (Marder et al., 1984; Grebanier et al., 1978; Reisfeld et al., 1982). Like many chloroplast genes, no

simple trend in transcript abundance or gene control mechanisms has been observed among plant species.

The plastid genome also contains several open reading frames (ORFs) of unknown function. The largest of these ORFs has a size range able to encode a protein of 2131-2280 amino acids (Shinozaki et al., 1986; Hiratsuka et al., 1989; Ohyama et al., 1986). Recently, transcripts from this large ORF have been reported in tomato (Richards et al., 1991) and in broad bean (Herdenberger et al., 1988). Translation products from the large ORF were observed in spinach, tobacco and Oenothera (Glick and Sears, in press) and in tomato fruits (Richards, unpublished). Data on ORF 2280 transcripts and protein is still scarce and there is need to determine if the ORF 2280 is transcribed and translated in other plant species.

Squash (Cucurbita pepo L.) has been chosen as a system for the current study for four reasons. First, the species has multiple tissues (leaves, fruits, flowers) in which different plastids develop normally. The differentiation of plastids could be a useful tool for studying the role played by tissue specificity in the regulation of plastid development and plastid functions.

Second, most plants in which plastid gene expression has been studied such as pepper and tomato display unidirectional plastid transformation during fruit ripening. Squash offers the following advantages: (1) plastid interconversion is

bidirectional; for example, chloroplasts can developed into chromoplasts and chromoplasts back to chloroplasts, (2) in the YY B^+B^+ isogenic line the proplastids develop into chloroplasts which later develop into chromoplasts, (3) while in the Bicolor gourds chromoplasts and chloroplasts simultaneously develop from proplastids on the same fruit. Each of these developmental pathways has advantages in addressing different biological questions. For example, the gene regulation during organelle differentiation can be investigated by studying the transcription and translation in chromoplasts derived from proplastids or chloroplasts. Gene regulatory mechanisms can also be studied by looking at transcription and translation products during the reversible differentiation of plastids (i.e. chloroplasts to chromoplasts to chloroplasts) (Boyer, 1989).

A third reason squash has been examined in the present study is that genetic control of chromoplast development in squash is already established (Boyer, 1989, Appendix 1). Two loci B and Y control the plastid developmental pathways in squash fruits. The B gene acts on the proplastids early in ovary/fruit development, while the Y gene acts later in the fruit development. The B^+ allele (as in B^+B^+), causes proplastids to develop into chloroplasts, which are maintained in the presence of a recessive Y° allele (as in $Y^\circ Y^\circ$ B^+B^+ in Zucchini) (Schaffer, 1982). B+ allele (for example in B^+B^+), induces proplastids to develop into

chloroplasts which, in the presence of a Y allele (as in YY B^+B^+), develop into chromoplasts. The B alleles (e.g BB), induces proplastids to develop into chromoplasts early in fruit development (e.g YY BB genotype of Precocious type of Early Prolific).

The fourth reason why squash was examined was that a system for studying plastid gene expression has already been developed. Establishment of ultrastructural differences during plastid differentiation, construction of the chloroplast DNA restriction and genetic maps, and determination of protein profiles during plastid differentiation have been done (Lim, 1990).

Ultrastructural differences were observed between the two isogenic lines, YY BB and YY B^+B^+ (Lim, 1990). Before flowering, proplastids with few internal membranes were noticed in YY B^+B^+ ovaries. At flowering the chloroplasts of YY B^+B^+ contained smaller stacked grana. After flowering the grana disappeared and chromoplasts developed. In contrast, the proplastids of the YY BB ovaries transformed directly into chromoplasts and there were no observable grana structures in the YY BB fruits.

Having established the above structural differences between squash lines, an attempt was made to correlate the structural differences, with protein profiles during plastid differentiation. It was notable that Rubisco large subunit (LSU) protein was undetectable in immature fruits but

detectable from 10 to 20 days after pollination. This was unusual because the later fruit developmental stages contain non-photosynthetic chromoplast tissue, while the early fruit developmental stages (where LSU was undetected) contain chloroplasts. In order to complement the preceding study, there is need to determine the presence of rbcL transcripts and ascertain if the observed Rubisco in the fruit is enzymatically active.

Several membrane bound proteins were also detected in squash plastids, of which the 35 kDa protein (Lim, 1990) is of interest because its molecular weight matches the D1 precursor protein. This putative D1 protein was not verified immunologically in squash fruits. No study has been done to determine the presence of psbA transcripts corresponding to the previously observed 35 kDa membrane protein patterns.

Squash plastid ORF 2280 was putatively located in the inverted repeat region (Lim et al., 1990). No study has been done to determine whether ORF 2280 of squash plastid DNA is transcribed and translated.

Research Objectives

In this study are analyzed the abundance of psbA, rbcL and ORF 2280 transcripts and translation products during

squash fruit development. Rubisco protein patterns and the corresponding enzymatic activities were also determined. In addition, the partial sequencing of the 9.8 kb fragment containing the ORF 2280 is reported. These objectives were accomplished in the following sequential steps:

1. Preliminary work
   (a) Isolation and cloning of 5 out of 12 squash cpDNA X PstI fragments.
   (b) Confirmation of the location of the psbA, rbcL genes X and ORF 2280 on the chloroplast restriction map.
   (c) Mapping and sub-cloning of the 9.8 kb PstI (fragment of cpDNA containing the ORF 2280) with BamHI, EcoRI and SalI.
2. RNA abundance
   (a) Isolation of total RNA and assessment of developmental variation in rbcL and psbA transcripts.
   (b) Preparation of probes internal to ORF 2280 and examination of transcripts from ORF 2280 during plastid development.
3. Protein study
   (a) Determination of Rubisco proteins patterns and enzymatic activities in skin and leaves of squash at different developmental stages.
4. Partial sequencing of PstI 9.8 kb fragment of squash ptDNA containing the ORF 2280.

Chapter 2

ANALYSIS OF STEADY-STATE mRNA TRANSCRIPT LEVELS FROM THE PLASTID GENOME

Literature Review

General control of plastid gene expression

In general, the regulation of gene expression can occur at four stages: transcription, post-transcription, translation and post-translation (Mullet, 1988; Gruissem, 1988; Shinozaki et al., 1988). The role played by the above gene regulatory mechanisms may vary in plastids: in tissue or organ specific manner (Kuntz et al., 1989), with the developmental stage of the plant (Nikolau and Klessig, 1985), from species to species (Kuntz et al., 1989) and even among cultivars within a species (Richards et al., 1991). Light (Gamble and Mullet, 1989; Klein, 1991) and the nuclear genome (Klein and Mullet,1990; Link, 1982) have also been reported to modulate the expression of plastid genes.

Species influence on the levels of plastid transcripts have been studied in sunflower and pepper (Kuntz et al., 1989). The levels of rbcL mRNAs remained constant during the

transformation from chloroplasts to chromoplasts in pepper and sunflower, while levels of psbA increased. In contrast, psaA decreased in pepper but remained constant in sunflower during chloroplasts to chromoplasts transformation (Kuntz et al., 1989).

Variation in plastid transcripts have been reported in cultivars of the same species (Richards et al., 1991; Piechulla et al., 1985, 1986). For example, in one tomato, cultivar (VFNT), the levels of psbA and rbcL mRNAs decreased with fruit ripening, while in another cultivar (Firstmore), psbA remained constant while rbcL increased (Piechulla et al., 1985, 1986). In another study, psbA, psbC/D and rbcL transcripts remained constant in one cultivar (Traveler 76), while in 'Count II,' both psbA and psbC/D decreased, and rbcL remained constant during fruit ripening (Richards et al., 1991).

The influence of tissue or organ specificity on plastid gene expression has been reported in sunflower and pepper (Kuntz et al., 1989) and in maize (Jolly et al., 1981). For example, Jolly et al., (1981) reported that atpB transcripts were present in similar amounts in the chloroplasts of the mesophyll and the bundle sheath cells whereas rbcL transcripts were much more abundant in the bundle sheath chloroplasts. In sunflower petals, the amounts of steady-state transcripts from psaA and rbcL genes remained constant and psbA mRNAs increased during chloroplast to chromoplast

differentiation (Kuntz et al., 1989). Levels of psbA mRNAs decreased, psaA increased while rbcL transcripts remained constant during chloroplasts to chromoplasts differentiation in pepper fruits.

The role of developmental signal on the regulation of plastid gene expression has been studied in barley (Deng and Gruissem, 1987) and amaranth (Nikolau and Klessig, 1985). During germination of amaranth seedlings the Rubisco large (LSU) and small subunit (SSU) proteins were observed in the cotyledons from 3 days and reached a maximum at 10 days. However, the LSU and SSU proteins were observed in amaranth leaves at 8 days and reached a maximum at 20 days, while in the stem LSU and SSU proteins were first detected at 5 days and reached a maximum at 24 days (Nikolau and Klessig, 1987). In barley seedlings, Deng and Gruissem (1987) observed a 3-fold increase in mRNAs from 10 plastid genes (including psbA and rbcL), in greening cotyledons and a 5-fold increase during leaf maturation.

The influence of the nuclear genome on plastid gene expression was highlighted by Ellis (1977) when he proposed the "cytoplasmic control principle." In general this principle states that cytoplasmic translations products control the transcription and translation of plastid gene products but not vice versa. Evidence in support of this principle was reported in two studies with barley seedlings (Gamble and Mullet, 1989; Klein, 1991). Gamble and Mullet

(1989) compared the translation of a chlorophyll binding protein, CP47, and D1 protein, in a barley nuclear mutant, viridis-115, lacking photosystem II (PSII) with wild-type plants. Translation of the two proteins occurred in the wild-type but not in the mutants. They concluded that the nuclear mutation inhibited the translation and stability of these proteins. Similarly, when 8-day-old dark-grown barley seedlings were incubated with cycloheximide, an inhibitor of cytoplasmic protein synthesis, light-activated transcription of rbcL, psbA and psaA was inhibited. In contrast, when the seedlings were incubated with chloramphenicol, an inhibitor of plastid protein synthesis, light-activated transcription of rbcL, psbA and psaA was stimulated. This led to the conclusion that light-induced plastid transcription is modulated by cytoplasmic (nuclear genome) and organelle (plastid genome) protein synthesis (Klein, 1991).

Function, structure and regulatory mechanisms of psbA gene

The psbA gene codes for the 32 kDa D1 protein of photosystem II (PSII). The D1 protein contains five membrane spanning alpha-helices which form binding sites for pheophytin, chlorophyll, carotenoids, quinones and Fe^{2+} (Kim et al., 1991). The D1 apoprotein stabilizes the reduced

quinone, which acts as a secondary electron acceptor during the PSII electron transport (Kyle, 1985). The D1 protein has been implicated as a primary target of several herbicides including triazine and diuron (Pfister et al., 1981; Steinback et al., 1981). Presumably, these herbicides displace the bound quinone molecules thus blocking the electron transport (Reith and Straus, 1987). DNA sequences of herbicide resistant biotypes have correlated the herbicide resistance to a single base mutation which results in one amino acid substitution (Hirschberg and McIntosh, 1983; Hirschberg et al., 1984). This type of resistance has been applied in agriculture. For example, a triazine resistant biotype of Brassica campestris has been used to introduce triazine resistance into agriculturally important Brassica cultivars by backcrossing methods (Beversdorf et al., 1980).

Complete nucleotide sequence of the psbA gene of several plant species including spinach, Nicotiana debneyi (Zurawski et al., 1982), Amaranthus hybrida (Hirschberg and McIntosh, 1983), and Nicotiana tobaccum (Shinozaki et al., 1986), show an exceptionally high degree of homology with no more than three amino acid differences among these proteins (Hanley-Bowdoin and Chua, 1988). The psbA gene in algae (Karabin et al., 1984), and in cyanobacteria (Curtis and Haselkorn, 1984), shows 87 to 93% homology with higher plants (Hanley-Bowdoin and Chua, 1988). The promoter region of the psbA gene has 90% (at the DNA sequence) and 99% (at the protein

sequence), homology in six dicots including spinach (Zurawski et al., 1982) and tobacco (Shinozaki et al., 1988). A DNA sequence homology of 72% in the 5' region of psbA gene was reported between wheat (monocot) and six dicots (Hanley-Bowdoin and Chua, 1988). The 5' region of the psbA gene of wheat and dicots contain sequences that resemble the prokaryotic -35 and -10 promoter elements (Hanley-Bowdoin and Chua, 1988). The -10 box diverges by one nucleotide from the canonical prokaryotic promoter. A perfect -35 element is located at 18 bp upstream of the -10 element (Hanley-Bowdoin and Chua, 1988). Apart from the structural similarities of the psbA gene between species, there is also high correlation in the patterns and control of expression of psbA gene among plant species.

From run-on transcription assays in spinach chloroplasts, the relative transcription rates of three chloroplast genes were ordered as psbA > rbcL > atpB (Deng et al., 1987). The relative transcription of psbA, rbcL, atpB genes corresponded with their respective promoter strengths. It was therefore suggested, that the expression of these genes was partly regulated by the promoters (Deng and Gruissem, 1987). In barley, the changes in the levels of psbA mRNAs were not paralleled with the changes in transcription. This lack of correlation between transcription and steady-state mRNAs levels, implied that high RNA stability was important in regulating the expression of the psbA gene at the post-

transcription level (Mullet and Klein, 1987). Similarly, in pepper fruits, the three plastid genes were ranked as psbA > rbcL > psaA based on their relative transcription rates during chloroplasts to chromoplasts differentiation (Kuntz et al., 1989). However, unlike in the above study, the relative transcription rates of these genes followed their steady-state levels, but the relationships were not parallel. It was therefore, suggested that both transcription and RNA stability were important in maintaining the respective steady-state mRNA levels during chloroplasts to chromoplasts differentiation (Kuntz et al., 1989). The three preceding studies suggest that psbA gene is relatively highly expressed in chloroplasts and chromoplasts when compared to other genes and this high expression could be due to either the strength of the promoter of the psbA gene or stability of the psbA transcript, or both.

Variation in levels of psbA transcripts have also been reported during plastid differentiation. For example, in ripening tomato fruits, the level of psbA mRNA decreased during the transformation from chloroplasts to chromoplasts, but still some were detectable in the red fruit (Piechulla et al., 1985). In contrast, in ripening tomato fruits, the psbA mRNA level remained constant (Richards et al., 1991). In another study with tomato fruits, the highest psbA mRNA was observed at 25 days after anthesis and decreased with fruit ripening (Piechulla et al., 1986). In the above studies no

attempt was made to correlate the psbA mRNA levels with the D1 protein patterns. In contrast to preceding studies, Bathgate et al., (1981) reported reduction or absence of psbA mRNA in the chromoplasts of tomato fruits. However, the absence of psbA mRNA, was inferred from lack of detectable D1 protein from in vitro translation assays of chromoplast RNA extracts, rather than a direct determination of psbA transcripts. They suggested that lack of D1 protein synthesis was due to absence of chlorophyll in ripe fruits, because the D1 protein usually associated with the thylakoids (Hollingsworth et al., 1984), which break down during the chloro-plasts to chromoplasts transformation (Rosso, 1968). Experimental evidence in support of this deduction was provided in green-flesh tomato mutants. In these mutants, a section of the fruit retains chloroplasts while other parts convert into chromoplasts during ripening (Rosso, 1968). The D1 protein was synthesized in the green section but not in the yellow portions of the fruit (Bathgate, 1986), suggesting that either the thylakoid membrane or the chlorophyll was essential for the synthesis of the D1 protein.

Several other studies have correlated the absence of D1 protein in plastids with either lack of light or chlorophyll. For example, the D1 protein was not detected in the etioplasts of dark-grown barley even though the psbA mRNA accumulated. However, upon illumination the polysomes accumulated on the psbA mRNA (Klein et al., 1988).

Similarly, Spirodella plants placed in darkness showed reduced synthesis of the D1 protein without a corresponding decrease in levels of psbA mRNA (Fromm et al., 1985). Evidence from barley studies demonstrated that chlorophyll regulates the accumulation of the D1 protein by increasing its stability (Mullet et al., 1990). These preceding studies suggest that the expression of the psbA gene is modulated at the level of translation directly through chlorophyll or indirectly through light causing the development of chlorophyll. In fact, higher plants grown in darkness lack chlorophyll and chlorophyll apoproteins, although protochlorophyllide, a chlorophyll precursor accumulates (Klein and Mullet, 1986a, 1986b; Klein et al., 1988a). When plants are illuminated, protochlorophyllide is reduced to chlorophyllide by proto-chlorophyllide reductase in a light and NAD(P)H-dependent reaction. Chlorophyllide is subsequently esterified with pyrophosphate in a light-dependent step to chlorophyll (Castelfranco, 1983).

The precursor D1 protein of 33-36 kDa (Vermaas et al., 1983; Herrin and Michaelis, 1985) is post-transcriptionally processed by removal of an approximately 1.5-2.0 kDa fragment from the C-terminus (Marder et al., 1984; Grebanier et al., 1978; Reisfeld et al., 1982). In addition the N-terminus of the D1 is modified by removal of N-terminal methionine and conversion of the penultimate amino acid to N-acetyl-o-phosphothreonine (Michel et al., 1988). D1 is also reversibly

palmitoylated in the stromal lamellae during synthesis (Mattoo and Edelman, 1987). The fatty acid modification may facilitate the transfer of newly synthesized D1 into the grana stacks or assembly of D1 with other PSII proteins (Kim et al., 1991).

The expression of the psbA gene is also regulated by the nuclear genome. For example, a nuclear mutation in barley seedlings, which caused lack of PSII, resulted in reduced levels of D1 protein and its stability. However, the amounts of psbA mRNA in the wild-type were equal to the mutant (Gamble and Mullet, 1989). In another study, the inhibition of cytoplasmic protein synthesis, abolished light-induced synthesis of psbA mRNA in barley seedlings (Klein, 1991). In addition, inhibition of plastid protein synthesis, caused increased translation of the D1 protein, while inhibition of plastid transcription using tagetin did not induce plastid translation (Klein, 1991). It is not surprising that the regulation of the psbA gene is under nuclear control. This is because the D1 protein encoded by the psbA gene is one of the 12-18 different proteins encoded by the nuclear and the plastid genomes that are part of the PSII (Gounaris et al., 1986). Thus, the synthesis and assembly of PSII requires coordination between nuclear and chloroplast gene expression (Gamble and Mullet, 1989).

The regulatory mechanisms of psbA gene are therefore variable with no simple trend prevalent among plants. Among

the several membrane bound plastid proteins which increased with squash fruit maturity (of YY BB and YY B^+B^+) was the 35 kDa protein (Lim, 1990). The same protein was abundant in the leaves but absent in immature fruits. Its molecular weight matched the size of the precursor D1 protein, although the study did not verify its identity immunologically. The presence of concomitant psbA mRNA in squash fruits have not been determined and correlated with the previously reported 35 kDa protein.

Function, structure and regulatory mechanisms of rbcL gene

Ribulose-1,5-bisphosphate carboxylase (Rubisco) is located in the chloroplasts of all higher plants and catalyzes the initial step of photosynthetic carbon fixation. It is often the most abundant protein in leaf cells. Rubisco enzyme has a molecular weight of about 550 kDa and consists of eight large subunits (LSU, each with a molecular weight 50-60 kDa) and eight small subunits (SSU, 12 to 20 kDa each) (Kwanji et al., 1975; Kirchanski and Park, 1976). The LSU protein has the substrate binding site and is encoded by the plastid gene, rbcL, while SSU protein is encoded by an 8 to 15 nuclear gene family, rbcS (Berry-Lowe, et al., 1982), and its function is unknown.

The *rbcL* gene has been sequenced in several plant species including maize (McIntosh *et al.*, 1980) and spinach (Zurawski *et al.*, 1982). The 5' region of the spinach *rbcL* gene is analogous to the prokaryotic -35 and -10 consensus elements (Zurawski *et al.*, 1982). In contrast, the 5' region of the maize *rbcL* gene does not show homology to the prokaryotic -35 and -10 consensus sequence (McIntosh *et al.*, 1980).

The influence of the promoter on the expression of *rbcL* gene in spinach has been documented (Hanley-Bowdoin *et al.*, 1985; Gruissem and Zurawski, 1985a, 1985b). Based on run-on assays in spinach leaf chloroplasts, the relative transcription rates of chloroplast genes were ordered as *rrn* > *psbA* > *rbcL* > *psaA* > *rpl2* > *psaB*-*petB*-*petD* > *atpB* > *rpoA* > (Deng *et al.*, 1987). In another study, the promoter strengths of three chloroplast spinach genes were ranked as *psbA* > *rbcL* > *atpB* (Gruissem and Zurawski, 1985b). The levels of transcripts from these genes closely correlated with their respective promoter strengths, leading to the suggestion that plastid genes are partially controlled at the level of transcription (Deng *et al.*, 1987). *RbcL* gene expression is regulated at the post-transcriptional level in barley (Mullet and Klein, 1987) and *Petunia hybrida* (Grinsven *et al.*, 1986) For example, in young barley seedlings, the amounts of *rbcL* transcripts correlated with the level of transcription, while in older seedlings *rbcL* mRNA abundance

exceeded transcription (Mullet and Klein, 1987), suggesting that in the older seedlings RNA stability partially determined the levels of this transcript. Similarly, in barley and maize, the stability of rbcL transcript played a role in determining the amounts of the transcript (Mullet and Klein, 1987). The stability of plastid transcripts has been attributed to the inverted repeat in the 3' region as demonstrated by Stern and Gruissem (1987).

The expression of rbcL gene has been reported to vary depending on plastid differentiation. Analysis of the rbcL mRNAs in two plastid systems, proplastids like organelles from cell suspension culture and mature leaf chloroplasts of petunia, showed no significant difference in the levels of the mRNAS (Grinsven et al.,1986). However, an assay of in vivo LSU protein levels showed vast quantities of LSU in the leaves and hardly any from the cell cultures. They concluded that the regulation of the rbcL gene in the two systems must be post-transcriptional. In sunflower petals and pepper fruits, the levels of rbcL, remained fairly constant during chloroplasts to chromoplasts transformation (Kuntz et al., 1989). Similarly, Richards et al. (1991), reported constant levels in rbcL mRNAS during tomato fruit ripening. Also in tomato, Piechulla et al. (1986) reported the presence of rbcL in ripe tomato but at lower levels than in the green fruit. In contrast, Livne and Gepstein (1988), reported that rbcS and rbcL increased during chloroplast maturation and

disappeared after transition of chloroplasts into chromoplasts, in tomato fruits. Kuntz et al.(1989) carried their study further to analyze LSU protein and observed only small amounts of LSU protein in tomato chromoplasts. In conjunction with inhibition studies, they concluded that protein synthesis does not occur in the chromoplasts or occurs at very low levels and the mechanism of plastid gene regulation is at the level of translation (Kuntz et al., 1989).

Regulation of rbcL gene expression at post-translation via turnover of the protein (Mishkind and Schmidt, 1983) as well as activation (Portis et al., 1986) and inhibition (Gutteridge et al., 1986) of the enzyme activity has been reported. Activation and inhibition are reviewed later in Chapter 3.

Being a photosynthetic gene, the influence of light on the expression of rbcL gene is not surprising. Evidence of light-mediated translational control of plastid gene expression has been observed in several plant species including amaranths cotyledons and leaves (Berry et al., 1988, 1990), in barley and maize seedlings (Klein and Mullet, 1986a, 1990), and in tomato fruits (Piechulla and Gruissem, 1987). For example, when amaranths seedlings were transferred from darkness to light, the amounts of LSU proteins increased without a correlated increase in rbcL transcripts (Berry et al., 1988). When cotyledons of 8-day-

old amaranth seedlings were transferred into darkness the synthesis of LSU protein was inhibited (Berry et al., 1990). When transferred back to light, LSU and SSU proteins were synthesized without a change in rbcS and rbcL mRNAs. Both these studies demonstrate light-mediated translation control of rbcL and rbcS mRNAS in the cotyledons and in seedlings of amaranth. Light-induced transcription of rbcL gene has been documented in barley and maize seedlings (Klein and Mullet, 1990). When 8-day-old barley and 9-day-old maize seedlings were transferred into light, transcription of rbcL gene increased. In contrast to the amaranth study by Berry et al. (1988, 1990), etioplasts of 5-day-old barley seedlings synthesized most soluble and membrane proteins found in illuminated plants, including Rubisco (Klein and Mullet, 1986a). The influence of light on the expression of the rbcL gene is not universal among plant species. For example, in maize (Nelson et al., 1984) and cucumber (Walden and Leaver, 1981), the amount of Rubisco from dark-grown seedlings was equal to light grown seedlings. In mustard (Link, 1982) and in maize (Crossland et al., 1984) only minute differences could be observed between rbcL transcripts in light-grown and etiolated plants. In pea, the amount of rbcL transcripts were more abundant in light-grown seedlings than in etiolated plants (Inamine et al., 1985; Sasaki et al., 1986). Analysis of tomato fruits (harvested from 7-35 days after anthesis), showed that the amounts of rbcL accumulated at night and

decreased to low levels in the afternoon (Piechulla and Gruissem, 1987).

There is a close coordination between the expression of the plastid encoded *rbcL* and the nuclear encode *rbcS*. This is expected because of the stoichiometric composition of the Rubisco multi-subunit enzyme of eight LSU (encoded by *rbcL*) and eight SSU proteins (encoded by *rbcS*). Approximately equal amounts of *rbcL* mRNA and *rbcS* have been reported to accumulate in the leaves of wheat (Dean and Leech, 1982), in maize (Nelson *et al.*, 1984), and in barley (Nivison and Stocking, 1983), suggesting that there is a coordinated expression of chloroplastic and nuclear genes. It was also shown that the polypeptides of LSU and SSU were coordinated in the leaves of corn (Nelson *et al.*, 1984), wheat (Dean and Leech, 1982), and barley (Nivison and Stocking, 1983). On the contrary, there appear to be no coordination between *rbcS* and *rbcL* mRNAs in tomato fruits and in the leaves and stems of amaranth (Nikalau and Klessig, 1987). It was therefore concluded that the coordination of *rbcL* and *rbcS* gene expression was not universal in all species and organs (Nikalau and Klessig, 1987).

The influence of the stage of developmental regulation on the expression of *rbcL* gene has been reported in tomato fruits (Piechulla and Gruissem, 1987) and in amaranths seedlings and cotyledons (Nikolau and Klessig, 1987). For example, *rbcL* transcripts were high in early stages of fruit

development, reached a maximum at 15 days and declined during fruit growth and ripening (Piechulla and Gruissem, 1987). In contrast: rbcL levels in pepper (Kuntz et al., 1989) and tomato (Richards et al., 1991) remained constant during fruit maturation.

Tissue specific regulation of rbcL has also been documented (Schuster et al., 1986; Link et al., 1978; Jolly et al., 1981). For example, Jolly et al. (1981) reported that atpB transcripts were present in similar levels in the chloroplasts of the mesophyll and the bundle sheath cells whereas rbcL transcripts were much more abundant in the bundle sheath chloroplasts.

Similar to psbA gene, no simple trend has been observed in the patterns of the expression of rbcL in several plant species so far studied. The regulatory mechanisms of the expression of the rbcL gene seem to vary from control at the transcriptional level to the production of the final active enzymatic product. The variations in expression of rbcL are also influenced by developmental and environmental (light) signals, plastid types, nuclear genome (rbcS), type of tissue and plant species.

Previous study in Early Prolific squash detected no LSU protein during early fruit development. Some detectable amounts of LSU protein were observed 10 days and maximum levels at 20 days post pollination (Lim, 1990). Lack of a detectable level of LSU in the immature fruit and elevated

level at 20 days was contrary to the expected. It was also observed that LSU protein in the green Zucchini fruit was much less than in the yellow Early Prolific fruit. These results were unusual because early fruit developmental stages contain chloroplasts while later fruit stages contain chromoplasts.

The presence rbcL transcripts and the Rubisco enzymatic activity during progressive stages of squash development has not been determined. The current study determines the rbcL transcripts whose protein product is functional in the stroma, in contrast to the psbA gene, which is representative of the light reaction of photosynthesis.

Materials and Methods

Plant materials

Two cultivars of Cucurbita pepo L., 'Early Prolific' and 'Fordhook Zucchini,' were grown in pots in the greenhouse, while the Bicolor breeding line was grown at the Horticulture farm at Rock Springs. Early Prolific was grown during spring 1990, while Zucchini was grown in spring 1991. The potting media consisted of peat: perlite: soil (3:2:1). The plants were later fertilized with nitrogen: phosphorus: potassium (20:20:20) as appropriately required. Fresh ovary/fruit

tissues from the YY BB and the YY B^+B^+ were harvested at -2, 0, 7, 10, 14 and 21 days.

Early Prolific consisted of two isogenic lines (YY BB and YY B^+B^+) with contrasting plastid developmental patterns (Fig 2.1). The YY B^+B^+ lines possessed green skin color (due to chloroplasts) prior to pollination. The skin color of YY B^+B^+ progressively turned yellow and by three days after pollination the entire fruit was yellow. The YY BB lines in contrast to YY B^+B^+, precociously developed yellow skin color (due to chromoplasts) from the time the ovary differentiated. At about 14 days and thereafter, both the YY BB and YY B^+B^+ lines had identical orange skin color.

Zucchini Y^oY^o B^+B^+, cultivar 'Fordhook Zucchini' ovaries and fruits possessed the green chloroplast color prior to pollination up to 21 days after pollination when they were harvested (Fig 2.2). There was, however, a slight contrast in the intensity of the green color, in that young ovaries/fruits of Zucchini (before pollination to about 3 days after pollination) had light green skin color, which later intensified into a dark green color with fruit maturity.

The Bicolor gourds were grown at the Penn State Horticulture farm (Rock Springs) during summer 1992. Fruits from Bicolor gourds from a specific plant portrayed three plastid developmental patterns. In one pattern chloroplasts developed which covered the entire fruit, while in others the

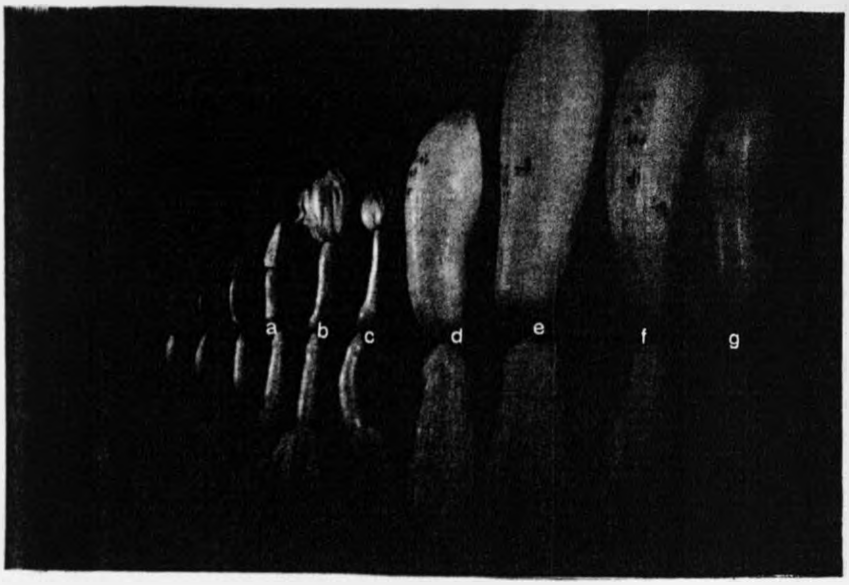


Fig 2.1. Ovaries /fruits of the YY B^+B^+ (top) and YY BB (bottom) lines of Early Prolific squash. Stage "a" shows ovaries before pollination while "b" is at pollination. Stages d, e, f, g are 7, 10, 14, 21 days after pollination, respectively.

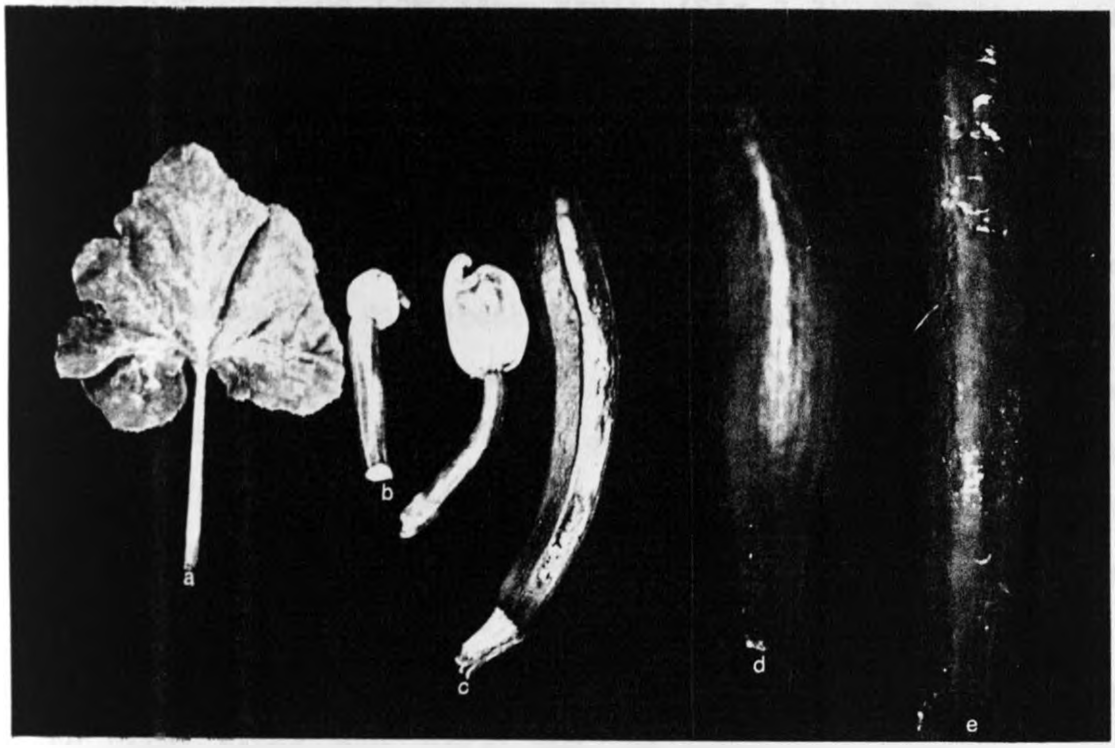


Fig 2.2. Leaf, ovaries and fruits of Fordhook Zucchini squash. "a" is a young leaf while "b" are ovaries at pollination. Stages c, d and e are fruits at 3, 10 and 21 days postpollination, respectively.

whole fruit skin developed chromoplasts. A third pattern showed both chromoplasts and chloroplasts developing simultaneously on the same fruit (Fig 2.3). Fruits which showed this later plastid developmental pattern were harvested at 14 and 21 days postpollination and used for RNA determinations.

Ovaries and fruits from the three varieties of squash were harvested at different stages of development and placed on ice. The skins were peeled off (to about 1 mm thickness) and immediately frozen in liquid nitrogen. Young leaves (with a lamina size of about 5 by 7 cm) were concurrently harvested and frozen in liquid nitrogen. The frozen tissues were stored at -70°C until the time of RNA extraction.

Two RNA assay experiments were done on EP fruits. In the first experiment total RNA was extracted from the skins of fruits from plants grown early in spring. In experiment two, the RNA was extracted from fruits grown late in spring. Plants grown late in spring were under slightly warmer and better illumination compared to plants grown early in spring. RNA was isolated from skins of Early Prolific ovaries/fruits harvested at -2, 0, 3, 7,10, 14, 21 days before and after pollination. In Zucchini, RNA was extracted from skins of ovaries/fruits at -2, 0, 3, 14, 21 days pre- and postpollination. Total RNA was also extracted from young leaves and used as a control. These fruit developmental

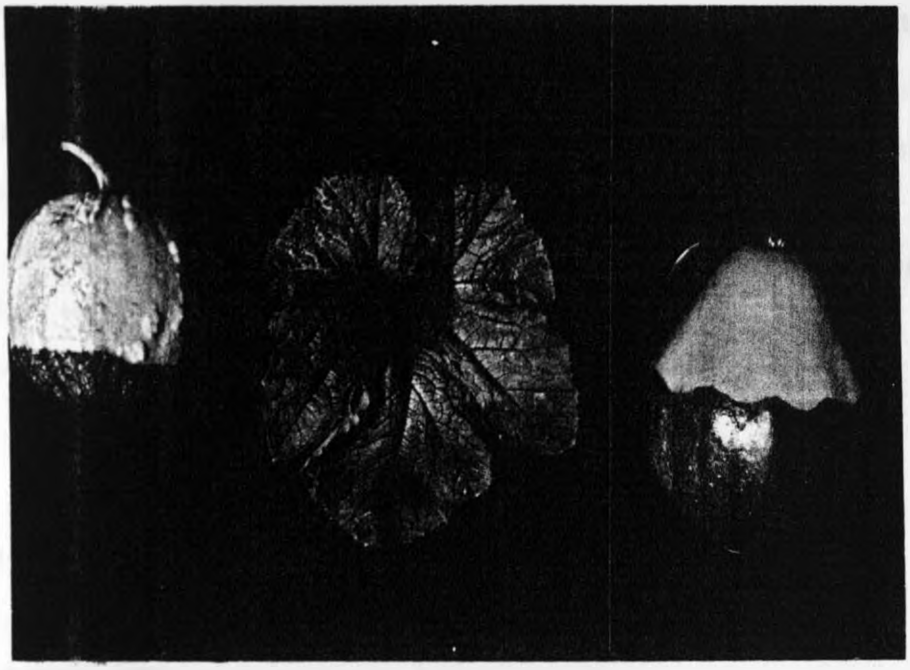


Fig 2.3. Fruits and leaf of Bicolor gourds of squash. The fruits show the simultaneous development of chromoplasts and chloroplasts.

stages were chosen so as to include the previously investigated maturation stages (of 0, 10, 20 days).

In order to obtain good extractions of mRNA, it was necessary to minimize the activity of the RNAases liberated during cell lysis or from other potential sources of contamination in the laboratory such as glassware, plasticware and reagents. The methods for achieving RNAase free working conditions as described by Sambrook et al. (1990), were applied in all RNA experiments described in the subsequent sections.

Extraction of total RNA

Total RNA were isolated by selective LiCl precipitation (Piechulla et al.,1986), from the pericarps of squash fruits and leaves which had been frozen in liquid nitrogen and stored at -70°C. The frozen tissue was first ground in a pre-cooled mortar and pestle under liquid nitrogen, and resuspended in 50 ml homogenization buffer (0.35 M sorbitol, 50 M Tris-HCl pH 8.0, 25 mM EDTA, 15 mM 2-mercaptoethanol, 2 mM dithiotheital, 0.1% polyvinylpyrrolidone, 5 mM aurintricarboxylic acid). One-tenth lysis buffer (5% sodium lauroysarcosinate, 50 mM Tris-HCl pH 8.0, 25 mM EDTA) was added and the homogenate incubated for 5-15 minutes at room temperature. Incubation of the young tissue for over 5

minutes led to RNA degradation due to higher RNAase activity in the young than in the old tissue. After lysis an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the homogenate and mixed thoroughly using a Pasteur pipette. The mixed homogenate was centrifuged at 10,000G for 15 minutes to remove the protein. The phenol/chloroform extraction was repeated 3-4 times depending on the protein content in the tissue. Finally, the top aqueous phase was recovered and 1/10 volume of 5 M ammonium acetate (pH 5.2) and two volumes absolute ethanol were added and stored at -70°C for 1 hr or at -20°C overnight, to precipitate the total nucleic acids. It was observed that when RNA was precipitated from the total nucleic acids directly, the resultant RNA product was hard to dissolve. This problem was circumvented by redissolving the total nucleic acid pellet into TE buffer (10 mM Tris, 1 mM EDTA) and adding 1/4 volumes of 10 M ammonium acetate, followed by centrifugation at 8,000G for 30 minutes. The supernatant was saved and a white pellet of polyuronic acids was discarded as described by Richards *et al.*(1991). Ten molar LiCl was added to the collected supernatant (to give a final concentration of 2.5 M LiCl), to selectively precipitate high molecular weight RNA. The precipitated RNA was pelleted by centrifugation at 10,000G for 1 hour. The RNA pellet was vacuum dried, re-suspended in an appropriate volume of diethylpyrocarbonate (DEPC) treated water and stored in aliquots at -70°C.

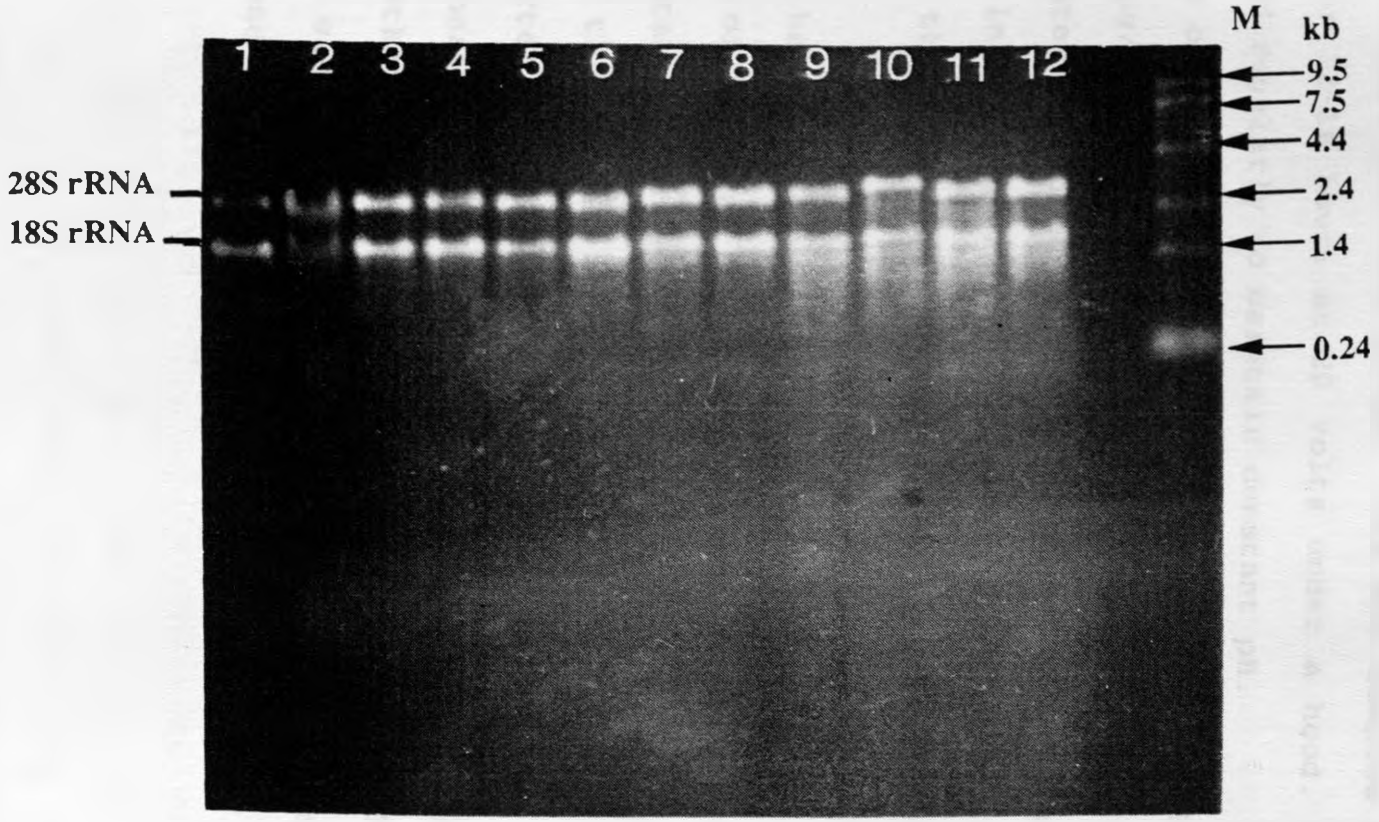
Normalization of RNA

Two approaches were used to ensure equal loading of plastid RNA prior to electrophoresis and blotting. First, the RNA was quantified based on the spectrometric absorption at 260 nm. Second, the RNA samples were normalized according to the 28S cytoplasmic rRNA band intensities as scanned by a densitometer (Fig 2.4). In order to show that the amounts of 28S rRNA did not significantly vary with fruit development, a simple linear regression analysis of days on the relative amounts of 28S rRNA signal/µg total RNA was done. A simple linear regression analysis of days on the relative amounts of normalized 28S rRNA was done in order to ascertain that the amounts of 28S signal was equal among the samples.

Formaldehyde gel electrophoresis of RNA and Northern blotting

Equal amounts of total RNA (5 µg) were denatured and fractionated on 1.2% agarose-formaldehyde gels according to the procedure described by Sambrook *et al.* (1990). This protocol, which was adapted from those of Lehrach *et al.* (1977), Goldberg (1980) and Seed (1982), was used to separate

Fig 2.4 Formaldehyde gel of total RNA from the EP fruits stained in ethidium bromide. The UV light-induced fluorescence illumination shows the 28S and 18S cytoplasmic rRNA bands. Odd numbered lines are RNA extracted from skins of YY BB ovaries and fruits at -2, 3, 7, 14, 21 days pre- and postpollination and in leaves. Even numbered lines refer to RNA extracted from skins of YY B^+B^+ ovaries and fruits and at -2, 3, 7, 14, 21 days pre- and postpollination and in leaves. "M" is the RNA marker


1 2 3 4 5 6 7 8 9 10 11 12
28S rRNA
18S rRNA
M
kb
9.5
7.5
4.4
2.4
1.4
0.24

RNA species according to their molecular weights. The loading of the RNA was adjusted such that the 28S cytoplasmic rRNA bands as scanned by a densitometer on photographic negatives, were equal for each stage. The RNA samples were fractionated for 12-16 hours at 20 volts under a hood. The buffer was mixed regularly to maintain constant pH.

At the end of the run, the gel was stained with ethidium bromide (0.5 µg/ml in 0.1 M ammonium acetate) for 45-60 minutes, and later destained in diethyl pyrocarbonate-treated water for at least two hours. A transparent ruler was aligned with the gel and photographed by ultraviolet illumination. The photograph was used to measure the distance from the loading well to each of the bands of the RNA. The $\log_{10}$ of the size of the RNA fragments were plotted against the distance migrated. The resulting curve was used to calculate the sizes of RNA species detected by hybridization after blotting.

The fractionated RNAs were transferred to nylon membrane by capillary method as described by Sambrook et al.(1990). The blotted RNA was immobilized by UV irradiation for 30s at 6 W/m^2 (Church and Gilbert, 1984).

Labelling of probes and hybridization

The following gene specific probes were used to identify and quantify individual transcripts: a 0.7 kb PstI fragment isolated from pZmB1A containing rbcL (Gatenby et al., 1981), psbA from cyanobacteria and the P7 fragment isolated from tomato generously provided by Dr. Donald Bryant and Christina Richards, respectively (both of Penn State).

^{32}P probes were prepared by the random primer labelling procedure developed by Feinberg and Vogelstein (1983). Typically, 50 ng of DNA probes were labelled to a specific activities of 10^5 to 10^8 cpm/µg DNA. Unincorporated precursor deoxyribonucleoside triphosphates were removed from the radiolabelled DNA by Quick Spin Columns (G-50 Sephadex).

Blots were prehybridized at 55°C (for heterologous probes) or 65°C (for homologous probes) in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulphate (SDS) for at least 40 minutes. Prior to hybridization, the purified probes were denatured in a boiling water bath for five minutes and immediately chilled on ice. Hybridization was performed overnight under similar conditions as prehybridization. The hybridized blots were washed once in 2.5% SEN (2.5% SDS, 1 mM EDTA and 40 mM Na phosphate, preheated to 45°C or 55°C as appropriately required) for 10 minutes, and twice in preheated (0.5% SDS, 1 mM EDTA and 40 mM Na phosphate) for 20 minutes each turn.

The blots were sealed in plastid bags and autoradiographed by exposing them to X-ray film (Kodak XAR-2) at -70°C with intensifying screens.

Chloroplast isolation and DNA extraction

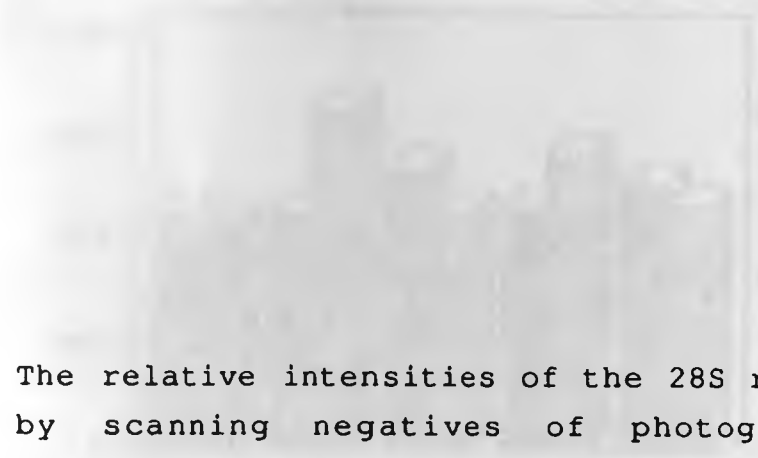
DNA was extracted from 2- to 3-week-old plants, chloroplast isolation and chloroplast DNA (cpDNA) extraction were carried out using a method developed by Lim (1990). The isolated plastid pellet was lysed in 5 ml homogenization buffer (0.35 M sucrose, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 30 mM 2-mercaptoethanol, 0.1% BSA, 0.2% polyvinylpyrrolidone) and 2 ml of sodium sarcosinate (3%) added. The suspension was incubated at room temperature for 1 hour for the chloroplasts to lyse. After one hour at room temperature, the preparation was extracted once with an equal volume of phenol, three times with phenol/chloroform and once with chloroform/2-butanol (4:1 v/v). Aqueous and organic solvents were separated by centrifugation at 10,000g for 10 minutes (JA-20 rotor) at each step. DNA was precipitated by adding 1/10 volume 7.5 M ammonium acetate and 2.5 volumes absolute ethanol and stored at -70°C for 1 hour. The precipitated DNA was collected by centrifugation at 10,000g for 10 minutes. The pellet was washed with 70% ethanol, dried in nitrogen gas and dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

Results

Variation in steady-state levels of *rbcL* transcripts in Early Prolific squash

The RNA was equalized as previously described in "Materials and Methods." Simple linear regression analysis of days on relative 28S rRNA/µg total RNA showed that the amounts of 28S rRNA did not significantly vary over the fruit developmental stages studied (Appendix B.1). The coefficient of determination, r^2 was 0.1. Figure 2.5 illustrates the intensities of 28S rRNA bands scanned from a photographic negative of an agarose gel. Simple linear regression analysis of days on relative amounts of 28S rRNA was insignificant and the coefficient of determination, r^2 was 0.12 (Appendix B.2).

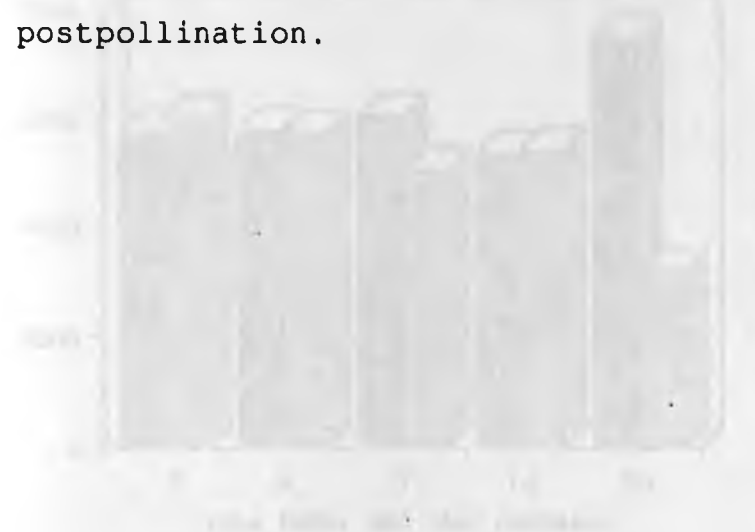
Steady-state levels of *rbcL* transcripts increased with fruit development, from undetectable amounts at 2 days before pollination (-2) to detectable levels at pollination (0), and culminated at 14 days postpollination (Fig 2.6). A similar trend in steady-state levels of *rbcL* transcripts was observed in experiment two, where the *rbcL* mRNA was first detected at day 3 postpollination and reached a maximum at 21 days (Fig 2.7). The steady-state amounts of *rbcL* transcripts in YY B^+B^+



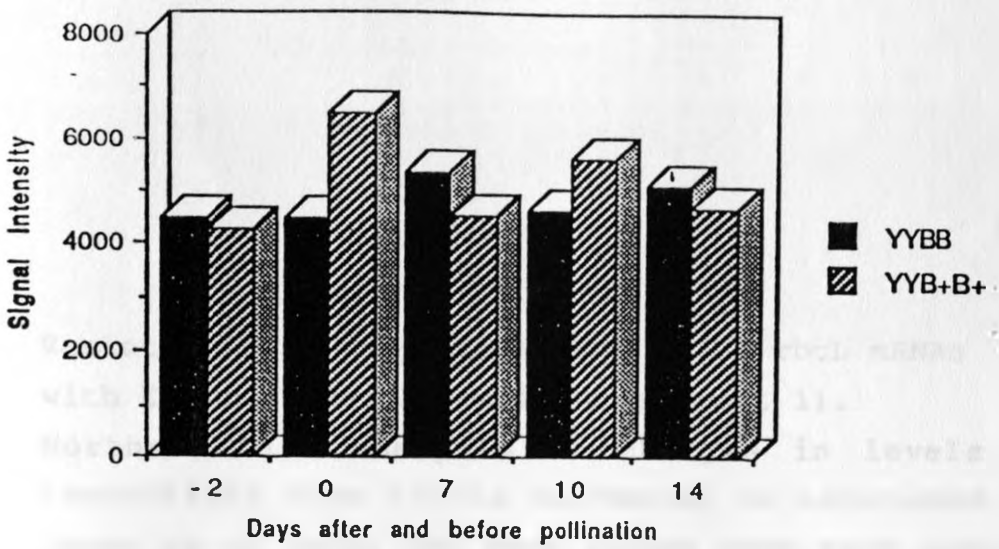
2.5 The relative intensities of the 28S rRNA obtained by scanning negatives of photographs of 2 formaldehyde gels prior to blotting.

A Levels of 28S rRNA from experiment 1 fruits harvested at -2, 0, 7, 10 and 14 days pre- and postpollination.

B Levels of 28S rRNA from experiment 2 fruits harvested at -2, 3, 7, 14 and 21 days pre- and postpollination.



A


8000
6000
4000
2000
0
Signal Intensity
-2
0
7
10
14
Days after and before pollination
YYBB
YYB+B+

B

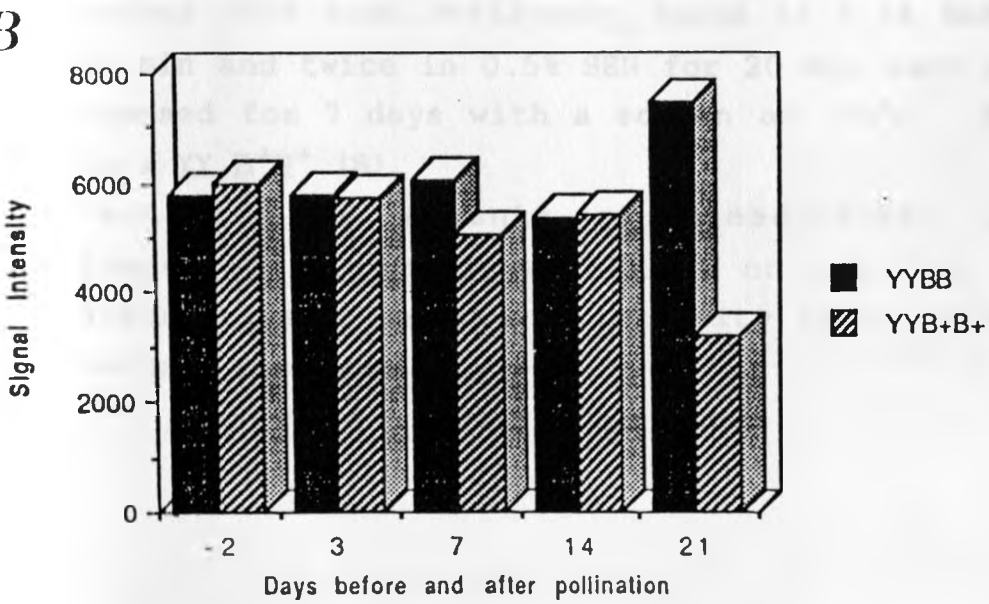
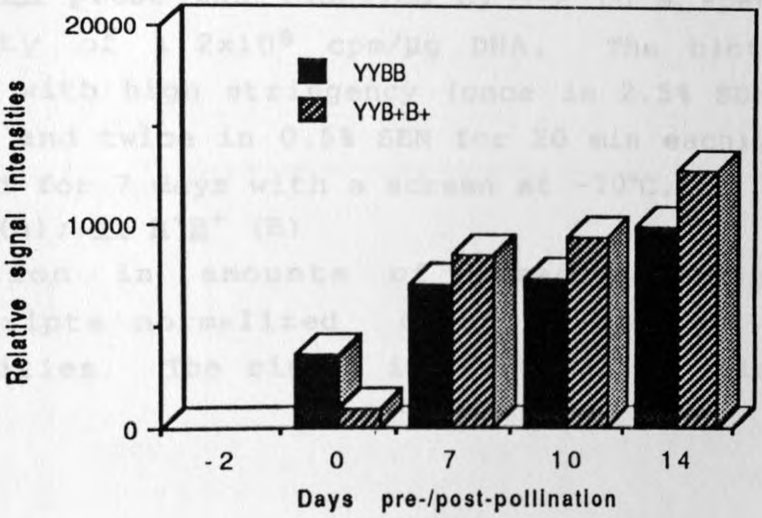

8000
6000
4000
2000
0
Signal Intensity
-2
3
7
14
21
Days before and after pollination
YYBB
YYB+B+

Fig 2.6 Variation in steady-state amounts of rbcL mRNAs with EP fruit development (experiment 1).

A Northern blot analysis of changes in levels of transcripts from fruits harvested in experiment 1. Seven µg of total RNA were loaded from each sample. The rbcL probe was labelled by ^{32}P to a specific activity of about 1.2×10^8 cpm/µg DNA. The blot was washed with high stringency (once in 2.5% SEN for 10 min and twice in 0.5% SEN for 20 min each), and exposed for 7 days with a screen at -70°C. YY BB (A); YY B^+B^+ (B)

B Variation in amounts of steady-state rbcL transcripts normalized based on the 28S rRNA intensities. The signal intensity is in relative units.

B


20000
10000
0
Relative signal intensities
YYBB
YYB+B+
-2
0
7
10
14
Days pre-/post-pollination

A

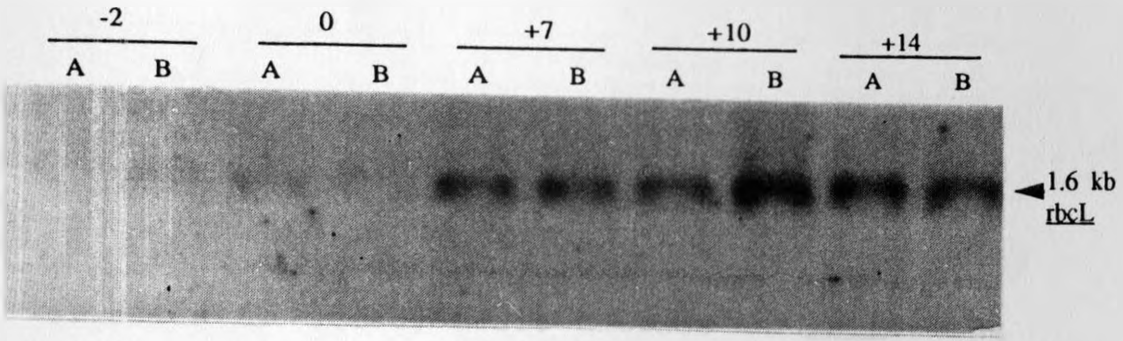
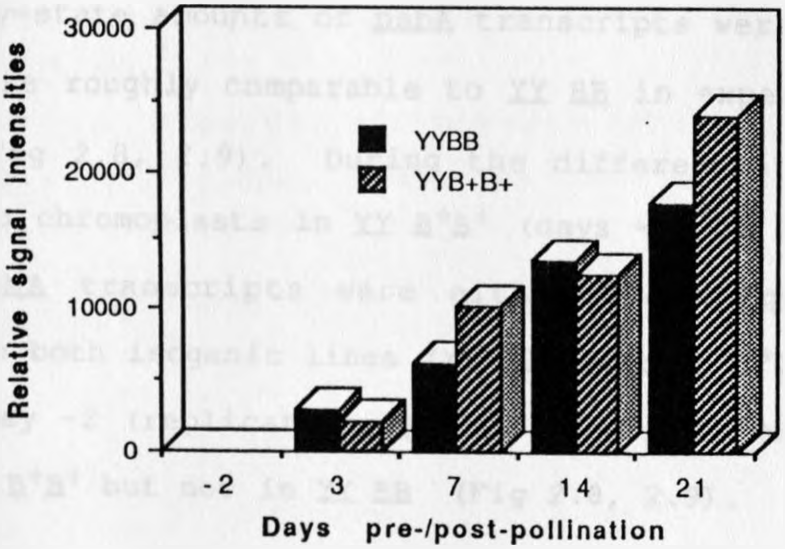


Fig 2.7 Variation in steady-state amounts of rbcL mRNAs with EP fruit development (experiment 2).

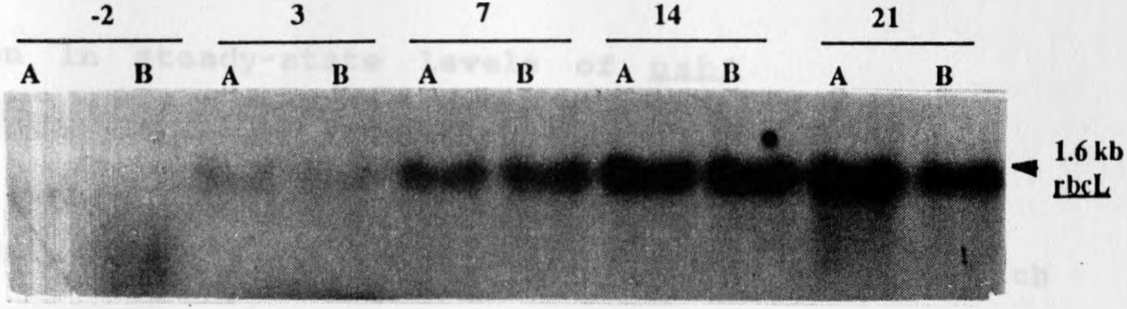
A Northern blot analysis of changes in levels of transcripts from fruits harvested in experiment 2. Seven µg of total RNA were loaded from each sample. The rbcL probe was labelled by ^{32}P to a specific activity of 1.2×10^8 cpm/µg DNA. The blot was washed with high stringency (once in 2.5% SEN for 10 min and twice in 0.5% SEN for 20 min each), and exposed for 7 days with a screen at -70°C.
YY BB (A); YY B^+B^+ (B)

B Variation in amounts of steady-state rbcL transcripts normalized based on the 28S rRNA intensities. The signal intensity is in relative units.

B


30000
20000
10000
0
Relative signal intensities
YYBB
YYB+B+
-2
3
7
14
21
Days pre-/post-pollination

A



and YY BB fruits were comparable in experiments one and two (Fig 2.6B, 2.7B).

Variation in steady-state levels of psbA transcripts in Early Prolific squash

Steady-state levels of psbA transcripts increased with fruit development, from slightly detectable amounts in YY B^+B^+ at 2 days before pollination (-2), to detectable levels at pollination (0), and culminated at 14 days postpollination (Fig 2.8). A similar trend in steady-state levels of psbA transcripts was observed in experiment two, where the psbA mRNA was first detected at 3 days postpollination and reached a maximum at 21 days (Fig 2.9).

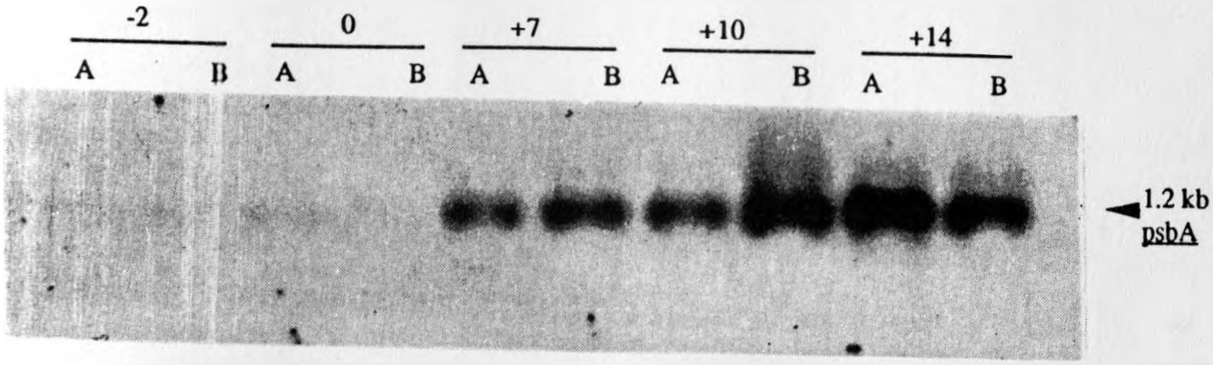
The steady-state amounts of psbA transcripts were in YY B^+B^+ fruits were roughly comparable to YY BB in experiments one and two (Fig 2.8, 2.9). During the differentiation of chloroplasts to chromoplasts in YY B^+B^+ (days -2, 0, 3), the amounts of psbA transcripts were either about equal or undetectable in both isogenic lines (YY BB and YY B^+B^+), with exception of day -2 (replication 2) when some psbA mRNA was detected in YY B^+B^+ but not in YY BB (Fig 2.8, 2.9).

ig 2.8 Variation in steady-state amounts of psbA mRNAs with EP fruit development. (experiment 1)

A Northern blot analysis of changes in levels of transcripts from fruits harvested in experiment 1. Seven µg of total RNA were loaded from each sample. The psbA probe was labelled by ^{32}P to a specific activity of about $1.2x10^8$ cpm/µg DNA. The blot was washed with high stringency (once in 2.5% SEN for 10 min and twice in 0.5% SEN for 20 min each), and exposed for 7 days with a screen at -70°C. YY BB (A); YY B^+B^+ (B)

B Variation in amounts of steady-state psbA transcripts normalized based on the 28S rRNA intensities. The signal intensity is in relative units.

A


-2
0
+7
+10
+14
A
B
A
B
A
B
A
B
A
B
1.2 kb
psbA

B

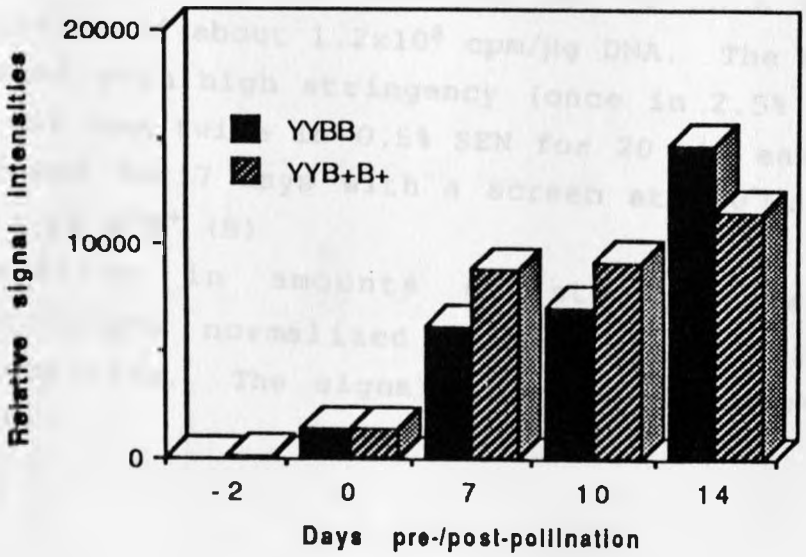
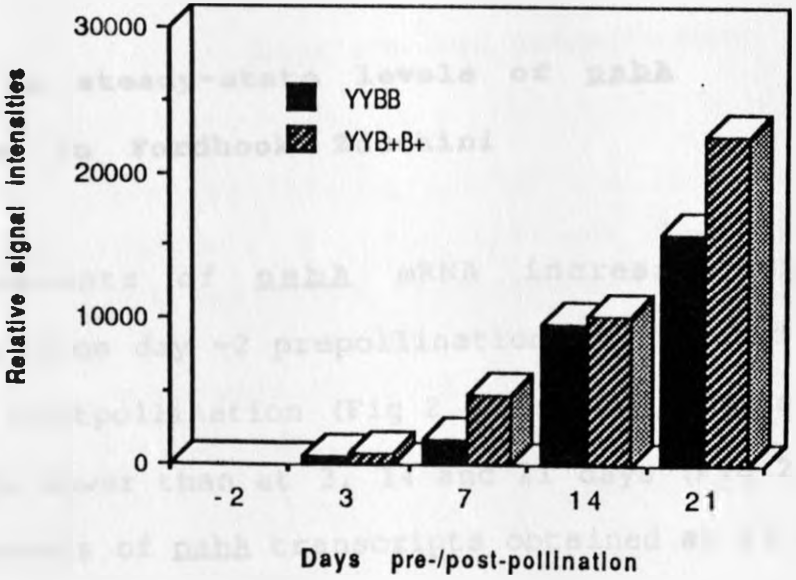

20000
10000
0
Relative signal intensities
YYBB
YYB+B+
-2
0
7
10
14
Days pre-/post-pollination

Fig 2.9 Variation in steady-state amounts of *psbA* mRNAs with EP fruit development. (experiment 2)

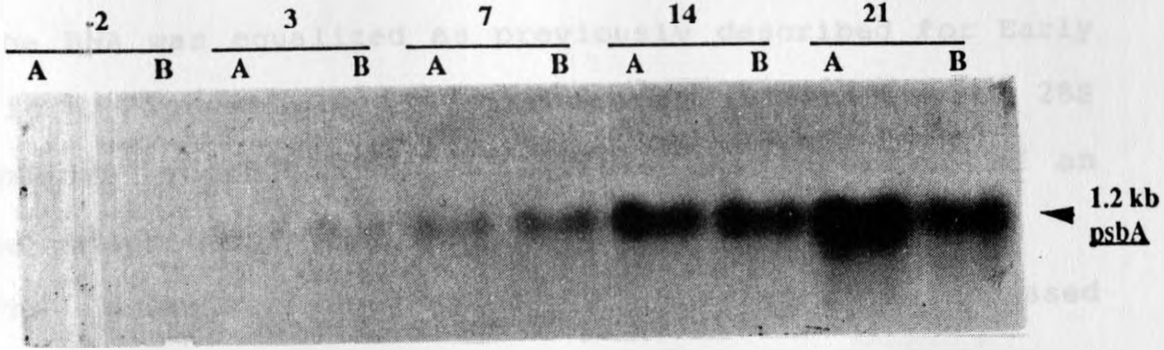
A Northern blot analysis of changes in levels of transcripts from fruits harvested in experiment 2. Seven µg of total RNA were loaded from each sample. The *psbA* probe was labelled by ^{32}P to a specific activity of about 1.2×10^8 cpm/µg DNA. The blot was washed with high stringency (once in 2.5% SEN for 10 min and twice in 0.5% SEN for 20 min each), and exposed for 7 days with a screen at -70°C. *YY BB* (A); *YY* B^+B^+ (B)

B Variation in amounts of steady-state *psbA* transcripts normalized based on the 28S rRNA intensities. The signal intensity is in relative units.

B


30000
20000
10000
0
Relative signal intensities
YYBB
YYB+B+
-2
3
7
14
21
Days pre-/post-pollination

A



Variation in steady-state levels of *rbcL* transcripts in Fordhook Zucchini

The RNA was equalized as previously described for Early Prolific. Figure 2.10 illustrates the intensities of 28S rRNA bands scanned from a negative of photograph of an agarose gel photograph with a densitometer.

The amounts of steady-state *rbcL* transcripts increased gradually from -2 days and reached a maximum at 3 days, before declining at 14 and 21 days (Fig 2.11). After adjustment based on 28S rRNA, the amounts maximum steady-state amounts of *rbcL* levels observed in the fruit was 17% of the amounts in the leaves.

Variation in steady-state levels of *psbA* transcripts in Fordhook Zucchini

The amounts of *psbA* mRNA increased with fruit development from day -2 prepollination and reached a maximum at 21 days postpollination (Fig 2.12). The amounts at -2 and 0 days were lower than at 3, 14 and 21 days (Fig 2.12). The maximum amounts of *psbA* transcripts obtained at 21 days after pollination were 50% of the amounts in the leaves after normalization based on 28S rRNA.

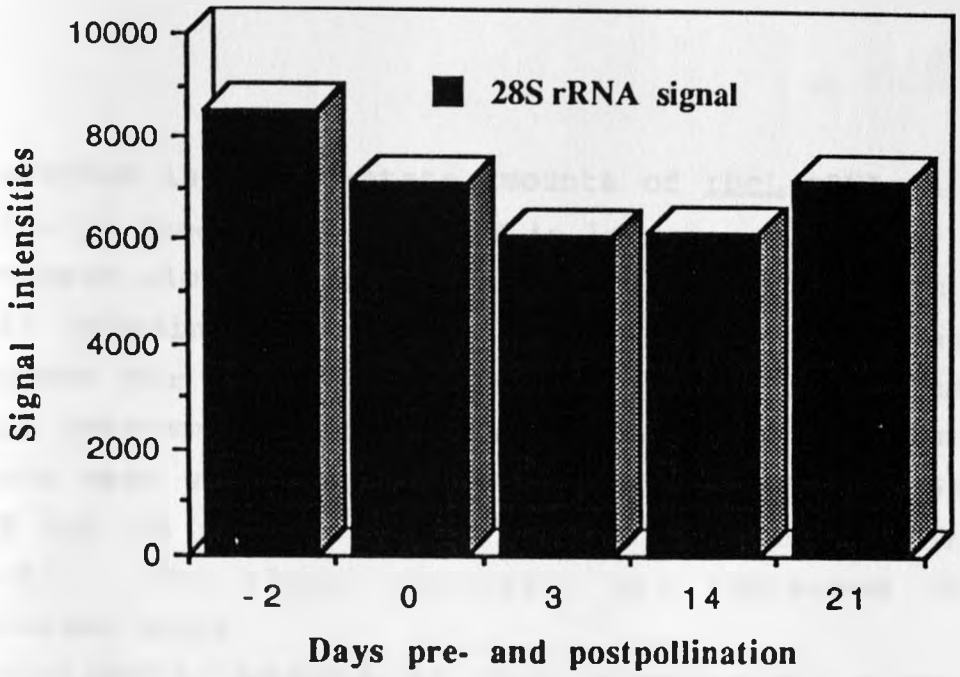


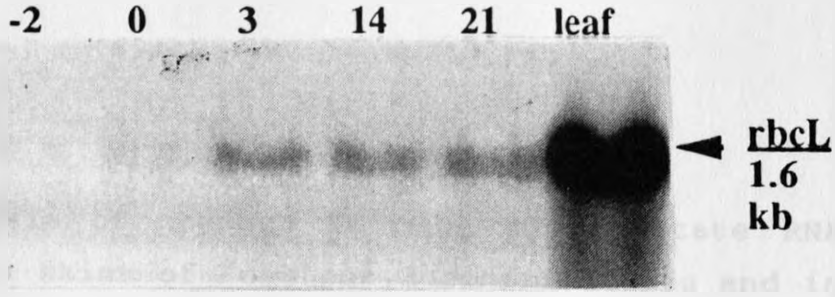
Fig 2.10 The relative intensities of the 28S rRNAs obtained by scanning a negative of formaldehyde gel photographed after electrophoresis. The graph the intensity of the 28S rRNA obtained from Zucchini fruits harvested at -2, 0, 3, 14 and 21 days pre- and postpollination.

Fig 2.11 Variation in steady-state amounts of rbcL mRNAs in skins of Zucchini fruits and in leaves.

A Northern blot analysis of rbcL steady-state. Each well contained about 7 µg of RNA. The blot was exposed for one day with a screen at -70°C. The rbcL gene-specific probe was labelled by ^{32}P. The blots were washed at high stringency once in 2.5% SEN for 10 min and twice in 0.5% SEN for 20 min each). The signal intensity are expressed in relative units.

B Variation in amounts of rbcL steady-state transcripts with fruit development normalized based on the 28S rRNA.

A


-2
0
3
14
21
leaf
rbcL
1.6
kb

B

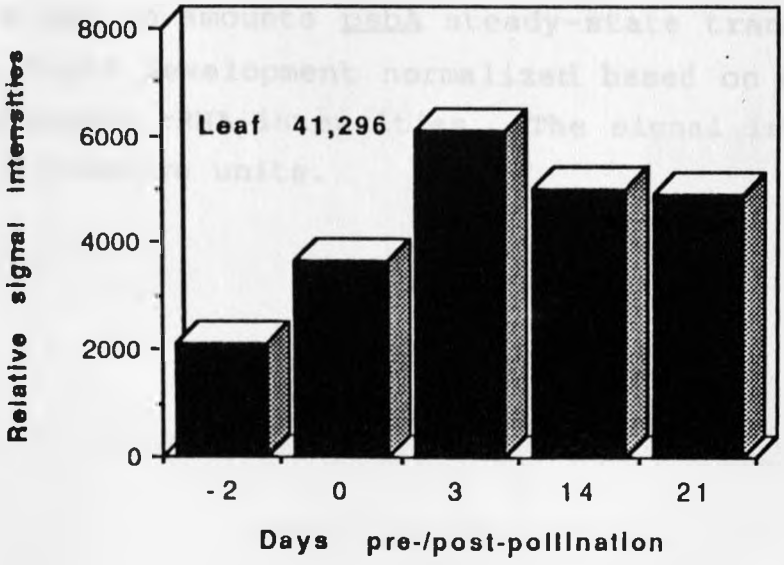
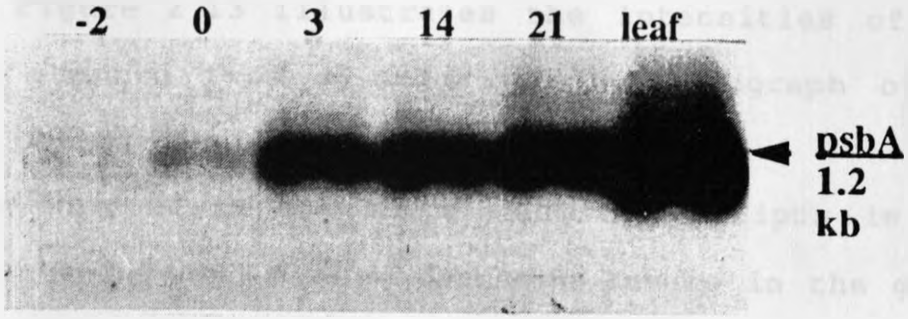

Relative signal intensities
8000
6000
4000
2000
0
Leaf 41,296
-2
0
3
14
21
Days pre-/post-pollination

Fig 2.12 An analysis of changes in psbA steady-state RNA levels in skins of Fordhook Zucchini fruits and in leaves.

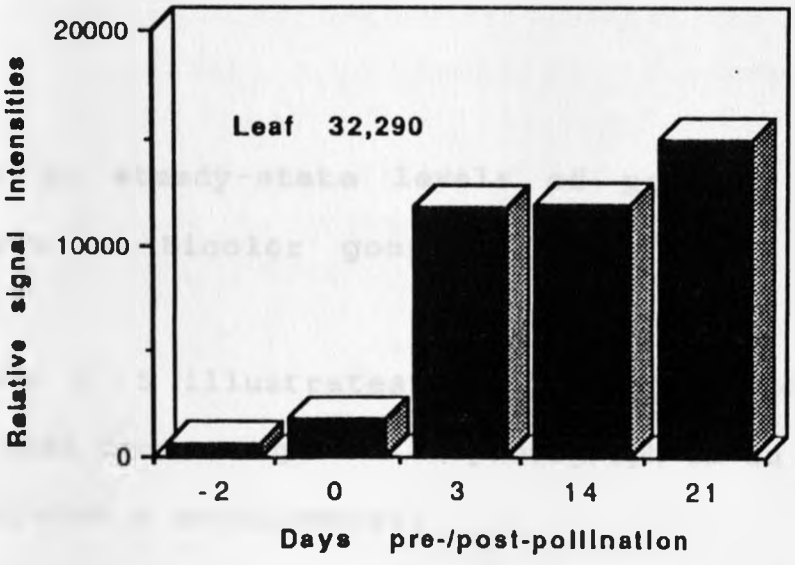
A Northern blot showing the changes in psbA mRNA with fruit development. Each well contained about 4.5 µg of RNA. The blot was exposed for 1 days with a screen at -70°C. The psbA gene-specific probe was labelled with ^{32}P. The blots was washed with high stringency (once in 2.5% SEN for 10 min and twice in 0.5% SEN for 20 min each).

B Variation in amounts psbA steady-state transcripts with fruit development normalized based on the 28S cytoplasmic rRNA intensities. The signal intensity is in relative units.

A


-2
0
3
14
21
leaf
psbA
1.2
kb

B


20000
10000
0
Relative signal Intensities
Leaf 32,290
-2
0
3
14
21
Days pre-/post-pollination

Variation in steady-state levels of rbcL transcripts in Bicolor gourds

The RNA was equalized as previously described for Early Prolific. Figure 2.13 illustrates the intensities of 28S rRNA bands scanned from a negative of photograph of an agarose gel photograph with a densitometer.

The amounts of steady-state rbcL transcripts in the chromoplast tissue were higher than the levels in the green tissue in fruits at 14 and 21 days postpollination (Fig 2.14, Table 2.1). Levels of rbcL mRNAs at days 14 exceeded the amounts at day 21 in chloroplasts but were about equal in the chromoplasts (Fig 2.14, Table 2.1). Levels of steady-state rbcL mRNAs in the fruit range from 3.5% to 7.2% compared with leaves (Table 2.1).

Variation in steady-state levels of psbA transcripts in Bicolor gourds

Figure 2.15 illustrates the intensities of 28S rRNA bands scanned from a negative of photograph of an agarose gel photograph with a densitometer.

Levels psbA mRNAs at day 21 slightly exceeded the amounts at day 14 in the yellow tissue by 1.3-fold, while in

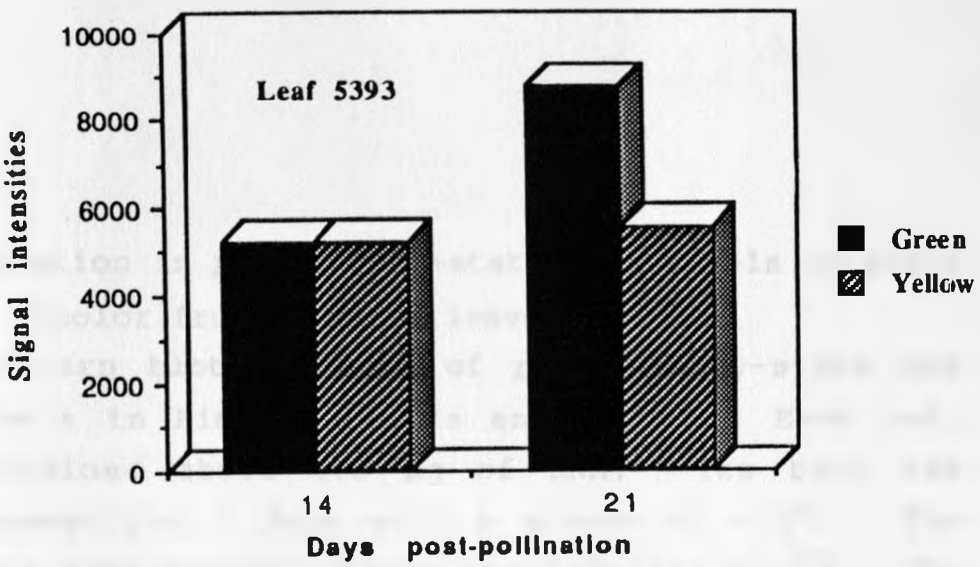


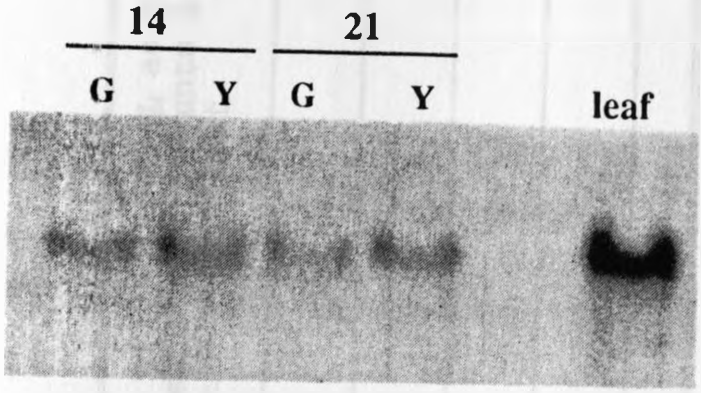
Fig 2.13 The relative intensities of the 28S rRNAs obtained by scanning a negative of formaldehyde gel photographed after electrophoresis. The graph shows the intensities of the 28S rRNA obtained from Bicolor harvested at 14 and 21 days pre- and postpollination.

Fig 2.14 Variation in rbcL steady-state mRNA levels in skins of Bicolor fruits and in leaves.

A Northern blot analysis of rbcL steady-state RNA levels in Bicolor fruits and leaves. Each well contained about 4.5 µg of RNA. The blot was exposed for 1 days with a screen at -70°C. The rbcL gene-specific probe was labelled by ^{32}P. The blots was washed with high stringency (once in 2.5% SEN for 10 min and twice in 0.5% SEN for 20 min each). Green portion of fruit (Gr), Yellow portion of fruit (Y).

B Changes in amounts of rbcL steady-state transcripts with fruit development normalized based on the 28S cytoplasmic rRNA intensities. The signal intensities are expressed in relative units.

A


14
21
G
Y
G
Y
leaf
rbcL
1.6 kb

B

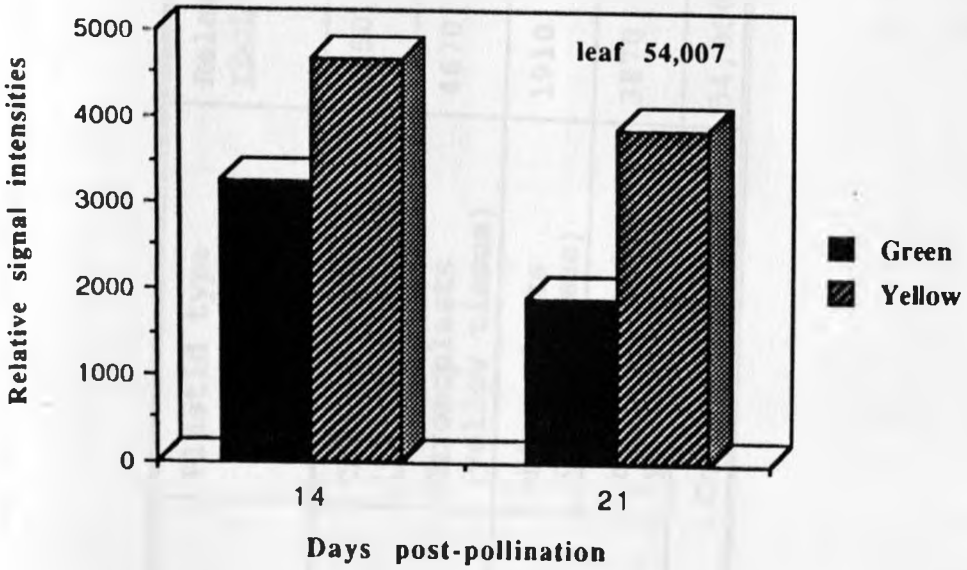

leaf 54,007
5000
4000
3000
2000
1000
0
Relative signal intensities
14
21
Days post-pollination
Green
Yellow

Table 2.1 Comparisons of the relative amounts of rbcL steady-state transcripts in the yellow and green portions of Bicolor squash fruits and leaves.

Tissue	Plastid type	Relative rbcL signal	rbcL as % of yellow tissue on same fruit	rbcL as % of amounts in leaf
Fruit (14 days)	Chloroplasts (green tissue)	3250	69.6	6
	Chromoplasts (yellow tissue)	4670	100	6.7
Fruit (21 days)	chloroplasts (green tissue)	1910	49.4	3.5
	Chromoplasts (yellow tissue)	3870	100	7.2
Leaves	Chloroplasts	54,000		100

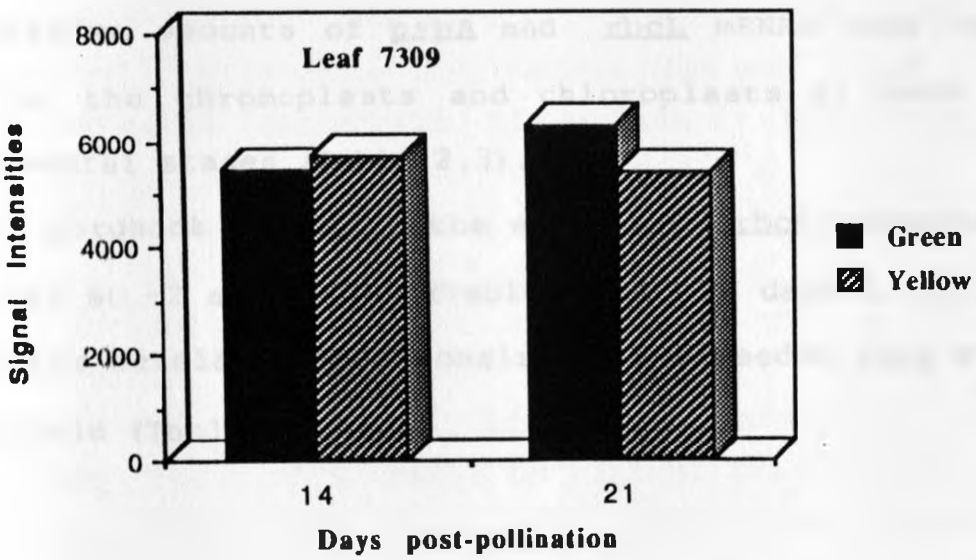


Fig 2.15 The relative intensities of the 28S rRNAs obtained by scanning a negative of a photograph of formaldehyde gel. The graph shows the intensities of the 28S rRNA obtained from Bicolor harvested at 14 and 21 days pre- and postpollination.

the green tissue the amounts decreased by 2.4-fold (Fig 2.16, Table 2.2). Levels of steady-state psbA mRNAs in the fruit ranged from 12.5% to 30% compared with leaves (Table 2.1).

Relative abundance of steady-state rbcL and psbA transcripts in Bicolor gourds and Fordhook Zucchini

The relative amounts of psbA mRNAs exceeded rbcL by 3.3 to 4.2-fold in Bicolor fruits at 14 and 21 days (Table 2.3). The relative amounts of psbA and rbcL mRNAs were roughly equal in the chromoplasts and chloroplasts at both fruit developmental stages (Table 2.3).

In Fordhook Zucchini, the amounts of rbcL exceeded psbA by 2-fold at -2 and 0 days (Table 2.4). In days 3, 14 and 21 fruits the levels of psbA consistently exceeded rbcL by 2.9- to 3.9-fold (Table 2.4).

Relative abundance of steady-state rbcL and psbA transcripts in EP, Fordhook Zucchini and Bicolor gourds

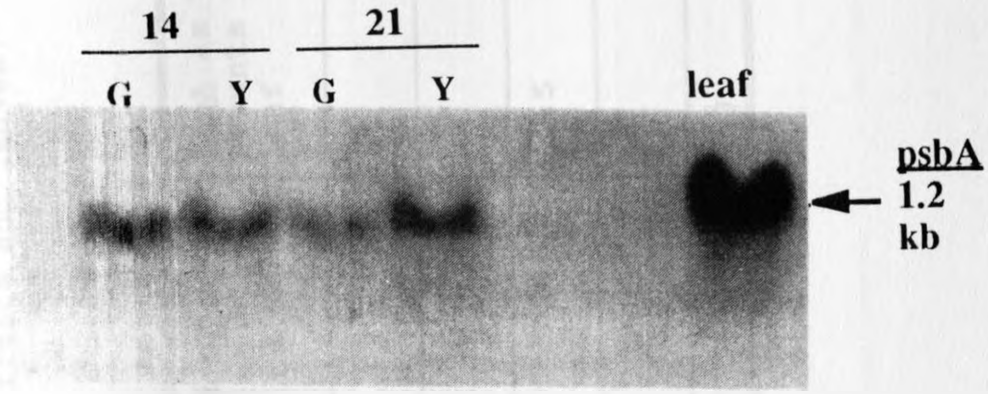
The relative amounts of steady-state rbcL transcripts in 21-day-old fruits of 3 varieties were ranked as Bicolor (yellow portion) > Bicolor (green portion) > Zucchini > EP (Fig 2.17).

Fig 2.16 Variation in psbA steady-state mRNA levels in skins of Bicolor fruits and in leaves.

A Northern blot analysis of psbA steady-state RNA levels in Bicolor fruits and leaves. Each well contained about 4.5 µg of RNA. The blot was exposed for 1 days with a screen at -70°C. The psbA gene-specific probe was labelled by ^{32}P. The blot was washed with high stringency (once in 2.5% SEN for 10 min and twice in 0.5% SEN for 20 min each).

B Changes in amounts psbA steady-state transcripts with fruit development normalized based on the 28S cytoplasmic rRNA intensities. The signal intensity is in relative units.

A


14
21
G
Y
G
Y
leaf
psbA
1.2
kb

B

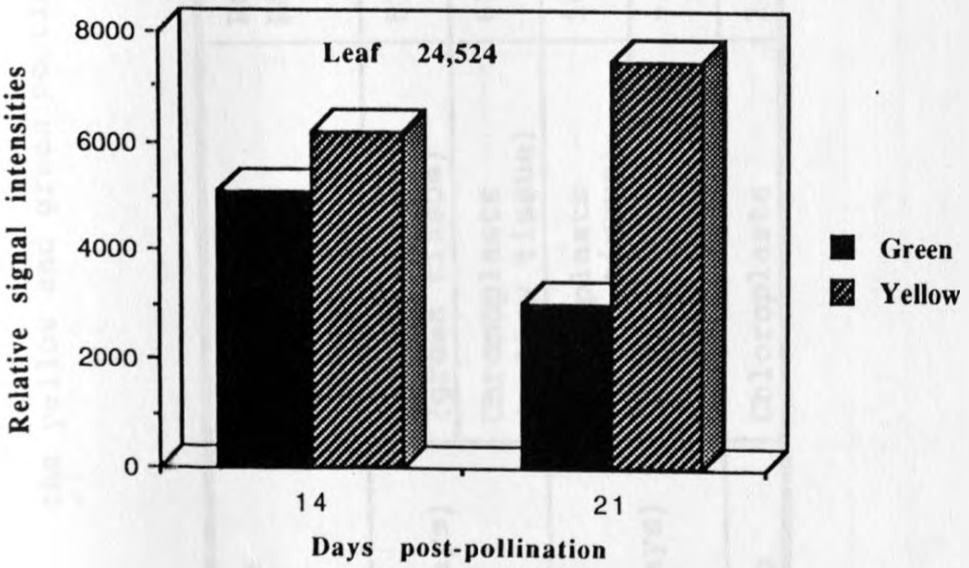

Relative signal intensities
8000
6000
4000
2000
0
Leaf 24,524
14
21
Days post-pollination
Green
Yellow

Table 2.2 Comparisons of the relative amounts of psbA steady-state transcripts in the yellow and green portions of Bicolor squash fruits and leaves.

Tissue	Plastid type	Relative psbA signal	psbA as % of yellow tissue on same fruit	psbA as % of amounts in leaf
Fruit (14 days)	Chloroplasts (green tissue)	5111	82.6	20
	Chromoplasts (yellow tissue)	6189	100	25
Fruit (21 days)	chloroplasts (green tissue)	3063	40.9	12.5
	Chromoplasts (yellow tissue)	7499	100	30
Leaves	Chloroplasts	24,524		100

Table 2.3 Comparisons of the relative amounts of psbA steady-state transcripts to rbcL mRNAs in Bicolor squash fruits.

Tissue	Plastid type	Relative amounts of psbA mRNAs in fruit compared to leaf (%)	Relative amounts of rbcL mRNAs in fruit compared to leaf (%)	Amounts of psbA mRNAs compared to rbcL in folds
Fruit (14 days)	Chloroplasts (green tissue)	20	6	3.3
	Chromoplasts (yellow tissue)	25	6.7	3.7
Fruit (21 days)	chloroplasts (green tissue)	12.5	3.5	3.6
	Chromoplasts (yellow tissue)	30	7.2	4.2
Leaves	Chloroplasts	100	100	

Table 2.4 Comparisons of the relative amounts of psbA steady-state transcripts to rbcL mRNAs in Fordhook Zucchini fruits.

Age of Fruit (days)	Plastid type	Relative amounts of psbA mRNAs in fruit compared to leaf (%)	Relative amounts of rbcL mRNAs in fruit compared to leaf (%)	Amounts of psbA mRNAs compared to rbcL in folds
-2	Chloroplasts	1.7	4.0	0.4
0	Chloroplasts	4.4	7.0	0.5
3	chloroplasts	28.7	11.6	2.5
14	Chloroplasts	29.3	9.3	3.2
21	Chloroplasts	36.3	9.3	3.9
leaves	Chloroplasts	100	100	

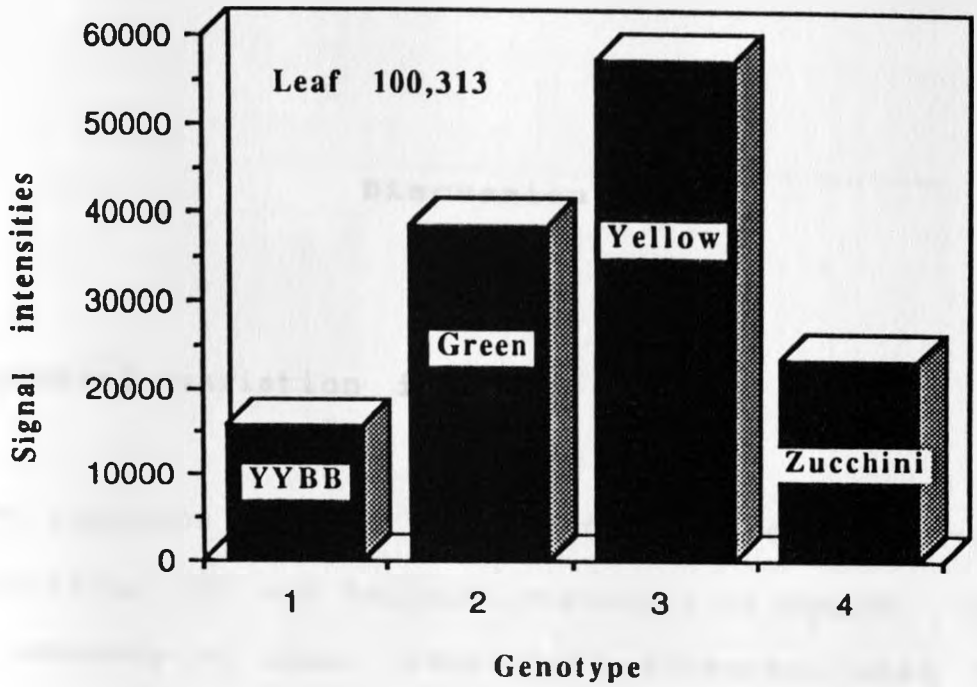


Fig 2.17 A plot of the relative amounts of rbcL steady-state transcripts in Early Prolific (YY BB), Bicolor (green and yellow portions) and in Zucchini fruits.

The relative amounts of steady-state psbA transcripts in 21-day-old fruits of 3 varieties were ranked as EP > Bicolor (yellow portion) > Bicolor (green portion) > Zucchini (Fig 2.18).

Discussion

Developmental variation in rbcL

Two patterns of rbcL gene expression were observed in Early Prolific (EP) and Zucchini varieties of squash. In the EP the amounts of rbcL transcripts increased with fruit developmental up to 21 days without a decline, while in Zucchini the rbcL transcripts increased to a maximum at 3 days and slightly declined at 14 and 21 days postpollination. The developmental variation in rbcL transcripts observed in Fordhook Zucchini fruit chloroplasts was quite similar to those observed in chloroplasts of amaranth leaves, cotyledons, and stem (Berry et al., 1986, Nikolau and Klessig, 1987), in spinach leaves (Deng and Gruissem, 1987) and in barley leaves (Mullet and Klein, 1987). For example, in cotyledons of dark-grown amaranth seedlings the rbcL steady-state mRNAs were first observed at 2 days after germination and reached a peak at day 6, decreased slightly

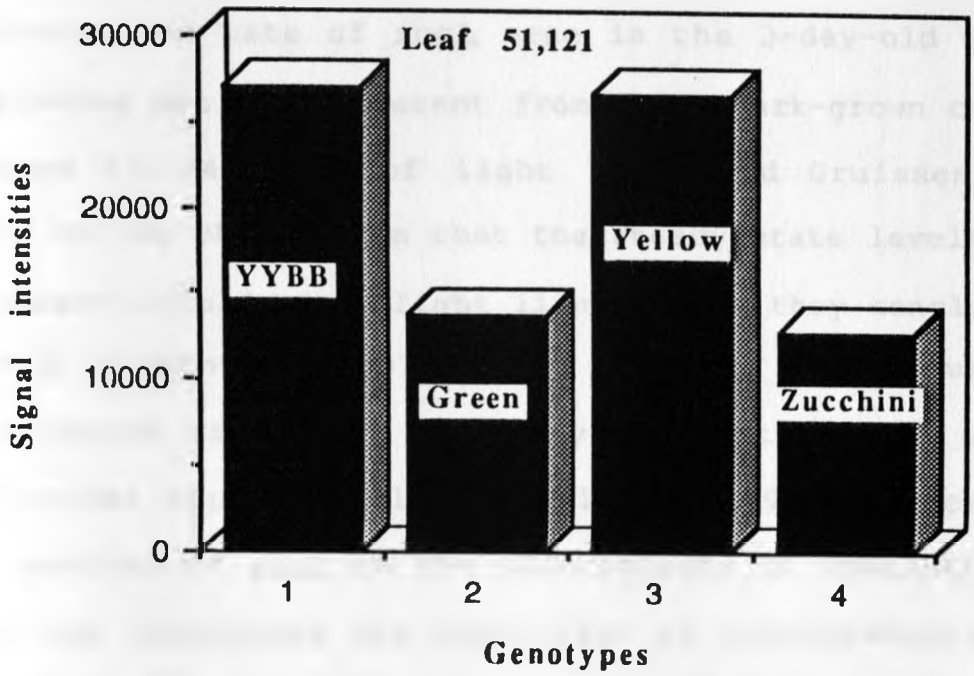


Fig 2.18 A plot of the relative amounts of psbA steady-state transcripts in Early Prolific (YY BB), Bicolor (green and yellow portions) and in Zucchini fruits.

at day 7, and drastically at day 8. In contrast, the rbcL, levels in cotyledons of light-germinated seedlings were first observed at day 2, reached a peak at day 6 and remained high through day 8. In spinach the rate of transcription of rbcL gene in the chloroplasts of mature and young leaves were quite similar (Deng and Gruissem, 1987). Similarly, the transcription rate of rbcL gene in the 3-day-old etiolated cotyledons was not different from 2-day-dark-grown cotyledons exposed to 24 hours of light (Deng and Gruissem, 1987). Based on the observation that the steady-state levels of rbcL increased 2-fold after light illumination they concluded that this developmental regulation of the rbcL mRNAs could be due to enhanced transcript stability in light than in darkness. In another study, Nikolau and Klessig (1987) suggested that the control of rbcL in the chloroplasts of amaranth leaves, stem and cotyledons was controlled by developmental signals acting at an organ-specific level. The developmental regulations of rbcL gene in the above discussed systems closely match the rbcL pattern observed in Zucchini variety of squash, in that the levels of rbcL transcript increased with development to a maximum at day 3 and slightly declined by day 21. Another reason for the similarity in rbcL gene expression in Zucchini, amaranth and spinach was that the rbcL gene expressions were all assayed in chloroplasts. However, although the patterns of rbcL steady-state transcripts appear similar, in that the levels first

increased and later dropped with development, it is possible that the specific gene regulatory mechanisms could be different in the chloroplasts of fruits, leaves and cotyledons. It is likely that different tissue-specific developmental gene regulatory mechanisms such as a trans-acting factor may be modulating rbcL gene expression in leaf chloroplasts differently from fruit chloroplasts. It is also possible that the regulation of rbcL gene expression in Zucchini (a C_3 plant) is different from amaranths (C_4 plant). A special kind of regulation of rbcL gene expression is found in C_4 plants such as maize (and possibly in amaranth). Mesophyll cells of C_4 plants which trap CO_2 in form of C_4 acids, are reported to lack rbcL mRNA, whereas bundle sheath cells which fix CO_2 through the Calvin cycle contain rbcL mRNA (Link et al., 1978). This aspect of tissue-specific regulation is likely to be operative in amaranth chloroplasts but not in chloroplasts or chromoplasts of Zucchini and Early Prolific respectively.

A second distinction in rbcL gene structure between C_3 and C_4 plants which would have a profound effect on the gene expression, is the difference in rbcL gene promoter regions. The rbcL promoter regions of C_3 plants (spinach and tobacco) have been reported to be highly homologous to the prokaryotic -10 and -35 consensus elements (Shinozaki and Suguira, 1982) while the corresponding maize (C_4) putative promoter did not match the prokaryotic promoter (McIntosh et al., 1980; Mullet

et al., 1985). There was also heterogeneity in the position of the 5' region of the rbcL between C_3 plant (spinach and tobacco) and C_4 plant maize, in that the 5' region of rbcL mRNAs in spinach and tobacco was at -180, while in maize it was at -300.

Although in both Zucchini chloroplasts and EP chromoplasts the amounts of steady-state rbcL mRNAs increased with fruit development, in EP the levels did not drop even after 21 days. Developmental changes in levels of rbcL transcripts in chromoplasts of ripening fruits have been reported in tomato and pepper. In developing tomato fruits rbcL transcripts were detected at day 7, culminated at days 11 to 19, dropped drastically at days 23 to 27 and were undetected at days 31 and 35 (Piechulla et al., 1987). In contrast, in two cultivars of tomato, the amounts rbcL mRNAs remained stable during fruit ripening (Richards et al., 1991). Similarly, in ripening pepper fruits and maturing sunflower petals the amounts of steady-state rbcL mRNAs remained constant during fruit ripening (Kuntz et al., 1989). These studies and the current study affirm that the transcriptional machinery for the expression of rbcL gene was fully functional in the chromoplasts of tomato, pepper and squash fruits and that the amounts of rbcL mRNAs were developmentally regulated. A possible reason why in the thesis study the amounts of rbcL increased continuously with fruit development while in tomato and pepper the levels were

constant was due to differences in fruit maturities in which the transcripts were determined. It is possible that in this study, the rbcL level would have dropped if more mature fruits had been examined.

Another possible cause of the discrepancy between the developmental patterns of rbcL in squash in comparison to pepper and tomato is differences in the time of onset of fruit ripening and accompanying physiological processes. In tomato fruits, the onset of chromoplasts development occurs at the terminal stage of fruit development, while in squash it occurs early in fruit development. The chloroplasts-to-chromoplasts transition in tomato involves synthesis of carotenoids such as lycopene (Kushwana et al., 1970, Rosso, 1968), while in squash fruits the main carotenoids synthesized are xanthophylls (Schaffer, 1982). Because the transition of chromoplasts into chloroplasts in tomato occurs at the terminal stage of fruit development, it is accompanied by other ripening processes such as cell wall degradation and softening (Grierson, 1981) and synthesis of polygalacturonase (Sawamara et al., 1978). In contrast, chromoplast development in squash commences early in fruit development and proceeds into fruit maturity. It is very unlikely that cellular degradative processes such described above in tomato occur during early fruit development in squash. It is possible that such signals of senescence could affect the rbcL levels in ripening tomato fruits, thus attributing to

the slight differences in the expression pattern of rbcL gene in squash fruits in comparison to pepper and tomatoes.

Varietal differences in rbcL and psbA transcripts between the two EP isogenic lines

The YY BB and YY B^+B^+ isogenic lines had comparably equal steady-state amounts of rbcL and psbA transcripts. In contrast to results in the current study, plastid transcripts have been reported to differ between cultivars of the same species (Richards et al., 1991; Piechulla et al., 1985, 1986). For example, in one tomato, cultivar (VFNT), the levels of psbA mRNAs and rbcL mRNAs decreased with fruit ripening, while in another cultivar (Firstmore), psbA remained constant while rbcL decreased (Piechulla et al., 1985, 1986). In another study, the levels of psbA mRNAs remained constant in tomato cultivar 'Traveler 76' and decreased in 'Count II' while rbcL transcripts remained constant in both cultivars during fruit ripening (Richards et al., 1991). The two squash EP isogenic lines have been reported to have identical chromoplast structures later in fruit development (Lim, 1990) and in carotenoid composition (Schaffer, 1982). The only discrepancy is that the YY B^+B^+ fruits grow slightly faster than the YY BB (Schaffer, 1982).

Changes in *psbA* transcripts with fruit development

Amounts of the *psbA* transcripts increased with fruit developmental in both EP and Zucchini fruits. Developmental regulation of plastid genes has been documented in spinach and in barley (Deng and Gruissem, 1987). In 3-day-old etiolated spinach cotyledons, the rate of transcription of the *psbA* gene was approximately equal to transcription in 2-day-old cotyledons illuminated for 24 hours (Deng and Gruissem, 1987). In the same study the rate of transcription of *psbA* in young spinach leaves slightly exceeded the rate in mature leaves. This study involved the expression of about nine other chloroplast genes whose rate of transcription was similar although the genes were differentially expressed. The rate transcription could not fully account for the relatively higher steady-state levels of *psbA* transcripts compared to the other nine genes. It was concluded that RNA stability partly accounted for differential expression of *psbA* gene. The *psbA* mRNA plus other chloroplast genes have inverted repeats (IR) in the 3' region which can from stem and loop structures. It was suggested that the IR are responsible for the stability of the transcript (Deng and Gruissem, 1987). Kuntz *et al*. (1989) reported a similar gradual elevation of *psbA* RNA in chromoplasts during pepper fruit maturation and maturing sunflower petals. In a run-on assay, they reported transcription of *psbA* gene in the

chromoplasts and chloroplasts of bell pepper fruits. However, the transcription rate of the psbA gene followed the variation in steady state amounts of the transcript, suggesting that both transcription activity and RNA stability attributed to the increases in levels of psbA transcripts in the chromoplasts.

Influence of plastid differentiation on expression of rbcL and psbA genes

The ultrastructural differences and the distinction in plastid types between the YY BB and YY B^+B^+ did not result in detectable difference in rbcL and psbA transcripts between the two isogenic lines at -2 and 0 days.

Comparative structural changes during plastid biogenesis in the YY BB and YY B^+B^+ was intensively studied (Lim, 1990). Two days prepollination the green ovaries of the YY B^+B^+ isogenic line began to turn yellow starting with the proximal end and proceeded to the distal end. Developing grana, internal lamellae and starch granules were noticeable. At pollination (0 days), small stacks of grana with several extended thylakoid membranes were observed. At pollination Huang and Boyer (unpublished) observed substantial stacks of grana in fruit of greenhouse-grown plants. Three days postpollination the grana and the thylakoid membranes had disappeared, while several lamellae and plastoglobuli were

apparent. Ten days postpollination distinct chromoplast structure consisting of concentric internal membranes, increased plastoglobuli, similar to ripe pepper chromoplasts (Harris and Spurr, 1969) and to pepper (Camara and Brangeon, 1981) were observed. Twenty days postpollination the internal membranous chromoplast structure had transformed into globuli chromoplasts consisting of few internal membranes interspersed between considerable numbers of plastoglobuli.

In contrast, with the exception of a few internal membranes, no granal structure was observed in the yellow YY BB ovaries 2 days prepollination. At pollination the YY BB ovaries contained plastoglobuli and a few single internal membranes. Three days postpollination the YY BB chromoplasts developed several single internal membranes with plastoglobuli. Twenty days after pollination no structural difference were observed between YY BB and YY B^+B^+ chromoplasts (Lim, 1990). Given the above described structural differences in plastids of the YY BB and YY B^+B^+, it was reasonable to expect these changes to influence the expression of the rbcL and psbA genes. This is because the psbA gene encodes the D1 protein, a thylakoid membrane structure involved in electron transport (a process which leads to production ATP and NAD(P)H, used in the light reaction of photosynthesis) while Rubisco, a plastid stromal enzyme encoded by the rbcL gene uses the products of the

light reaction (ATP and NAD(P)H) to fix CO_2. Hypothetically, the *rbcL* and *psbA* genes should have been more highly expressed in the *YY* B^+B^+ ovaries prepollination (due to the presence of chloroplasts) and possibly decline with fruit development. The expression of these genes in the *YY BB* ovaries at prepollination, if any should have been less than *YY* B^+B^+. However, the levels of *rbcL* and *psbA* mRNAs did not correlate with plastid differentiation patterns in EP.

Relative abundance of *psbA* and *rbcL* transcripts in Fordhook Zucchini and Bicolor gourds

The levels of the *psbA* transcripts were consistently higher than the *rbcL* in the fruit and leaf tissue of the 3 squash varieties studied. Similarly, in spinach the *psbA* gene was relatively more highly expressed than the *rbcL* and the *atpB* gene (Deng *et al.*, 1987). In a study of spinach, the high expression of *psbA* gene was attributable to the strong promoter of the *psbA* gene. Sequences of the promoter region of *psbA* gene in wheat and six dicots closely resembled the prokaryotic -35 and -10 consensus elements (Hanley-Bowdoin and Chua, 1988). The -10 box diverged by one nucleotide while the -35 region located 18 bp upstream of the -10 region, perfectly matched the prokaryotic -35 consensus element (Hanley-Bowdoin and Chua, 1988).

Relative abundance of *rbcL* and *psbA* transcripts among three squash varieties

The relative amounts of steady-state *rbcL* transcripts in 21-day-old fruits of three squash varieties in order of decreasing magnitude was Bicolor (yellow) > Bicolor (green) > Fordhook Zucchini > EP. For *psbA* the fruits were ranked as EP > Bicolor (yellow) > Zucchini > Bicolor (green) > Zucchini. The results are difficult to interpret because of the different environmental conditions under which the three varieties were grown. Although Early Prolific and Zucchini were planted in the greenhouse in the spring of 1991 and 1992, respectively, the environmental conditions, especially natural light and temperature, vary substantially in the greenhouse. The Bicolor gourds on the other hand were planted in the summer of 1992, in the field under natural light. Richards *et al.* (1991) observed higher plastid transcripts in 'Count II' grown in the field compared to 'Traveler 76' planted in the greenhouse. They suggested that these differences in the levels of transcripts between the two tomato cultivars was possibly due to the influence of the different environments in which they were grown. In the current study the difference in amounts of the transcripts between the two isogenic lines was insignificant, possibly

because the varieties were grown under the same environmental conditions.

Conclusions

The steady-state amounts of psbA and rbcL transcripts in EP, Fordhook Zucchini and Bicolor gourds squash were regulated by the fruit developmental patterns. The levels of these two transcripts did not appear to be influenced by the different plastid types in EP. There were slightly more steady-state levels of psbA and rbcL mRNAs in the YY B^+B^+ than in the YY BB at later fruit developmental stages. This difference could be attributed to the more vigorous growth YY B^+B^+ fruits than the YY BB.

In Bicolor gourds the steady-state levels of psbA and rbcL mRNAs in the yellow tissue of Bicolor gourds slightly exceeded the amounts in the green tissue. The steady-state levels of psbA transcripts exceeded the rbcL mRNAs in the chromoplasts and chloroplasts of fruit and leaf tissues of Bicolor gourds and Fordhook Zucchini.

The psbA gene expression exceeded the rbcL gene expression and the expression of these genes was greater in the leaves than in the fruits of Fordhook, Zucchini and Bicolor gourds.

Chapter 3

DETERMINATION OF CHANGES IN LEVELS OF RUBISCO LARGE SUBUNIT AND RUBISCO ACTIVITY DURING SQUASH FRUIT DEVELOPMENT

Literature Review

Variation in Rubisco LSU protein and Rubisco activity in plastids

Several studies have examined the changes in Rubisco large subunit (LSU) protein and Rubisco activity in plants with a purpose of determining the regulatory mechanisms of the rbcL gene expression. Three of these studies have been done in amaranth chloroplasts and tomato fruit chromoplasts (Piechulla et al., 1987; Berry et al., 1986).

When dark-grown amaranth seedlings were transferred to light the syntheses of SSU and LSU proteins were rapidly initiated before any increases in corresponding mRNAs were observed. On the other hand when light-grown seedlings were transferred into darkness, syntheses of LSU and SSU proteins decreased before any detectable decrease in mRNAs was observed. It was concluded that the expression of rbcL gene was determined over a short time span at the level of

translation but over a long period through changes in the mRNA accumulation (Berry et al.,1986).

Rubisco activity (3.2 nmol CO_2 /min/mg protein) was detected in extracts of cotyledons of amaranth seedlings 3 days after germination, which coincided with high levels of both LSU and SSU polypeptides (Berry et al., 1986). A maximum level of activity, (20-fold of the initial activity), was observed at day 10 which also closely matched the levels of SSU and LSU proteins. In the stem, moderate Rubisco activity of 3 to 8 nmol CO_2/min/mg protein was observed. However, the amounts were 8- to 20-fold lower than in the cotyledons. SSU protein accumulation in the stem coincided with Rubisco activity. The amounts changed only moderately with development while LSU protein remained relatively constant. When leaves first appeared at day 8, Rubisco activity was 13 nmol CO_2 /min/mg protein, and rose to a maximum of 85 nmol CO_2 /min/mg protein (at day 19). SSU proteins varied in coordination with the activity while LSU remained fairly constant.

Hansmann and Sitte (1984) reported reduced amounts of LSU and SSU proteins in the chromoplasts versus chloroplasts of daffodils. Similarly, Piechulla et al. (1987) detected very little rbcL mRNAs in the red tomato fruit pericarps. They suggested that there was reduced or no significant synthesis of Rubisco in the chromoplasts and that the Rubisco activity detected was residual from previous synthesis due to

stability. However, in addition to the fruit being a sink, there is evidence that green fruits contribute to the photosynthetic capacity of the plants. For example, pepper fruits contributed 12% to the total CO_2 fixed by the plant (Steer and Pearson, 1976). Furthermore, in green tomato fruit pericarp substantial levels of Rubisco activity, the reaction center proteins PSI, and PSII, the light harvesting complex proteins of PSII, plastocyanin and Fd-NADP-oxidoreductase were detected (Piechulla et al., 1987). This lead to the suggestion that chloroplasts in tomato fruits were photosynthetically active (Piechulla et al., 1987). Compared to the leaf tissue (with activity of 9.32 nmol CO_2 /min/mg protein), the photosynthetic activity in the green pericarp was 41.4% (3.25 nmol CO_2 /min/mg protein) while in intermediate fruit (45 days after pollination) was 13% (0.20 nmol CO_2 /min/mg protein) (Piechulla et al., 1987). These results were consistent with the previous report of CO_2 fixation in the chloroplasts and chromoplasts of tomatoes (Iwatsuki et al., 1984).

Due to the presence of plastocyanin in the chromoplasts of tomato fruits, it was speculated that perhaps the electron transport chain exists in the chromoplasts which could be used to channel electrons from other chemical reactions to provide energy in form of NAD(P)H (Piechulla et al., 1987). Several studies, reviewed below, have investigated the regulatory mechanisms of Rubisco activity.

Regulation of Rubisco activity

The initial reactions in the photosynthetic CO_2 reduction and photorespiratory carbon oxidation are catalyzed by Rubisco (Ogren, 1984). Catalysis of carboxylation (photosynthesis) and oxygenation (photorespiration) occurs only when Rubisco is in the activated state (Werneke et al., 1988). Rubisco activation in vitro can be achieved by spontaneous addition of CO_2 and Mg^{2+} to form a carbamylated enzyme (Lorimer et al., 1976; McCurry et al., 1981). However, even with fully activated enzyme, the initial rate of carboxylation upon addition of ribulose-1,5-bisphosphate (RUBP) is not maintained and this is why most in vitro assays for Rubisco activity are conducted for one minute or less (Robinson and Portis, 1989). The decline in activity, a phenomenon termed "Fallover," is not due to product accumulation, substrate exhaustion (Andrew and Hatch, 1969), or binding of RUBP to the decarbamylated enzyme (Robinson and Portis, 1989). The decline is due to the binding of a phosphorylated inhibitor to the active site of Rubisco (Robinson and Portis, 1988b). "Fallover" has been attributed to the binding of misfire products: xylulose-1,5-bisphosphate (XUBP) and 3-Keto-arabinitol-1,5-bisphosphate to the catalytic sites of Rubisco (Miziorko and Lorimer, 1983; Edmondson, et al., 1989a, 1989b; Zhu and Jensen, 1991). Under

in vivo conditions, Rubisco is activated by Rubisco activase (Salvucci *et al.*, 1985). The ability of Rubisco activase to activate Rubisco, was further demonstrated *in vitro* using Rubisco activase protein isolated from *E. coli* transformed with a spinach Rubisco activase cDNA (Werneke *et al.*, 1988) and also independently by Robinson and Portis (1989).

Rubisco activity has been reported to fluctuate diurnally. This diurnal variation in Rubisco activity was correlated to increased concentration of an organic phosphate ester in chloroplasts in the dark (Sharkey and Vanderveer, 1989). The Rubisco inhibitor was purified from the chloroplasts of dark-treated potato (*Solanum tuberosum*) leaves and was established as 2-carboxyarabinitol-1-phosphate (CA1P), a molecule which resembles 2-carboxy-3-keto-arabinitol-1,5-bisphosphate, an intermediate in the carboxylase reaction of Rubisco (Gutteridge *et al.*, 1981; Berry *et al.*, 1987). This product has been reported to inhibit Rubisco activity under dark or reduced light conditions (Gutteridge *et al.*, 1986; Berry *et al.*, 1987). CA1P is a naturally occurring inhibitor of Rubisco present in several agronomically important plants such as soybean, beans, potato and tobacco (Vu *et al.*, 1984; Servaites, 1985; Seemann *et al.*, 1985). CA1P accumulates in the dark and by early morning the inhibitor occupies 50-100% of Rubisco active sites (Servaites *et al.*, 1985; Seemann *et al.*, 1985). CA1P inhibits Rubisco by binding tightly to the active site

forming a stable enzyme-inhibitor complex (Servaites et al., 1984; Seemann et al., 1985). This is the site where RUBP binds on Rubisco (Robinson and Portis, 1988a). Addition of RUBP and 6-phosphogluconate partially reversed CA1P inhibition by displacing the inhibitor from Rubisco. Rubisco activase also partially reversed the inhibition, while both RUBP and Rubisco activase in combination almost completely reversed the inhibition. Rubisco activase does not metabolize CA1P. Rubisco activase and RUBP cooperatively interact with Rubisco thereby increasing the dissociation rate of the bound CA1P from the enzyme (Robinson and Portis, 1988b). Carboxyarabinitol 1-phosphatase (2'-carboxyl-D-arabinitol 1-phosphate phosphohydrolase) isolated from the stroma of tobacco chloroplasts has been reported to hydrolyze CA1P giving 2'-carboxylarabinitol (Salvucci and Holbrook, 1988; Gutteridge and Julien, 1989; Holbrook et al., 1991).

It has been suggested that the regulatory mechanisms of Rubisco may vary among plant species. Certain species may rely predominantly upon the metabolism of CA1P for the photo flux density (PFD)-dependent regulation of Rubisco activity. Other species may use some combination of activation and CA1P for this regulation. Finally, others may use activation and tight-binding inhibitor to carry out this regulation (Seemann and Kobza, 1988). There is evidence that species differ in their rates of CA1P synthesis but not in the rates of degradation.

A previous study in squash did not detect Rubisco protein in the skin of squash fruit at prepollination (Lim, 1990). However, substantial amounts of Rubisco large (LSU) protein were observed 20 days after flowering. In the current study, the presence of Rubisco LSU protein was confirmed and Rubisco activity determined.

Materials and Methods

Plant materials

Two isogenic lines of the cultivar Early Prolific were grown in pots under natural environmental conditions during the summer of 1991 at Penn State. The plant growth media consisted of peat: perlite: soil (3:2:1). The plants were later fertilized with nitrogen:phosphorus:potassium (20:20:20) as appropriately required. Early Prolific, YY BB and YY $B^{+}B^{+}$ fresh ovary/fruit tissues were harvested at -2, 0, 7, 10 and 14 days relative to pollination. The amounts of illumination during the time of harvest was between 1400-1500 µmols. The Zucchini plants were grown in the greenhouse at Penn State during spring (1992). The fruits were harvested at -2, 0, 10, 14 and 21 days at pre- and postpollination and

the leaves were concurrently harvested. The light intensity in the greenhouse was about 250-300 µmols.

The Bicolor gourds were grown at the Penn State Horticulture farm (Rock Springs) during summer, 1992. Fruits were harvested at 14 and 21 days postpollination and placed on ice prior to freezing in liquid nitrogen. Young leaves (of leaf lamina of size of about 5 cm by 7 cm) were concurrently harvested and treated similar to the fruits. Skin tissues from the three squash varieties were removed with a potato peeler from the fruits and immediately frozen in liquid nitrogen and stored at -70°C until the time of protein and enzymatic assays. Young leaves were concurrently harvested and frozen at -70°C.

Extraction of soluble plastid proteins

Total soluble proteins were extracted using a procedure similar to that developed by Vu et al. (1984). Frozen tissue of 0.4 gm was weighed and ground with a precooled pestle and mortar, while immersed in liquid nitrogen. The ground tissue was transferred into a beaker and 4 ml of extraction buffer (consisting of 50 mM Bicine (pH 8.0), 20 mM $NaHCO_3$, 20 mM $MgCl_2$, 0.1 mM EDTA, 5.0 mM DTT, 10 µM leupeptin sulfate, 1 mM phenylmethyl sulfonyl flouride [PMSF]) was added. The tissue was homogenized for 60 seconds using a polytron (Brinkman

instrument, model PCU-2-110) at a setting of five. The homogenized tissue was filtered through one layer of miracloth. The filtrate was centrifuged in a microfuge at 10,000 rpm for 10 minutes. The supernatant was aliquoted and used for SDS-PAGE and determination of total Rubisco activity.

SDS-PAGE of proteins

The supernatant obtained from the preceding extraction procedure was used to determine the amounts of Rubisco large subunit (LSU). Prior to SDS-PAGE, total proteins from each sample were quantified using Peterson's (1977) modification of the Lowry procedure (Lowry, et al. 1951).

Fifteen µg of total protein (from each sample) was added to SDS-PAGE loading buffer (containing: 50mM Tris.HCl, 100 mM DTT, 2% SDS, 0.15% bromophenol blue, 10% glycerol) and heated in a boiling water bath for 3 minutes, to denature the proteins (Sambrook et al., 1990). Denatured samples were electrophoresed using the discontinuous buffer system of Laemmli (1970) of 15% resolving gels and 5% stacking gels (Sambrook et al., 1990) for 2-3 hours at 250 mA. In order to estimate the molecular weight of the protein under study, protein markers of known sizes were run in each gel. The "Rainbow" protein molecular weight markers from Amersham

were: Myosin (200 kDa), Phosphorylase b (97.4 kDa), BSA (69 kDa), Ovalbumin (46 kDa), Carbonic anhydrase (30 kDa), Trypsin inhibitor (21.5 kDa) and Lysozyme (14.3 kDa). The rainbow markers were run along with samples in gels intended for electroblotting and immunological study. The following molecular weight markers from Sigma were used to determine molecular weights of proteins intended for Coomassie Brilliant Blue staining: Albumin (bovine plasma 66 kDa), Ovalbumin (albumin, egg 45 kDa), Pepsin (34.7 kDa), Trypsinogen (18.4 kDa), Lysozyme (14.3 kDa).

Staining of proteins with Coomassie Brilliant Blue

After electrophoresis the gels were stained overnight in Coomassie Brilliant Blue stain (0.15% Coomassie blue, 50% methanol, 10% glacial acetic acid). The background staining was removed by destaining 4 times in 200 ml solution containing 45% water, 45% methanol and 10% glacial acetic acid. The gels were photographed and preserved in 50% glycerol.

Staining proteins immobilized on nitrocellulose membranes

After electroblotting the blots were stained with Ponceau's stain [2% Ponceau S, 30% (w/v), trichloroacetic acid, 30% (w/v), sulfosalicylic acid (dissolved in deionized water)]. The blots were stained with Ponceau S in order to visualize the electrophoretic transfer of the proteins. Positions of the molecular markers were marked on the nitrocellulose with a pencil. The western blot was then probed with antibody as described below.

Immunoblotting

After electrophoresis the proteins were blotted on a nitrocellulose membrane using the method described by Towbin et al. (1979). Immunological detection of proteins was done using specific antibodies. The anti-Rubisco was generously provided by Dr. Eva Pell of Penn State. The anti-Rubisco activase was provided by Dr. William Ogren and Dr. Archie Portis from the University of Illinois.

Assay of total Rubisco activity

Part of the supernatant extracted as described above for total proteins was used for Rubisco activity assay. Assay reactions were performed at 30°C in a total volume of 500 µl in eppendorf tubes. The reaction mixture consisted of 400 µl assay buffer (similar to extraction buffer minus DTT, leupeptin, PMSF), 25 µl [0.2 µCurie/mol ^{14}C-$NaHCO_3$] and 25 µl 10 mM RuBP. The reaction was initiated by addition of 50 µl of crude extract and terminated after 30 s by addition of 100 µl of 2 N HCl. The tubes were then vortexed and opened in the hood to allow unfixed labelled ^{14}C to escape. One hundred µl of the reaction product was dried for 2 hr using an infra-red lamp. The samples were cooled and resuspended in 500 µl of water. Four ml of Ready-Safe scintillation fluid was added to the suspension and acid-stable ^{14}C radioactivity determined by liquid scintillation spectrophotometry for 10 minutes per sample using the Tri-Carb liquid scintillation counter.

Results

Variation in Rubisco large subunit protein and absence of Rubisco activase in EP fruits

Figure 3.1 shows a Coomassie Blue stained SDS-PAGE of total soluble proteins from EP fruits at -2, 14, and 21 days pre- and postpollination, and from leaves. The Rubisco LSU protein was immunologically undetectable at 2 days before pollination but detectable at 14 and 21 days after pollination in Early Prolific (EP) fruits (Fig 3.2). Young leaves had about 6 times more LSU than the highest amounts of LSU protein which was observed in fruits at 21 days postpollination.

Rubisco activase was neither detected in ovaries and fruits of EP from -2 days prepollination to 21 days postpollination, nor in the leaves.

In Zucchini the LSU protein was detectable throughout the fruit developmental stages examined. The amounts of LSU protein increased from -2 days to a maximum at day 21 post-pollination (Fig 3.3). Similar to EP, the amounts of LSU protein in the leaves exceeded the levels in the fruit but only by about 1.2-fold.

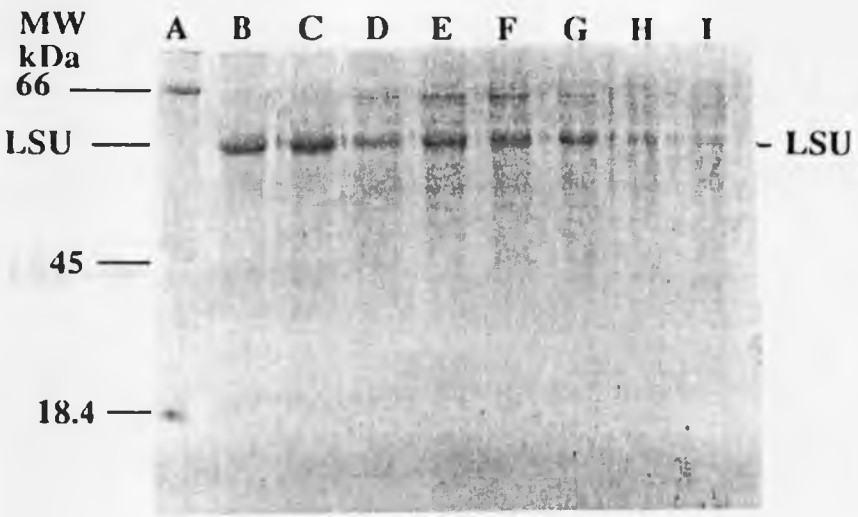


Fig 3.1 SDS-PAGE showing a profile of changes in total soluble proteins in the skins of ovaries and fruits of Early Prolific isogenic lines YY BB (B, D, F) and the YY B^+B^+ (C, E, G) with development. Total proteins were extracted from ovaries at 2 days prepollination (A, B) and 14 days (C, D) and 21 days old fruits (E, F), and young leaves (H: YY BB), (I: YY B^+B^+)

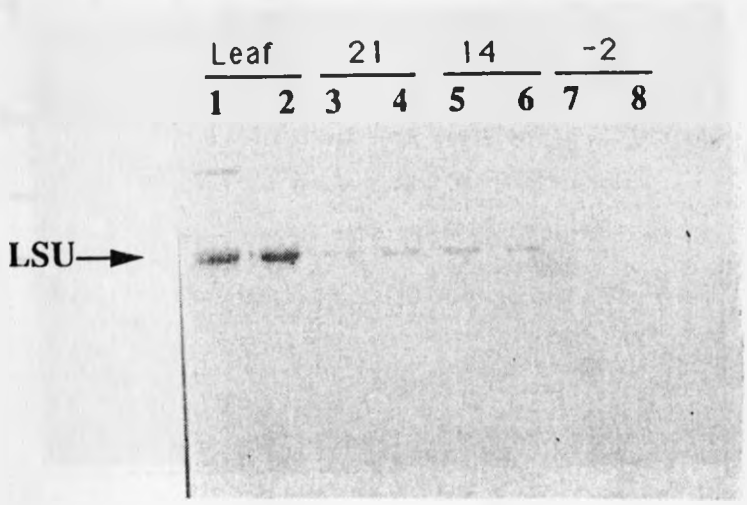


Fig 3.2 Western blot showing changes in Rubisco LSU protein in ovaries, fruits and in leaves of Early Prolific isogenic lines YY BB (lanes 1, 3, 5, 7) and YY B^+B^+ (lanes 2, 4, 6, 8) with development. The ovaries/fruits were harvested at -2, 14 and 21 days pre- and postpollination.

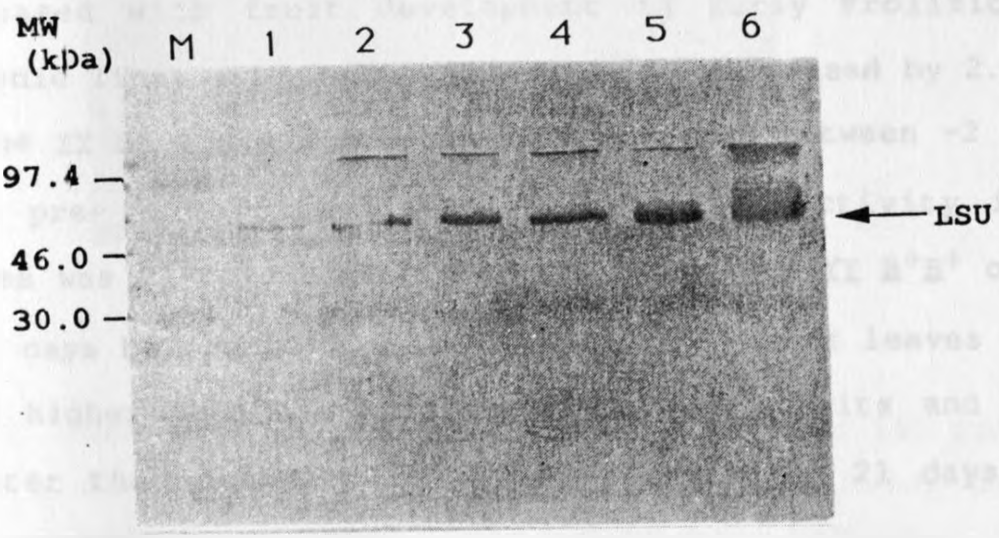


Fig 3.3 Western blot showing changes in Rubisco LSU protein in the ovaries, fruits and leaves of Fordhook Zucchini with development. The ovaries/fruits were harvested at -2, 0, 3, 14 and 21 days pre- and postpollination (lanes 1, 2, 3, 4 and 5, respectively). Lane 6 shows Rubisco LSU protein from leaf tissue. Lane M contains molecular weight markers.

Amounts of Rubisco LSU in day 21 fruits exceeded the amounts in day 14 fruits (Fig 3.4). Levels of LSU protein in the chromoplasts exceeded the amounts in the chloroplasts at 14 and 21 days postpollination. Levels of LSU protein in the leaves exceeded the amounts in the fruits by about 5-fold.

Rubisco activity in squash varieties

Rubisco activity, expressed on fresh weight basis, increased with fruit development in Early Prolific (EP) isogenic lines (Fig 3.5). The activity increased by 2.6-fold in the YY BB and 4.2-fold in YY B^+B^+ lines between -2 and 21 days pre- and postpollination. Rubisco activity in the leaves was 21-fold higher than the YY BB and YY B^+B^+ ovaries at 2 days before pollination. Activity in the leaves was 8-fold higher than the activity in YY B^+B^+ fruits and 5-fold greater than activity in (YY BB) fruits, at 21 days post-pollination.

Based on the general F-test the Rubisco activities were significantly different at the different fruit developmental stages assayed. However, mean separation using the Duncan's multiple range test showed that activity at 21 days significantly exceeded activities in less mature fruits ($p=0.01$) (Fig 3.6). However, the activities at -2, 0, 7 and 14 days pre- and postpollination were not significantly different.

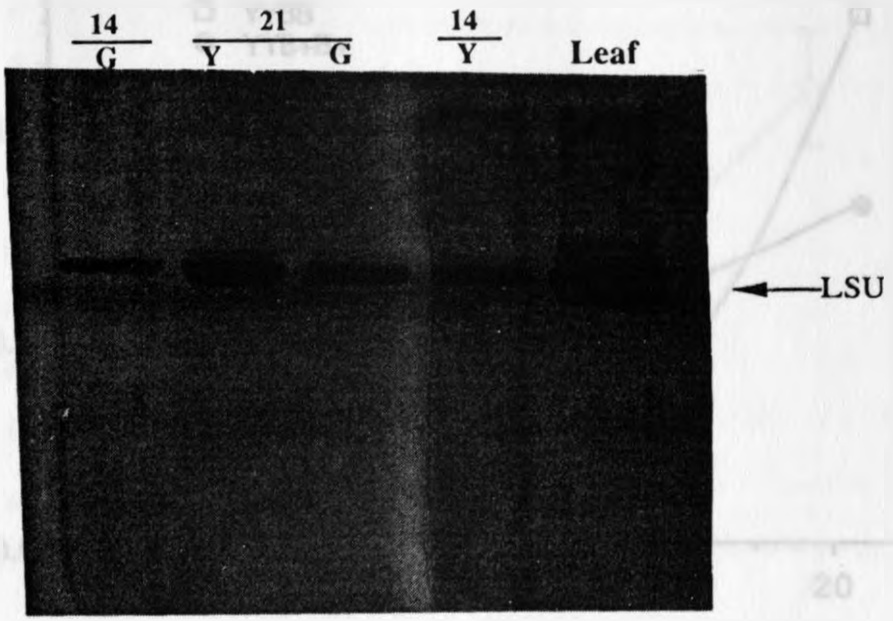


Fig 3.4 Western blot showing changes in Rubisco LSU protein in the fruit skins and leaves of Bicolor gourds with development. The fruits were harvested at 14 and 21 days postpollination.

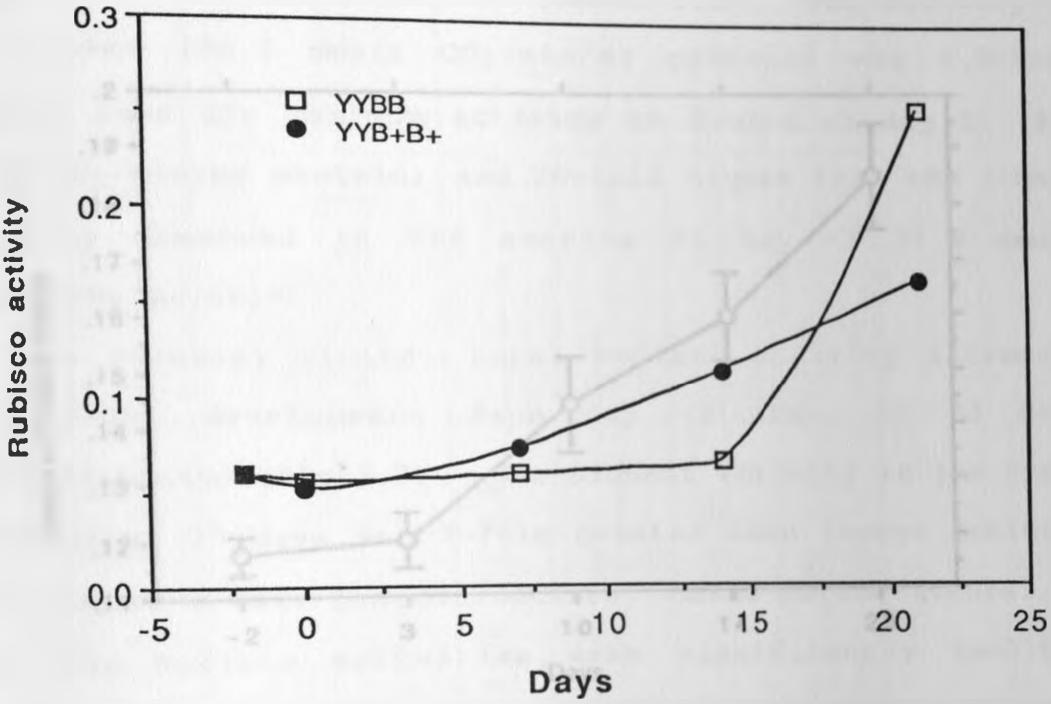


Fig 3.5 Rubisco activity in the fruit skins of EP isogenic lines YY BB and YY B^+B^+ measured at -2, 0, 7, 14 and 21 days pre- and postpollination. The assay was replicated three times and the radioactivity counts were done in duplicate for each assay.

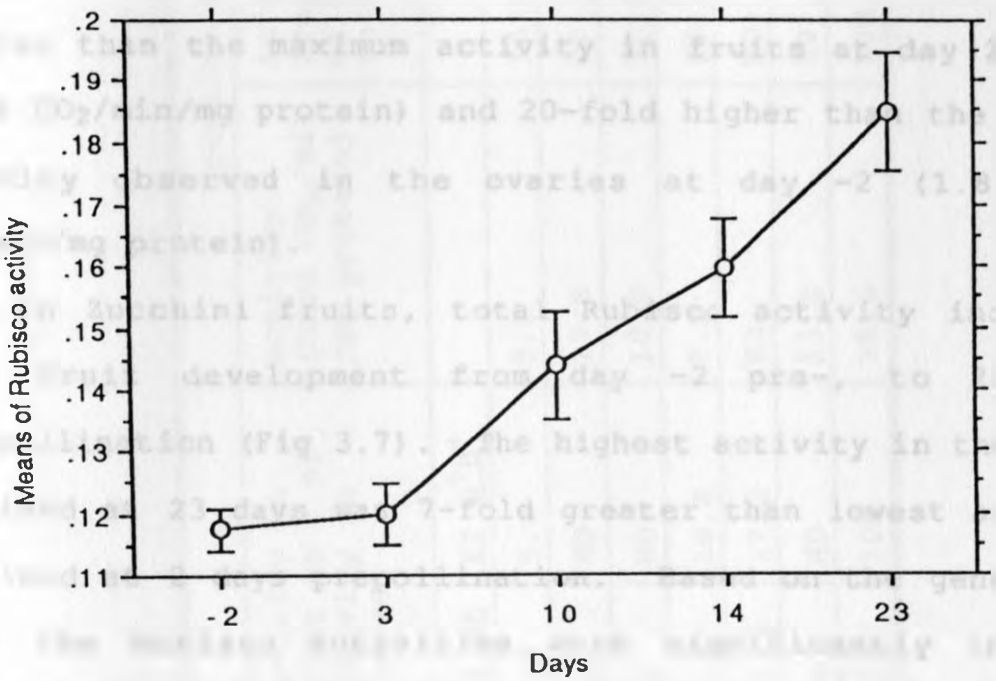


Fig 3.6 A plot of the mean Rubisco activity in EP isogenic lines YY BB and YY B^+B^+ fruit skins. The means were based on three replications. Activity at day 21 was significantly greater than the activities at other days ($p=0.01$).

Rubisco activity expressed on a protein basis (in nmols CO_2/min/mg protein) increased with fruit development reaching a maximum at day 21 (Table 3.1). The activity in the leaves (36.1 nmols CO_2/min/mg protein) was 4.2-fold greater than the maximum activity in fruits at day 21 (8.6 nmols CO_2/min/mg protein) and 20-fold higher than the lowest activity observed in the ovaries at day -2 (1.8 nmols CO_2/min/mg protein).

In Zucchini fruits, total Rubisco activity increased with fruit development from day -2 pre-, to 23 days postpollination (Fig 3.7). The highest activity in the fruit obtained at 23 days was 7-fold greater than lowest activity obtained at 2 days prepollination. Based on the general F-test the Rubisco activities were significantly ($p=0.01$) different at different fruit developmental stages assayed. Mean separation using the Duncan's multiple range test showed that activity at 23 days significantly exceeded the activities in less mature fruits (Fig 3.7). The activities at -2 and 0 days were significantly different from activities at 10 and 14 days pre- and postpollination. However, the mean activities at -2 and 0 days were not significantly different from one another. Similarly, the mean activity at 10 days did not significantly differ from activity at 14 days. Activity in leaves was 1.3-fold higher than the activity in 23-day-old fruits.

Table 3.1 Rubisco activity in Early Prolific and Fordhook Zucchini fruits and leaves expressed per total proteins.

Days pre- and post-pollination	Early Prolific				Zucchini Fordhook	
	Activity (nmol CO_2 /min/mg protein)		Activity in fruit as % of activity in leaves		Activity (nmol CO_2/min /mg protein)	Activity in fruit as % of activty in leaves
	YY BB	YY B^+B^+	YY BB	YY B^+B^+		
-2	1.8	2.1	6.2	5.8	5.8	16.3
3	ND[a]	ND	ND	ND	13.8	38.8
10	ND	ND	ND	ND	10.9	30.6
14	1.9	3.9	6.5	10.8	9.2	25.8
21	8.6	6.2	29.5	17.2	ND	ND
23	ND	ND	ND	ND	13.9	39
Leaves	29.2	36.1	100	100	35.6	100

[a]ND.-Not determined

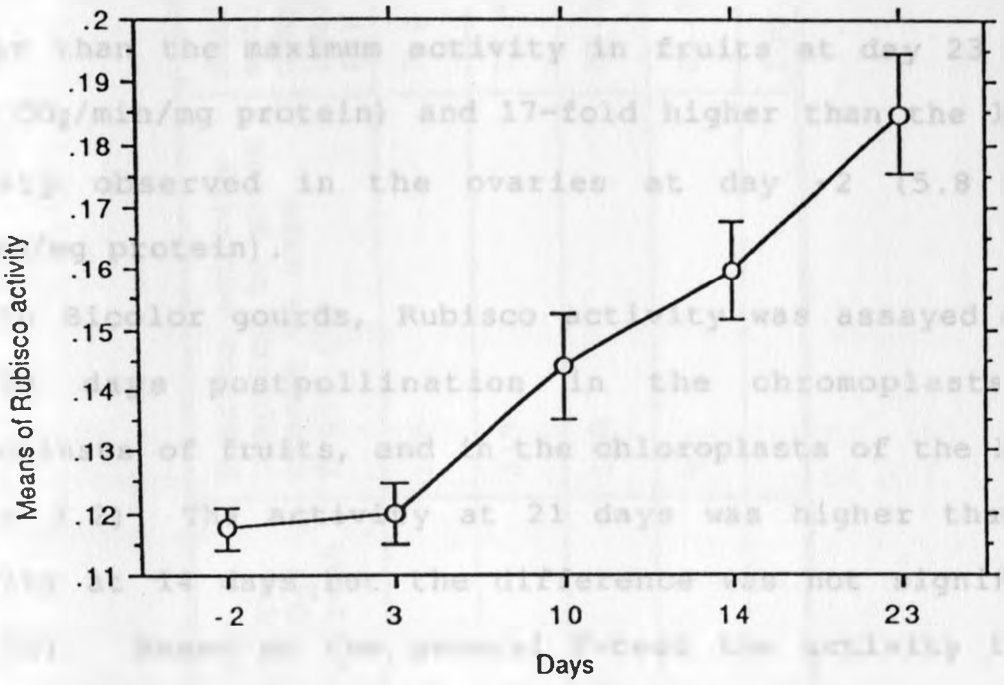


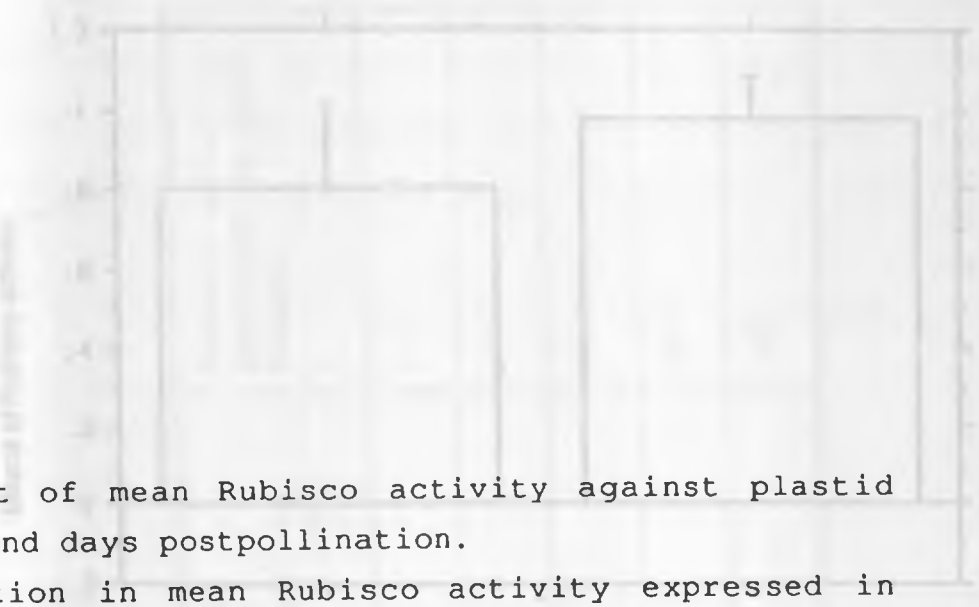
Fig 3.7 Rubisco activity in the fruit skins of Fordhook Zucchini measured at -2, 0, 10, 14, 21 days pre- and postpollination. The assay was replicated four times and the radioactivity counts were done in duplicate for each assay. Activity at day 21 was significantly greater than activities at other days ($p=0.01$).

Activity expressed on protein basis did not show a continuous increase with fruit development (Table 3.1). The maximum activity was observed at day 3 and 21. Activity in the leaves (35.6 nmols CO_2/min/mg protein) was 2.6-fold greater than the maximum activity in fruits at day 23 (13.8 nmols CO_2/min/mg protein) and 17-fold higher than the lowest activity observed in the ovaries at day -2 (5.8 nmols CO_2/min/mg protein).

In Bicolor gourds, Rubisco activity was assayed at 14 and 21 days postpollination in the chromoplasts and chloroplasts of fruits, and in the chloroplasts of the leaves (Table 3.2). The activity at 21 days was higher than the activity at 14 days but the difference was not significant ($p=0.05$). Based on the general F-test the activity in the chromoplasts significantly exceeded the activity in the chloroplasts ($p=0.01$). At 14 days the activity in the chromoplasts was twice the activity in the chloroplasts, while at 21 days the activities were about equal in both plastid types (Fig 3.8). Rubisco activity in the leaves was about five times higher than the activity in both fruit chromoplasts and chloroplasts. The trend in variation in relative Rubisco activity with fruit age and plastid types expressed on a protein basis (in nmols CO_2/min/mg protein) was similar to that obtained when activity was expressed per gram fresh weight (Table 3.3).

Table 3.2 Comparison of Rubisco activity in yellow and green portions of Bicolor 14 and 21 day old fruits and leaves expresed on fresh weight basis.

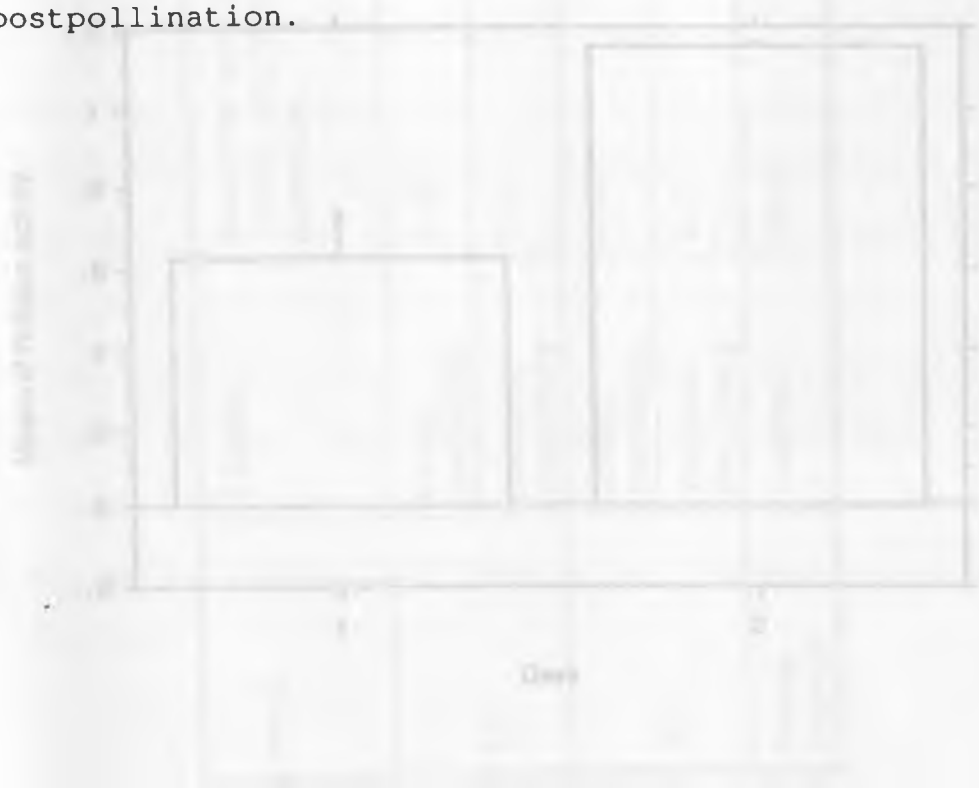
Tissue	Plastid type	Rubisco activity (umol CO_2/ min/g fresh wt).	Rubisco activity compared to green tissue on same fruit (%)	Rubisco activity compared to leaf tissue (%)
Fruit (14 days)	Chloroplasts (green tissue)	0.38	100	7.2
	Chromoplasts (yellow tissue)	0.81	213	15.4
Fruit (21 days)	chloroplasts (green tissue)	1.16	100	22.1
	Chromoplasts (yellow tissue)	1.15	97	21.9
Leaves	Chloroplasts	5.25		100



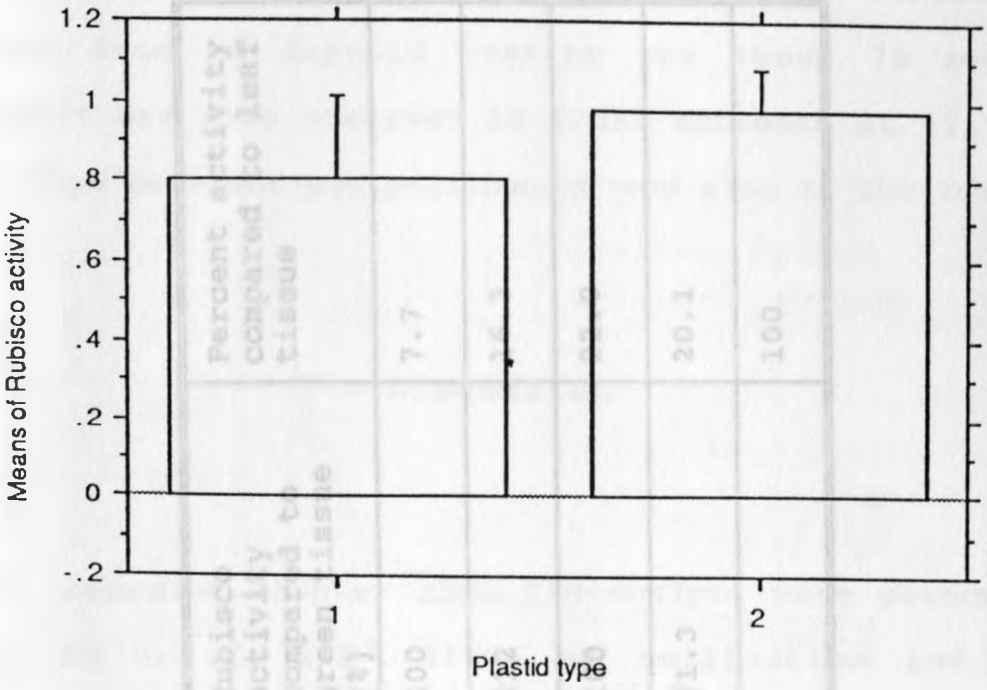
3.8 A plot of mean Rubisco activity against plastid type and days postpollination.

A Variation in mean Rubisco activity expressed in nmol CO_2/min/g fresh weight in the chloroplasts (1) and chromoplasts (2) of a Bicolor fruit.

B Variation in mean Rubisco activity expressed in nmol CO_2/min/g fresh weight at 14 (1) and 21 (2) days postpollination.



A


1.2
1
.8
.6
.4
.2
0
-.2
Means of Rubisco activity
1
2
Plastid type

B

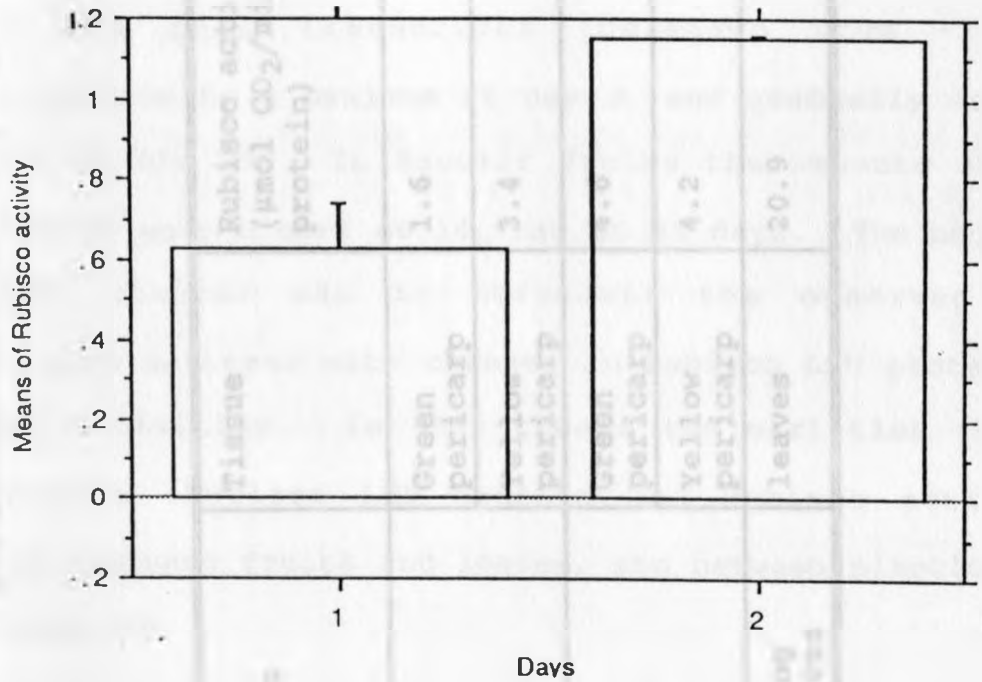

1.2
1
.8
.6
.4
.2
0
-.2
Means of Rubisco activity
1
2
Days

Table 3.3 Rubisco activity in Bicolor fruits harvested at 14 and 21 days postpollination and in young leaves expressed on protein basis.

Days	Tissue	Rubisco activity (µmol CO_2/min/mg protein)	Rubisco activity compared to green tissue (%)	Percent activity compared to leaf tissue
14	Green pericarp	1.6	100	7.7
	Yellow pericarp	3.4	212	16.3
21	Green pericarp	4.6	100	22.0
	Yellow pericarp	4.2	91.3	20.1
Young leaves	leaves	20.9		100

There was a drop in Rubisco activity, "Fallover," with time of assay (Fig 3.9). The half-life of the "Fallover" in extracts from -2 day-old ovaries was about 75 seconds. "Fallover" was also observed in fruit extracts at -2, 7, 14 and 21 days pre- and postpollination and also in the leaves.

Discussion

In the preceding chapter *rbcL* transcripts were detected in the *YY BB* and *YY* B^+B^+ lines at pollination and these transcripts increased with fruit development reaching a maximum at 21 days postpollination. In Fordhook Zucchini fruits the *rbcL* transcripts increased from -2 days prepollination to a maximum at day 3, and gradually declined at days 14 and 21. In Bicolor fruits the amounts of *rbcL* transcripts were higher at 14 than at 21 days. The objective of this chapter was to correlate the observed *rbcL* transcripts patterns with changes in Rubisco LSU protein and Rubisco activities. In this thesis the variation in *rbcL* transcripts, Rubisco LSU protein and Rubisco activities profiles between fruits and leaves, and between plastid types were compared.

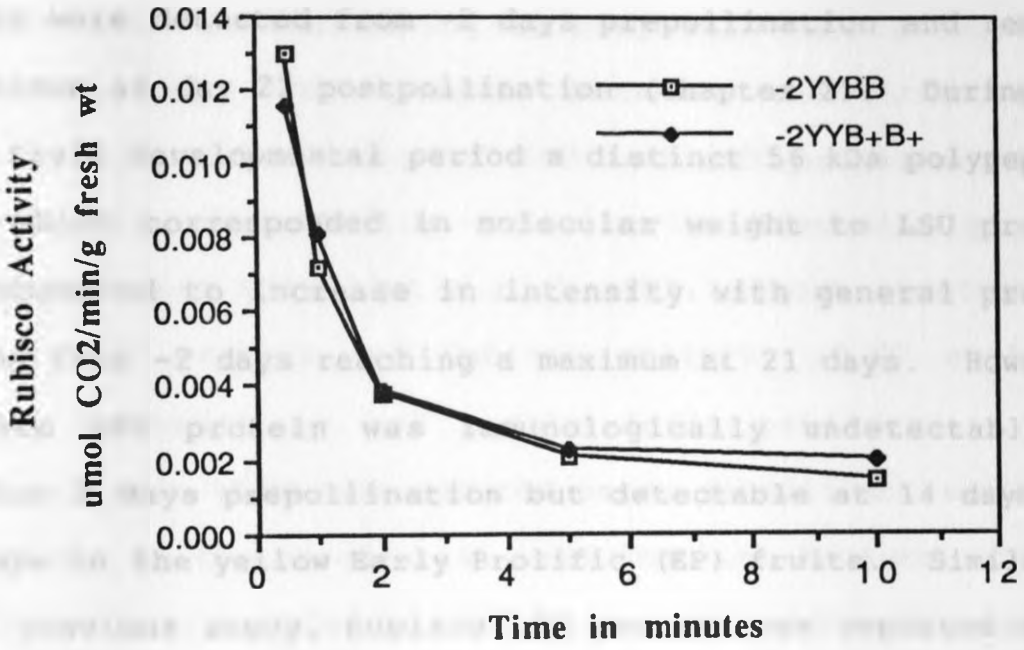


Fig 3.9 The inhibition of Rubisco activity during in vitro catalysis in EP ovaries. Crude extracts from ovaries at -2 days prepollination were assayed for Rubisco activity for durations of 1/2, 1, 2, 5, 1 minutes.

Patterns of Rubisco LSU Protein and Rubisco activity in EP

Steady-state *rbcL* transcripts in Early Prolific (EP) fruits were detected from -2 days prepollination and reached a maximum at day 21 postpollination (Chapter 2). During the same fruit developmental period a distinct 56 kDa polypeptide band which corresponded in molecular weight to LSU protein was observed to increase in intensity with general protein stains from -2 days reaching a maximum at 21 days. However, Rubisco LSU protein was immunologically undetectable in ovaries 2 days prepollination but detectable at 14 days and 21 days in the yellow Early Prolific (EP) fruits. Similarly, in a previous study, Rubisco LSU protein was reported in EP fruits in 0, 10, 20-day-old fruits based on molecular weight while immunological assay detected Rubisco LSU protein only in 20-days-old fruits (Lim, 1990). Based on information in the previous study (Lim, 1990) and in the current study, the *rbcL* gene is transcribed and translated into Rubisco LSU protein in ovaries and fruits of EP from -2 days to 21 days postpollination. The distinct 56 kDa polypeptide bands observed early in fruit development were due to co-migrating proteins and the amounts of LSU proteins in these bands was below immunological detection. This suggestion is supported by the presence of Rubisco activity in young EP ovaries of

1.8 nmol CO_2 /min/mg protein and in fruits of 8.6 nmol CO_2/min/mg protein at day 21.

Several studies reported results which are contrary to ours in that the levels of Rubisco LSU protein and activity decreased in fruits during chloroplasts to chromoplasts transformation. For example, Hansmann and Sitte (1984) reported reduced amounts of LSU and SSU proteins in the chromoplasts of daffodils. Similarly, Piechulla et al., (1987) detected very little rbcL mRNA, almost undetectable Rubisco LSU protein and Rubisco activity (0.07 nmol CO_2 /min/mg protein) in red tomato fruit pericarps. They suggested that there was reduced or no significant synthesis of Rubisco in the chromoplasts and that the Rubisco activity detected was residual from previous synthesis due to stability of the protein. A comparison of Rubisco activities in EP chromoplasts to those obtained in chloroplasts of green tomato fruits (Piechulla et al., 1987), and in cotyledons, stems, and leaves of amaranth seedlings, shows that the activities obtained in EP fruits were within the range obtained by other researchers. For example, in tomato leaf tissue a 3-fold Rubisco activity (9.32 nmol CO_2 /min/mg protein) was observed compared to the green pericarp (3.25 nmol CO_2 /min/mg protein) (Piechulla et al., 1987). In the current study, young EP leaves had activity of 36.1 nmol CO_2 /min/mg protein, which was 20-fold higher than the minimum obtained in fruit and 4.2-fold greater than the maximum

obtained in fruits. In amaranth seedlings, Rubisco activity ranges of 3.2 to 64, 3 to 8, 13 to 85 nmol CO_2 /min/mg protein were detected in the cotyledons, stems and leaves respectively seedlings. The levels of Rubisco activity in squash fruits and leaves are higher than those obtained by Piechulla et al. (1987) in tomato fruits but fall within the range observed in amaranth stems, cotyledons and leaves (Berry et al., 1986). Therefore, the current study provides evidence that there was substantial Rubisco LSU protein synthesis and Rubisco activity in squash EP fruit chromoplasts.

A vital question that was previously addressed by Piechulla et al. (1987) is whether the chromoplasts are photosynthetically active. The dark stage of photosynthesis requires two products of the light reaction, ATP and NAD(P)H, for CO_2 fixation. Substantial levels of polypeptides participating in the photosynthetic light and dark reactions such as Rubisco, the reaction center proteins of photosystem (PS) I and II, the light harvesting protein of PSI and PSII, Fd-NADP-oxidoreductase were detected in the green tomato fruit but not in red fruit, except for plastocyanin which was detected at both fruit developmental stages. Because of the presence of plastocyanin in the red fruit they did not rule out the possibility that an electron transport exist in the chromoplasts which could be used to channel electrons from

other chemical reactions to provide in the energy in form of NAD(P)H.

Part of the objective of this study was to correlate developmental plastid structural differences with Rubisco LSU and Rubisco activity patterns in EP fruits. Prior to pollination the YY B^+B^+ ovaries possess chloroplasts with grana structure while the YY BB have chromoplasts (Lim, 1990). By pollination and thereafter the YY B^+B^+ have chromoplasts and at 20 days postpollination the YY BB and the YY B^+B^+ have chromoplasts that are structurally indistinct. There was no difference in LSU protein levels in the ovaries of the two isogenic lines prior to pollination. Therefore, the fact that YY B^+B^+ possessed chloroplasts and YY BB had chromoplasts did not cause a distinction in the amounts of LSU protein and Rubisco activity. The fact that Rubisco LSU protein and Rubisco activity increased with fruit development suggests that the expression of the *rbcL* gene in EP fruits was developmentally regulated rather than dependent on plastid differentiation.

Lack of correspondence between the patterns of expression of *rbcL* gene and the different plastids in EP fruits (chromoplasts versus chloroplasts) suggested that these two processes (*rbcL* gene expression and plastid differentiation) are regulated independently from one another. The timing of the appearance of chloroplasts and chromoplasts is controlled by the B and Y genes but not the

structure or the content of the plastids (Schaffer, 1982; Boyer, 1989; Lim, 1990). In addition Shiffris (1981) hypothesized that the B gene acts as a timer gene which, with the Y gene, controls the blockage or breakdown of chlorophyll synthesis. The results reported here for EP YY B^+B^+ and YY BB lines support the action of Y and B genes as timers for development.

Patterns of Rubisco LSU Protein and Rubisco activity in Fordhook Zucchini

Steady-state rbcL transcripts in Fordhook Zucchini increased with fruit development from -2 days prepollination to a maximum at 3 days, and gradually declined at 14 and 21 days postpollination. During the same fruit developmental period the LSU protein increased in the fruit to a maximum at 21 days, while Rubisco activity reached a maximum at 23 days. These results show that there was a close correlation between the LSU protein profile and the Rubisco activity during fruit development. However, the levels of steady-state rbcL transcripts in Zucchini chloroplasts did correlate with the LSU protein and Rubisco activity from day -2 to day 3 but not thereafter. The quantities of LSU proteins continued to accumulate in 14 and 21 day old fruits although the amounts of rbcL transcripts declined during this period. Lack of

correlation between the rbcL transcripts and LSU proteins had been observed in amaranth chloroplasts (Berry et al., 1986). When dark-grown amaranth seedlings were transferred to light the synthesis of SSU and LSU proteins were rapidly initiated before any increase in corresponding mRNAs were observed. On the other hand when light-grown seedlings were transferred into darkness, synthesis of LSU and SSU proteins decreased before any detectable decrease in mRNAs was observed. Berry et al. (1986) concluded that the expression of rbcL gene was determined over a short time span at the level of translation but over a long period through changes in the mRNA accumulation. Results in the current study suggest that there was substantial regulation of rbcL gene in Zucchini fruit chloroplasts at the translation level of gene expression. The accumulation of Rubisco LSU protein, while the rbcL mRNAs levels dropped later in fruit development, could be attributed to either low turnover of the protein or enhanced stability of the protein or both.

A high correlation between the LSU protein to Rubisco activity similar to that observed in the current study was reported in amaranth chloroplasts. In amaranth cotyledons, Rubisco activity was detected 3 days after germination, reached a maximum at day 10 and the LSU and SSU polypeptides levels closely matched the activity (Berry et al., 1986). However, the correlation of Rubisco LSU protein and activity was not maintained in the stems and leaves of amaranth

seedlings. For example, activity in the amaranth stem was 8- to 20-fold lower than in the cotyledons, and the SSU protein accumulation in the stem coincided with Rubisco activity, while LSU protein remained relatively constant. In amaranth leaves, Rubisco activity increased 10-fold reaching a maximum at 19 days. SSU protein varied in concert with the activity while LSU protein remained fairly constant. Based on these results the authors suggested that the accumulation of Rubisco LSU protein in amaranth was influenced by tissue type. The high correlation between Rubisco LSU protein and Rubisco activity observed in squash implies that the observed LSU protein was being converted into active Rubisco (in conjunction with SSU protein)

The presence of Rubisco LSU protein and Rubisco activity in the green Zucchini fruits is attributable to the subsidiary photosynthetic function of the fruit. The capability of green fruits to photosynthesize has been reported in tomatoes, where green fruits contributed approximately 12% to the net photosynthesis of the entire plant (Iwatsuki et al., 1984). Further evidence in support of the photosynthetic function of Zucchini fruits is the presence of grana similar to the leaves (Lim, 1990). Green Zucchini fruits have grana and thylakoid membranes similar to those observed in EP leaves. The only difference is that the partition length of the thylakoid in Zucchini fruit chloroplasts are more extensive than leaf chloroplasts. In

a study in tomato, substantial levels of Rubisco activity, the reaction center proteins PSI and PSII, the light harvesting complex proteins of PSII, plastocyanin and Fd-NADP-oxidoreductase were detected in green tomato fruit pericarp (Piechulla et al., 1987). This lead to the suggestion that chloroplasts in tomato fruits were photosynthetically active (Piechulla et al., 1987). The photosynthetic activity in the tomato leaf tissue, was 2.5-fold and 7.7-fold greater than in the green pericarp and intermediate fruits (45 days after pollination), respectively (Piechulla et al., 1987). Similar comparable Rubisco activities were obtained in Fordhook Zucchini, where the activity in leaves exceeded activity in the -2 day ovaries by 6.1-fold and day 23 fruits by 2.4-fold.

Patterns of Rubisco LSU Protein and Rubisco activity in Bicolor gourd fruits

Bicolor gourds showed three plastid developmental patterns where the fruits were either entirely green or yellow, and where the chromoplasts and chloroplasts developed simultaneously on the same fruit. The objective of this study was to use this later unique plastid developmental pattern to study changes in plastid transcripts, Rubisco LSU protein and Rubisco activity because: (1) the two distinct

plastid developmental patterns on the same fruit are constant for nuclear and plastid genotypes; (2) the environment, e.g light, nutrient is similar for fruits on the same plant.

In the current study, the changes in rbcL transcripts (Chapter 2), Rubisco LSU protein and Rubisco activity in the yellow and green portion of the same fruit have been compared at 14 and 21 days postpollination. The steady-state levels of rbcL mRNAs were higher at 14 days than at 21 days. The amounts of rbcL transcripts therefore decreased with fruit development, indicating the role played by developmental signals in the regulation of rbcL gene expression in Bicolor fruits. This contrasted with the EP, fruits where the amounts of rbcL mRNAs increased in the fruits culminating at 21 days.

The ratio of rbcL transcripts in the chloroplasts to chromoplasts was 10:11 while the ratio for Rubisco activity was 10:21 in day 14 fruits. This suggests that there was some post-transcriptional adjustment mechanism in the expression of rbcL gene, causing higher Rubisco activity in the chromoplasts than in the chloroplasts. Similarly, in 21-day-old fruits, the ratio of rbcL transcripts in chloroplasts:chromoplasts was 1:2, while the ratio of Rubisco activity was 1:1, which also suggests that the expression of the rbcL gene was adjusted at the post-transcriptional level.

At 14 days the yellow tissue showed more Rubisco activity than the green tissue, while at 21 days the

activities were almost identical. The expression of rbcL gene in Bicolor fruits correlated with the plastid type but in an unexpected manner, in that both the rbcL mRNAs and Rubisco activity were more highly expressed in the chromoplasts than in the chloroplasts. Contrary to these results in Bicolor fruits, Grinsven et al. (1986) found no difference in the rbcL mRNA in the proplastids of cell suspension cultures and chloroplasts of petunia. However, there was no LSU protein in proplastids (cell culture) but vast quantities in the leaves. They concluded that the rbcL gene was regulated at the post-transcriptional level. Another contrast to the current study was reported in pepper, where there was no change in rbcL mRNAs during chloroplast to chromoplast differentiation. However, the synthesis of LSU protein was greatly reduced in the chromoplasts (Kuntz et al., 1989). It was logical to attribute the presence of LSU protein in petunia chloroplasts and absence of LSU protein in proplastids, to the photosynthetic function of the chloroplasts. Similarly, the reduction of LSU protein in the chromoplasts of pepper can be explained by the non-photosynthetic function of chromoplasts. However, the presence of higher rbcL mRNAs, Rubisco LSU protein and Rubisco activity in the chromoplasts than in the chloroplasts of Bicolor fruits is rather unusual. Chloroplast to chromoplast transformation in tomato, pepper and squash fruits is similar in that carotenoids are synthesized and

chlorophyll degraded. However, distinct differences occur between chloroplast to chromoplast transition in pepper and tomato as compared to squash fruits. For example, chloroplast to chromoplast transition in pepper and tomato occurs at the terminal stage of fruit development and is unidirectional. In contrast, chloroplast to chromoplast development in squash fruits occurs early in fruit development and under certain conditions is bidirectional. For example, under light, stress, or age the chromoplasts can revert into chloroplasts (Boyer, 1989). Perhaps the discrepancies in the expression of rbcL and psbA genes observed in the current study as compared to observation in other fruit such as tomato and pepper was because of differences in fruit ripening processes in tomato and pepper as compared to fruit development of squash. The two processes involve different developmental patterns, with the transition of chloroplasts to chromoplasts in squash fruits being a more complex developmental mechanism than the unidirectional plastid transition found in ripening tomato and pepper (Lim, 1990).

Developmental regulation of rbcL gene expression

In general, the expression of rbcL gene in fruits of EP, Fordhook Zucchini and Bicolor fruits correlated with fruit development. In EP the rbcL mRNA, Rubisco LSU protein and

Rubisco activity increased with fruit development. In Fordhook Zucchini fruits the rbcL mRNAs increased to a maximum at three days postpollination and then declined. However, both Rubisco LSU and Rubisco activity increased during fruit development up to 23 days postpollination. In Bicolor fruits, the rbcL mRNA levels were higher in 14-day-old fruits than day 21 fruits. However, the Rubisco activity was higher at 21 than 14 days postpollination. The role of developmental signals in the regulation of rbcL gene in the chloroplast of amaranths seedlings have been reported (Nikolau and Klessig, 1987). During the germination of seedlings, the Rubisco LSU and SSU proteins were observed from three days in the cotyledons and reached a maximum at 10 days. In the leaves the LSU and SSU proteins were observed at eight days and reached maximum at 30 days, while in the stem LSU and SSU proteins were first observed at five days and reached a maximum at 24 days. They concluded that the expression of the rbcL gene was controlled by developmental signals acting in at organ-specific level.

The expression rbcL gene in the non-photosynthetic chromoplast tissue and in the photosynthetic chloroplast tissue is not surprising for a gene such as rbcL with a complex gene regulatory mechanism. As previously reviewed post-translation regulation of rbcL can occur by activation by Mg^{2+} on CO_2, through inhibition with xylulose-1,5-bisphosphate (XUBP) and 3-Keto-arabinitol-1,5-bisphosphate

(causing fallover), through alleviation of fallover by Rubisco activase, or by inhibition with 2-carboxyarabinitol-1-phosphate (CA1P) (a nocturnal inhibitor of Rubisco), and by absence of phosphatase (an enzyme which inactivates CA1P). Perhaps the rbcL gene expression is not stringently controlled at the transcriptional and translation levels in the chromoplasts of EP and Bicolor squash but controlled post-translationally.

Lack of detection of rbca mRNA or Rubisco activase in fruits of EP and the observed fallover during Rubisco activity assays are tentative evidence in support of post-translation control of rbcL. However, these inferences are only speculative since rbca and Rubisco activase were not detected in any of the squash tissue.

Conclusions

The expression of rbcL gene in the three varieties of squash was developmentally regulated. In EP fruits the different plastid types did not show a differential influence the amounts of Rubisco LSU proteins and Rubisco activities. In Bicolor gourds the plastid type had an unusual influence on the amounts of Rubisco LSU proteins and Rubisco activities, in that these two parameters were higher in

chromoplasts than in the chloroplasts. There was evidence of post-transcriptional and post-translational regulation of rbcL gene expression in Bicolor gourds. Regulation of the rbcL gene expression at the post-transcriptional level was observed in Fordhook Zucchini fruits.

Chapter 4

RESTRICTION MAPPING AND PARTIAL NUCLEOTIDE SEQUENCE OF THE PSTI 9.8 kb FRAGMENT CONTAINING "ORF 2280"

Literature Review

The large open reading frame, ORF 2280

Previous studies on plastid gene expression mainly have focused on plastid genes with known functions. In addition to these genes, the plastid genome contains many unidentified open reading frames (ORFs) (Shinozaki et al., 1986; Ohyama et al., 1986; Hiratsuka et al., 1989). Recent studies have focused on a large ORF, capable of encoding a protein of 2100-2280 amino acids. This large ORF has been identified in several plant species including: tobacco, ORF 2280 (Palmer, 1991; Shinozaki et al., 1986), spinach, ORF 2132 (Zhou et al., 1988), liverwort, ORF 2136 (Glick and Sears, submitted) and broad bean ORFx (Herdenberger et al., 1988). The rice chloroplast genome is an exception in that it lacks this large ORF (Hiratsuka et al., 1989, Shimada and Suguira, 1991).

In addition to the prevalence of the large ORF among plant species, there is also sequence homology between the large ORFs of different species. For example, the partial

sequence of the tomato large ORF matched the tobacco ORF 2280 at 96 out of 97 position with an exception of a nine nucleotide deletion in tomato or insertion in tobacco (Richards et al., 1991). The predicted protein from ORFx of broad bean had 80% amino acid homology with the predicted protein from tobacco ORF 2280, 50% homology with the predicted protein from liverwort ORF 2135 and high homology with the predicted protein from spinach ORF 2132 (Herdenberger et al., 1988). High amino acid homology has also been reported between tobacco and liverwort ORFs (80-90%), while spinach and liverwort ORFs contain a region of high homology (71-73%) and low homology (35 to 50%). The prevalence of the large ORF 2280 in several plant species including the parasitic beechdrops (Epiphagus virginiana) and the high sequence homology among plant species led to the suggestion that the ORF 2280 is a functional gene (Glick and Sear, submitted).

The initial steps to unravel the function of the large ORF involved a search for transcripts. Five ORF 2280 transcripts of sizes 1.7, 2.4, 2.7, 8.3 kb have been detected in tomato fruits and leaves (Richards et al., 1991). The amounts of the 8.3 kb transcript increased with fruit ripening in tomato cultivar 'Count II.' The amounts of this transcript also increased in 'Traveler 76' cultivar, while the levels of the 1.7 to 3.2 kb transcripts increased only slightly in both cultivars. The 8.3 transcript was present

in leaf RNA but at lower levels compared to small transcripts (Richards et al., 1991). Similarly, a 10 kb ORF transcript was detected in broad bean (Herbenberger et al., 1988).

Further steps to elucidate the function of ORF involved a search for ORF translation products. Potentially the ORF 2280 is capable of encoding a protein of about 300 kDa. However, immunological study of ORF translation products detected proteins with apparent molecular weights ranging from 170-180 kDa in tobacco, spinach and Oenothera chloroplasts (Glick and Sears, submitted). Similarly, Richards (unpublished communication) found a 170-180 kDa ORF 2280 protein in tomato fruits. The discrepancy between the observed ORF proteins (170-180 kDa) and the size of molecular weight of the protein the ORF is potentially capable of encoding (about 300 kDa), has been attributed to post-transcriptional and post-translational processing (Glick and Sears, submitted).

Whereas the function of the ORF remains an enigma, it is unlikely that the ORF encodes a protein that is functional in the photosynthetic apparatus, since no protein of that size has been detected in the electron transport chain of the thylakoid membrane (Glick and Sears, submitted; Anderson, 1986; Kaplan and Arntzen, 1982). Based on the observation that ORF transcripts were higher in the chromoplasts than in the chloroplasts of tomato, Richards et al. (1991) suggested that ORF of tomato may encode a protein that is mainly

functional in the chromoplasts. In the plastid DNA of beechdrops, a non-photosynthetic angiosperm, most of the photosynthetic genes and other ORFs are absent but genes for three rRNA, seven tRNAs and region homologous to ORF 2280 are present (dePamphilis and Palmer, 1990; Morden et al., 1991), suggesting that the ORF protein may not be a component of the photosynthetic apparatus (Glick and Sears, submitted). Similarities between predicted amino acid sequence ORF 2280 and the Beta subunit of the RNA polymerase point to the possibility that the ORF protein may be a partial product of the secondary ORF transcript in the chloroplasts (Glick and Sears, submitted).

The presence of the large ORF in several plant species, the detection of ORF transcripts and translation products, the sequence homology of ORF between species and the homology of ORF sequence with the Beta subunit of RNA polymerase are strong evidence that ORF 2280 is a functional gene. Although the specific function is still unknown, further study is needed in aspects such as: search for transcripts and translation products from ORF 2280 in other plant species and characterization of ORF by DNA sequencing. The squash plastid genome contains region which hybridized to the tomato ORF 2280 (which in this study will be referred to as "ORF 2280"). However, no study has been done to determined the presence of transcription and translation products from this region in squash. The object of this part of the study was

to examine the presence of "ORF 2280" transcripts and to characterize the squash "ORF 2280" by partially sequencing the PstI 9.8 kb fragment. These objectives were achieved in the following steps:

1. Make ORF 2280 specific probes internal to the tomato PstI 7.7 fragment.
2. Locate a region on the squash plastid DNA homologous to tomato ORF 2280
3. Preparation of homologous squash "ORF 2280" probes.
4. Probe the Northern blot of squash total RNA with "ORF 2280" probes.
5. Partially sequence the squash "ORF 2280" and compare with the tobacco ORF 2280 sequence.

Materials and Methods

Preparation of probes internal to the ORF 2280 from the tomato PstI 7.7 kb fragment

The DNA sequence at the terminal ends of the PstI 7.7 kb fragment of tomato perfectly aligned with the tobacco ORF sequence covering the 147,176 to 147,281 and 154,674 to 155,788) regions, except for a nine bp deletion which did not alter the reading frame (Richards et al., 1991). Using the published tobacco ORF sequence (Shinozaki et al., 1986), the

BamHI restriction sites were located in the tobacco ORF 2280 region that corresponded to the tomato PstI 7.7 fragment. The seven predicted restriction fragments after digestion with BamHI were: 0.2, 0.3, 0.7, 1.05, 1.11, 1.19 and 2.93 kbs (0.7 and 1.05 were flanking fragments with PstI and BamHI ends, respectively). Because of the high similarity between the tobacco ORF 2280 and the tomato PstI 7.7 fragment, it was presumed and demonstrated that digestion of the tomato PstI 7.7 fragment would produce similar fragments as predicted from the tobacco sequence. The tomato PstI 7.7 fragment was digested with BamHI and PstI and the 1.19 and the 0.7 kb fragments were isolated and cloned in pUC18 and Bluescript SK^+, respectively.

Extraction and cloning of the chloroplast DNA (cpDNA)

Chloroplast DNA was extracted from squash leaves harvested from 2- to 3-week-old plants using a similar protocol developed by Lim (1990). The extracted cpDNA was digested with PstI, SalI, PvuII and SacII and fractionated on 0.7% agarose gel. The DNA was immobilized on nitrocellulose membrane and probed with the 1.19 kb (ORF 2280 specific probe from tomato cpDNA) in order to locate the position of "ORF 2280" in squash cpDNA. The 9.8 kb fragment on which "ORF

2280" was located was isolated and cloned in pUC18 as described below (Appendix B).

PstI digested cpDNA was fractionated on 0.7% agarose and the 9.8 kb band dissected out of the gel. The DNA band was extracted by electroelution into aqueous solution of 1/2 TBE (TBE: 0.089 M Tris-HCl, 0.089 M borate, 0.001 M EDTA). DNA was purified in three steps extraction with phenol, phenol/chloroform/isoamyl alcohol (25:25:1), and once with chloroform/butanol (4:1).

The plasmid vector (pUC18) was digested with the PstI restriction enzyme at 37°C. The cpDNA/pUC18 fragments were ligated in a water bath at a temperature range of 6-14.5°C for 16 hours using T_4 ligase. The E. coli cells were made competent by treatment with $CaCl_2$ solution. The E. coli strain JM83 was used as the host cell. Transformed clones were selected using antibiotic ampicillin and screened for cpDNA insert using 5-bromo-4-chloro-3-indolyl-Beta-D-galactoside (X-gal). Isopropyl thio-galactoside (IPTG) was used as a non-metabolizable inducer. To ascertain the presence of the insert, the DNA was extracted from the clones, digested with restriction enzymes and run on a gel.

In order to characterize "ORF 2280" of squash cpDNA, the PstI 9.8 fragment was mapped by digestion with SalI (S), BamHI (B), EcoRI (E), double digested with SxB, ExB, and triple digestion with PxExB, PxBxS. Subfragments of 4.7, 3.2

and 1.9 kbs were cloned into pUC18 and partially sequenced (Appendix B).

Northern blotting and hybridization

The conditions for prehybridization, hybridization and washing of DNA immobilized on nitrocellulose membrane were essentially the same as described for RNA in Chapter 3.

Sequence analysis

DNA sequences of the 4.7, 3.2 and 1.9 fragments were accomplished by dideoxy chain termination method (Sanger et al., 1977) using Sequenase (T_7 DNA polymerase). The sequences were compared for homology with the tobacco ORF 2280 using the Intelligenetics Program.

Results and Discussion

Isolation of ORF 2280 specific probe from tomato cpDNA

The predicted fragments of sizes 0.2, 0.3, 0.7, 1.0, 1.1, 1.2, 2.6 (pUC18), and 3.0 kb were obtained from the digestion of the plasmids containing the PstI 7.7 fragment of tomato with PstI and BamHI (Fig 4.1). The 1.2 kb fragment was cloned in pUC18 (Fig 4.1 C) while the 0.7 kb fragment was clone in Bluescript SK^+ (Fig 4.1 A). The 1.2 fragment internal to "ORF 2280" of tomato was used as a probe to locate the squash "ORF 2280" on the cpDNA.

The plastid DNA was digested by the following restriction enzymes: PstI, PvuII, BglI and SacII. Probe of the southern blot with BamHI-1.19-BamHI kb fragment internal to tomato ORF 2280 localized the squash ORF in the inverted repeat on the following fragments: the 12.1 (inverted repeat B, IRB) and the 9.8 kb of PstI (IRA); the 27.5 kb of SacII; the 31.0 and the 20.3 kb BglI; the 56.7 and the 16.7 kbs of PvuII (Fig 4.2). These results agree with the results of Lim (1990).

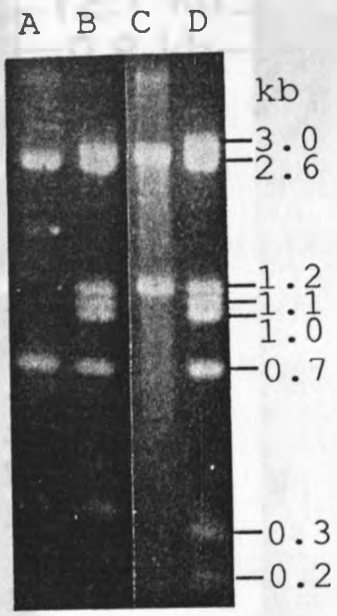


Fig 4.1 Subcloning of portions of tomato chloroplast DNA 7.7 kb PstI fragment. A. 0.7 kb fragment released from Bluescript vector by digestion with PstI and BamHI. B. 7.7 kb PstI fragment in pUC18 digested with PstI and BamHI. C. 1.2 kb fragment released from pUC18 by BamHI. D. Same as B.

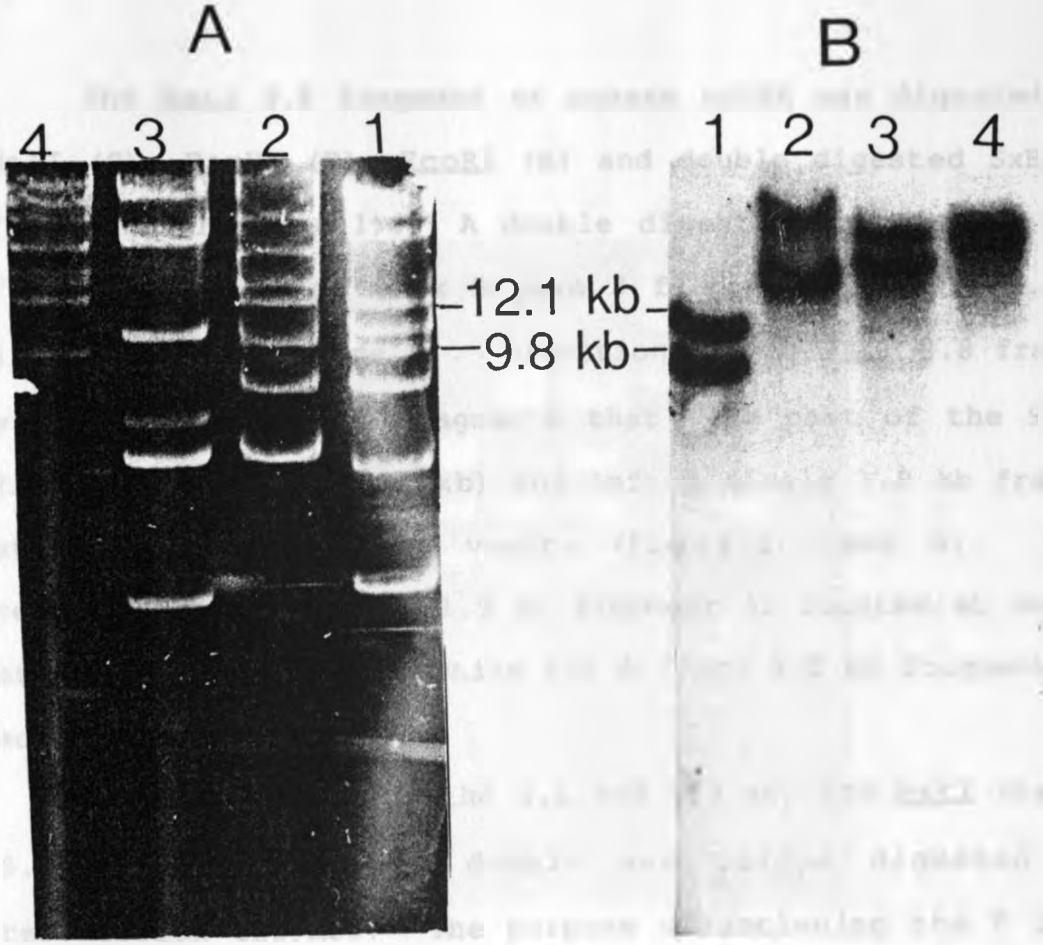


Fig 4.2 Southern blot of squash cpDNA digested with PstI (1), BgLI (2), PvuII (3) and SacII (4) restriction enzymes and probed with ^{32}P labelled tomato 1.2 kb probe internal to ORF 2280. Left side is a photograph of the gel prior to blotting.

Cloning and mapping of the 9.8 kb PstI fragment of cpDNA

The PstI 9.8 fragment of squash cpDNA was digested with SalI (S), BamHI (B), EcoRI (E) and double digested SxB, ExB (Fig 4.3, Table 4.1). A double digestion of the PstI 9.8 fragment with PstI (P)x B gave 3 fragments of 4.7, 3.2 and 1.9 kb (Fig 4.3, lane F). Digestion of the PstI 9.8 fragment with B alone gave 2 fragments that were part of the 9.8 kb fragment (i.e 4.7, 3.2 kb) and left a single 1.9 kb fragment still attached to the vector (Fig 4.3, lane G). These results imply that the 1.9 kb fragment is located at one end of the 9.8 kb fragment while the 4.7 and 3.2 kb fragments are adjacent.

In order to align the 3.2 and 4.7 kb, the PstI fragment 9.8 kb fragment was double and triple digested with restriction enzymes. The purpose of including the P in the digestion was to remove the insert from the vector, so it can be disregarded in the following analysis. The pUC18 vector (2.7 kb) was left intact and can also be disregarded. Double digestion with PxB gave 1.8, 3.2, 4.7 kb fragments (excluding pUC18). Triple digestion with PxExB produced 3.2 kb and 1.8 kb fragments common to the PxB digestion (Fig 4.3, lane B). However,. the 4.7 kb fragment was absent, replaced by two new fragments of 0.6 and 4.1 kbs (Fig 4.3, Table 4.1). It

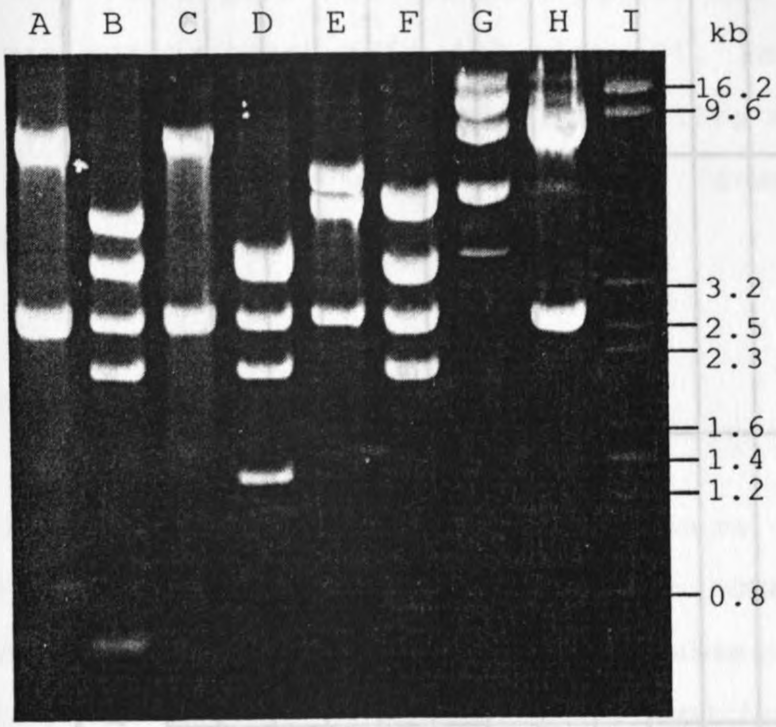


Fig 4.3 Mapping of the PstI 9.8 kb fragment of squash cpDNA by restriction enzyme digestion. The 9.8 kb fragment was cloned into the PstI site of pUC18. A: PstI x EcoRI x SalI; B: PstI x BamHI x EcoRI; C: PstI x EcoRI; D: PstI x BamHI x SalI; E: PstI x SalI; F: PstI x BamHI; G: BamHI; H: PstI; I: marker lambda DNA digested with BglI.

Table 4.1 Restriction endonuclease digestion of the PstI 9.8 kb fragment of squash chloroplast DNA containing the large ORF 2280 and the psbA gene using three restriction enzymes plus PstI which was used to remove the insert.

Restriction Enzymes				
SalI	BamHI	Sal1 x BamHI	EcoRI	EcoRI x BamHI
				0.6
		1.3		
	1.9	1.9		1.9
			2.5	
	3.2	3.2		3.2
		3.4		
				4.1
4.4				
	4.7			
5.4				
			7.3	
Totals	9.8	9.8	9.8	9.8

can be inferred from these results that 4.7 kb fragment is located between the 1.9 and 3.2 kb fragments.

Other double and triple digestions with P, S, and B confirmed the above alignment of fragments. A triple digestion with PxBxS gave 1.8, 3.2 kb fragment and the 4.7 kb fragment was again absent (Fig 4.3, lane D). Instead, two fragments of sizes 1.3 and 3.4 kb reappeared (Fig 4.3 lane D; Table 4.1). Based on the above results, a linear map was constructed (Fig 4.4).

Transcripts from the "ORF 2280"

Total plastid RNA from 3 squash cultivars was probed with heterologous 1.2 kb probe internal to "ORF 2280" of tomato and homologous 9.8 kb ORF 2280, and subsequently with smaller 4.7, 3.2 and 1.9 kb probes (that comprise the PstI 9.8 kb fragment). A 2.6 and 1.4 kb fragment was detected in Early Prolific fruits with the heterologous 1.2 kb probe (Fig 4.5). The X-ray film was exposed 6 days with screen due to the weakness of the signal. Similarly, 2.6 and 1.4 kb fragments were detected in the chromoplasts and chloroplasts of Bicolor fruits and in the leaves (Fig 4.6). The amounts detected in the leaves exceeded the amounts in the fruits. 2.6 kb and 1.2 kb transcripts were also detected in the EP fruits with a homologous 9.8 kb probe. However, the signal was rather weak

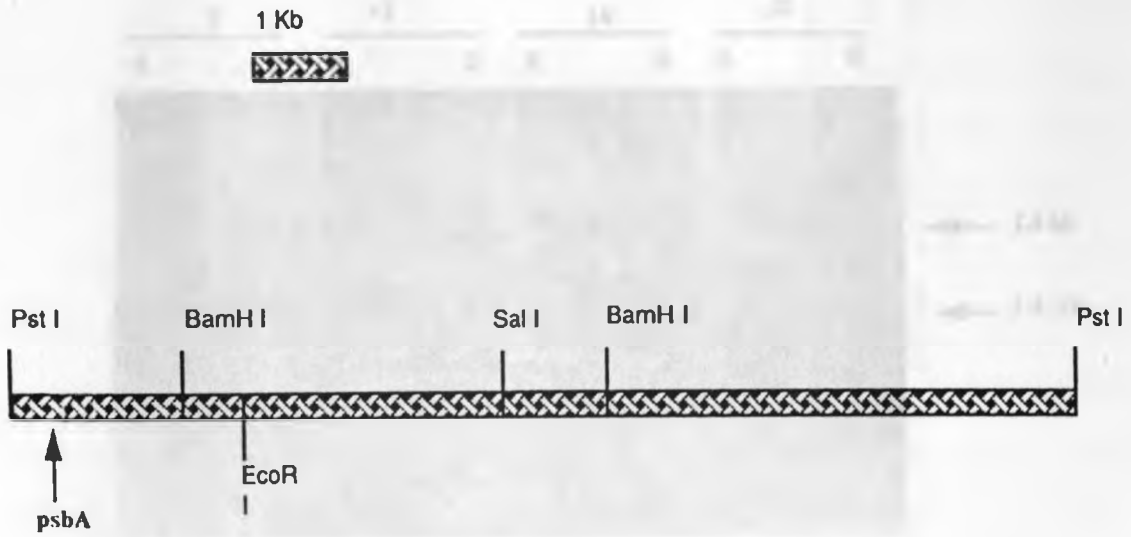


Fig 4.4 The restriction map of the 9.8 kb fragment of squash cpDNA constructed using 4 restriction enzymes. The psbA gene is located on the 1.9 kb PstI/BamHI fragment; part of "ORF 2280" is located in the 4.7 kb BamHI fragment.

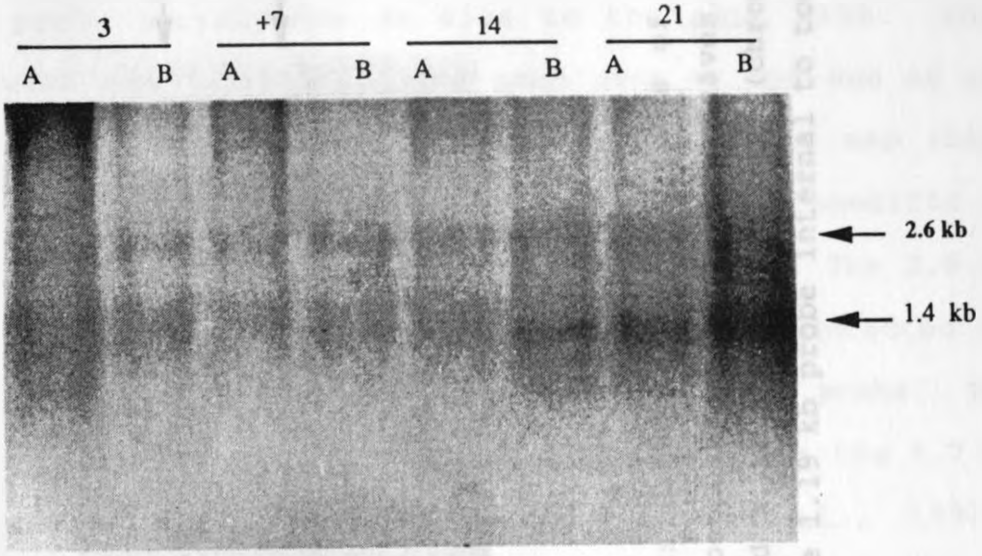


Fig 4.5 The 2.6 and 1.4 kb transcripts detected from the EP fruits harvested at 3, 7, 14, and 21 days postpollination. The transcripts were detected by the 1.19 kb probe internal to tomato ORF 2280. A: YY BB genotype; B: YY B^+B^+ genotype. The X-ray film was exposed for 6 days with a screen.

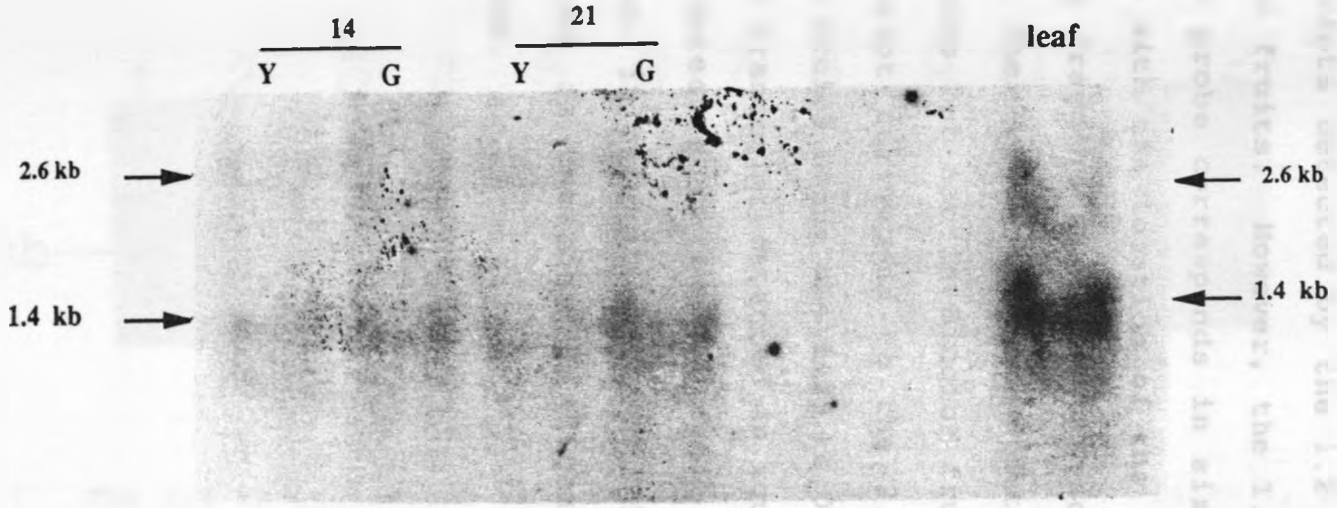


Fig 4.6 The 2.6 and 1.4 kb transcripts detected in the skins of Bicolor fruits harvested at 14 and 21 days postpollination and in leaves. G represents green tissue (chloroplast) and Y the yellow tissue (chromoplast). The transcripts were detected by the 1.19 kb probe internal to tomato ORF 2280.

and the X-ray film had to be exposed for 7 days with a screen. The 2.6 mRNA detected by the PstI 9.8 kb probe in the Early Prolific chromoplasts matched the size of transcripts detected by the 1.2 homologous probe in EP and Bicolor fruits. However, the 1.2 kb mRNAs detected by the 9.8 kb probe corresponds in size to the psbA mRNA. This agrees with the location of the psbA gene at one end of the 9.8 kb fragment on the squash cpDNA restriction map (Lim, 1990). The 2.6 kb transcript detected was fruit specific in EP fruits but not in Bicolor fruits (Fig 4.7). The 2.6 kb transcript correspond to the 2.7 kb fragment detected in tomato fruits using the 1.19 kb ORF 2280 internal probe. The 1.4 kb transcript detected in squash fruits match the 1.7 kb mRNA detected in tomato fruit (Richards et al., 1991). However, in this study the 8.3 kb ORF 2280 mRNA previously detected in tomatoes by Richards et al.(1991) was not detected.

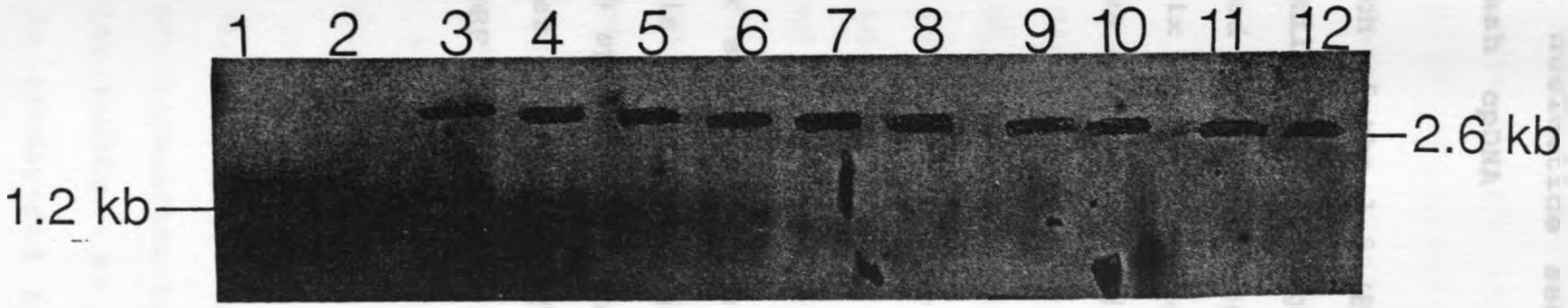


Fig 4.7 The 2.6 kb transcript detected in EP leaves and fruits. Lanes 1, 3, 5, 7, 9 and 11 contain RNA from YY B^+B^+ genotype leaves and 21, 14, 7, 3, and -2 days pre- and postpollination fruit, respectively. Lanes 2, 4, 6, 8, 10 and 12 contain RNA from YY BB genotype leaves and 21, 14, 7, 3, and -2 days pre- and postpollination fruit, respectively. Transcripts were detected with the 9.8 kb probe which also detects the psbA transcript of 1.2 kb. The X-ray film was exposed for 7 days with a screen.

Partial nucleotide sequence of the PstI fragment of squash cpDNA

Each of the 1.9 (PstI-BamHI), 3.2 (BamHI-PstI) and 4.7 kb (BamHI-BamHI) fragments from the PstI 9.8 kb squash discussed in the preceding section were cloned in pUC18 (Appendix C) and sequenced at their terminal ends. The sequences are reported in Appendix D.

Conclusions

Our study provides initial evidence that "ORF 2280" of squash is transcribed giving a 2.6 kb mRNA detected by both ORF 2280 specific tomato probe and the squash P 9.8 kb probe. A smaller transcript of 1.4 kb was also detected with the tomato ORF 2280 probe.

Chapter 5

SUMMARY OF THE STUDY AND RECOMMENDATIONS

Squash as a system for studying plastid gene expression

The presence of chloroplasts or chromoplasts in EP fruits did not have detectable influence on the expression of rbcL and psbA gene expression. The regulation of plastid differentiation did not appear to be synchronized with the regulation of the two photosynthetic genes studied. This suggested that the plastid differentiation and regulation of photosynthetic genes are under separate gene regulatory mechanisms. The two different pathways leading to chromoplast development affect only plastid structure and content. Plastid gene expression was under a separate gene control that was different between the two EP isogenic lines.

In contrast to EP YY BB and EP YY B^+B^+ fruits, the steady-state levels of rbcL and psbA transcripts gene were higher in the chromoplasts than in the chloroplasts of Bicolor fruits. This implied the presence of transcriptional regulatory mechanisms that were coordinated with plastid type in Bicolor fruits. As steady-state levels of rbcL mRNA were higher in 14-day-old fruits than in the 21-day-old fruits,

while the Rubisco activity and amounts Rubisco LSU proteins increased during this period, we also suggest the presence of post-transcription gene expression in Bicolor fruits.

In EP, Zucchini and Bicolor fruits the rbcL and psbA mRNAs, Rubisco LSU protein and Rubisco activity changed with fruit development but the relative changes differed among cultivars. The steady-state levels of these transcripts were however reduced in fruits when compared to leaves. The results demonstrate the complexity of tissue-specificity and developmental expression of psbA and rbcL genes in squash. Therefore, these squash cultivars are a rich potential system for studying the role of developmental and tissue-specific signals in regulation of plastid gene expression. In addition the Bicolor gourds are a potential system for investigating the presence of nuclear or plastid encoded factors which caused the differential expression of psbA and rbcL genes.

Two possible approaches to determining the cause of differential gene expression would be as follows:

(1) Do run-on transcription assays using chloroplasts and chromoplasts extracts from the same fruit. The objective would be to determine whether the differences in the expression of genes between the 2 tissues was either due to differences in the rates of transcription or mRNA stabilities or both.

(2) Do inhibition studies using inhibitor of plastid translation (chloramphenical) and inhibition of cytoplasmic translation (cycloheximide). The objective would be to determine if some nuclear or plastid encoded transcriptional factor caused the differences in rates of transcription of plastid genes in the chromoplasts as compared to chloroplasts.

"ORF 2280"

The "ORF 2280" of squash plastid DNA was expressed in fruits but not in leaves of EP fruits although the signal was faint. In Bicolor fruits the "ORF 2280" was expressed both in the fruits and in the leaves. However, the transcripts detected by the ORF 2280 specific probes were not full length of the entire ORF. Future investigation should examine the presence of "ORF 2280" translation products.

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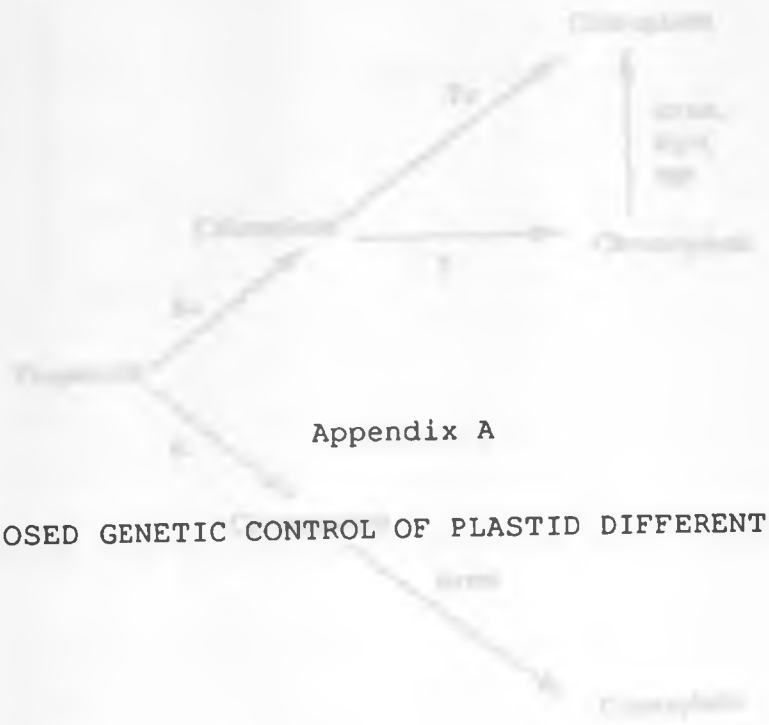


Appendix A

PROPOSED GENETIC CONTROL OF PLASTID DIFFERENTIATION

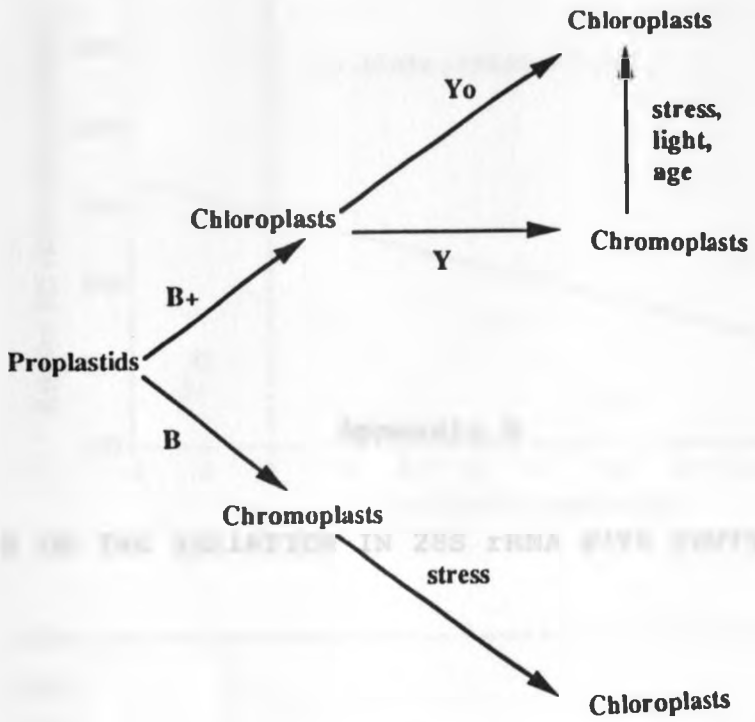


Fig A.1 Proposed points of genetic and environmental regulation of plastid development in the skins of Cucurbita pepo L.

Appendix B

ANALYSIS OF THE VARIATION IN 28S rRNA WITH FRUIT DEVELOPMENT

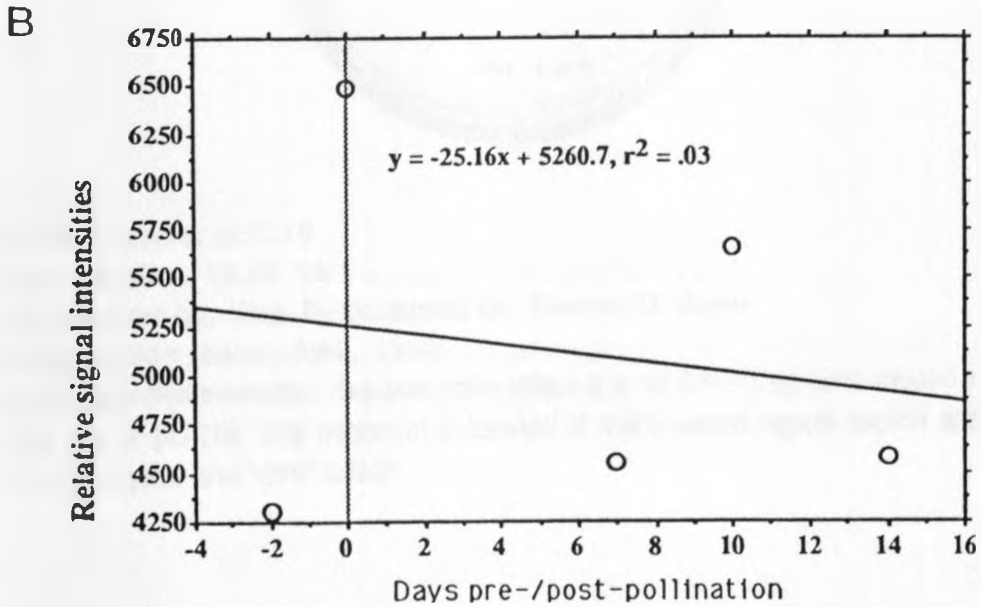
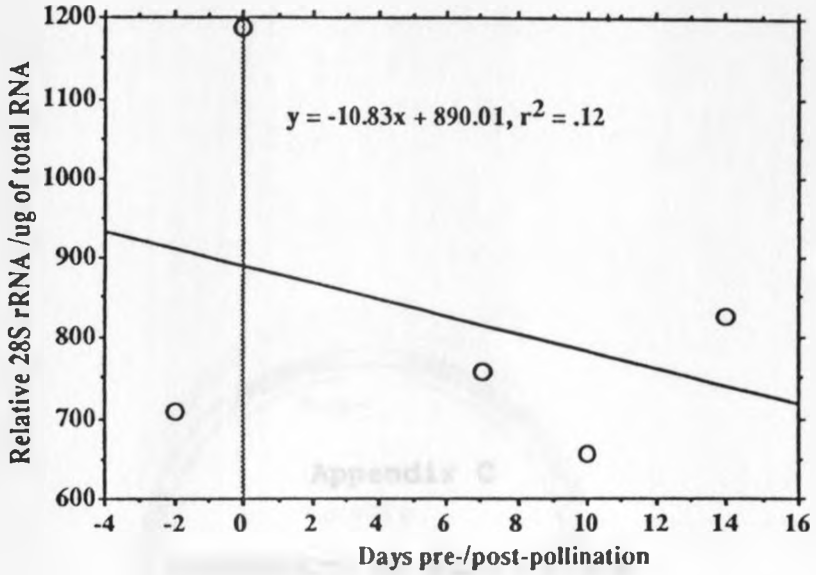


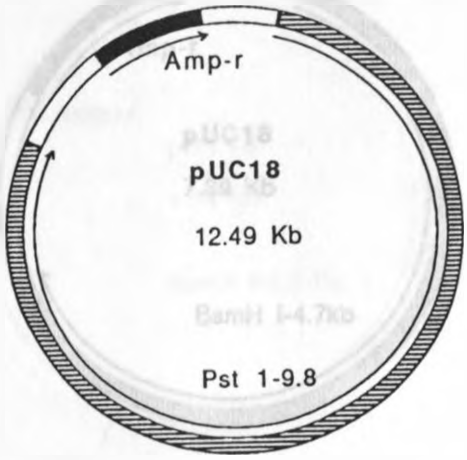
Fig B.1 Regression analysis of relative signal intensity for 28S rRNA versus fruit age.

A


Relative 28S rRNA /ug of total RNA
y = -10.83x + 890.01, r2 = .12
1200
1100
1000
900
800
700
600
-4
-2
0
2
4
6
8
10
12
14
16
Days pre-/post-pollination

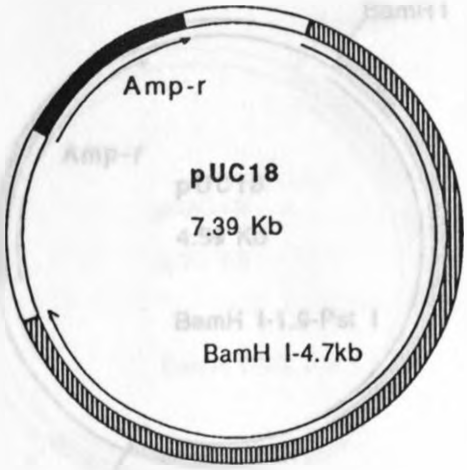
Appendix C

CONSTRUCTS OF PLASTID DNA



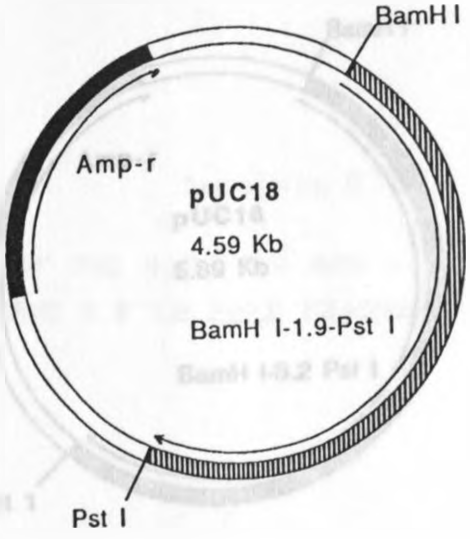
Plasmid Name: pUC18
Plasmid size: 12.49 kb
Constructed by: Silas D. Obukosia; Dr. Charles D. Boyer
Construction date: April 1992
Comments/References: Squash chloroplast 9.8 kb DNA fragment cloned in the PstI site of pUC18. The fragment is located in the inverted repeat region and contains the psbA gene and "ORF 2280"

Fig C.1 Plasmid construct containing the 9.8 kb PstI fragment of squash chloroplast DNA



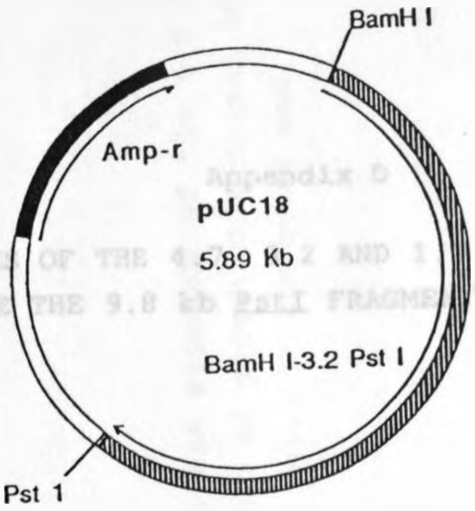
Plasmid Name: pUC18
Plasmid size: 7.39 kb
Constructed by: Silas D. Obukosia; Dr. Charles D. Boyer
Construction date: April 1992
Comments/References: Squash chloroplast 4.7 kb DNA fragment cloned in the BamHI site of pUC18. The fragment is located in the inverted repeat

Fig C.2 Plasmid construct containing the 4.7 kb BamHI fragment of squash chloroplast DNA



Plasmid Name: pUC18
Plasmid size: 4.59 kb
Constructed by: Silas D. Obukosia; Dr. Charles D. Boyer.
Construction date: April 1992
Comments/References: Squash chloroplast 1.9 kb DNA fragment cloned in the BamHI- Pst I sites of pUC18. The fragment is located at one end of the inverted repeat and contains the psbA and rpS 2 genes

Fig C.3 Plasmid construct containing the 1.9 kb BamHI/PstI fragment of squash chloroplast DNA



Plasmid Name: pUC18
Plasmid size: 5.89 kb
Constructed by: Silas D. Obukosia; Dr. Charles D. Boyer
Construction date: April 1992
Comments/References: Squash chloroplast 3.2 kb DNA fragment cloned in the Pst 1 and BamH1 sites of pUC18. The fragment is located in the inverted repeat region.

Fig C.4 Plasmid construct containing the 3.2 kb BamHI/PstI fragment of squash chloroplast DNA

Appendix D

DNA SEQUENCES OF THE 4.7, 3.2 AND 1.9 kb FRAGMENTS WHICH CONSTITUTE THE 9.8 kb PstI FRAGMENT OF SQUASH cpDNA

Figure D.1 The partial DNA sequence of the 4.7 kb BamHI fragment of the 9.8 kb PstI fragment of squash chloroplast DNA: A. 185 base pairs at one end; B. 215 base pairs at the other end.

A

```
          10         20         30         40         50         60         70
AGGGCATCGT TGAGCCTGAA AGCAGACAAG AAGATCCCGT CCGGCCTTAT GTCTTGGATC ACCTACGCGG
TCCCGTAGCA ACTCGGACTT TCGTCTGTTC TTCTAGGGCA GGCCGGAATA CAGAACCTAG TGGATGCGCC

          80         90        100        110        120        130        140
CGGTGCAATC TCTGGCGACT GCCTTGCGCG TACCGGTTAG CGATGAGCCG CTCCGGTGTG TAAGATTAAG
GCCACGTTAG AGACCGCTGA CGGAACGCGC ATGGCCAATC GCTACTCGGC GAGGCCACAC ATTCTAATTC

         150        160        170        180
CTAACGGTGC AACACCTGGA TTGGCGCTGA ACTGGTCGAA AGGGC
GATTGCCACG TTGTGGACCT AACCGCGACT TGACCAGCTT TCCCG
```

B

```
          10         20         30         40         50         60         70
CCGGCTAGTT CAGTTCGATT GGCATAGTCG CAGATCCTGT TAGGTTTGCG CGGCTGTTCC GGTCTAATCT
GGCCGATCAA GTCAAGCTAA CCGTATCAGC GTCTAGGACA ATCCAAACGC GCCGACAAGG CCAGATTAGA

          80         90        100        110        120        130        140
GCTTTCTTGA AGGCGTCTTG TTGTTAATAG AGAACGGTCG AGCTGTTGAA TGGCTGGAGA GTCGCTTGCT
CGAAAGAACT TCCGCAGAAC AACAATTATC TCTTGCCAGC TCGACAACTT ACCGACCTCT CAGCGAACGA

         150        160        170        180        190        200        210
GTCGGAGTAG TACTGAACCC TCGACAACTG GCTCTCATGC ACTGATCCTG CTCTTAAACC CGTATAACCG
CAGCCTCATC ATGACTTGGG AGCTGTTGAC CGAGAGTACG TGACTAGGAC GAGAATTTGG GCATATTGGC

CTATG
GATAC
```

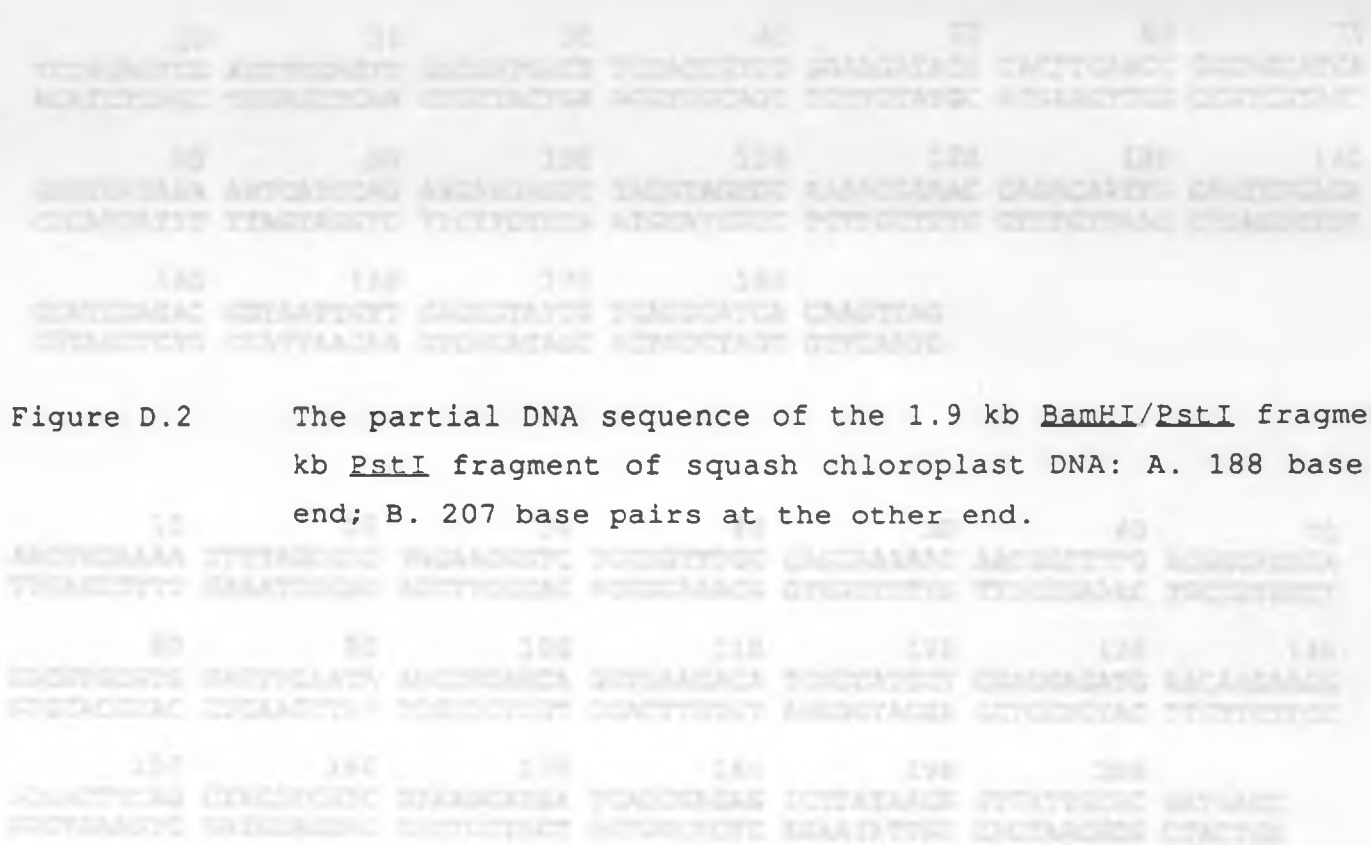


Figure D.2 The partial DNA sequence of the 1.9 kb BamHI/PstI fragment of the 9.8 kb PstI fragment of squash chloroplast DNA: A. 188 base pairs at one end; B. 207 base pairs at the other end.

A

```
          10          20          30          40          50          60          70
TCTAGAGTCG ACCTCGAGTT GGCGATGACT TCCACCGTCG GGAAGATACG TACTTCAACC GGCAGCATCA
AGATCTCAGC TGGAGCTCAA CCGCTACTGA AGGTGGCAGC CCTTCTATGC ATGAAGTTGG CCGTCGTAGT

          80          90         100         110         120         130         140
GGGTGATAAA AATCATCCAG AAGAAGAGGT TACGTAGCGG AAAACGAAAC CAGACAATTG GAATCGGAGA
CCCACTATTT TTAGTAGGTC TTCTTCTCCA ATGCATCGCC TTTTGCTTTG GTCTGTTAAC CTTAGCCTCT

         150         160         170         180
GCATCGAGAC GGTAATTGTT GAGCGTATCG TGACGCATCA CAAGTTAG
CGTAGCTCTG CCATTAACAA CTCGCATAGC ACTGCGTAGT GTTCAATC
```

B

```
          10          20          30          40          50          60          70
AACTGGAAAA CTTTAGCGCC TGGAACGGTC TGCCGTTTGC CAGCAAAAAC AACGGCTTTG ACGGCACGGA
TTGACCTTTT GAAATCGCGG ACCTTGCCAG ACGGCAAACG GTCGTTTTTG TTGCCGAAAC TGCCGTGCCT

          80          90         100         110         120         130         140
CGCGTGCGTG GAGTTCAATA AGCCGGAGCA GGTGAACACA TCGCCATGCT CGAGGAGATG AACAAGAAGG
GCGCACGCAC CTCAAGTTAT TCGGCCTCGT CCACTTGTGT AGCGGTACGA GCTCCTCTAC TTGTTCTTCC

         150         160         170         180         190         200
GCGACTTCAG CTACGTCGTC GTAAGGATGA TCACCGAGAG TCTTATAACG GTGATTGCGC GATGACC
CGCTGAAGTC GATGCAGCAG CATTCCTACT AGTGGCTCTC AGAATATTGC CACTAACGCG CTACTGG
```

Figure D.3 The partial DNA sequence of the 3.2 kb BamHI/PstI fragment of the 9.8 kb PstI fragment of squash chloroplast DNA: A. 231 base pairs at one end; B. 206 base pairs at the other end.

A

```
          10          20          30          40          50          60          70
TTGACCGACG GCGATGGCAG GGCTTGGACG AACGCAGCCA GGACAACATC ATCGTGCGCG CTGGGACGAA
AACTGGCTGC CGCTACCGTC CCGAACCTGC TTGCGTCGGT CCTGTTGTAG TAGCACGCGC GACCCTGCTT

          80          90         100         110         120         130         140
GGACAACAGT CCACGTTGCA AGAACTGCTG ACCAGACGCG TTCCGTGAGC TGTACCTGAA AGACGCATGT
CCTGTTGTCA GGTGCAACGT TCTTGACGAC TGGTCTGCGC AAGGCACTCG ACATGGACTT TCTGCGTACA

         150         160         170         180         190         200         210
ATTAGCAGAG ACCTGATGAG ATGCGGTTTG TTTCGGCTCT CTATATAACC TCGCTTCGCA TCCACGCAGT
TAATCGTCTC TGGACTACTC TACGCCAAAC AAAGCCGAGA GATATATTGG AGCGAAGCGT AGGTGCGTCA

         220         230
GTATAACTTG CAATATGCAT C
CATATTGAAC GTTATACGTA G
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B

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          10          20          30          40          50          60          70
GAAGACAACG ACGCACTTGC ACTTTCCGGA AAAATAAAAC TGGGTTTTCA GGCCAACGGA ACGGCTGGCG
CTTCTGTTGC TGCGTGAACG TGAAAGGCCT TTTTATTTTG ACCCAAAAGT CCGGTTGCCT TGCCGACCGC

          80          90         100         110         120         130         140
TCACGATCTG CCCATTTCGG TAGTAACGCG TAGTAACGAA GTCGATGTTT CTTTCAGGCG GCGATCTGCG
AGTGCTAGAC GGGTAAAGCC ATCATTGCGC ATCATTGCTT CAGCTACAAA GAAAGTCCGC CGCTAGACGC

         150         160         170         180         190         200
CGAGAATCTT CTCCGCGGTA ATACGTCGAG AGACGACGTG CGTACGTTTC AGCCGTCTGA CAACAG
GCTCTTAGAA GAGGCGCCAT TATGCAGCTC TCTGCTGCAC GCATGCAAAG TCGGCAGACT GTTGTC
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VITA

Silas Daniel Obukosia was born November 21, 1958, in Funyula, Kenya. After completion of his secondary education in Funyula, Mr. Obukosia entered the University of Nairobi in 1979. He graduated from the University of Nairobi with a B.Sc. degree in Agriculture with Second Class Honours (Upper Division) in 1983. Mr. Obukosia continued his studies at the University of Nairobi in the area of Genetics and Plant Breeding in the Department of Crop Science. In 1986, he received an M.Sc. degree in Plant Breeding. His research for the M.Sc. degree was on the interspecific hybridization of *Phaseolus vulgaris* L. lines adapted to Kenya and *P. acutifolius* L. As part of this research, Mr. Obukosia developed methods of embryo culture to rescue immature interspecific embryos. After completion of his M.Sc., Mr. Obukosia was employed as a Lecturer in the Department of Crop Science at the University of Nairobi. He entered the Graduate Program in Genetics at The Pennsylvania State University in 1988. During his studies at Penn State, Silas was part of a reciprocal student exchange program with the University of Nairobi. Upon completion of his studies in the U.S., Mr. Obukosia will be engaged in teaching and research at the University of Nairobi.