IDENTIFICATION AND CHARACTERIZATION OF cDNA CLONES FOR CHROMOPLAST-ASSOCIATED PROTEINS IN TOMATO FRUIT

By

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The chloroplast to chromoplast transition during tomato fruit ripening is characterized by a dramatic change in plastid structure and function. The thylakoid membranes of photosynthetically competent chloroplasts are broken down and the carotenoid pigment lycopene is deposited within the developing chromoplast. A goal of this investigation was to determine whether this process is mediated by an increase in the steady-state level of RNA for plastid-targeted proteins. Assays for import of radiolabeled translation products into isolated pea chloroplasts were used to monitor levels of chromoplast-targeted proteins at four stages of tomato fruit development. During development, striking increases in levels of translatable RNA for two such proteins were found. Additionally, the import of *in vitro* translation products was examined for seven individual cDNA clones known to encode RNA that increases

during fruit ripening. Three of these clones produced *in vitro* translation products that imported into pea chloroplasts. One clone, pTOM5, has recently been shown by others to encode the enzyme responsible for the first committed step in the synthesis of lycopene.

Subsequent sequence analysis of another cDNA, pTOM 111, revealed that it encodes a plastid-localized low MW heat shock protein. Not only does this transcript increase during development at normal temperatures, but it is increasingly inducible by elevated temperatures as ripening progresses. This is the first report of the developmental induction of a plastid-localized low MW heat shock protein. Identification of the induction of a heat stress protein during this transition suggests that either the plastid is subjected to an increasingly stressful environment or, as is consistent for the developmental induction of other heat stress proteins, a massive structural reprogramming is underway.

These studies imply that there is synthesis and import of new proteins during the transition from chloroplast to chromoplast, and that the plastid conversion is an active developmental program rather than a simple decline in synthesis of the photosynthetic apparatus. Furthermore, these results demonstrate the utility of this method for identification of structural genes involved in plastid morphogenesis.

CHAPTER 1
INTRODUCTION

Ripening of tomato fruit involves a wide variety of developmental changes that include but are not limited to changes in cell wall structure, in metabolism, in ultrastructure, and an especially dramatic change in the form and function of the plastid. Biochemical and molecular studies suggest that most or all of these changes are mediated by the enhanced expression of ripening specific genes. I have chosen this system as a means to investigate plastid development and interconversion.

We are interested in determining how the transition from chloroplast to chromoplast is regulated, e.g., does a subset of plastid proteins increase during this process? In an initial examination of this question, we have asked whether an increase in the accumulation of RNA for a subset of plastid proteins correlates with the chloroplast to chromoplast transition. Since transcription and/or translation of the plastid genome seems to diminish during chromoplast formation (Kobayashi, 1991), chromoplast-specific proteins are probably encoded in the nucleus. Nuclear-encoded plastid proteins are synthesized in the cytosol and posttranslationally imported into plastids. The import of proteins into plastids is a highly specific process (Keegstra, 1989). The proteins are made as precursors with transient amino terminal "transit peptides," which are both necessary and sufficient for the correct

targeting into this organelle. It is this latter observation that makes the import into plastids a potentially diagnostic assay for the identification of chromoplast associated proteins.

The approach described within this dissertation has been to monitor chloroplast import of *in vitro* translation products from RNA of tomato fruit isolated at different ripening stages. We have also studied translation products programmed with transcripts from cDNAs (Slater et al., 1985) whose steady state RNA level increases during ripening. Since individual cDNAs were analyzed, subsequent sequence analysis allowed further characterization of the proteins that mediate this transition. Eventually the role of chromoplast-associated proteins in chromoplast development can be assessed directly via antisense technology. The final outcome of such a study should be an understanding of the role new protein synthesis plays in this process and the isolation, identification and characterization of genes that mediate this transition.

CHAPTER 2
REVIEW OF LITERATURE

The manner in which the transition from chloroplast to chromoplast is regulated is largely unknown. Electron micrographic analysis has shown that a massive reordering of plastid structure occurs during the conversion (Thelander et al., 1986). In tomato fruit, this is characterized by the overall breakdown of chloroplast specific components, e.g. the thylakoid membrane system and starch, with the concomitant accumulation of membrane-associated deposits of the carotenoid lycopene (Thelander et al., 1986). It has not been determined whether the breakdown of chloroplast structures is caused simply by the decrease in synthesis of photosynthetic proteins or if increases in degradative enzymes actually enhance this process. An examination of nuclear-encoded, plastid-targeted, photosynthetic proteins has shown that their transcription stops 5 to 10 days before the fruit reaches full size (Piechulla et al., 1986). Analysis of plastid-encoded transcripts has demonstrated that some are greatly reduced, whereas others persist, and one appears to increase during chromoplast formation (Kobayashi, 1991; Richards et al., 1991). Similarly, little has been ascertained about the function or expression of genes involved in the synthesis of chromoplast structures as few clones specific to this developmental stage have been available for analysis (for example, Kuntz et al., 1992).

In considering what aspects of fruit ripening directly affect the proteins that are targeted to the plastid, it quickly became apparent that two types of functions could cause increases in proteins directed to the developing chromoplast. First, proteins involved in the degradation of the photosynthetic apparatus or the build up in carotenoids are excellent candidates for chromoplast-targeting and enhanced synthesis during fruit ripening. A broad description of plastid senescence will be considered, since that transition also involves the degradation of chloroplast structures and because an examination of RNA from senescing leaves has shown that many of the same transcripts are also present. Secondly, other aspects of maturation may have indirect effects on the environment in which the developing plastid resides. For example, the autocatalytic production of ethylene, the respiratory climacteric or an increase in oxidative stress during ripening could secondarily create increases in plastid-targeted proteins. Since the plastid is responsible for a number of basic biosynthetic functions, such as the synthesis of aromatic amino acids, fatty acids and δ-aminolevulinic acid these changes outside of the plastid could create a need for substrates synthesized within this organelle.

A consequence of the work described in this dissertation was the characterization of a cDNA that encodes a plastid-localized low molecular weight (MW) heat shock protein (hsp). Because transcript for this hsp increases during ripening at normal temperatures, it was necessary to assess how this unexpected finding fit into chromoplast development or fruit ripening. Since oxidative stress has been associated with senescence and hsps have been induced by oxidative stress in

animal tissues, a consideration of heat stress proteins and oxidative stress during fruit ripening will also be examined.

Morphological Changes

The overall changes that occur during the ripening of tomato fruit are as familiar to ones mother as they are to any post-harvest physiologist. Full sized green fruit harvested from the garden and brought into the kitchen will ripen normally. The color changes observed during the ripening of tomato fruit are due to the breakdown of chlorophyll and the deposition of lycopene within the fruit plastids. The breaker stage is identified by the appearance of external color at the blossom end. The exact fruit color that then develops is dependent on variety and consequently, the fruit may turn yellow, pink, or orange-red. As the fruit becomes uniformly red, the cell walls begin to soften. The characteristic flavor of ripe tomatoes is created by a combination of glucose, fructose, citric and glutamic acids, which accumulate in the vacuoles, along with the synthesis of hundreds of volatile compounds (Davies and Hobson 1981; Grierson, 1986).

The young fruit is green because it contains chlorophyll in photosynthetically functional chloroplasts. It is considered immature green (IMG) until it reaches its full size. Growth is complete at mature green (MG). A green tomato may appear morphologically quiescent; however, it is undergoing important ripening changes. Its locular tissue transforms from a solid to a gelatinous substance, it can respond rapidly to exogenous ethylene and the endogenous ethylene level also increases at

this time. By late MG, carotenoids begin to appear in the locules. Thus the color change begins in the interior of the fruit (Kader and Morris, 1976). After the fruit has passed through mature green, the transformation to a red ripe soft tomato proceeds rapidly.

Biochemistry and Molecular Biology

Although the change in fruit color is a convenient method of classifying tomato fruit ripening, a number of other biochemical changes also occur. For example, a dramatic rise in respiration and ethylene production takes place. This phenomenon is known as the climacteric. Climacteric ethylene levels reach about one half of their maximum by the breaker stage (Picton and Grierson, 1988). The respiratory peak occurs soon after the rise in ethylene (Grierson, 1986).

Alterations in Basic Metabolism During Fruit Ripening

Changes take place in the type of carbohydrate that accumulates as tomato fruit ripens. Starch granules transiently appear between 14-28 days postanthesis in the immature green chloroplasts (Davies and Cocking, 1965). Although these plastids can fix carbon at up to 35% of the rate attained by leaf plastids, the starch granules are mostly the product of sucrose imported from the leaves (Thelander et al., 1986). Since the import of carbohydrate from the leaves stops by MG (Thelander et al., 1986), fruit at this stage will develop normally if harvested from the plant. About 28-35 days after anthesis degradation of starch occurs as an increase in soluble

sugars takes place. Consequently, little starch remains in the mature green fruit (Yelle et al., 1988). The rapid degradation of starch precedes the climacteric rise in respiration (Davies and Cocking, 1965) and this carbohydrate is stored as glucose and fructose in the vacuole (Grierson, 1986). The degradation of starch seems to occur via phosphorylase (Robinson et al., 1988), and the further steps of hexose to triose occur via the pentose phosphate pathway and glycolysis (Thelander et al., 1986). Therefore, as the tomato fruit matures, a shift of stored carbohydrate occurs from starch in the plastid to hexose sugars in the vacuole. In fact, the red ripe tomato contains no measurable starch and 47% hexose by dry weight (Davies and Hobson, 1981). The changes in stored carbohydrate precede the breakdown of the thylakoid membranes and consequently, any plastid associated enzymes responsible for starch degradation are not expected to increase during chromoplast development.

It is unclear what causes the increase in CO_2 during ripening and, consequently, the molecular regulation of this increase is also unknown (Solomos, 1988). Fluctuations in enzymes involved in the tricarboxylic acid (TCA) cycle and glycolysis occur but it has not been determined whether these changes are due to a variation in the level of enzymatic effectors or are caused by alterations in the amount of de novo synthesis of these enzymes during ripening. Solomos (1988) reviewed the literature on respiration in ripening fruit and concluded that the respiratory burst measured is probably a result of an increase in substrate level phosphorylation caused by enhanced glycolysis. An increase in the activities of cytosolic NADP linked malic enzyme (Goodenough et al., 1986) and the glycolytic

enzyme phosphofructokinase (PFK) have been observed (Grierson, 1986). PFK is a control point in glycolysis and an increase in this enzyme activity probably results in an increase in carbon flow through this pathway (Solomos, 1988). Indeed, an increase in the accumulation of ATP is generally noted during the respiratory rise (Solomos, 1988). Investigation of mitochondrial electron transport in ripening fruit suggested that it remains unchanged (Solomos, 1988). However, the TCA cycle enzymes citrate synthase, malate dehydrogenase, NAD-linked malic enzyme, and isocitrate dehydrogenase decrease in ripening tomato fruit (Grierson, 1986; Jeffery et al.,1986). Overall, these data do not explain why an increase in respiration is measured during ripening. Since these changes are underway during the plastid conversion while photosynthesis is declining, it is unclear how this may effect the synthesis of proteins targeted to the plastid.

Other Changes Associated with Fruit Ripening

Apparently, the level of the anoxia-responsive enzyme, alcohol dehydrogenase (ADH), increases during ripening in tomato fruit (Longhurst et al., 1990; Van Der Straeten et al., 1991). Longhurst et al. (1990) concluded that the increase in ADH during ripening may be caused by the cytoplasmic increase in pH. It has been hypothesized that low oxygen levels could result within such a bulky organ, but by exposing ripening fruit to different levels of O_2 and CO_2 these authors surmised that the change in pH leads to an induction of this enzyme.

As fruit matures, the cell walls soften and an increase in soluble pectin occurs (Grierson, 1986). Characterization of the regulation of the enzymes involved in cell wall degradation has begun. These data suggest that the pattern of expression for enzymes involved in cell wall degradation is complex and can even involve more than one isozyme (DellaPenna et al., 1986; Grierson et al., 1986; Fisher and Bennett, 1991; Harriman et al., 1991). For example, antisense mutagenesis of the cell wall softening enzyme, polygalacturonase (PG), has shown that although this enzyme is dramatically induced during fruit ripening, loss of this enzymatic function has little overall effect on maturation (Sheehy et al., 1988; Smith et al., 1988 and 1990).

The synthesis of hundreds of volatile compounds occurs as tomato fruit ripens (Davies and Hobson, 1981). However, little is known about how these compounds are synthesized during maturation.

Oxidative Stress and Fruit Ripening

Since a chromoplast-associated low MW heat shock protein was identified in this work, it is important to understand the role this stress protein may play in fruit development. Oxidative stress occurs when the cell's ability to respond is exceeded by the level of reactive oxygen species present (Barja de Quiroga, 1992). The induction of hsps by compounds that cause oxidative stress has been shown in animal tissues (Drummond and Steinhardt, 1987; Cajone and Bernemmi-Zazzera, 1988; Courgeon et al., 1988). In addition, oxidative stress has often been associated with ripening and senescence. One theory postulates that a buildup in the deleterious

effects of free radicals results in the physiological manifestations of aging (Harman, 1992).

Free radical production during normal cellular metabolism

Reactive oxygen compounds can cause damage to proteins and especially membrane components of the cell. A number of normal cellular functions result in the production of these species, which can be a side effect of reactions that reduce molecular oxygen. For example, at least one percent of the oxygen utilized during respiratory electron transport is incompletely reduced (Fleming et al., 1992). Furthermore, an increase in basal metabolism can result in an increase in the amount of reactive oxygen generated even without invoking a breakdown in oxidative phosphorylation (Barja de Quiroga, 1992). Photosynthesis is another normal cellular function during which a large amount of free radicals can form (Scandalios, 1993). In addition, some enzymatic reactions directly produce reactive molecules. Examples of this latter group include xanthine oxidase (an ATP degrading enzyme) and beta oxidation of fatty acids (Scandalios, 1993). Finally, certain environmental stresses can enhance the level of reactive molecules. For example, radiation, herbicides, pathogens, hyperoxia, and temperature fluctuations can induce free radical production (Scandalios, 1993). Perhaps a buildup of these deleterious compounds occurs during the ripening of tomato fruit. Since any cellular function that involves the reduction of oxygen causes production of reactive oxygen, either autocatalytic ethylene synthesis or the respiratory climacteric would be excellent sources of free radicals.

One source of reactive oxygen is lipoxygenase activity, which increases during ripening (Thompson et al., 1987). This enzyme incorporates oxygen into polyunsaturated fatty acids (Hatanaka et al., 1992) and leads to a build up of the reactive oxygen compounds hydroperoxide or superoxide. Recently, Bowsher et al. (1992) have purified a membrane associated lipoxygenase from breaker stage tomato fruit. They find that the activity fractionates with the thylakoid membrane. Consequently, this could result in increases in the amount of reactive oxygen species in the developing chromoplast.

The change in membrane integrity during fruit ripening

A decrease in membrane fluidity, which may be created by oxidative stress, occurs during ripening and senescence along with a progressive loss of specialized membrane function (Thompson et al., 1987). Many aspects of membrane structure can affect the level of membrane viscosity. For example, the length or degree of unsaturation of the hydrocarbons and the level of sterols all can play a part. Although a decrease in membrane fluidity occurs in the plasma membrane of tomato fruit (Legge et al., 1986) and pepper microsomal membranes (Whitaker, 1991), no change in plastid membrane fluidity occurs in either tomato or pepper chromplasts. Neither fluorescent membrane probes, which directly assess the structure within specific environments of the membrane used by Legge et al. (1986), nor measurements of the free sterol or total sterol to phospholipid ratios determined by Whitaker (1991) could detect a change in the developing chromoplast membrane. Considering the increase in carotenoid biosynthesis during ripening, which is

associated with plastid envelope membranes, it is appropriate that the integrity of this membrane persists.

Free radical scavengers and oxidative stress

Initially, an increase in free radicals results in an increase in enzymes or antioxidants that neutralize these reactive species. For example, a family of superoxide dismutases (SOD) acts to scavenge superoxide and create oxygen and hydrogen peroxide. Five isozymes of this enzyme have been described in tomatoes. Two are cytoplasmic, one is mitochondrial and two occur in chloroplasts (Perl-Treves and Galun, 1991). Also, ascorbic acid and α-tocopherol can act as antioxidants (Leshem, 1988). A number of enzymes are also involved in reducing the levels of hydrogen peroxide. Catalase, peroxidase and glutathione peroxidase all act in this manner (Leshem, 1988). Additionally, glutathione reductase is required to regenerate glutathione lost by interaction with hydrogen peroxide (Leshem, 1988). Consequently,the increase in these enzymes is correlated with an increase in reactive oxygen molecules. Any decrease in these enzymes with senescence, therefore, could contribute to degeneration of membranes and disturbance of cellular function.

Increases in basal metabolism may induce SODs in plant tissue. Bowler et al. (1989) concluded that the mitochondrial SOD may respond to changes in the activity of respiratory electron transport, because the induction of this enzyme in tobacco correlated with increased activity for cytochrome oxidase.

In addition, oxidative stress in one compartment can lead to the induction of free radical scavengers in another part of the cell. This was shown when paraquat,

which is thought to act mainly on chloroplasts, was applied to tobacco and caused a strong induction of SOD transcript in the mitochondria, cytoplasm and chloroplast (Tsang et al., 1991).

The level to which SOD is induced within a cellular compartment may depend on the amount of SOD that is currently present. This was the explanation of Perl-Treves and Galun (1991) when they found that the addition of paraquat resulted in a greater induction of the cytoplasmic SOD in comparison to the plastid message in tomato leaves. The level of plastidial enzyme activity in mature leaves is already high in comparison with transcript level. They suggested that induction of plastidial SOD transcript may therefore be unnecessary.

Ethylene has been shown to induce cytoplasmic and plastidial SOD transcripts and enzyme activity in tomato leaves (Perl-Treves and Galun, 1991) and mitochondrial SOD message in tobacco leaves (Bowler et al., 1989). In response to ethylene, therefore, an increase in reactive oxygen species has been noted and enzymes within all three cellular compartments can be affected.

Free radical scavengers in ripening fruit

The role of free radical scavengers during fruit ripening, however, is somewhat confusing. Peroxidase has been shown to decrease during ripening (Rabinowitch et al., 1982) or peak during the pink stage in tomato fruit (Rothan and Nicolas, 1989). Perhaps the techniques used by Rabinowith et al. (1982) were not sensitive enough to detect this peak of activity. The transient increase in basic peroxidase activity paralleled the increase in ethylene (Rothan and Nicolas, 1989). Since the conversion

of 1-aminocyclopropane-1-carboxylate (ACC) to ethylene requires oxygen, this may lead to a transient increase in reactive oxygen molecules and an increase in this free radical scavenger.

Changes in SOD have been reported during tomato fruit ripening but results were contradictory. SOD activity has been found to peak at the pink or orange phase (Rabinowitch et al., 1982; Livine and Gepstein, 1988). However, no change in the two plastidial SOD isozymes was reported by Perl-Treves and Galun (1991), nor was a peak of the cytoplasmic or plastidial SOD message noted during ripening. In fact, they found that the plastid-associated message actually decreased with maturation. They explain these contradictory results by suggesting that the enzyme could be quite stable and turnover very slowly. It is hard to explain all of the varied data reported by these three groups. More work needs to be done before a conclusion can be made about free radical scavengers and ripening in tomato fruit. Since the messages for the additional plastidial and cytoplasmic SODs have not been examined, perhaps these additional data will help reconcile these dissimilar observations.

Pepper chromoplasts do appear to respond to an oxidative stress during fruit maturation (Romer et al., 1992). The antioxidant producing enzyme cysteine synthase was purified and a cDNA was isolated. They found not only did this enzyme increase during ripening but the activity of glutathione reductase, glutathione synthetase and glutathione content within chromoplasts also increased during their development. Since all these compounds protect organisms from oxidative damage and an increase in α-tocopherol also occurs during ripening, they concluded that these increases in

antioxidants and peroxide scavenging compounds may allow protection of the overaccumulated carotenoids and other membrane components from oxidative stress.

Since the results from tomato fruit on the induction of free radical scavengers is contradictory, information from other systems may help predict what occurs in tomato fruit chromoplasts. Leaf tissue responds to an increases in basal metabolism and the addition of ethylene by inducing SOD (Bowler et al., 1989; Perl-Treves and Galun, 1991). In addition, a compound expected to affect predominantly one compartment can induce SOD in other portions of the cell (Tsang et al., 1991). Finally, the level of induction may be dependent on current levels of enzyme activity (Perl-Treves and Galun, 1991). Perhaps the results from pepper chromoplasts where increases in a number of these free radical scavengers has been measured, explains how the chromoplast, unlike the plasma membrane, avoids oxidative damage and maintains membrane integrity (Whitaker, 1991; Romer et al., 1992). One might predict that the plastid can respond to an environment that contains an increasing amount of reactive oxygen and consequently it is able to maintain chromoplast membrane integrity during ripening. These data also suggest that the production of ethylene and/or the respiratory climacteric may induce the production of chromoplast-associated proteins involved in free radical scavenging.

Changes in Plastid Structure

Massive changes take place in plastid ultrastructure during the chloroplast to chromoplast transition. The thylakoid membranes are dismantled and chlorophyll

degrades (Thelander et al., 1986). Concomitantly, lycopene begins to accumulate in the stroma. The *green flesh* mutant, which is inhibited in chlorophyll degradation during ripening, is helpful in the determination of how pigment accumulates in the developing chromoplast. Electron micrographs show that the granal stacks persist in this mutant even as pigment is deposited (Goodwin, 1980), and suggests that lycopene builds up around the disintegrating thylakoids. By the time a normal tomato is fully ripe, the chromoplasts contain little else but large stores of lycopene, which accumulate as lipophilic globules and crystals of pigment (Harris and Spurr, 1969).

The type of chromoplast that develops may be dependent on the type of carotenoids that accumulate. The '*r*' mutant, for example, with its low level of both lycopene and ß-carotene, contains only plastoglobules (Goodwin, 1980).

Regulation of Photosynthetic Proteins

Transcripts for the nuclear-encoded components of photosynthesis decrease long before the breakdown of the thylakoid membranes. The mRNA for rbcS and cab (Rubisco small subunit and chlorophyll a/b binding protein respectively) are not detected after 30 days post anthesis (Piechulla et al., 1985, 1986). This loss of nuclear transcripts occurs 5 to 10 days before MG, and precedes the end of chlorophyll synthesis and the disassembly of the thylakoid membranes by 2-3 weeks.

The plastid encoded messages rbcL and psbA (Rubisco large subunit and the 32kD photosystem II protein), however, persist throughout development (Piechulla et al., 1986). Most of the other plastid transcribed proteins, such as the photosystem

I and II proteins (psaA psbB psbC or psbD), all diminish in message levels throughout development.

Once ripening has progressed to an intermediate stage of development beyond mature green, levels of photosynthetic activity have greatly diminished and this is reflected in decreased amounts of photosynthetic proteins. For example, by the yellow stage of development, the amount of LHCP protein has declined to 10% of the level present in IMG fruit (Livine and Gepstein, 1988).

At the yellow-orange stage, the level of Rubisco activity has diminished to 2% of the level present at MG (Piechulla et al, 1987) while the amount of measurable protein for the large subunit has also decreased. The level of photosynthetic electron transport has decreased to 13% of what was observed at MG (Piechulla et al., 1987). Only plastocyanin and the 32kD protein were detected by this stage of development.

These data underscore the dramatic change in the protein components of the plastid that occurs between mature green and this intermediate stage of ripening. It is unclear from this type of analysis whether the loss of photosynthetic proteins is simply due to a decrease in synthesis or is also caused by an enhanced production of degradative enzymes.

Examination of plastid gene expression during chromoplast development has shown a reduction in the level of plastid transcript during this process. It is uncertain whether this is the result of posttranscriptional regulation (Gruissem et al., 1989; Marano and Carillo, 1991), or whether the rate of transcription is decreased due to methylation of the plastid genome (Ngernprasirtsiri et al., 1988; Kobayashi et al.,

1990). The level of translation is also dramatically reduced in *Capsicum* plastids (Kuntz et al., 1989) where ultrastructural analysis has shown that red fruit contain no ribosomes or rRNA (Carde et al., 1988). Richards et al. (1991) have identified a plastid transcript that is more abundant in chromoplast RNA than chloroplast RNA of tomato fruit. However, given the lack of functional translation in pepper chromoplasts and the decreased amount of transcripts present in tomato chromoplasts, it is expected that most proteins that are increasingly present in chromoplasts will be nuclear-encoded.

The Role of Ethylene in Tomato Fruit Ripening

The rise in ethylene during the climacteric is mediated by de novo synthesis of at least the final two enzymes in the biosynthetic pathway (Holdsworth et al. 1987; Olson et al., 1991; Rottmann et al., 1991). To date, only the genes for the enzymes that catalyze these final two steps in ethylene biosynthesis have been isolated.

Ethylene is synthesized from methionine via S-adenosyl-L-methionine and 1-aminocyclopropane-1-carboxylic acid (ACC). The enzyme that synthesizes ACC (ACC synthase) is thought to be a pyridoxal 5'-phosphate utilizing enzyme (Yip et al., 1990). The final step in the synthesis of ethylene is mediated by ethylene oxidase (EFE), requires oxygen, and can be inhibited by the addition of free radical quenchers (Matoo and Aharoni, 1988). The synthesis of ACC is considered the rate limiting step in ethylene biosynthesis. However, EFE is also increasingly synthesized

during ripening in tomato and avocado fruit, (Holdsworth et al., 1987, McGarvey et al., 1992) and during the senescence of carnation petals (Wang and Woodson, 1991).

Production of transgenic tomato plants that contain antisense transcripts for the enzyme ACC synthase has shown that ripening involves both ethylene dependent and independent pathways (Theologis, 1992). A number of laboratories have produced transgenic tomato plants with reduced levels of ethylene (Hamilton et al., 1990, Klee et al., 1991, Oeller et al., 1991). The most dramatic decrease occurred in a transgenic plant carrying ten antisense copies of part of a gene for ACC synthase. The authors reported that the plant has a 99.5% reduction in the level of ethylene (Oeller et al., 1991) and produces fruit that remain firm and green until exogenous ethylene is applied. If no ethylene is added, the mutant fruit still express RNA for the ripening induced cell wall softening enzyme, polygalacturonase (PG) (Oeller et al., 1991). This result was unexpected because ethylene is required for the expression of PG. This suggests that the regulation of ripening by ethylene is more complex than just simple transcriptional control. While the transcription of PG is ethylene independent, the expression of the enzyme is dependent on the continued presence of this hormone.

Transcriptional Regulation of Fruit Ripening

A molecular analysis of fruit ripening in tomato has led to the conclusion that the de novo synthesis of ripening specific proteins occurs and is responsible for many of the changes associated with maturation. Several researchers have characterized

changes in the profile of *in vitro* translation products that occur during development of tomato fruit (Rattanapanone et al., 1978; Speirs et al., 1984; Biggs et al., 1986; Lincoln et al., 1987). A number of groups have constructed cDNA libraries enriched in ripening specific sequences (Mannsson et al., 1985; and Slater et al., 1985; Lincoln et al., 1987). Generally, the onset of ripening in tomato fruit is mediated by an increase in a specific subset of transcripts, including those for PG, EFE and ACC synthase (DellaPenna et al., 1986; Grierson et al., 1986; Holdsworth et al., 1987; Olson et al., 1991; Rottman et al., 1991).

Examination of the clones isolated by Grierson's group has shown that, for most of their cDNAs, RNA fails to accumulate when the fruit is held at high temperature (Picton and Grierson, 1988). High temperature (35^0C) has an inhibitory effect on fruit ripening, depressing the synthesis of lycopene, PG, and ethylene (Picton and Grierson, 1988). Perhaps this effect of high temperature represents a heat shock response (Picton and Grierson, 1988). This was not an overall effect for all clones, however, since RNA increased initially, for some clones, in response to an increase in temperature. Unfortunately, many of these clones have yet to be assigned a function.

Processes Associated with Chromoplast Development

Upon review of the work characterizing the molecular changes that occur during the chloroplast to chromoplast transition, it is notable that mainly photosynthetic proteins have been examined during this phase (Piechulla et al., 1985,

1986, 1987; Livine and Gepstein, 1988). To undertake the investigation of proteins that increase during chromoplast development, a method must be found to identify proteins specific to this process. Isolation of chromoplasts and the subsequent comparison of proteins on SDS-PAGE from different developmental stages has been attempted and proteins that increase in amount have been identified (Iwatsuki et al., 1984, Bathgate et al., 1985, Wrench et al., 1987, Hadjeb et al., 1988). Using this method, specific chromoplast-associated proteins have been isolated from only pepper chromoplasts (Hadjeb et al., 1988). Antibody has been purified for two proteins that increase during *Capsicum* chromoplast development (Newman et al., 1989). One of these proteins binds carotenoids and is a major constituent of *Capsicum* chromoplast membranes (Cervantes-Cervantes et al., 1990). However, this protein probably binds capsanthin since the antibody does not react to any protein present in chloroplasts or tomato fruit chromoplasts (Cervantes-Cervantes et al., 1990). While this type of approach has resulted in the characterization of a few proteins involved in chromoplast function, only abundant proteins can be identified in this manner.

The plastid is involved in the synthesis of many compounds necessary for cell function. For example, the synthesis of fatty acids, aromatic amino acids and other substances such as δ-aminolevulinic acid have all been localized to plastids. Many of these compounds are precursors to a wide array of secondary metabolites, so it is possible that an increased synthesis of these plastid-synthesized compounds is required during chromoplast development. A recent review by Hrazdina and Jensen

(1992) however documented that in many instances isozymes for the synthesis of amino acids involved in secondary metabolism may be present in the cytoplasm rather than in the plastids. More information on the compartmentalization of these pathways is needed before conclusions about the localization of enzymes for secondary metabolism can be drawn.

Carotenoids

Enzymes responsible for the synthesis of carotenoids are obviously the most likely candidates for proteins that may be increasingly produced during chromoplast development. Therefore, it is necessary to consider what enzymes and cofactors may be involved in this pathway and how it may be regulated.

Carotenoid function

In the chloroplast, carotenoids are an integral part of the photosynthetic apparatus, where they act as accessory pigments funneling light energy into the reaction centers. If light intensity becomes too great, the photosynthetic carotenoids can also trap excessive light energy. Without these peripheral pigments, the plants may photobleach (Mayfield et al., 1986). The necessity of carotenoids to forestall this deleterious event is apparent when synthesis of carotenoids is blocked by inhibitors or mutation.

Another function of carotenoids, however, is the pigmentation of many flowers and fruits. Most of the orange, yellow or red pigments found in these tissues can be attributed to carotenoids, which accumulate in the chromoplasts.

Carotenoid structure

Carotenoids are long chain hydrocarbons. The conjugated double bonds give them their color. The synthetic pathway produces carotenes, xanthophylls and abscissic acid. The desaturations that occur in the biosynthesis of carotenoids can create either a cis or trans configuration. Cyclization at one or both ends can occur (Goodwin, 1980). A beta, epsilon or gamma ring forms based on the position of the double bond (Goodwin, 1980). The end groups can be acyclic in the case of lycopene. If the pigment is a long chain hydrocarbon then it is a carotene. Xanthophylls on the other hand, are carotenoids that contain oxygen.

A number of different modifications are possible to this basic long hydrocarbon chain; 500 different structures have been described. The complement of carotenoids found in green leaves are relatively uniform throughout the higher plant kingdom (Goodwin and Mercer, 1983). This may be expected considering the importance these compounds play in photosynthesis and the conservation of structure required of the proteins and pigments of the thylakoid membranes. However, wide structural variety occurs in carotenoids found in petals and fruit. The type of molecule that accumulates is species dependent.

Carotenoid synthesis

The basic scheme for the synthesis of higher plant carotenoids is shown in Figure 2-1. The production of phytoene represents the first committed step in the synthesis of carotenoids. Usually, step-wise desaturation of phytoene leads to all-trans-lycopene. The normal product of phytoene synthase is 15-cis-phytoene. Where

does the cis-trans isomerization take place? Beyond zeta-carotene, trans intermediates are involved. Four stepwise removals of 2 hydrogen molecules occur from phytoene to lycopene, as oxygen is reduced in this reaction. Proton attack of the C1 double bond of lycopene is involved in the formation of the ring structures. Different enzymes are responsible for the different rings that are formed. Little interconversion of these rings occurs. Xanthophyll formation results from the addition of hydroxyl groups.

Location of carotenoid biosynthesis

Where are the enzymes of carotenoid synthesis located? All activities after the synthesis of isopentyl pyrophosphate (IPP) have been shown to occur in the plastids (Gray, 1987). Compartmentalization of the enzyme activity has been best analyzed in chloroplasts as opposed to chromoplasts, since they are most amenable to fractionation. Mayfield (1986) examined the chloroplasts of spinach, and concluded that phytoene desaturase is located on the inner envelope membrane. Ultrastructural examination of developing chromoplasts suggests that pigments associate with membrane as they are produced in the inner envelope, which is followed by vesiculation of this membrane pigment complex (Camara and Brangeon, 1981). In addition, examination of the lipid component of these membranes confirms that they are a product of the inner envelope (Sitte et al., 1980).

Genetic studies have mapped all the known tomato mutants to the nuclear genome (Tomato Genetic Cooperative Report, 1987), which suggests that the genes involved in the synthesis of carotenoids are nuclear-encoded. Furthermore, inhibition

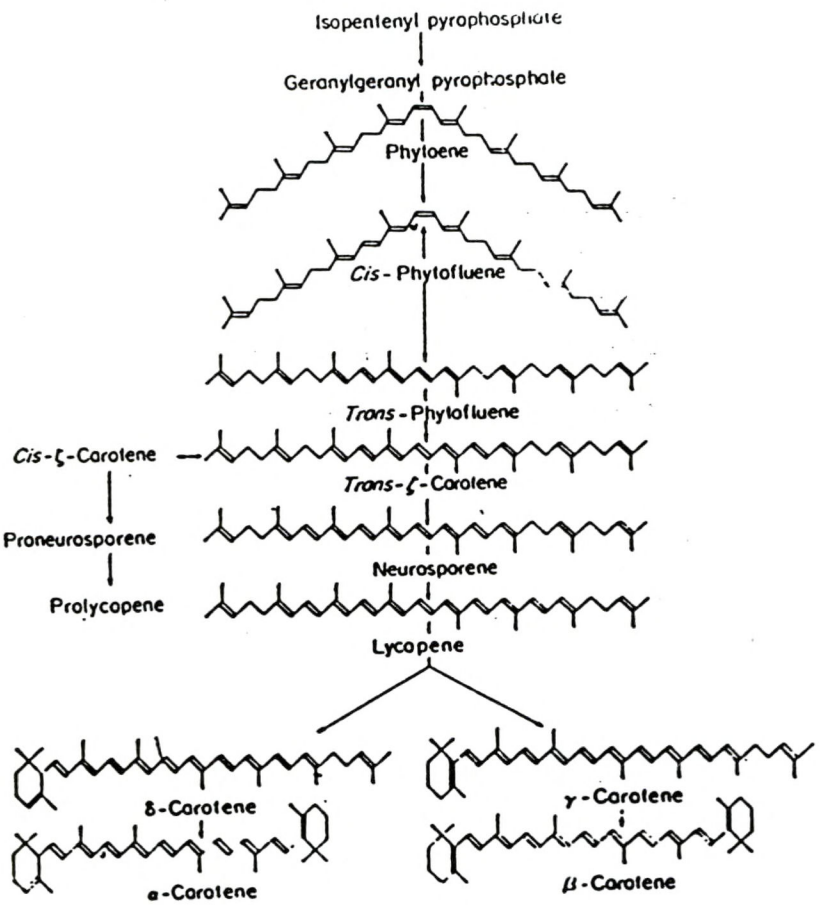


Fig. 2-1 Pathway for the Biosynthesis of Carotenoids in Higher Plants

of plastid translation has demonstrated that this process is not necessary for carotenoid accumulation (Camara, 1984). Finally, the enhanced synthesis of phytoene caused by the addition of CPTA is blocked by the cytoplasmic translation inhibitor cycloheximide. Therefore, the evidence indicates that the enzymes required for carotenoid synthesis are imported into the plastid.

Enzymology of carotenoid biosynthesis in tomato fruit

The purification of the carotenoid biosynthetic enzymes is difficult because all the enzymes in this pathway beyond the synthesis of phytoene are associated with plastid membranes. Consequently, studies that characterize the number and type of enzymatic steps responsible for the synthesis of these compounds in higher plants have relied on adding radioactive precursors to solubilized crude enzyme preparations. The distribution of radioactivity between the different carotenoids is then measured. These experiments have shown that when, for example, red tomato extracts are fed labeled lycopene, cyclic carotenoids are produced (Quershi et al., 1974). This suggests that the enzymes for the production of these compounds are present, but do not normally accept the lycopene that is produced *in vivo*. Perhaps an inhibitor is present *in vivo* that has been diluted out *in vitro*. Alternatively, a spatial separation between these enzymes may exist *in vivo* that is disrupted *in vitro* (Papastephanou et al., 1973). These *in vitro* studies involved the addition of quantities of substrate and cofactors that may drastically differ from the situation *in vivo*. These unexpected results demonstrate that the pathway for the synthesis of

carotenoids is complicated and a more thorough understanding of the production of carotenoids requires analysis in less disrupted systems.

Examination of mutants in the synthesis of tomato fruit carotenoids has predicted that the photosynthetic pigments are produced by different isozymes. A number of tomato mutants have been studied (for review see Goodwin and Goad, 1970). Many have been mapped, their complement of carotenoids has been measured and the position of the genetic lesion in the biosynthetic pathway has been predicted (Goodwin and Goad, 1970; and Khudari, 1972; Tomato Genetic Cooperative Report, 1987). Many of these mutants affect only fruit color, while the photosynthetic pigments found in the leaves are completely normal (Thelander et al., 1986).

Genetic evidence suggests that fruit carotenoids are produced by two separate pathways. One pathway produces the abundant amount of lycopene generally associated with red ripe tomatoes, while the other pathway appears to make a small amount of ß-carotene. Evidence that two pathways exist includes the tomato mutant *apricot*, which is deficient in the accumulation of lycopene without affecting the level of ß-carotene in the fruit (Goodwin, 1980). Additional evidence includes the fact that the production of lycopene is sensitive to high temperatures in normal red tomatoes (Thelander et al., 1986), while the *high ß* mutant has reduced lycopene and enhanced temperature sensitive synthesis of ß-carotene. In other words, the dominant *high ß* tomato seems to have used the same temperature sensitive pathway to augment the levels of ß-carotene. Both the existence of the *apricot* mutant and the residual non-

temperature sensitive amount of ß-carotene found in *high ß* fruit imply that a second pathway exists.

The residual, but ever present, level of total carotenoids found in some mutants, the presence of temperature sensitive ß-carotene, and enzymes that function *in vitro* but fail to accumulate their product *in vivo* suggest that a major ripening enhanced inducible pathway for carotenoid biosynthesis may exist along with a minor, possibly constitutive, path. If the minor pathway is responsible for the production of the cyclic carotenoids, this predicts that enzymes for the production of cyclic carotenoids may not necessarily be enhanced during ripening in normal red tomatoes.

Characterization of carotenoid biosynthesis in bacteria: a model for higher plant analysis

The cloning and subsequent complementation of genes for carotenoid biosynthesis in bacteria demonstrates how powerful such a systematic analysis can be in the understanding of how the pathway functions. These genes are organized into clusters in the bacteria *Erwinia herbicola* and *Erwinia uredovora*. In addition, the carotenoids in these species are similar to the pigments that accumulate in higher plants. A number of investigators have used these genes to transform *E. coli*, *Agrobacterium* and *Zymomonas* (Misawa et al., 1990; Hundle et al., 1991; Misawa et al., 1991; Naagawa and Misawa, 1991). Deletion analysis of the cluster transformed into *E. coli* allowed an unambiguous ordering of the pathway by analysis of the carotenoids that accumulated in the resulting mutants (Misawa et al., 1990).

The power of the characterization of carotenoid biosynthesis in *E. coli* is demonstrated by the analysis of individual genes from different species by Linden et al. (1991). A plasmid containing the genes responsible for the synthesis of phytoene in *Erwinia uredovora* can be transformed into *E. coli* and sequentially cotransformed with a plasmid containing a phytoene desaturase (PDS) gene. The type of pigment that is produced by the specific PDS enzymes can then be monitored. The PDS cDNAs cloned from *Synechococcus*, *Rhodobacter* and *Erwinia uredovora* were tested. Analysis of the carotenoids produced in these transgenic *E. coli* showed that the different enzymes produced a different complement of products. For example, the *Synechococcus* enzyme was able to introduce two double bonds in phytoene while the *Rhodobacter* enzyme introduces three double bonds. This analysis also shed some light on the level of cis-trans isomerizations that accumulate in the various complemented bacteria. The authors concluded that a gradual shift from cis to trans carotenoids occurs with an increase in the number of conjugated double bonds produced by the different enzymes. This suggests that a specific cis-trans isomerase may not be required for this interconversion. Finally, since the *Rhodobacter* PDS adds 3 double bonds, the specificity of the *Erwinia* lycopene cyclase and beta-carotene hydroxylase on the asymmetrical molecule formed could be assessed. This experiment was performed by the introduction of a plasmid containing the entire *Erwinia* gene cluster except for a mutant PDS gene. Cotransformation of this plasmid with the *Rhodobacter* PDS created unique carotenoids. This experiment demonstrated that the cyclase or the hydroxylase recognizes half of the carotenoid molecule. This

type of analysis can allow a careful identification of the number and types of enzymes required to produce a specific complement of carotenoids. The sequential cloning and analysis of the different carotenoid biosynthetic genes in transgenic bacteria demonstrate how powerful such an approach could be for the dissection of this pathway in higher plants. If the higher plant carotenoid genes were cloned, a similar series of experiments could be undertaken.

Molecular biology of carotenoid biosynthesis in higher plants

The gene for phytoene synthesis has been isolated from tomato fruit. Originally, the clone for the phytoene synthase cDNA (pTOM5) was isolated by Slater et al. (1985) simply because it recognized increasing amounts of RNA as ripening progressed. Significant homology to the deduced amino acid sequence of the tomato cDNA, pTOM5, to a clone for phytoene synthase in *Rhodobacter* was identified (Armstrong et al., 1990a). Bartley et al. (1992) used this information to clone a similar cDNA from tomato fruit and provided evidence, by complimenting a mutant *Rhodobacter*, that it indeed coded for phytoene synthase. Recently, a normal red variety of tomato has been transformed with a constituitively expressed antisense version of pTOM5 (Bird et al., 1991). This resulted in yellow fruit and pale flowers. Chlorophyll a and b levels were normal in the leaves of the transformed plants, which suggested that the leaf carotenoids were unaffected by the constitutively expressed antisense message. These results confirm that pTOM5 codes for a phytoene synthase that is specific for ripening fruit. A more complete analysis of the transgenic plants by Bramley et al. (1992) confirmed that antisense pTOM5 alters

carotenoid accumulation in the flower and fruit but not the leaves of transgenic plants.

The gene for geranylgeranyl pyrophosphate (GGPP) synthase has been cloned from pepper fruit (Kuntz et al., 1992). The enzyme was purified, antibody was produced, and a cDNA was isolated from a ripe pepper fruit expression library. Both the mRNA and the protein increased during fruit ripening. Immunological examination has shown that this stromal enzyme is mainly found concentrated around chromoplast plastoglobuli (Cheniclet et al., 1992).

The deduced amino acid sequence from the cyanobacterium *Synechococcus* PDS allowed the cloning of a cDNA from soybean cotyledon by Scolnik's lab (Bartley et al., 1991). Similar work by Pecker et al. (1992) produced a PDS from ripening tomato fruit. An interesting observation resulting from this work is that, while the cyanobacterium and higher plant PDSs share more than 65% deduced amino acid identity, no homology beyond a short amino terminal region that contains a conserved dinucleotide-binding motif exists with the fungal, *Rhodobacter* or *Erwinia* PDS. This suggests that separate PDSs may have evolved for higher plants and green algae in contrast with other photosynthetic organisms. Following a different approach, Camara's lab purified a phytoene desaturase from *Capsicum* and used antibody to isolate a cDNA clone (Hugueney et al., 1992). The deduced amino acid sequence of that clone has striking homology to the tomato fruit sequence. Only slight increases in the steady-state level of RNA that hybridizes to this clone occur at MG. This is in contrast with the analysis of the protein and the enzyme activity,

which increases significantly with ripening. It appears from the evidence provided by both laboratories (Hugueney et al., 1992; Pecker et al., 1992) that the quantity of this mRNA is low during ripening. Perhaps during chromoplast development large increases in mRNA occur only for the early enzymes involved in carotenoid biosynthesis.

Mayer et al. (1992) have purified an oxidoreductase that is specific for chromoplasts and crucial for desaturation of carotenoids. The peripheral nature of the involvement of this enzyme in carotenoid synthesis is supported by the fact that antibody to this protein does not inhibit the synthesis of carotenoids. However, oxidized quinones are required for carotenoid desaturation, so it is assumed that this enzyme acts to affect the redox state of the chromoplast membranes where a large amount of oxidized quinones and tocopherols found. Perhaps the oxidoreductase and the increased amount of antioxidants in pepper chromoplasts point to an environment that contains an escalating amount of reactive oxygen (Romer et al., 1992). The desaturation of phytoene requires the transfer of electrons to oxygen. This might cause an increase in reactive oxygen and lead to the induction of enzymes that generate antioxidants.

Two pathways are predicted for the synthesis of carotenoids in ripening tomato fruit. None of the genes for the enzymes responsible for steps in the pathway beyond the synthesis of zeta-carotene have been cloned. It is interesting that only minor increases in the steady-state level of RNA for PDS occur during fruit ripening. Since large increases in steady-state levels of RNA accumulate for both phytoene

synthase in tomato and GGPP synthase in pepper fruit (Slater et al., 1985; Kuntz et al., 1992), perhaps only the very early steps of this pathway are transcriptionally induced during ripening, while transcript for the later steps will be constitutively expressed at a low level. Conceivably the dominant mutants *del+* and *b+*, which produce increased amounts of δ and ß carotenoids respectively, will contain increased levels of RNA for the the enzymes responsible for these later steps in the biosynthetic pathway.

Chloroplast Senescence

Chloroplast senescence involves the degradation of chlorophyll and the dismantling of photosynthetic membranes. Considering that the analysis of chloroplast breakdown during chromoplast formation has relied on ultrastructural analysis and characterization of mostly the decrease in steady-state levels of photosynthetic proteins, perhaps an understanding of how this process occurs during leaf sensecence will allow predictions for the plastid transition in ripening fruit. Indeed, 7 out of 12 fruit ripening enhanced clones share homology to transcripts that increase in senescing leaves (Davies and Grierson, 1989). During ripening and senescence starch, chlorophyll and photosynthetic proteins degrade (Davies and Grierson, 1989). A burst in ethylene synthesis has been correlated with senescence in leaf tissue (Davies and Grierson, 1989) and respiration also increases.

Photosynthetic protein synthesis during leaf senescence

A decrease in the synthesis of photosynthetic proteins occurs during chloroplast senescence (Roberts et al., 1987). This loss of proteins follows a similar pattern as described for chromoplast development by Piechulla. The synthesis of protein in different ages of *Phaseolus* leaves was measured, and the amounts of cytochrome f, cytochrome b_6, a and b subunits of the ATPase, 68kD PSI protein, and LHCP decline in leaves. However, the 32 kD protein persists throughout senescence.

Ultrastructural analysis of chloroplast senescence

Plastid senescence is a gradual ordered process, which occurs within the plastid. It was initially assumed that chloroplasts were engulfed and degraded within vacuoles (reviewed by Gepstein, 1988). These early studies concluded that senescing leaves actually contained fewer plastids. However, reappraisal of this work suggested that the isolation of fewer plastids in senescing leaves was due to the increased difficulty in isolating these fragile organelles (reviewed by Gepstein, 1988). Additional ultrastructural analysis showed that plastids in senescing leaves undergo a transition in which thylakoids become unstacked and plastoglobuli accumulate (Gepstein, 1988). This suggests that the degradation of plastid components during senescence occurs within the plastid itself (Thayer et al., 1987), and is gradual and ordered. The plastid envelope maintains its integrity until very late in senescence, long after the degradation of thylakoid membranes is complete (Thomson and Platt-Aloia, 1987). Furthermore, this means that if enhanced degradation of plastid

components occurs during sensecence then it is possible that new proteins would be imported into the plastid for this purpose.

The degradation of chlorophyll during chloroplast senescence

The degradation of chlorophyll has been suggested to be the trigger for the proteolysis of the protein components of the thylakoid membranes. Unfortunately, the pathway for the catabolism of chlorophyll is not well understood. Work with the non-yellowing mutant of the temperate grass *festuca* suggested that an initial step in thylakoid degradation involves dephytlation of the chlorophyll molecule while it remains in the pigment-protein complex (Thomas et al., 1989). A polar green chlorophyll degradation product can be isolated from purified light harvesting complexes (LHCs) of these mutant plastids. This degradation product is also produced in normal plastids exposed to anoxic conditions. The buildup of this intermediate occurs in the mutant plant because a downstream step in the chlorophyll catabolism pathway is missing. Since the product could be isolated from purified LHCs the authors concluded that the first step in the degradation of chlorophyll occurs within the protein-pigment complexes of the thylakoid membrane. Chlorophyllase has been isolated from chloroplasts and could perform this function (Matile et al., 1989). However, the protein does not seem to increase during senescence. Alternatively, the enzyme could remain latent in the plastid and be activated when necessary. Perhaps the activation of the chlorophyllase requires a newly synthesized protein?

The next step in the catabolism of chlorophyll is probably an oxidation. However, it is not clear whether this occurs in the plastid or vacuole (Matile et al., 1989). One theory suggested that a thylakoid associated chlorophyll oxidase is induced by an increase in linolenic acid during senescence (Luthy et al., 1984), which triggers the catabolism of chlorophyll and the resulting cascade of degradative reactions. This idea may require reappraisal in light of the fact that oxidized pyyroles have been found in vacuoles and not isolated from senescing plastids (Matile et al., 1989).

Degradation of the thylakoid membranes during chloroplast senescence

The analysis of stay-green mutants in soybean and the previously described mutant in *festuca* demonstrates that the degradation of the thylakoid membrane is mediated by at least two separate pathways and is the result of both nuclear and plastid-encoded proteins.

For example, the cytoplasmic mutant cytG of soybean specifically retains the (LHCs) (Guiamet et al., 1991). Clearly the breakdown of the core proteins involves a separate path from the degradation of the LHCs. Since chlorophyll a and b are still catabolized in this plant, these data suggest that a plastid-encoded protease is lacking and is involved in LHC breakdown.

When two homeologous recessive nuclear-encoded genes (d_1d_2) of soybean are present in this tetraploid plant, most of the chlorophyll is retained and all of the thylakoid proteins appear to be present, in contrast with nonmutant leaves (Guiamet

et al., 1991). These data imply that the degradation of the thylakoid membrane in soybean is also mediated by nuclear-encoded genes.

Evidence from the mutant *festuca* underscores the complexity of the degradation of thylakoids. The phenotype of this recessive mutant is a selective maintenance of components of the photosynthetic membranes. For example, the level of LHCP is maintained in the mutant plant long after the amount of this protein has begun to diminish in normal plants (Thomas and Hilditch, 1987). Additionally, the degradation of the plastid D1 protein, which normally turns over in either the light or the dark, is inhibited in the dark, but not the light (Thomas and Hilditch, 1987). The 33Kd protein, however, degrades normally in this mutant (Thomas and Hilditch, 1987). These mutant plants are also able to photobleach if they are subjected to high light intensities (Thomas and Hilditch, 1987). These data suggest that two separate protease systems are responsible for the degradation of thylakoid membranes. One system is light activated and unaffected by this mutation and another functions in the dark and is inhibited by this genetic lesion.

Degradation of Rubisco

An examination of the degradation of Rubisco suggests how a stromal protein may be broken down during senescence. The breakdown of Rubisco can be induced by oxidative stress, in a process that appears to mimic natural senescence (Mehta et al., 1992). A specific and highly conserved cysteine is oxidized by the addition of $CuSO_4$ and leads to dimerization of the protein. This is followed by a transient accumulation of this stromal protein on the membrane, and, finally, degradation. *In*

vitro studies have also demonstrated that the oxidized form of Rubisco is inactive and more susceptible to proteolytic digestion (Penarrubia and Moreno, 1990). This suggests that the turnover of stromal protein is selective and may involve membrane associated proteases. It is uncertain whether this response to oxidative damage is analogous to senescence, however, it does demonstrate that an ordered system of stromal protein degradation exists in the plastid. It remains to be seen if natural senescence involves a build up of oxidized protein that enters into this degradation pathway, or if it is developmentally manifested in some other manner.

Changes in other components of the chloroplast during senescence

A decrease in the level of galactolipids has been noted in senescing chloroplasts (Woolhouse, 1984). However, Woolhouse (1984) pointed out that it is uncertain whether this change in lipid content is caused by decreased biosynthesis or increased degradation.

The isolation of proteases involved in plastid protein degradation has been fairly unsuccessful (Dalling, 1987). The majority of proteases are found in the vacuole, and their presence masks an examination of changes in plastid proteases during senescence.

Control of chloroplast senescence

Overall, plastid senescence requires continued protein synthesis, since the process can be inhibited by cycloheximide (reviewed by Gepstein, 1988). Although plastid senescence can be triggered by a number of environmental stimuli such as darkness, oxidative compounds, or nitrogen deprivation, it is still unclear whether this

process is mediated by an increase in de novo synthesis of specific proteins. In the case of Rubisco, turnover is slow until a nuclear encoded event (assumed since it is inhibited by cycloheximide or kinetin) triggers the rapid degradation of this abundant photosynthetic protein (Thayer et al., 1987).

Perhaps the breakdown of chlorophyll acts as a signal for the degradation of photosynthetic membrane proteins (Nock et al., 1992). This has been suggested by the results from studies of the mutant *festuca* where a mutation in chlorophyll catabolism inhibits the breakdown of the protein components of the thylakoid membranes. The production of the dephytlated chlorophyll degradation product requires protein synthesis (Thomas et al., 1989), which implies that the synthesis of a cytoplasmic protein is required for induction of chloroplast senescence.

Conclusions about chloroplast senescence

To date, ultrastructural analysis has suggested that the senescence of chloroplasts is a gradual event within the organelle. The degradation of plastid proteins is a complex process involving multiple proteases with different properties. Since the breakdown of chloroplasts is inhibited by cycloheximide, this suggests that senescence requires continued protein synthesis (Gepstein, 1988). Additionally, the rapid degradation of Rubisco during senescence implies that this upsurge in protein loss may require a de novo increase in proteins involved in this degradative process rather than simply continued constitutive expression.

It is difficult to draw generalizations about what may trigger senescence from the analysis of the few mutants available. The mutant phenotype could simply result

from the accumulation of particular plastid components due to loss of a single function and may not represent a protein that is developmentally induced during senescence. It seems safe to assume that the non-yellowing *festuca* demonstrates that although the thylakoid membranes can degrade in response to photooxidative stress, the more gradual and perhaps dark associated breakdown requires proteases that are not active until the pigment component has been modified.

It would be extremely helpful if proteins or the genes responsible for the senescence of chloroplasts could be isolated. An understanding of their regulation could suggest how senescence is controlled. It is possible that the conversion from chloroplast to chromoplast may represent an ideal system for the isolation and identification of cDNA clones for proteins involved in this degradative process since the chromoplast transition does not take place in a sensecing tissue.

Heat Shock Proteins and Oxidative Stress

The identification of a chromoplast-associated low MW heat shock proteins (hsp) during this study provoked an investigation into the literature on why this hsp may have been induced during the chloroplast to chromoplast transition. One possibility is that the low MW hsp is induced during certain developmental stages. Historically, the induction of low MW hsps occurs during times when rapid restructuring of the tissue is underway (Arrigo, 1987). It is also feasible that the hsp is induced because the tissue is enduring an oxidative stress. Hsp induction by compounds that create oxidative damage has been described in animal tissues

(Drummond and Steinhardt, 1987; Cajone and Bernelli-Zazzera, 1988; Courgeon et al., 1988). Both the induction of hsps during development and oxidative stress will be considered.

Introduction to Heat Shock Proteins

Heat shock proteins were first identified by their massive induction upon increases of about 10^0C above ambient temperature (recently reviewed for plants by Vierling, 1991; and generally by Jaattela and Wissing, 1992). Hsps have been found in a diverse range of organisms from bacteria to mammals. These proteins are broadly categorized by their molecular weight in SDS-PAGE. An individual organism may contain a number of spatially and functionally distinct proteins from each size class. For example, a number of different hsp 70 proteins have been described, some of which are present constitutively (hsc).

The function of these proteins has been best characterized for the constitutive members of the hsp60 and 70 classes. The 60 kD protein plays a role in the assembly of the multisubunit Rubisco enzyme complex (Roy, 1989). Involvement in the assembly of multimeric proteins in the mitochondria has also been shown (Cheng et al., 1989). The 70 kD proteins have been implicated in moving newly synthesized proteins from ribosomes to the cellular compartment in which they function (Weber, 1992). The role of the hsps during heat shock has been inferred from the function of their constitutive forms. It is thought that the hsps bind to proteins denatured by superoptimal temperatures and may facilitate refolding (Weber, 1992). A role for

hsps in providing thermotolerance has been suggested by experiments that create protection from severe heat shock by the induction of these proteins with a brief or moderate increase in temperature (reviewed by Lindquist and Craig, 1988).

The induction of hsp messages is rapid and transient (reviewed by Neumann et al., 1989). Examination of the steady-state level of RNA for a number of different hsps in soybean revealed that levels decay more quickly at normal temperatures than during heat stress and the absolute amount of hsps induced is lower in plants that have been previously exposed to a small heat stress (Kimpel et al., 1990). This is reminiscent of the thermotolerance response in that the reaction to heat shock is reduced by heat pretreatment. The transient nature of this response is apparent in the fact that the hsp message had already begun to decay while the temperature was still high, and the message was undetectable within 17 hours after the peak temperature (Kimpel et al., 1990). The induction of heat shock proteins in field grown plants has also been demonstrated (Burke et al., 1985; Kimpel and Key, 1985). Kimpel and Key (1985) found that a transient increase in the message for both low and high MW hsps occurs under fairly normal Georgia summer field conditions with soybean. This induction is dramatically enhanced in non-irrigated plants. Burke et al. (1985) also showed similar responses in material from irrigated versus non-irrigated fields. However, they compared the canopy temperatures and found that the non-irrigated field was 10^0C above the temperature in the irrigated field. This is an important point since the quantity of hsp message produced increases with increasing temperature (Chen et al., 1990; Kimpel et al., 1990).

Low MW Heat Shock Proteins

In plants the low MW hsps are generally not present at detectable levels in leaves under nonstress conditions (Vierling, 1991). The low MW hsps represent a small multigene family that has been subdivided on the basis of amino acid sequence similarity (Vierling, 1991). Classes of this family have been found in the cytosol, plastid, and endoplasmic reticulum. In fact, one class of low MW hsp is induced during meiosis (Bouchard, 1990). Upon induction, the low MW hsps can accumulate to massive amounts and form large crystalline deposits within the cytoplasm.

Low MW Heat Shock Proteins and Development

The low MW hsps have been found to increase transiently at different times during development. The best characterized developmental induction of these proteins is found during *Drosophila* embryonic development. Arrigo (1987) suggested that the period of development in which these proteins are most highly induced represents a time of massive developmental change when the cell may need carefully controlled mechanisms for overall transformation. Four low MW hsps (ranging in size from 22 to 27 kD) follow a complex pattern of expression that appears to be mediated by the molting hormone ecdysterone. Addition of this hormone to embryonic cell lines induces these proteins (reviewed in Bond and Schlesinger, 1987). Deletion analysis of their promoters indicated that regions of the DNA responsive to hormone and heat induction reside in separate portions of the gene. The same hsp is, therefore, induced developmentally and by heat stress. Since heat causes

destabilization and unfolding of proteins and embryo development suggests rapid protein biogenesis, the induction of the same group of proteins during both events implies that they are involved in either stabilizing or degrading incorrectly folded proteins.

Evidence for a developmental change in low MW hsps has begun to accumulate in higher plants. The 14 and 40 kD hsps are present in dry embryos of wheat and their mRNA has also been isolated from quiescent wheat embryos not subjected to heat stress (Helm and Abernethy, 1990). These authors propose that these hsps may act to protect the seed early in development from the effects of excess heat. Since the germinating wheat embryo has fewer mechanisms to control ambient temperature in comparison to an older seedling, maybe the production of hsps allows the organism to tolerate this transiently harsh environment (Helms and Abernethy, 1990).

The wheat embryo hsps also demonstrate how the genes for hsps may respond differently to heat stress at different stages of development. These proteins are heat inducible at early times during imbibition. However, they are not induced after as little as 12 hours of imbibition (Helm et al., 1989). The wheat seed is initially quite tolerant to heat stress, such that pretreatment with a low but hsp inducing temperature does not improve this thermotolerance (Abernethy et al., 1989). By 9 hours of imbibition, however, a pretreatment can improve the survival of the seed to heat stress (Abernethy et al., 1989). Another interpretation of these data from wheat, however, could be that rather than protecting the young embryo from heat

stress perhaps the hsps are present in preparation for development and consequently, additional induction by heat may be unnecessary.

Messages for low MW hsps have been found in dry embryos in other plant species. The mRNA to a 17.6 kD hsp is present in dry seed and accumulates during embryo development of sunflower (Almoguera and Jordano, 1992). This cDNA also hybridizes to mRNA from 3-day-old seedlings subjected to ABA, mannitol or heat stress. The mRNA for an alfalfa hsp 18.1 has been found to be present in microcallus suspension and increases transiently during embryo formation at normal temperatures (Gyorgyey et al., 1991). This cDNA also hybridizes to RNA from callus subjected to $CdCl_2$, sucrose and heat stress. These data demonstrate that transcripts for low MW hsps have been found in plant embryos and appear to be developmentally produced at nonheat stress temperatures. However, it is unclear from these data whether the transcript induced by stress is encoded by the same developmentally induced gene or represents another member of a multigene family.

Plastid-Localized Low MW Heat Shock Proteins

A structural analysis of plastid-localized low MW heat shock proteins may suggest a possible function. Chen and Vierling (1991) have compared the deduced amino acid sequences of 5 of these plastid proteins, and have reported three conserved regions. Two of these regions are also present in the low MW hsps found in the cytoplasm. A third carboxyl terminal consensus region, which is abundant in methionine, is unique to the plastid-localized low MW proteins. Computer analysis

of the tertiary structure of the protein in this methionine rich region suggested that an alpha helix with a hydrophobic and hydrophilic face could form (Chen and Vierling, 1991). This same type of three dimensional structure is predicted for three such methionine rich regions of the SRP 54 (signal recognition particle), which is involved in transporting newly synthesized proteins in the endoplasmic reticulum (Lutke et al., 1992). Careful structure/function analysis of this region in SRP 54 and its interaction with signal peptides using crosslinking reagents, antibodies produced against specific fragments of the SRP 54 and deletion analysis of this protein revealed that binding of signal sequences of nascent proteins occurs here (Lutcke et al., 1992). Binding experiments with the 7S RNA showed that this same region is involved in interaction with the RNA. High and Dobberstein (1991) suggested that the SRP 54 interacts with signal peptide on the methionine rich hydrophobic face and with RNA on the hydrophilic face. It is tempting to speculate that the low MW plastid-localized hsp is involved in a similar protein-protein interaction. Perhaps the need for these hsps is increased during times when excessive incorrectly folded proteins are present.

The sublocalization of the plastid-localized low MW hsp has been controversial. Work by Kloppstech and colleagues suggested that this hsp becomes associated with thylakoid membranes upon heat stress (Kloppstech et al., 1985). However, this occurs only with plants grown at low light intensities (Glaczinski and Kloppstech, 1988). Binding of hsp to the membrane occurs most abundantly at a light intensity below the level at which net carbon is fixed. Considering the transient

nature of these proteins, and that their transcription usually requires heat stress, this combination of circumstances could occur at dusk in summer or on the floor of a tropical rain forest. Chen et al. (1990) have shown that under heat stress and higher light levels this hsp remains predominantly in the stroma. They reported that the only plastid-localized low MW hsp from pea associates into 200 kD stromal complexes. Clarke and Critchley (1992) showed that one of the two low MW plastid-localized hsps, the 32 kD form, from heat stressed barley leaves associates into 250 to 265 kD complexes within the stroma and contains no RNA, DNA or the α-subunit of the Rubisco binding protein. This protein is reminiscent of an hsp 28 found in soybean plastids, which also contains two low MW hsps of 22 and 28 kD (Clark and Critchley, 1992).

The idea that the methionine rich region could interact with denatured proteins during heat stress suggests that these complexes could sequester unfolded proteins. The hsp 60 also associates into an 800-900 kD oligomer of 14 subunits (Roy, 1989). Perhaps the role of the plastid-localized low MW hsp will be easier to resolve if, like the hsp 60 and 70s, a constitutive or developmentally associated form can be identified. Maybe the chromoplast-associated low MW hsp described within this work is induced by the degradation of proteins for the photosynthetic apparatus that occurs between mature green and intermediate stages of fruit ripening.

Induction of Heat Shock Proteins By Other Stresses

Hsps can also be induced by heavy metals, ethanol, amino acid analogues and glucose starvation (Vierling, 1991). Arsenite and cadmium produced the greatest

induction of hsps (Czarnecka et al., 1984; Edelman et al., 1988). At lower concentrations of these compounds, the response, like the heat stress response, was also transient. By 12 hours of exposure, the messages had begun to diminish (Edelman et al., 1988).

Oxidative stress and the induction of hsps

The initial correlation between heat and oxidative stress was made in an examination of *Drosophila* salivary glands. Puffs, representing high levels of transcription, may occur at the same loci along the chromosome when either a heat or oxidative stress is applied (reviewed by Loven, 1988). A combination of heat treatment and application of an oxidizing agent can act synergistically to reduce the deleterious effect of a subsequent heat stress (Issels et al., 1987). This thermotolerance developed from oxidative stress inducers can be blocked by the addition of catalase during the induction period (Issels et al., 1987).

Studies in *Drosophila* cells (Drummond and Steinhardt, 1987; Courgeon et al., 1988) and rat hepatocytes (Cajone and Bernelli-Zazzera, 1988) show a mild induction of some hsps by oxidative stress. In addition, different hsps were induced by different chemicals (Cajone and Bernelli-Zazzera, 1988). Hsps are also induced by oxidative stress in *Drosophila* cells (Courgeon et al., 1988) where hsp 70 and hsp 23 were induced by hydrogen peroxide. These experiments point to the conclusion that oxidative stress and heat shock, which induce many of the same proteins, do not provoke an identical response.

Heat stress and the induction of free radical scavenging enzymes

It still is not entirely clear how heat and oxidative stress are related. Heat shock uncouples oxidative phosphorylation and this creates an increased level of reactive oxygen species (Loven, 1988). Additionally, a link has been made between the application of a heat stress and an induction of free radical scavenging enzymes. A hyperthermia induced increase in SOD, CAT and glutathione levels occurs with exercise training of skeletal muscle (reviewed in Barja de Quiroga, 1992). The implication is that these antioxidants are induced by mild heat stress produced during exercise. These additional antioxidants reduce the long term deleterious effects of free radicals produced in this tissue from the uncoupling of oxidative phosphorylation.

The heat induced induction of plant SOD was also noted by Tsang et al. (1991) in tobacco. The cytosolic form of SOD was induced most dramatically compared with the mitochondrial or plastid form in tobacco. This infers that plants also may respond to heat stress by inducing enzymes involved in oxidative stress.

Conclusions about the relationship of heat and oxidative stress

Since both heat and oxidative stresses produce a similar response, a number of hypotheses have been developed to explain how they are related. Some authors have suggested that the deleterious effects of heat stress are due solely to oxidative damage (Loven, 1988). This idea may be a bit extreme in view of the fact that hsps can be induced under anoxia in *Drosophila* cells (Drummond and Steinhardt, 1987). Rather than the induction of antioxidants, therefore, this type of assertion requires

that direct oxidative damage be measured, which can be done by examining the amount of lipid peroxidation. However, few studies have been preformed in which hyperthermia is directly correlated with oxidative stress (Barja de Quiroga, 1992). A corollary to this idea was developed in Ame's lab (Lee et al., 1983), in which the synthesis of bisnucleoside polyphosphates, also termed alarmones, acts as the signal for oxidative stress induction of hsps. Apparently an increase in these compounds is observed during both of these stresses. However, it was soon demonstrated that the synthesis of alarmones is inhibited by heat stress rather than induced. A buildup in these compounds occurs because their degradation is actually inhibited by heat. Addition of alarmones to cells was also shown to have no effect on the induction of hsps and hsps could actually be induced without an increase in the amount of these compounds (Guedon et al., 1986).

The hypothesis that is currently favored for the induction of hsps by oxidative stress is that both heat and free radicals create aberrant proteins that lead to the production of hsps (Neumann et al., 1989). This probably explains why the two stress responses do not completely overlap. Since hyperthermia creates unstable proteins and uncouples oxidative phosphorylation, the increase in free radicals and the resulting response may occur secondarily. Oxidative stress may mainly induce free radical scavengers, which can forestall the production of large numbers of aberrant proteins.

Conclusions

The molecular characterization of fruit ripening has shown that the events that characterize the maturation of tomato fruit involve the de novo synthesis of a subset of ripening associated transcripts. At this point, however, only a few genes involved in this process have been identified and their mode of regulation assessed.

Even though the conversion of chloroplasts into chromoplasts involves the degradation of chlorophyll and the breakdown of thylakoid membranes along with a rapid synthesis of carotenoids, only a small number of genes have been identified that encode proteins that may be involved in this transformation. To date, two genes in tomato fruit and one in pepper that code for proteins involved in carotenoid biosynthesis have been isolated. In addition, only a single gene for synthesis of cysteine synthase has been identified in pepper chromoplasts. Therefore, it is unclear how many proteins may be involved in this process and whether their synthesis increases during development.

Historically the characterization of carotenoid biosynthesis in higher plants involved following the incorportaion of radiolabeled precursors into crude enzyme preparations or the identification of the compliment of carotenoids produced by mutants in the biosynthetic pathway. This type of examination was complicated by the presence of multiple pathways and by the complexity inherent in assays of disrupted tissue. The analysis of carotenoid biosynthesis, as demonstrated in bacteria, in which individual genes can be cloned and the activity can be assessed by complementation in *E. coli* may now be possible in higher plants. In this way, an

unambiguous assessment of the role an individual enzyme plays on the synthesis of carotenoids can finally be evaluated. The determination of how many enzymes are involved in the biosynthesis of fruit carotenoids can be determined. In addition, the cloning and characterization of the genes involved in this pathway will allow an understanding of how the synthesis of carotenoids is controlled during chromoplast formation.

The senescence of chloroplasts in leaves involves the breakdown of photosynthetic structures, in a gradual process within the plastid. It is clear from evidence accumulated from the analysis of stay-green mutants that the degradation of thylakoid membranes involves at least two different systems of protein degradation, portions of which are encoded in both the nucleus and the cytoplasm. However, it is uncertain whether de novo synthesis of new proteins occurs during chloroplast senescence in the leaves.

During the transition of chloroplast to chromoplast in pepper chromoplasts, an increase of compounds that scavenge free radicals occurs. Since the induction of hsps has been observed during oxidative stress, it is possible that the induction of an hsp during fruit ripening is caused by such an increasingly harsh environment. Clearly, the ripening-associated hormone ethylene can induce SOD in tomato and tobacco leaves. However, the evidence for an increase in enzymes involved in oxidative stress in tomatoes remains contradictory. More information is needed before conclusions can be drawn about what role oxidative stress plays during tomato fruit ripening.

Clearly the transition within the developing chromoplast involves a rapid degradation of proteins. The period between mature green and yellow fruit appears to be one in which a particularly rapid degradation of plastid proteins occurs. Since an increase in hsps has been associated with developmental stages where a great deal of structural change occurs, the chloroplast to chromoplast transition may be an ideal environment for the induction of these proteins.

CHAPTER 3
CHROMOPAST-TARGETED PROTEINS IN TOMATO FRUIT

Introduction

The transformation of chloroplasts into chromoplasts results in a dramatic alteration of organellar structure. The thylakoid membranes are dismantled in a ordered process while carotenoids build up within the plastid (Thelander et al., 1986). The structural changes that occur during this transition have been documented by electron microscopy (Harris and Spurr, 1969). However, it is unclear how this event is regulated.

One of the objectives of this project was to determine whether the development of chromoplasts during tomato fruit ripening is mediated by an increase in nuclear-encoded transcripts of chromoplast-associated proteins. Since plastid transcription and translation is greatly reduced during chromoplast development (Kobayashi, 1991) we have focused on proteins that are nuclear-encoded, translated in the cytoplasm and imported into the plastid. The import of proteins into plastids is highly specific and requires an amino-terminal transit peptide, which is necessary and sufficient for targeting into the organelle (Keegstra, 1989). We have monitored chloroplast import of *in vitro* translation products from RNA of tomato fruit isolated at different ripening stages. We have also examined translation products from

transcripts encoded from cDNAs (slater et al, 1985) whose steady-state RNA level increases during ripening.

Materials and Methods

Plant Material

Tomato plants (*Lycopersicon esculentum* Mill. cv. Rutgers) were grown in a greenhouse in Gainesville, Florida. Fruit were harvested at four ripening stages. MG1 was characterized by full-size green fruit with firm locular tissue; MG3 fruit had completely developed locular gel; the 20-50% ripe fruit were red over less than half of the outer pericarp; and >80% ripe fruit were red over 80% of the outer pericarp. The pericarp was frozen in liquid nitrogen immediately after harvesting.

Preparation and Translation of RNA

Total RNA was extracted by combining the protocols of McCarty (1986) and Grierson et al.(1985). Briefly, this involved grinding the frozen tissue with a coffee grinder and homogenizing in extraction buffer with a polytron. The extraction buffer consisted of 100 mM Tris-HCl pH 9.0, 200 mM NaCl, 5 mM DTT, 1% SDS and 20 mM EDTA. The insoluble material was pelleted and reextracted with 1/2 volume of extraction buffer. The supernatant was extracted 2 times with phenol/chloroform (1:1) and one time with chloroform. The final supernatant was brought to 0.1M KCl and precipitated overnight with 2 volumes of ethanol at 4^0C. The precipitate was

washed several times as described in Grierson et al. (1985) and the soluble material was applied to a cellulose (Sigmacell type 50) column to remove substances that interfere with extraction of poly $(A)^+$ RNA (Grierson et al., 1985). Poly $(A)^+$ RNA was isolated from total RNA by one passage over oligo(dT)-cellulose (BRL).

Individual pTOM clones pTOM 5, 25, 36, 41, 92, 111, and 114 (Slater et al., 1985) were subcloned into the *in vitro* transcription vector pSport (BRL) at the *PstI* site. Plasmid DNA was isolated by the alkaline lysis method of Lee and Rasheed (1990). The plasmid DNA was linearized and transcribed with either SP6 polymerase or T7 polymerase as described in Cline (1988). The poly $(A)^+$ RNA and the *in vitro* transcribed RNA were translated (see Figure legends for specific experiments) with either a wheat germ system (Cline, 1988) or a rabbit reticulocyte lysate system from Promega (Madison,Wi.) in the presence of $[^{35}S]$-methionine.

Chloroplast Isolation and Import Assay

Chloroplasts were isolated from pea (*Pisum sativum* L. cv Laxton's Progress 9) shoots by a combination of differential centrifugation and Percoll gradient centrifugation (Cline et al., 1989). Import assays were conducted as described by Cline (1988) except that translation products were diluted 1:3 with unlabelled methionine in import buffer; 50 µl of the diluted translation products were added to 100 µl of chloroplasts (1 mg chlorophyll/ml). Import assays were conducted for 20 min at 25^0 with white light and 10mM Mg-ATP. After incubation, intact chloroplasts were reisolated by centrifugation through 35% Percoll with or without a treatment

of the protease thermolysin to remove surface bound proteins (Cline, 1988). Repurified plastids were lysed in 30 µl of 10mM Hepes/KOH pH 8.0. Experiments testing the requirement of ATP were conducted in foil wrapped tubes to maintain darkness.

Subfractionation and Treatment of Plastids after Import

In experiments examining import of translation products from total poly $(A)^+$ RNA, the repurified plastids were lysed in 10mM Hepes/KOH pH 8.0 and subsequently subfractionated into stromal (soluble) and membrane (pellet) fractions by centrifugation for 10 min at 10,000 x g. In experiments with translation products from individual cDNA clones, the soluble fraction was isolated from the membranes by lysing the plastids in 10mM Hepes/KOH pH 8.0 on ice for 5 min and centrifuging 30 min at 37,500 x g. The membranes were washed one time with 1 ml of 10mM Hepes/KOH pH 8.0 and resuspended in the same solution. Alternatively, the membrane fraction was washed with 0.1 N NaOH (Cline, 1986). Envelopes were separated from thylakoid membranes as described in Cline (1986), however, 1.3 ml of plastids (1 mg chlorophyll/ml) were used for each import reaction.

Sensitivity of the imported proteins to thermolysin was tested as follows: A standard import assay was carried out and divided into two equal fractions. These fractions were either resuspended in import buffer or lysed with 10mM Hepes/KOH pH 8.0. Aliquots of 25 µl (1mg chlorophyll/ml) were incubated with 0 or 20 µg of thermolysin for 45 min at 4^0 C. EDTA was added to 5mM and the samples were

immediately frozen. An equal volume of 2X-SDS PAGE buffer (Cline, 1986) was added to freshly thawed samples and the tubes were heated to 98^0 C for 3 min.

Analysis of Import Products

Import products from total poly $(A)^+$ RNA were examined by SDS-PAGE and fluorography on 7.5%-20% gradient gels (Cline, 1986). Gel lanes received equivalent amounts of protein as determined by chlorophyll estimation of the recovered plastids. Import products from *in vitro* transcribed RNA were examined on 12.5% SDS PAGE gels.

Results and Discussion

Profiles of *in vitro* translation products of poly $(A)^+$ RNA differed markedly between ripe fruit and mature green tissue (Fig.3-1A). The profiles of the two later ripening stages closely resembled one another, as did the translation products from the two samples of mature green tissue. Speirs et al. (1984) reported a similar result from a more detailed analysis of the polypeptide profiles of total mRNA from ripening tomato fruit.

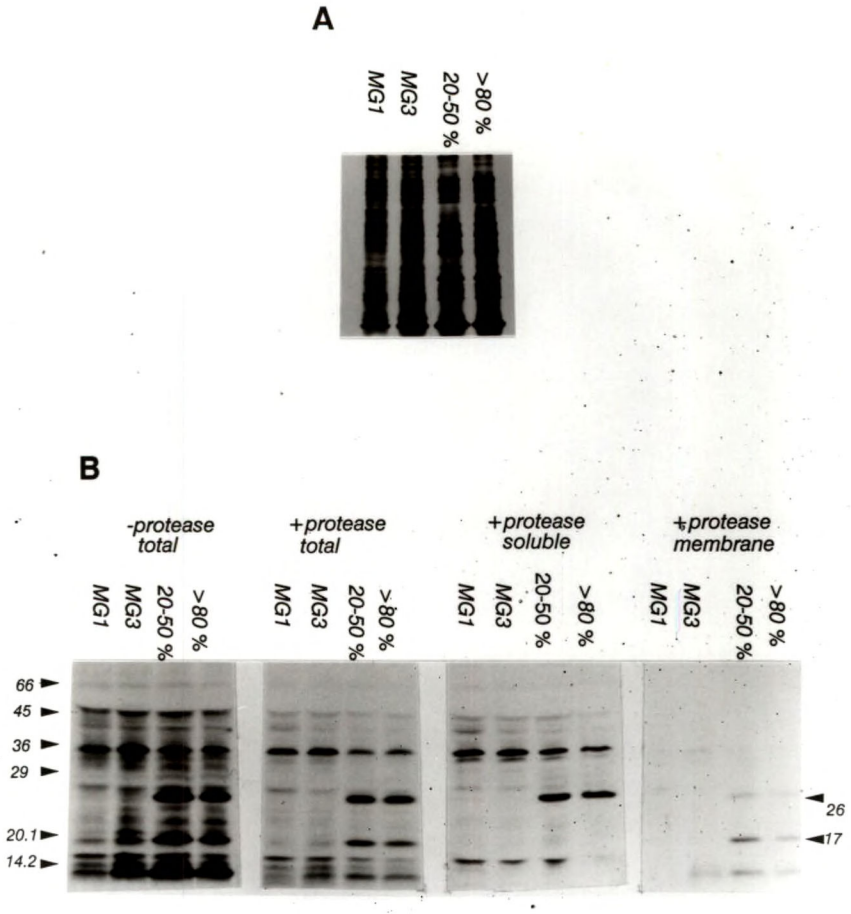
Pea chloroplasts were chosen to analyze import into plastids because of their ease of isolation. The isolation of tomato chromoplasts has been reported by others (Iwatsuki et al., 1984; Bathgate et al., 1985; Hunt et al., 1986; Wrench et al., 1987), but we were unable to isolate pure and intact fruit chromoplasts that withstand the handling required to demonstrate successful import. However, *in vitro* import of an

amyloplast specific protein into chloroplasts (Klosgen et al., 1989) and the *in vivo* import of a chloroplast protein into non-green plastids using transgenic plants (deBoer et al., 1988) implied that chromoplast proteins should also be imported into pea chloroplasts.

Changes in the profile of imported proteins during chromoplast development were detected (Fig.3-1B). A number of proteins decreased during this period (Fig. 3-1B). For example, a 30kD and a 14kD soluble fraction protein diminished as ripening progressed. Two imported translation products dramatically increased in the later ripening stages. A 26 kD protein fractionated with the soluble plastid proteins, whereas a 17 kD protein was associated with the membrane fraction. These results imply that there is synthesis and import of new proteins during the transition from chloroplast to chromoplast and that the plastid conversion may be an active developmental program rather than a simple decline in synthesis of the photosynthetic apparatus.

We next asked whether an import assay could be used to identify individual clones coding for chromoplast-targeted proteins. A specific cDNA clone, pTOM5, was selected to evaluate this system. Slater et al. (1985) originally isolated this cDNA because its RNA becomes more abundant as ripening proceeds. Armstrong et al. (1990) recognized that the deduced protein sequence shares approximately 25% identity with the predicted polypeptide of a bacterial prephytoene synthase. Bird et al. (1991) confirmed the involvement of pTOM5 in carotenoid synthesis by creating

Figure 3-1. Profiles of *in vitro* translation products and import products are altered during tomato fruit ripening. A, *In vitro* translation of total poly $(A)^+$ RNA with four different ripening stages from tomato fruit pericarp. 1 ug of total poly $(A)^+$ RNA per 50 ul reaction was translated *in vitro* in a wheat germ system in the presence of $[^{35}S]$-methionine. Translation products were examined by SDS-PAGE and fluorography on 7.5%-20% gradient gels. (MG1) mature green 1, (MG3) mature green 3, (20-50%) 20-50% red over the outer pericarp, (>80%) greater than 80% red over the outer pericarp. B, Import into pea chloroplasts with *in vitro* translation products of total poly $(A)^+$ RNA extracted from tomato fruit pericarp at four different ripening stages. Import assays were conducted and fractionated as described in Materials and Methods. The (soluble) fraction was isolated from the (membranes) of the re-purified lysed plastids by centrifugation for 10 min at 10,000 x g. Equivalent amounts of plastid protein (as determined by chlorophyll estimation) from the +/- protease treated plastids were separated on 7.5%-20% SDS-PAGE gradient gels and fluorographed. $[^{14}C]$-labelled 10 to 70 kD molecular weight standards (Sigma) appear as arrows on the left side of the figure. The size of the two import products were derived from a standard curve and are designated by arrows on the right side of the figure.


A
MG1
MG3
20-50 %
>80 %
B
-protease total
+protease total
+protease soluble
+protease membrane
MG1
MG3
20-50 %
>80 %
66
45
36
29
20.1
14.2
26
17

an antisense pTOM5 tomato transformant that produces yellow rather than normal red fruit. The transgenic fruit are blocked in the synthesis of phytoene from its immediate precursor, implying that pTOM5 encodes phytoene synthase (Bramley et al., 1992). We selected this clone because enzymological data suggest that synthesis of this nonpigmented carotenoid occurs in plastids (Lutke-Brinkhaus et al., 1982; Mayfield et al., 1986; Dogbo et al., 1987;).

The major translation product of the pTOM5 transcript was 46 kD (Fig. 3-2, lane 1); this is in agreement with the size of the hybrid-selected translation product for pTOM5 identified by Slater et al. (1985). Minor lower molecular weight translation products were also produced from this transcript and may represent commencement of translation from internal methionines because these products are not imported into plastids. Incubation with pea chloroplasts resulted in the appearance of a 41 kD polypeptide that fractionated with intact plastids and was resistant to protease treatment of the chloroplasts (Fig. 3-2, lane 4). Protease resistance was not due to an inherent property of the 41 kD protein because it was degraded by thermolysin if the plastids were lysed prior to treatment (data not shown). This result is expected of a protein produced in the cytosol, imported into the plastid, protected from protease by the organellar envelope, and cleaved of its transit peptide.

Additional criteria must also be demonstrated for the confirmation of import into plastids. Import is an energy dependent process (Keegstra, 1989); white light or

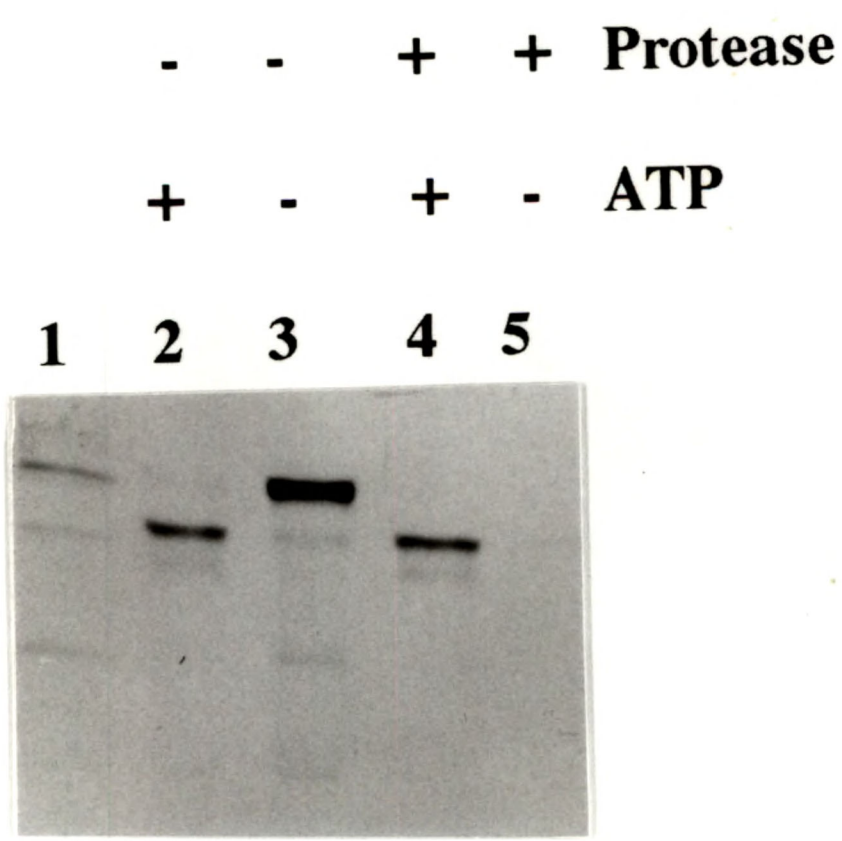


Figure 3-2. The 41 kD form of pTOM5 is protected from protease digestion by the plastid envelope and its appearance requires the addition of ATP. Lane 1) *In vitro* transcribed RNA was translated with a rabbit reticulocyte lysate system in the presence of [^{35}S]-methionine. Import assays and post assay treatments were conducted as described in Materials and Methods. Lanes 2-5) Import of pTOM5 *in vitro* translation products was conducted with (lanes 2,4) or without (lanes 3,5) 10mM Mg-ATP in darkness. Intact chloroplasts were re-isolated with (lanes 4,5) or without (lanes 2,3) protease post-treatment. Proteins from lysed plastids were separated on 12.5% SDS-PAGE gels and flurographed.

exogenous ATP must be provided for the successful import of proteins. Fig. 3-2, lanes 2 and 3 show that processing of the 46 kD protein into the 41 kD peptide required exogenous ATP. In the absence of ATP, the 46 kD putative precursor bound to the chloroplasts but was destroyed by the protease (Fig. 3-2, lanes 3 and 5). The import into plastids should also be time dependent; this was found to be the case for the production of the 41 kD protein (Fig. 3-3). Thus, the successful import of the pTOM5 translation product verified the utility of import assays for the identification of genes encoding chromoplast-targeted proteins.

Pea chloroplasts were fractionated into soluble and membrane fractions to allow analysis of the sublocalization of the 41 kD protein. The protein distributed between these two compartments (Fig 3-3). Parallel import assays with the thylakoid localized light-harvesting chlorophyll a/b protein (LHCP) and the stromal small subunit of Rubisco (SS) were used to assess the quality of our subfractionation. LHCP was exclusively found in the membrane fraction whereas virtually all of the SS was recovered in the soluble fraction (data not shown). During preparation of this manuscript, Bartley et al. (1992) have reported the isolation of a cDNA from a tomato fruit library by hybridization with a PCR fragment produced with oligonucleotides derived from the sequence of pTOM5. They found that their clone differs from pTOM5 in 19 amino acids that reside 5 amino acids away from the deduced carboxyl terminal end. They also demonstrated that their *in vitro* translation product imports via an ATP requiring process and that their import product also appears in both stromal and membrane fractions of pea chloroplasts.

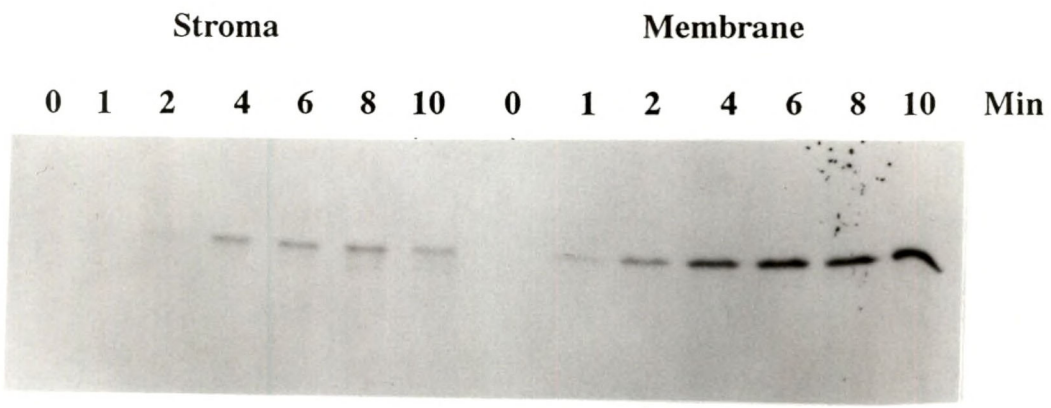


Figure 3-3. The production of the 41 kD form of pTOM5 was time dependent. This protein associated with both stromal and membrane fractions. pTOM5 import assays were conducted as described in Materials and Methods. One half of a standard import assay was immediately repurified on a 35% Percoll cushion after 0, 1, 2, 4, 6, 8, or 10 minutes of incubation. Chloroplasts were protease treated, repurified and subfractionated as described in the Materials and Methods. Samples were analyzed by SDS-PAGE and fluorography.

The membranes of pea chloroplasts were further subfractionated into envelopes and thylakoid membranes (Cline, 1986); upon fractionation of the membranes after import of pTOM5, virtually all of the mature-sized protein was found to be associated with the thylakoid fraction (data not shown). A mild NaOH wash removed the pTOM5 protein from the membrane (data not shown). This suggested that the protein was only peripherally associated with this fraction (Cline, 1986).

Previous attempts by others to localize the site of phytoene synthesis within the plastid via enzyme assays have resulted in conflicting conclusions. Lutke-Brinkhaus et al. (1982) concluded that the production of phytoene is associated with the plastid envelope. However, Mayfield et al. (1986), and independently, Dogbo et al. (1987) deduced that phytoene is synthesized in the stroma. The unexpected association of the protein with thylakoids in import assays and its peripheral association with the membranes demonstrated by the NaOH extraction may have provided additional evidence that this protein is functionally associated with the stroma. Alternatively, perhaps the 41 Kd protein has not folded or localized correctly in pea chloroplasts. Bartley et al. (1991) reported the import of phytoene desaturase from soybean leaf into pea chloroplasts. Although this protein imported effectively, Bartley et al. found that it does not also fractionate into the plastid compartment expected from biochemical analysis. Immunocytolocalization may clarify these contradicting results. Therefore, it appears that import assays can be useful for the identification of chromoplast-targeted proteins; however, it is currently unclear what

conclusions can be made from the *in vitro* organellar sublocalization when using heterologous systems.

Six additional ripening enhanced pTOM cDNA clones (pTOM 25, 36, 41, 92, 111, 114) of unknown function (Slater et al., 1985) were tested to determine whether they encode plastid targeted proteins. These particular clones were selected because a comparison of the published sizes of the cDNA inserts with the size of their respective mRNA suggested that they might be full length (Slater et al., 1985; Maunders et al., 1987). *In vitro* transcription of the cDNAs was carried out in both directions because the orientation of the clones within the vector was unknown. *In vitro* translation products of these transcript pairs failed to import in four of the six clones. Fig. 3-4, lanes 1 to 7 shows examples of the translation products and lack of import products produced from these clones. However, since the transit peptide, which is required for successful import of the protein, is located on the amino terminal end, and since *in vitro* translation can begin from any internally situated methionine, any cDNAs that produced an *in vitro* translation product but not an imported product may simply not include the entire coding sequence. Translation products from pTOM41 and pTOM111 were imported successfully into pea chloroplasts (Fig. 3-4, lanes 9 and 11). The imported product from both proteins was smaller than the translation product, which is consistent with processing of the plastid transit peptide. These proteins were sensitive to protease treatment (see chapter 4), but resistant after import into plastids (Fig. 3-4). As with pTOM5, import of pTOM111 and pTOM41 translation products were ATP and time dependent (see

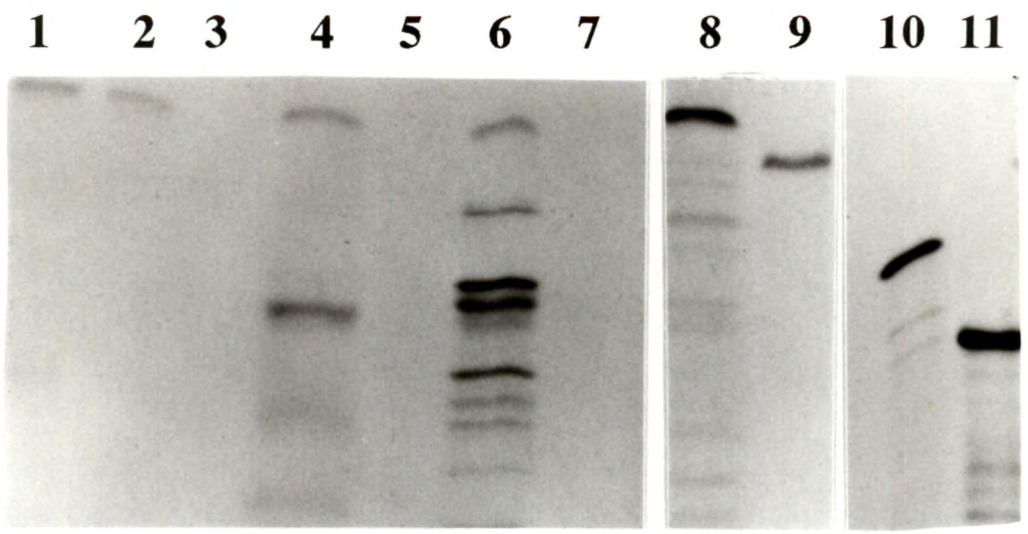
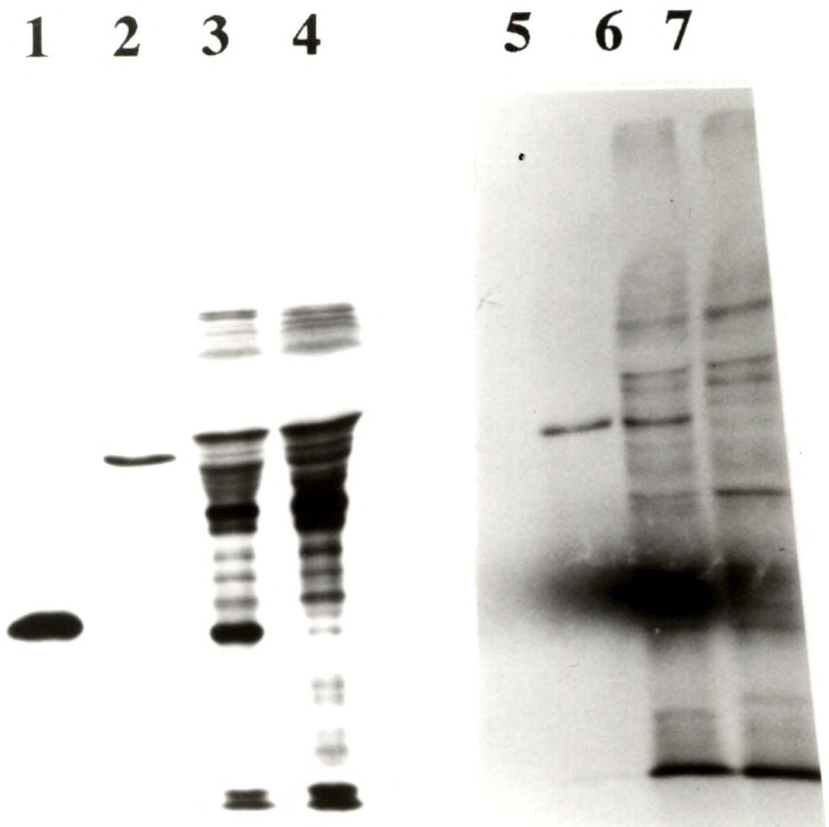


Figure 3-4. Translation products from transcripts of additional pTOM clones were tested for their ability to import into isolated pea chloroplasts. *In vitro* translation products from pTOM41 and pTOM111 imported into isolated pea chloroplasts. *In vitro* transcribed RNA was translated with a rabbit reticulocyte lysate system in the presence of [^{35}S]-methionine. Import assays were conducted as described in Materials and Methods. Samples were analyzed by SDS-PAGE and fluorography. *In vitro* translation with no added RNA (lane 1). pTOM92 transcribed *in vitro* with SP6 polymerase, translated *in vitro*, (lane 2) and imported (lane 3). pTOM92 transcribed *in vitro* with T7 polymerase, translated *in vitro*, (lane 4) and imported (lane 5). pTOM36 transcribed *in vitro* with T7 polymerase, translated *in vitro*, (lane 6) and imported (lane 7). pTOM41 transcribed *in vitro* with T7 polymerase, translated *in vitro*, (lane 8) and imported (lane 9). pTOM111 transcribed *in vitro* with T7 polymerase, translated *in vitro*, (lane 10) and imported (lane 11).

chapter 4). These data support the idea that the cDNAs do indeed code for plastid-targeted genes. We have begun sequence analysis (see chapter 4) and antisense transformation experiments of these cDNAs (see Appendix) to investigate their role in chromoplast function.

We wondered whether our initial examination of import from total poly $(A)^+$ translation products (Fig. 3-1B) might underestimate the number of chromoplast-targeted proteins. Therefore, we asked whether the pTOM import products could be recognized among the total poly $(A)^+$ RNA import products. The pTOM5 protein comigrated with a band shown to increase in the import products from the membrane fraction of late stage fruit (Fig. 3-5, lanes 5 and 6). It is interesting that the 26kD soluble import product (Fig. 3-1B) migrates similarly to the pTOM111 import product (Fig. 3-5). However, analysis of these two proteins on several gels suggested that the pTOM111 product was slightly larger. Consequently, it is unclear if pTOM111 represents a different isozyme or a completely dissimilar protein. Since the import of the *in vitro* translation product of pTOM41 associates with the stromal fraction, this was compared with the soluble fraction of total poly A^+ import products. No protein appears to increase in the later stages of ripening fruit which comigrates with this band (Fig. 3-5, lanes 2 and 3). We concluded, therefore, that not all of the chromoplast-targeted proteins could be detected in our analysis of total poly $(A)^+$ RNA import products.

Figure 3-5. Import products corresponding in size to those for pTOM111 and pTOM5 were enhanced in late stage tomato fruit. However, no polypeptide was seen that coincided to the pTOM41 import product. pTOM41, pTOM5 and pTOM111 were transcribed *in vitro* with T7 polymerase. RNA was translated with rabbit reticulocyte lysate in the presence of [^{35}S]-methionine. *In vitro* translation, import assays and subfractionation were conducted as described for analysis of individual translation products in Materials and Methods. Lane 1) Soluble fraction of the pTOM111 import product. Lane 2) Soluble fraction of the pTOM41 import product. Lane 3) Soluble fraction of imported *in vitro* translation products of total poly $(A)^+$ RNA from >80% ripe fruit. Lane 4) Soluble fraction from imported, *in vitro* translation products of immature green total poly $(A)^+$ RNA. Lane 5) Membrane fraction of the pTOM5 import product. Lane 6) Membrane fraction from imported, *in vitro* translation products of total poly $(A)^+$ RNA from >80% red ripe fruit. Lane 7) Membrane fraction from imported, *in vitro* translation products of immature green total poly $(A)^+$ RNA.


1 2 3 4
5 6 7

It is presently unclear how many plastid targeted proteins increase during the chloroplast to chromoplast transition. The data presented in Fig. 3-4 and 5 clearly demonstrate that our original examination of chromoplast targeted proteins shown in Fig. 3-1 underrepresents the number of proteins that increase during this process. In fact, this is not surprising when considering the limited sensitivity of import of total poly (A^+) translation products. Therefore, we believe that individual testing of ripening enhanced cDNA clones, with the inherent increase in the quantity of the individual protein product, is required to clearly identify all the chromoplast targeted proteins whose RNA increases during this developmental process.

Little detailed analysis of the regulation of ripening enhanced genes has been undertaken. Lincoln and Fisher (1988) and DellaPenna et al. (1989) concluded that increases in transcription rate were mainly responsible for the increases in steady-state RNA levels for the ripening enhanced genes they have analyzed. However, they also invoked post-transcriptional mechanisms to explain differences between the rate of transcription and accumulation. Although we cannot exclude post-transcriptional mechanisms for the increase in steady-state levels of the RNAs we have analyzed, the simplest interpretation for our data that is consistent with other ripening enhanced genes is that the processes involved in the development of the chromoplast are directed at a transcriptional level in the nucleus.

Utilizing an *in vitro* assay for the import of proteins into plastids we have identified two proteins from *in vitro* translation products of total poly $(A)^+$ RNA that increase during the ripening of tomato fruit. Additionally, we have shown that the

in vitro translation products from three individual ripening enhanced cDNA clones import into plastids. Therefore, the transition from chloroplast to chromoplast coincides with an increase in steady-state levels of RNA for proteins destined to this organelle. This suggests that this process is not a simple decline in the synthesis of photosynthetic proteins that causes a breakdown of the thylakoid membranes and a shunting of substrates into the carotenoid pathway, but an active developmental program.

CHAPTER 4
CHARACTERIZATION OF TWO cDNAS FOR CHROMOPLAST-TARGETED PROTEINS

Introduction

As tomato fruit ripens, cell walls soften, hexoses are deposited into vacuoles, and carotenoids accumulate in developing chromoplasts. The transformation of chloroplasts into chromoplasts is distinguished by the breakdown of the photosynthetic apparatus and a massive synthesis and deposition of carotenoids. Little is known about how the catabolism of thylakoid membranes or the synthesis of carotenoids is regulated. Only two tomato genes for early steps in the carotenoid biosynthetic pathway have been cloned (Bird et al., 1991; Bartley et al., 1992; Pecker et al., 1992). Besides proteins involved in the synthesis of carotenoids, only the plastidial superoxide dismutase, characterized by Livine and Gepstein (1988), has been shown to increase during tomato fruit ripening. However, Romer et al. (1992) have also isolated and cloned a cysteine synthase from pepper chromoplasts. These latter findings suggest, therefore, that the chromoplast may be responding to an increase in oxidative stress during development.

In order to understand how the transformation from chloroplast to chromoplast is mediated one objective of the present study was to identify and clone

genes for proteins involved in chromoplast development. We have identified plastid proteins that may be increasingly synthesized during chromoplast development by examining cDNAs isolated from a ripening enhanced tomato fruit library, translating their transcripts *in vitro* and testing whether the resulting proteins can be imported into isolated pea chloroplasts. This chapter describes the characterization of two cDNAs that code for transcripts whose steady-state level increases during chromoplast development and whose proteins can be imported into plastids. The characterization of these two cDNAs represents a novel use of import assays to examine chromoplast development. Since this type of analysis is free of functional bias, the identification of unexpected components of chromoplast development is possible.

Materials and Methods

Plant Material

Tomato plants (*Lycopersicon esculentum* Mill cv. Sunbeam) were grown in the field at the Horticultural Unit of the University of Florida in Gainesville, Florida. Fruit were harvested at 9:00 am on June 19, 1992 from five different ripening stages. Mature green 2 (MG2) was characterized by full size green fruit with fully developed gel in at least one but not all of the locules; mature green 3 (MG3) fruit had completely developed locular gel; breaker stage fruit were characterized by the appearance of carotenoids at the blossom end; 20-50% ripe fruit were red over less

than half but more than 20% of the outer pericarp; and >80% ripe fruit were red over 80% of the outer pericarp. Pericarp from fruit of each stage was frozen in liquid nitrogen immediately after harvesting at -70°C for analysis at a later date.

For the examination of ripening under controlled temperature, MG fruit were placed in jars with a flow through system of humidified air and allowed to ripen at 22°C in the dark. This prevented water stress or a buildup of respiratory CO_2. Fruit from three different ripening stages (MG3, breaker, and 20-50% ripe) were selected and exposed for 6 hours to 36°C in this humidified environment. Following the incubation period, pericarp was frozen in liquid nitrogen.

Preparation of RNA

Total RNA was extracted by combining the methods of Chomczyinski and Sacchi (1987) and Cathala et al. (1983). Briefly, this involved grinding 30 g of frozen tissue in a coffee grinder and homogenizing in 60 ml of extraction buffer with three 30 sec bursts of a Brinckman polytron. The extraction buffer contained 4M guanidine isothiocyanate (Bethesda Research Laboratories), 0.5% sarkosyl, 25 mM sodium citrate pH 7.0, and 0.1M 2-mercaptoethanol. Sodium acetate pH 4.0 was added to 0.18M, followed by one volume of phenol and 1/10 volume chloroform. The homogenate was shaken for 10 sec after each addition and then cooled on ice for 15 min. The phases were separated by centrifugation at 10,000 x g for 20 min, the aqueous phase filtered through 2 layers of Miracloth (Calbiochem), and nucleic acids precipitated with an equal volume of isopropanol for one hour at -20°C. The

precipitate was collected by centrifugation for 45 min at 10,000 x g. The pellet was dissolved in extraction buffer and RNA was precipitated with 5 volumes of 4M LiCl for 15 hours at 4^0C. Following centrifugation for 90 min at 10,000 x g, the pellet was washed with 3M LiCl and recovered by centrifugation for 1 hour. The RNA was dissolved in 0.1% SDS, extracted with 1 volume of phenol/ chloroform (1:1), then reextracted with 1 volume chloroform, and precipitated with 0.3M sodium acetate and 2.5 volumes of ethanol. Precipitated RNA was dissolved in sterile water and stored at -70°C.

Template Preparation, In Vitro Transcription and In Vitro Translation

A cDNA for the hsp 21 from heat stressed pea leaves, pHSP 21, was provided by E. Vierling (Vierling et al., 1988). The pTOM 111 and pTOM 41 cDNA were provided by D. Grierson (Slater et al., 1985). The pTOM cDNAs were digested with *Pst1* and ligated into pSport 1 (Bethesda Research Laboratories) at the *PstI* site, and transformed into *E. coli* strain TB1. Plasmid DNA was isolated by the alkaline lysis method of Lee and Rasheed (1990). The pHSP 21 plasmid was linearized with *Bam HI* and *in vitro* transcribed with SP6 polymerase as described in Cline (1988). The pTOM 41 and pTOM 111 plasmid DNAs were linearized with *Eco RI* and *in vitro* transcribed with T7 polymerase (Bethesda Research Laboratories). The *in vitro* transcribed RNA transcripts were translated with a rabbit reticulocyte lysate system from Promega (Madison, Wi.) in the presence of either [^{35}S]-Met or [^{3}H]-Leu according to procedures described in the Promega manual.

Northern Hybridization

Total RNA, quantified by spectrophotometric absorbance at A_{260}, was loaded (15 μg/lane) onto a formaldehyde denaturing gel (Sambrook et al., 1989). After electrophoresis the gel was washed in sterile water and stained in ethidium bromide (1μg/ml) for 30 min and destained in 20X SSC, which contained 0.3 M sodiun citrate and 3 M sodium chloride, for 45 min. The amount of ribosomal RNA was visually assessed to ensure that approximately equivalent amounts of RNA were loaded. The RNA was capillary blotted onto nylon membrane (Amersham Corp.) in 20X SSC overnight. The blot was washed with 10X SSC and exposed to 1.5 J/cm^2 UV light with a transilluminator (Bios).

For hybridization probes, the entire cDNA insert was excised with *PstI*, isolated by gel electrophoresis, and electro-eluted. 100 ng of DNA was radiolabeled with 75 μCi of α^{32}P-dCTP by random primer labelling with a kit (Boehringer Mannheim).

Prehybridization and hybridization conditions were as described in Church and Gilbert (1984). The prehybridization buffer contained 0.5M sodium phosphate pH 7.2, 7% SDS and 1% bovine serum albumin (Sigma, fraction V) at 65°C. Blots were prehybridized in 40 ml of buffer for at least 1 hour at 65°C. The labeled probe was denatured at 100°C for 5 min and added to the blot with 5 ml of prehybridization solution. This was incubated overnight at 65°C. The blot was washed one time in 1X SSC and 0.1% SDS at room temperature and then 3 times in 0.2X SSC and 0.1% SDS for 30 min. X-ray film (Kodak, X-OMAT) was exposed to the blot overnight

with two intensifying screens (DuPont) to locate bands. Bands were excised from the membrane and quantified by liquid scintillation counting.

DNA Sequencing

The *PstI* excised pTOM 111 cDNA insert was subcloned into pBluescript (SK) plasmid (Stratagene) at the *PstI* site. This was restricted with either *XbaI* and *SstI* or *KpnI* and *Eco RV* for production of ExoIII/Mungbean nested deletion plasmids following the protocol described in the manual (Stratagene). The resulting plasmids were precipitated, resuspended in TE, ligated directly, and transformed into *E. coli* strain TB1. Plasmids containing nested deletions from either direction were selected and sequenced using the T3 or M13-40 primer of the pBluescript vector. Double stranded plasmid DNA was used as a template and prepared as described by Lee and Rasheed (1990). DNA sequencing procedures were as described in the Sequenase sequencing kit manual (US Biochemical).

The pTOM 41 cDNA was blunt end ligated (Sambrook et al., 1989) into pGEM-4Z (Promega) at the *SmaI* site. Both the pGem constructs described above and the pSport constructs described in the section on *in vitro* transcription were used as template for double stranded DNA sequencing as described above. Initially the T7 or M13-40 sites of the pSport or pGem vectors were used as primer sites. Oligomers (15mers) specific to pTOM 41 were synthesized by the UF-ICBR DNA Synthesis Core and used in subsequent sequencing reactions. Some portions of the sequence were generated by the ICBR DNA Sequencing Core. Sequences were

analyzed with the University of Wisconsin Genetics Computer Group computer programs (Devereux et al., 1984). Protein sequence comparisons were performed at the National Center for Biotechnology Information using the GENINFO(R) program of the Experimental Blast Network Service (Altschul et al., 1990) made available by the ICBR Biological Computing Facility. The petunia hsp 21 sequence (Chen and Vierling, 1991) is available as EMBL accession #X54103. The pTOM66 sequence (Fray et al.,1990) is available as EMBL accession #X56138. The sequence for the hypothetical 30.9 Kd protein from *E. coli* (Post et al., 1992) is available in GenBank accession #M77236.

Chloroplast Isolation and Import Assays

Chloroplasts were isolated from pea (*Pisum sativum* L. cv Laxton's Progress 9) shoots by a combination of differential centrifugation and Percoll gradient centrifugation (Cline et al., 1989). Import assays were conducted as described by Cline (1988) except that translation products were diluted 1:3 with unlabelled methionine or leucine in import buffer; and that 50 µl of diluted translation products were added to 100 µl of chloroplasts (1 mg chlorophyll/ml) for a 300 µl total assay volume. Import assays were conducted for 15 minutes at 25^{0}C with white light and 10mM Mg-ATP unless indicated otherwise. Experiments testing the requirement of ATP for import of pTOM 111 protein involved incubation of 200 µl of the translation mixture with or without 4 units of apyrase (Sigma) for 5 min at 25^{0}C. The import assays were conducted in foil wrapped tubes to maintain darkness at 25^{0}C.

After incubation, intact chloroplasts were reisolated by centrifugation through 35% Percoll with or without a treatment of the protease thermolysin (Sigma) to remove surface bound proteins (Cline, 1988). Plastids were treated with 50 μg thermolysin for 40 min at 4°C unless indicated otherwise. Import products were diluted 1:1 with 2X-SDS PAGE buffer, incubated 8 min at 60°C and analyzed by SDS-PAGE and fluorography (Cline, 1986) on 12.5% gels.

In order to demonstrate that the imported protein was sensitive to thermolysin, an import assay was performed and the chloroplasts were lysed before the addition of the protease. One fraction of a standard assay was resuspended in 100 μl import buffer, the other fraction was lysed with the same amount of 10mM Hepes/KOH pH 8.0. Thirty μl aliquots (0.5mg chlorophyll/ml) were incubated with 0, 12, and 24 μg of thermolysin for 45 min at 4°C. For the pTOM 41 protein, [^{3}H]-leucine was used in the *in vitro* translation and 1/2 of a standard import reaction (50 μl of plastids at 1mg chlorophyll/ml) was resuspended in 25 μl 10mM Hepes/KOH pH 8.0. This sample was divided in half and 5 μg of thermolysin was added to an aliquot. After 40 min incubation at 4°C, EDTA was added to a final concentration of 5mM and the samples were immediately frozen. An equal volume of 2X-SDS PAGE buffer (Cline, 1986) was added to freshly thawed samples, the tubes were heated at 98°C for 3 min and 10 μl was loaded onto 12.5% SDS-PAGE gels.

For analysis of the location of imported proteins, chloroplasts recovered from assays were lysed in 50 μl 10 mM Hepes/KOH pH 8.0, and incubated for 5 min. Stroma was separated from membranes by centrifugation for 30 min at 37,500 x g.

The membranes were washed with 1 ml of import buffer, centrifuged for 30 min at 37,500 x g. and finally resuspended in 40 μl of 10mM Hepes/KOH pH 8.0.

Results

The tomato fruit cDNAs pTOM 111 and 41 were selected by Slater et al. (1985) because transcripts homologous to these clones increased during ripening. Data presented in the previous chapter have shown that *in vitro* transcripts from these clones can be translated *in vitro* and the translation products can be imported by pea chloroplasts. Further studies were performed in order to ensure that all the criteria for successful import were met, such as the energy and time dependence of the reaction along with sensitivity of the mature form of the protein to protease.

Import Studies of pTOM 111 Protein

The pTOM 111 *in vitro* transcript was translated into a 30 kD protein and processed by the isolated plastids into a 24 kD form in an energy (Fig.4-1A) and time (Fig.4-1C) dependent reaction. The production of this processed form was virtually prevented by the addition of apyrase, an ATPase, to the translation mix in the no ATP assay (Fig. 4-1A lanes 1 and 2 versus lanes 3 and 4). Only the 30 kD precursor, which remains outside of the plastid, was removed by the protease treatment (Fig. 4-1A lanes 1 and 4). The mature protein was sensitive to the protease only after the plastids had been lysed prior to addition of this enzyme (Fig. 4-1B).

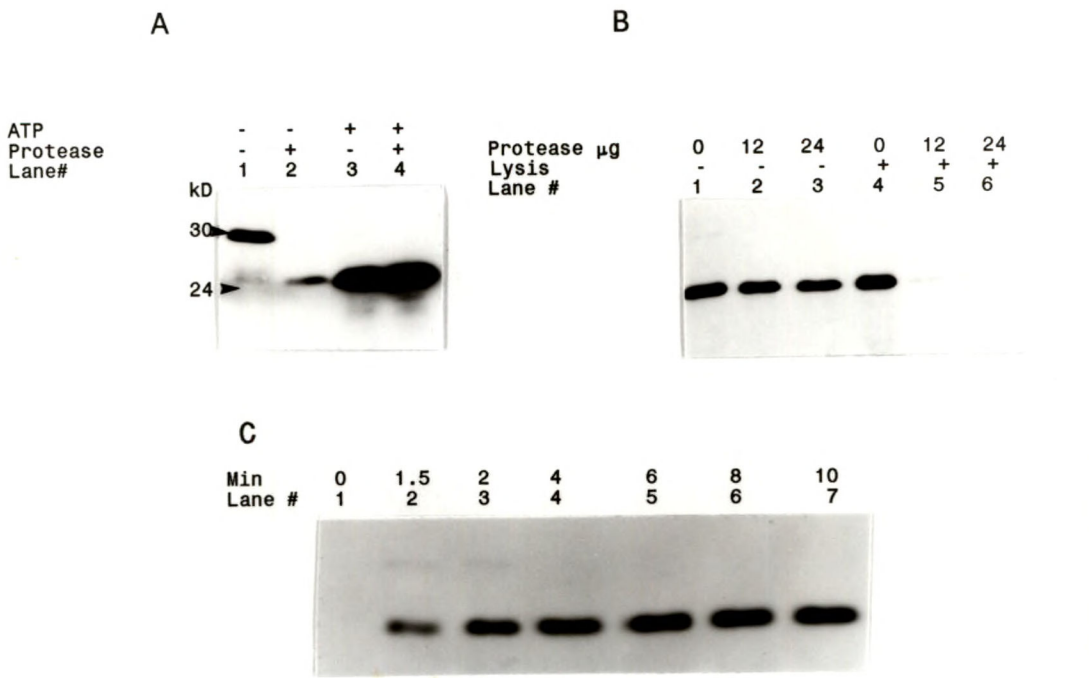


Figure 4-1 The 30 kD pTOM 111 translation product can be imported into pea chloroplasts and processed into a 24kD form in an energy and time dependent process. *In vitro* transcribed RNA was translated with a rabbit reticulocyte lysate system in the presence of [^{35}S]-Met. Import assays were conducted as described in the Materials and Methods. A) Production of the mature form of the protein requires ATP and is protected from protease digestion. A standard import assay was conducted with (lanes 3 and 4) or without (lanes 1 and 2) 10mM Mg-ATP. Plastids were pelleted and resuspended with (lanes 2 and 4) or without (lanes 1 and 3) protease. B) The plastid membrane protects the mature form of pTOM 111 from the added protease. Plastids were (lanes 4, 5 and 6) and were not lysed (lanes 1, 2, and 3) after import with 0 (lanes 1 and 4) 12 µg (lanes 2 and 5) or 24 µg (lanes 3 and 6) of protease. C) The production of the mature form of pTOM 111 is time dependent. Assays were of 100 µl of plastids (1mg/ml chlorophyll) for each time point. The reactions were terminated with 3.3 mM $HgCl_2$ to quickly stop the reaction.

When $HgCl_2$ was used to terminate the assays, a small amount of protease-resistant precursor was observed in the early time points (Fig. 4-1B). This may reflect a stage in the reaction prior to cleavage of the transit peptide but after import into the organelle.

As will be discussed in the following section, sequence analysis identified pTOM 111 as encoding a plastid-localized low MW heat shock protein (hsp). Consequently, we asked whether our hsp would localize *in vitro* in the same manner as a plastid localized low MW hsp that was isolated from heat stressed pea leaves. Chloroplasts recovered from import assays were separated into membrane and soluble fractions in Fig. 4-2. The proteins from both pTOM 111 and the pea cDNA were recovered in the soluble fraction. Even though the tomato cDNA was isolated from ripening fruit, the protein behaves in a manner similar to one isolated from heat stressed leaves.

Import Studies of pTOM 41 Protein

A similar analysis of the import of an in vitro translation product from pTOM 41 was performed (Fig 4-3). In this case, a 47 kD protein was produced upon *in vitro* translation of the pTOM 41 transcript (Fig. 4-3B). When the translation product was incubated with isolated pea chloroplasts, a 41 kD protein was generated (Fig. 3B). This predicts that a 6 kD transit peptide is cleaved upon import into the plastid. Little of the 47 kD precursor was associated with chloroplasts recovered from assays without added ATP, even without protease post-treatment (Fig. 4-3A, lane 1). Since

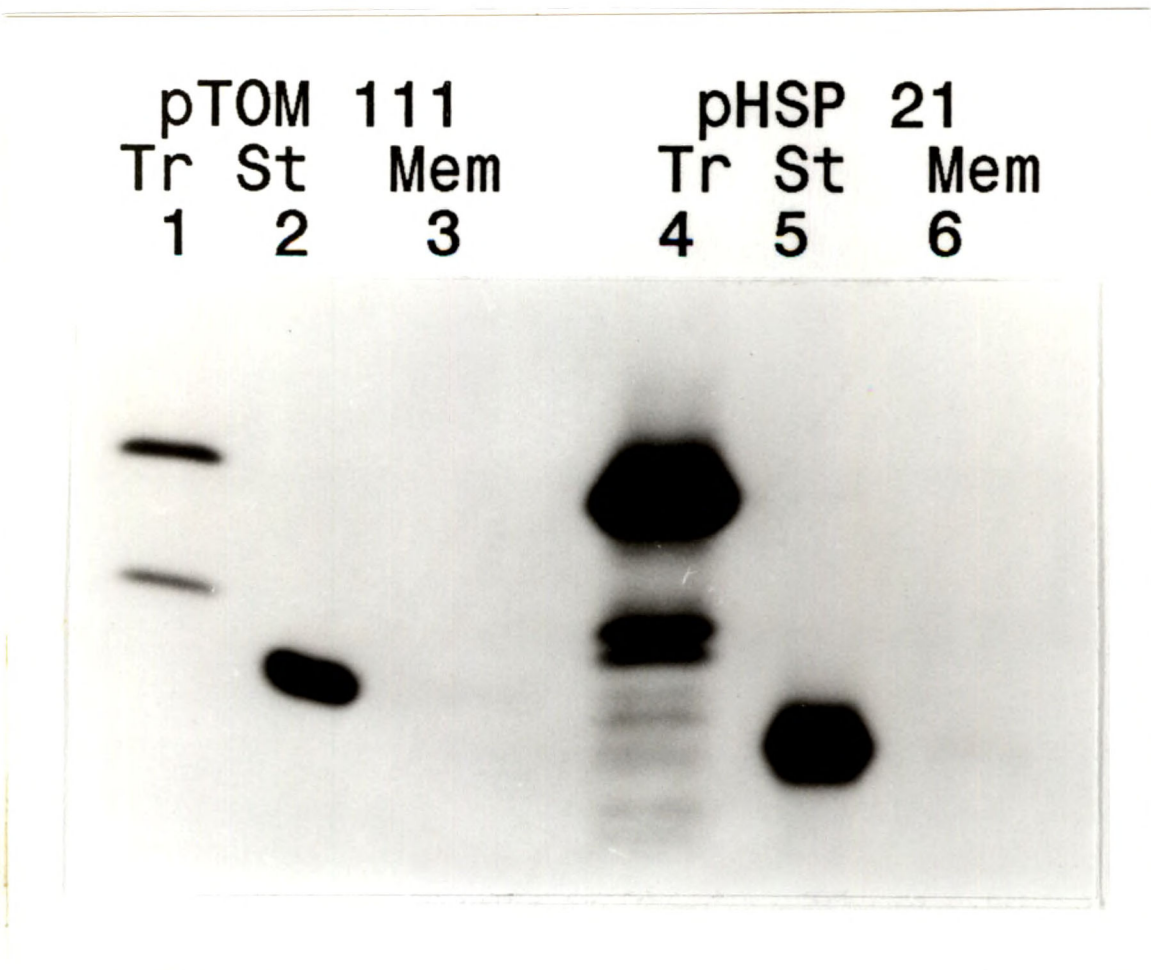


Figure 4-2. The pTOM 111 and an hsp 21 from heat stressed peas localize predominantly to the stroma. In vitro transcript was translated with a rabbit reticulocyte lysate system in the presence of [^{35}S]-Met and import assays were conducted as described in the Materials and Methods. Translation products (Tr), stroma (St) and membrane (Mem) fractions were analyzed by SDS-PAGE gels and fluorography.

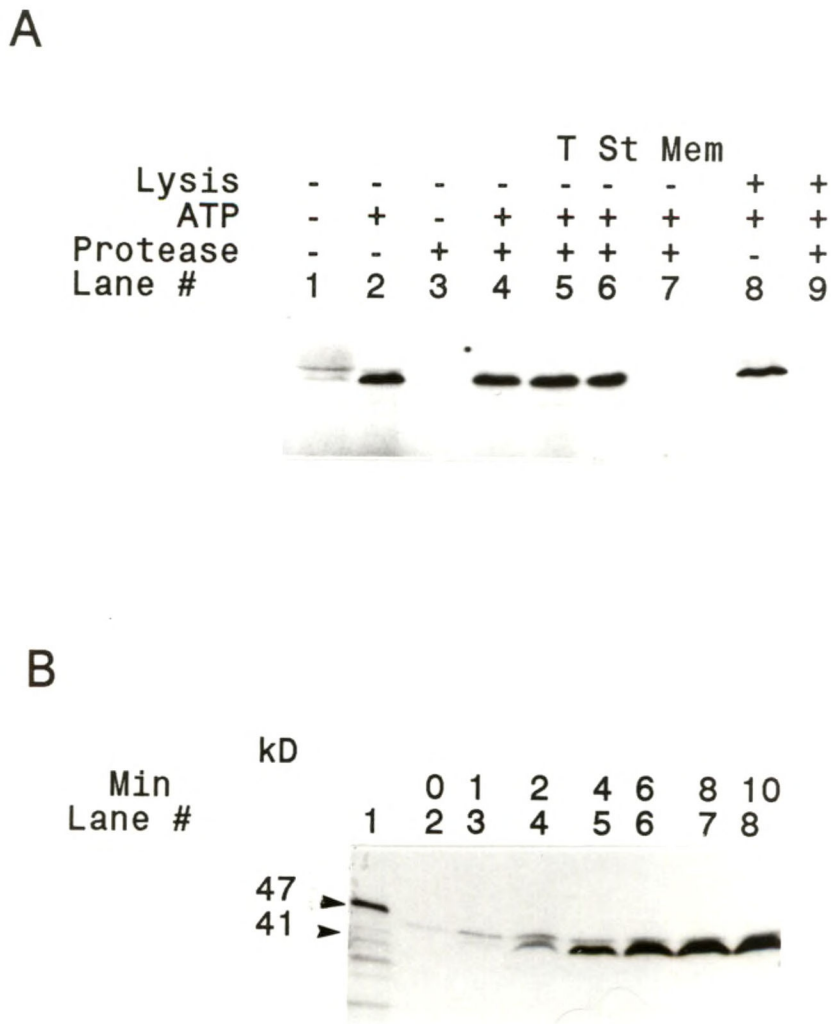


Figure 4-3. The 47 kD pTOM 41 translation product can be imported into pea chloroplasts and processed into a 41 kD form in an energy and time dependent process. A) Production of the mature form of the protein requires ATP and is protected from protease digestion. The mature form of PTOM 41 can be digested by the protease if the plastids are lysed. Import assays contained 10mM Mg-ATP (lanes 2, 4, 5, 6, 7, 8, and 9). Thermolysin was added after import (lanes 3, 4, 5, 6, 7, and 9). Plastids were lysed after import in 10mM Hepes/ KOH pH 8.0 and treated with thermolysin (lane 9). Stromal (St) and membrane (mem) fractions were separated from total (T) plastids following an import reaction as described in the Materials and Methods. B) The production of the mature form of pTOM 41 is time dependent. Assays contained 50 µl of plastids (1mg/ml chlorophyll) for each time point. [^{3}H]-leucine replaced [^{35}S]-methionine in the *in vitro* translation reactions.

the plastids were repurified before the assays were analyzed, this suggests that little of the pTOM 41 translation product attaches to the outside of the plastid. Subfractionation of recovered chloroplasts showed the pTOM 41 imported protein to be in the soluble fraction (Fig. 4-3A, lane 7). If plastids were lysed after import and treated with protease, the pTOM 41 mature protein was digested (Fig.4-3A, lane 9 versus lane 8), which demonstrates that it is not inherently resistant to this treatment. A small amount of protease-resistant precursor was also found in the early time points after import of the pTOM 41 protein (Fig. 4-3B).

These data demonstrate that the *in vitro* translation products of both pTOM 41 and 111 are imported into the plastid, are protected from protease by the organellar envelope and are cleaved of their transit peptides. In order to further characterize these chromoplast-associated proteins, the cDNAs were sequenced and the corresponding amino acid sequences deduced (Materials and Methods).

The pTOM 111 cDNA Codes for a Low MW HSP

The pTOM 111 sequence (Fig. 4-4) shows significant homology to those of low MW plastid-localized heat shock proteins isolated from heat stressed leaves. Proteins from soybean, pea (Vierling et al., 1988) Arabidopsis, petunia (Chen and Vierling, 1991) and maize (Mieto-Sotelo et al., 1990) have been cloned and sequenced. The pTOM 111 sequence is 81% homologous to a cDNA isolated from heat stressed petunia leaves. Low MW hsps from plants have been categorized on

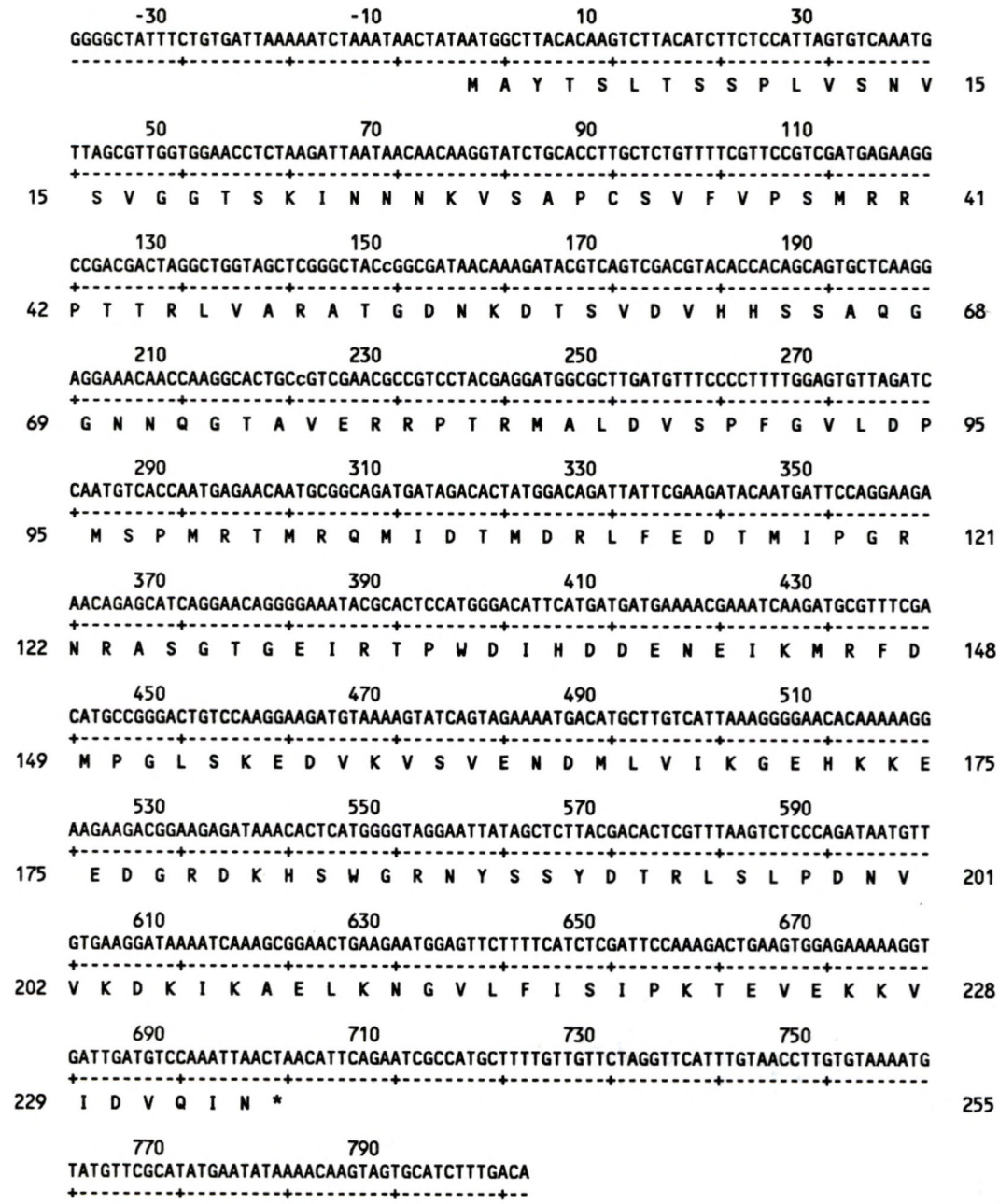


Figure 4-4. Nucleotide and deduced amino acid sequence of pTOM 111. The sequence was analyzed using the University of Wisconsin Genetics Computer Group programs. The position of the specific nucleotides appears above the sequence while the position of the specific amino acid appears to the side.

the basis of deduced amino acid sequence similarity (Vierling, 1991). There are three classes of cytoplasmically-localized low MW hsps, one class associated with the chloroplast, and another affiliated with the endoplasmic reticulum. A comparison of the deduced amino acid sequence of pTOM 111 to these proteins revealed the greatest similarity with the petunia plastid hsp (77% identity) (Fig. 4-5). This is not surprising since these species are evolutionarily most alike. Comparison to the only other characterized low MW hsp from tomato fruit, pTOM 66 (Fray et al., 1990), a cytoplasmically-localized hsp, revealed only 32% identity to pTOM 111 (Fig. 4-5). The pTOM 66 protein shares more sequence similarity to cytosolically localized hsps from other species (data not shown). Overall these hsp classes share more sequence identity among members from different species than they do with hsps from other families within the same species. The homology of pTOM 111 to a cDNA for a known plastid-localized protein supports the use of import assays to identify cDNAs for chromoplast-associated proteins.

Chen and Vierling (1991) have identified 3 conserved regions in 5 plastid localized low MW hsps they have examined. While the first two regions are found in all low MW hsps, the third region is unique to the plastid-localized proteins. It is in these three regions that pTOM 111 has the highest homology to other low MW hsps. A 90% amino acid identity to the petunia protein is found in these regions in contrast with the 77% overall homology (Fig. 4-5).

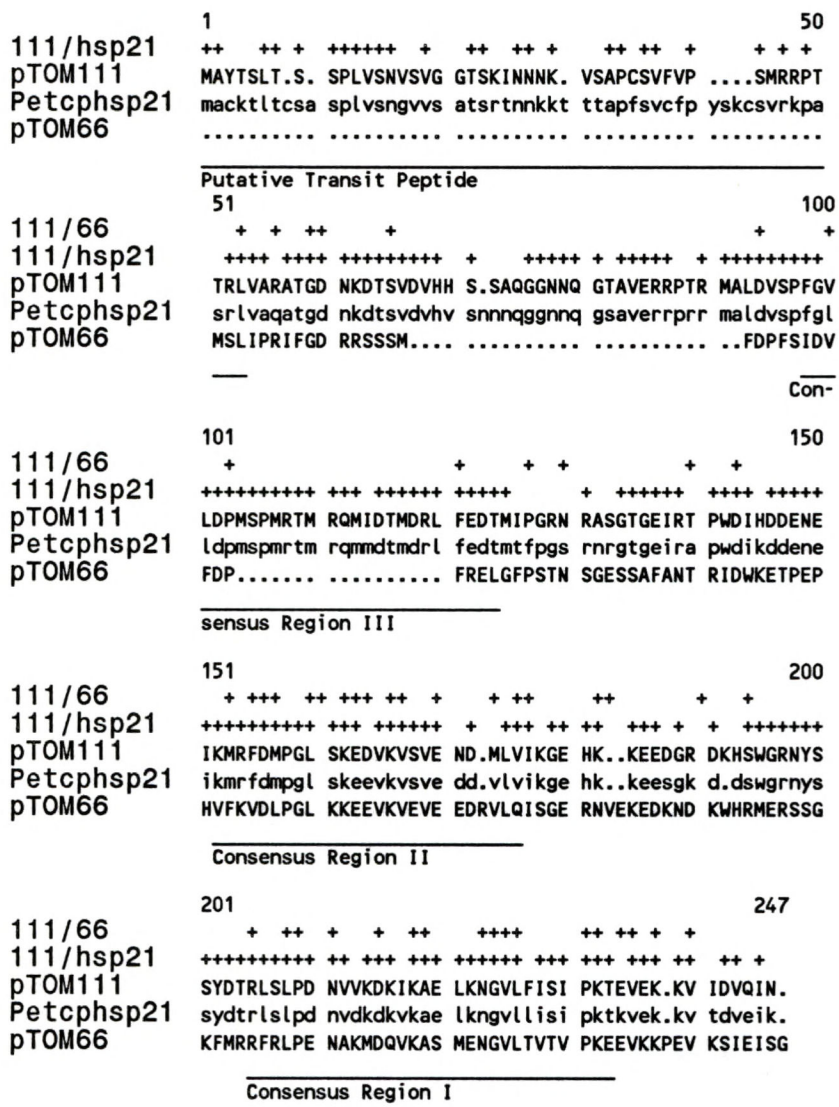


Figure 4-5. The pTOM 111 shares 77% amino acid sequence identity to an hsp 21 from petunia and 32% identity to pTOM 66 from tomato fruit. The deduced amino acid sequences were compared with the University of Wisconsin GCG program, "pileup", as described in the Materials and Methods. The + symbol represents exact matches between proteins, while dots represent spaces built into the protein by the pileup alignment. The putative transit peptide (Vierling, 1991) is delineated by a solid underline. The three consensus regions described by Chen and Vierling (1991) are also marked with a solid line.

The putative transit peptide of pTOM 111 protein is identified in Fig. 4-5. (Vierling, 1991). Although this part of the protein is the least similar to the petunia hsp shown in Fig. 4-5 (63% amino acid identity), the region still contains a number of features characteristic of transit peptides (Keegstra et al., 1989). For example, it is rich in serine, valine and alanine and deficient in the acidic amino acids glutamic and aspartic acid. In addition, some basic amino acids (arginine and lysine) are also present. Where the sequence varies from the petunia hsp within this region the substitutions are generally those residues characteristic of transit peptides. The observation that this portion of the protein is least similar to other plastid localized low MW hsps is not surprising, since an examination of transit peptides for the 5 plastid-localized low MW hsps analyzed by Chen and Vierling (1991) has also shown that they are the most diverged portion of the protein.

Even though the structure and function of the chromoplast differs drastically from the chloroplast, a similar hsp is produced. Our data agree with the conclusion of Vierling (1991) that localization to the plastid appears to predict more sequence conservation than does the tissue or organism in which the protein is expressed. Although the increases in low MW hsps pTOM 66 and pTOM 111 may be a general result of ripening, sequence conservation suggests that their role may be more pertinent to the compartment in which they function.

Sequence Analysis of pTOM 41

The sequence obtained for pTOM 41 is shown in Fig 4-6. The cDNA is 1423

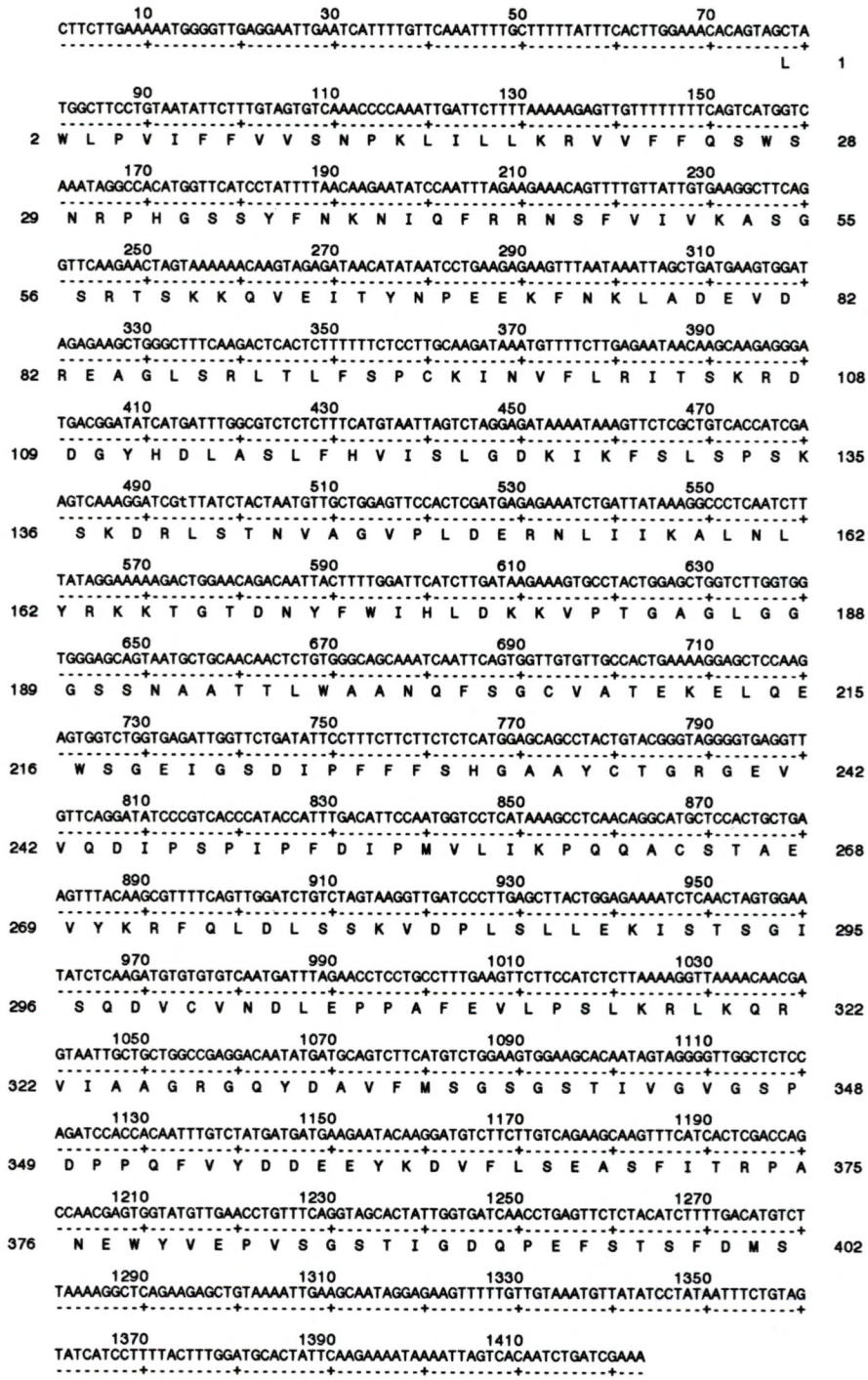


Figure 4-6. Nucleotide sequence and deduced amino acid sequence of pTOM 41. Numbers appearing above the sequence refer to the nucleotide sequence. Numbers appearing to the side of the sequence refer to the deduced amino acid sequence.

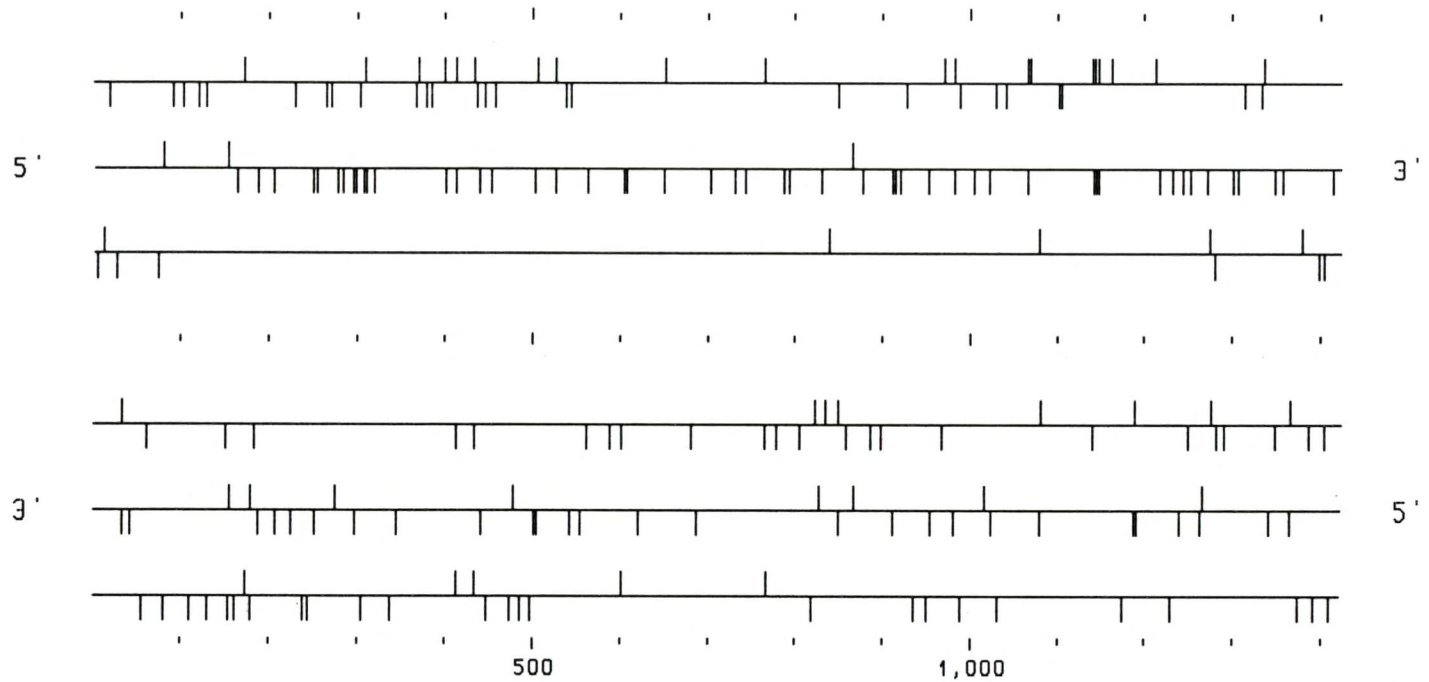


Figure 4-7. Graphic representation of potential reading frames of pTOM 41. Methionines are represented by lines appearing above the reading frame. Stop codons are represented by lines appearing below the reading frame. The top three frames were produced by reading the sequence from an arbitrary 5' end. The bottom three frames refer to the reading frames produced from the opposite strand.

bases long. Only 1 long open reading frame (ORF) is present, but it starts with a leucine, not a methionine (Fig. 4-6 and 4-7). This is hard to understand, given that the pTOM 41 transcript directed production of a 47 kD translation product. A methionine does occur within 12 nucleotides of the beginning of the long reading frame; however, two stop codons are encountered within the next 57 bases. Another small ORF of 28 residues is found two nucleotides 3' of the large ORF. This begins with a methionine in a favorable initiation context (Kozak, 1991). Two possible explanations for the observed anomaly are: 1) a sequencing error, or 2) a +1 frameshift during translation. The first possibility was addressed by additional sequence analysis of the areas in the vicinity of the 5' and 3' ends of the long ORF.

The cDNA sequence in Fig. 4-6 shows that the long ORF starts at 78 bases and ends at 1280. It is clear that an ORF of approximately this length is required to produce the protein observed on SDS-PAGE, all other potential reading frames are littered with stop codons, therefore, if a simple sequence error was generated, it would be found within the first few hundred bases. The first 290 bases were reexamined using a number of different modifications to the sequencing reactions. Figure 4-8 is a compilation of these individual sequencing results. For example, both strands of the sequence (R1 and F1) were examined by comparing incorporation of dITP instead of dGTP to the standard Sequenase double stranded sequencing reactions. This region of the sequence was also analyzed by the ICBR DNA sequencing core. They used either Klenow (R4) or TAQ polymerases (F2 and R2) in generating these sequences. Since the template that was used for most of the

Figure 4-8. Individual sequencing results of the first 290 bases of pTOM 41. Numbers appearing above the line refer to the position of the nucleotide within the sequence. The (consensus) sequence was deduced from the individual runs. Inconsistencies to the consensus sequence are underlined. (F1) was obtained by comparing sequence reactions run with dGTP or dITP in the forward direction or (R1) from the opposite strand. (F2) and (R2) were obtained in sequence reactions with primers specific for either strand using TAQ polymerase at the ICBR DNA sequencing core. (F3) and (R3) were obtained with primers specific for either strand using a standard Sequenase sequencing reaction. (F3a) was obtained with a pSport based template while (F3b) was derived from a pGEM-4Z based template. (R4) was obtained by the ICBR DNA sequencing core using Klenow polymerase.

```
     1                                                  50
Consensus  CTTCTTGAAA AATGGGGTTG AGGAATTGAA TCATTTTGTT CAAATTTTGC
F1         cttcttgaaa aatggggttg aggaattgaa tcattttgtt caaattttgc
R1         ...cttgaga aatggggttg aggaattgaa tcattttgtt caaattttgc
F2         CTTGGTNAAA AATGGGGTTG AGGAATTNAA TCATTTTNTN CAAATTTTGC
R2         CTTCTTGAAA AATGGGGTTG AGGAATTGAA TCATTTTGTT CAAATTTTGC
F3a        CTTCTTGAAA AATGGGGTTG AGGAATTGAA TCATTTTGTT CAAATTTTGC
F3b        .ttcttgaaa aatggggttg aggaattgaa tcattttgtt caaattttgc
R3         gctcttgaaa aatggggttg aggaattgaa .cattttgtt caaattttgc
R4         cttcttgaaa aatggggttg aggaattgaa tcattttgtn caaattttgc


           51                                                 100
CONSENSUS  TTTTTATTTC ACTTGGAAAC ACAGTAGCTA TGGCTTCCTG TAATATTCTT
F1         tttttatttc acttggaaac acagtagcta tggcttcctg taatattctT
R1         tttttatttc acttggaaac acagtagcta tggcttcctg taatattctT
F2         TTTTNATTTC ACTNGGAAAC ACAGTAGCTA TGGCTTCCTG TAATATNCTT
R2         TTTTTATTTC ACTTGGAAAC ACAGTAGCTA TGGCTTCCTG TAATATTCTT
F3a        TTTTTATTTC ACTTGGAAAC ACAGTAGCTA TGGCTTCCTG TAATATTCTT
F3b        tttttatttc acttggaaac acagtagcta tggcttcctg taatattctt
R3         tttttatttc acttggaaac acagtagcta tggcttcctg taatattctT
R4         tttttatttc acttggaaac acagtagcta tggcttcctg taatattctT

           101                                                150
CONSENSUS  TGTAGTGTCA AACCCCAAAT TGATTCTTTT AAAAAGAGTT GTTTTTTTTC
F1         tgtagtgtca aaccccaaat tgattctttt aaaaagagtt gttttttttc
R1         tgtagtgtca aaccccaaat tgattctttt aaaaagagtt gttttttttc
F2         TGTAGTGTNA AACCCCAAAT TGATTCTTTT AAAAAGAGTT GTTTTTTTTC
R2         TGTAGTGTCA AACCCCAAAT TGATTCTTTT AAAAAGAGTT GTTTTTTTTC
F3a        TGTAGTGTCA AACCCCAAAT TGATTCTTTT AAAAAGAGTT GTTTTTTTTC
F3b        tgtagtgtca aaccccaaat tgattctttt aaaaagagtt gttttttttc
R3         tgtagtgtca aaccccaaat tgattctttt aaaaagagtt gttttttttc
R4         tgtagtgtca aaccccaaat tgattctttt aaaaagagtt gttttttttc

           151                                                200
CONSENSUS  AGTCATGGTC AAATAGGCCA CATGGTTCAT CCTATTTTAA CAAGAATATT
F1         agtcatggtc aaataggcca catggttcat cctattttaa caagaatatc
R1         agtcatggtc aaataggcca catggttcat cctattttaa caagaatatc
F2         AGTCATGGTC AAATAGGCCA CATGGTTCAT CCTATTTGAA CAAGANTATC
R2         AGTCATGGTC AAATAGNCCA CATGGTTCAT CCTATTTTAA CAAGAATATC
F3a        AGTCATGGTC AAATAGGCCA CATGGTTCAT CCTATTTTAA CAAGAATATC
F3b        agtcatggtc aaataggcca catggttcat cctattttaa caagaatatc
R3         agtcatggtc aaataggcca catggttcat cctattttaa caagaatatc
R4         agtcatggcc aaatagncca catggttcat cctattttaa caagaatatc

           201                                                250
CONSENSUS  CAATTTAGAA GAAACAGTTT TGTTATTGTG AAGGCTTCAG GTTCAAGAAC
F1         caatttagaa gaaacagttt tgttattgtg aaggcttcag gttcaagaac
R1         caatttagaa gaaacagttt tgttattgtg aaggcttcag gttcaagaac
F2         CAATTTAGAA GAAACAGTTT TGTTATNGTG AAGNCTTCAG GTTCAGGAAC
R2         CAATTTAGAA GAAACAGTTT TGTTATTGTG AAGGCTTCAG GTTCAAGAAC
F3a        CAATTTAGAG GAAACAGTTT TGTTATTGTG AAGGCTTCAG GTTCAAGAAC
F3b        caatttagaa gaaacagttt tgttattgtg aaggcttcag gttcaagaac
R3         caatttagaa gaaacagttt tgttattgtg aaggcttcag gttcaagaac
R4         caatttagaa gaaacagttt tgttattgtg aaggcttcag gttcaagaac

           251                                          290
CONSENSUS  TAGTAAAAAA CAAGTAGAGA TAACATATAA TCCTGAAGAG
F1         tagtaaaaaa caagtagaga taacatataa tcctgaagag
R1         tagtaaaaaa caagtagaga taacatataa tcctgaaga.
F2         TAGTAAAAAN CAAGTAGGGA TAACATATAA TCCTGA....
R2         TAGTAAAAAA CAAGTAGAGA TAACATATAA TCCTGAAGAG
F3a        TAGTAAAAAC CAGGTGGAGA TAACATNTAA TCCCGAGGGG
F3b        tagtaaaaaa caagtagaga taacatataa tc.tgaagag
R3         tagtaaaaaa caagtagaga t......... ..........
R4         tagtaaaaaa caagtagaga taacatataa tcctgaagag
```

sequencing was cloned into pGEM, and therefore represents a second generation away from the pSport cDNA that was used for *in vitro* transcription, the pSport cDNA (F3a) was also sequenced in this region. Small single base differences were obtained with the different sequence reactions. However, no difference in the sequence was observed between the reaction that used the pSport template and the consensus sequence derived from other individual sequencing reactions (Fig. 4-8). These results verify that no simple sequence error was made within the first 290 bases.

To establish that an error at the end of the sequence had not produced a prematurely smaller ORF, the 100 bases around the stop codon of the long ORF were also reexamined (Fig. 4-9). The sequences shown in Fig. 4-9 have all been generated by standard Sequenase sequencing. Introduction of any changes to the consensus, (found in individual sequencing reactions) singly or in combination does not alter the length of the ORF. In addition, since adjustments to the first 300 bases are required to place a methionine in the long ORF, any changes to the end of the sequence cannot significantly alter the conclusions. These data suggest that this particular cDNA generates a transcript that is either altered during in vitro transcription or is translated in an unusual fashion.

Attempts to directly sequence the in vitro transcript in this region of pTOM 41 were not successful (data not shown). This area of the cDNA is extremely A:T rich (67.6%). For example, an 8 base run of thymines between 142 and 149 nucleotides completely stops further RNA sequence characterization downstream.

```
          1263                                                  1312
Consensus ACATCTTTTG ACATGTCTTA AAAGGCTCAG AAGAGC.TGT AAAATTGAAG
Gam43     acatcttttg acatgtctta aaaggctcag aagagc.tgt aaaattgaag
Gam41b    acatcttt.g acatgtctta aa.ggctcag aagagc.tgt aaaattgaag
Gam31b    acatcttttg acatgtctta aaaggctcag aagagcctgt aaaattgaag
Gam31     acatcttttg acatgtctta aaag.ctcag aagagcctgt aaaattgaag

          1313                                                  1362
CONSENSUS CAATAGGAGA AGTTTTTGTT GTAAATGTTA TATCCTATAA TTTCTGTAGT
Gam43     caataggaga agtttttgtt ctaaatgtta tatcctataa tttctgtagt
Gam41b    caataggaga .gttt..gtt gta.atg.ta tat.ctata. tttctgtag.
Gam31b    caataggaga agtttttgtt gtaaatgtta tatcctataa tttctgtagt
Gam31     caataggaga agtttttgt. gt..... ..... .....
```

Figure 4-9. Individual sequence results obtained by sequencing both strands of the pTOM 41 cDNA between nucleotide 1263 and 1362 using standard Sequenase sequencing reactions. The (consensus) sequence was obtained by comparison to the individual sequencing results. Gam 43, 41 and 31 represent specific primers for the pTOM 41 sequence.

This result may explain how an error by the reverse transcriptase could have been made when the pTOM 41 cDNA was originally produced.

The pTOM 41 ORF Shares Homology to an *E. Coli* ORF

Comparison of the long ORF of pTOM 41 to other deduced amino acid sequences revealed 51% sequence similarity to an ORF from *E. coli* (Fig. 4-10). A glycine rich region between peptide 183 and 196 is particularly striking. Post et al. (1992) identified this region in the *E. coli* ORF as a nucleotide binding domain based on similarity to other proteins. The sequence similarity between these two proteins starts at the 88th amino acid of pTOM 41. However, it runs throughout the entire bacterial sequence. If the first 87 amino acids of the pTOM 41 ORF are removed, a 10 kD transit peptide and a 34.5 kD mature protein would be predicted. The molecular weight on SDS-PAGE suggested that a 6 kD transit peptide would be produced. Bartley et al. (1992) showed a similar result when they analyzed a tomato phytoene synthase they isolated. A 50 amino acid transit peptide would be cleaved upon import into plastids while homology to the bacterial phytoene synthase predicted a transit peptide of 120 amino acids. These authors suggested that perhaps the amino terminal end of the plant protein is simply longer than the bacterial peptide.

Steady State RNA Analysis of the Chromoplast-Associated Hsp

It was important to determine whether the accumulation of pTOM 111

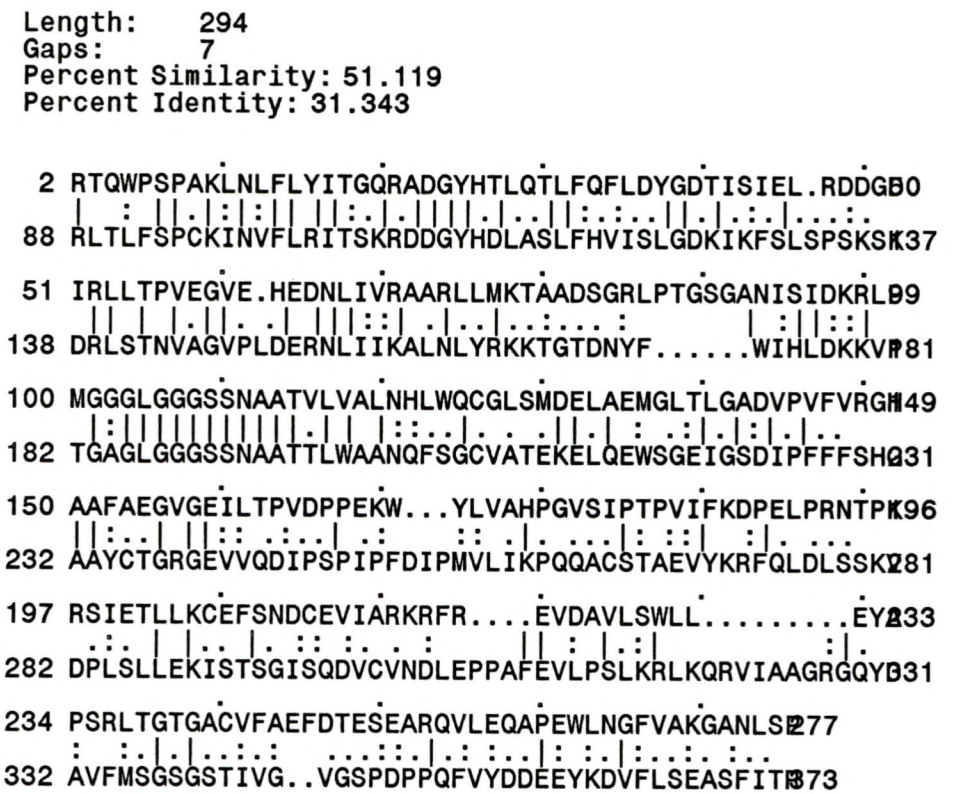


Figure 4-10. Comparison of the deduced amino acid sequences of the putative 30.9 kD protein from *E. Coli* to pTOM 41 using Bestfit from the University of Wisconsin Genetics Computer Group programs. The *E. coli* sequence appears at the top, while the pTOM 41 sequence is underneath. Identical matches are noted by a solid line. Conservative substitutions are denoted by two dots, while amino acids broadly sharing similar properties are identified by a single dot.

transcripts in ripe fruit was the result of ripening or of heat stress. This was accomplished by measuring the steady state level of transcript in fruit harvested from the field and comparing that to transcript from fruit that had been picked and held at 22^{0}C during ripening or alternatively elevated to 36^{0}C. The field grown fruit represented tissue that presumably differs only in ripening stage. Fruit was isolated from field grown plants at 9:00am in order to avoid prior heat induction. The pTOM 111 transcript was increasingly abundant in field-ripened fruit as development proceeded (Fig. 4-11, lanes 1-5). For example, breaker fruit contained 8 times as much transcript as MG.

Fruit was also harvested and stored in jars with a flow through system of humidified air at 22^{0}C. No change in internal temperature was observed during ripening. Fruit maintained at 22^{0}C took 2 days to reach 50% ripe from the breaker stage. Examination of the steady-state RNA that hybridizes to pTOM 111 revealed that an increase in transcript occurred as development progressed (Fig. 4-11, lane 6-8). About 2.5 to 3 times more pTOM 111 specific RNA was present by the 50% ripe stage in fruit held at nonstress temperatures (Fig. 4-11, lanes 6-8).

Approximately 4 times more RNA hybridized to pTOM 111 from field grown breaker stage tissue in comparison to fruit held at 22^{0}C (Fig. 4-11, lane 3 versus lane 7). More pTOM 111 hybridizing RNA was found in all later stage field-grown tissue in comparison to fruit held at 22°C. This difference may be caused by an increase in some stress inducing factor in the field-grown fruit. Clearly, conditions in the field

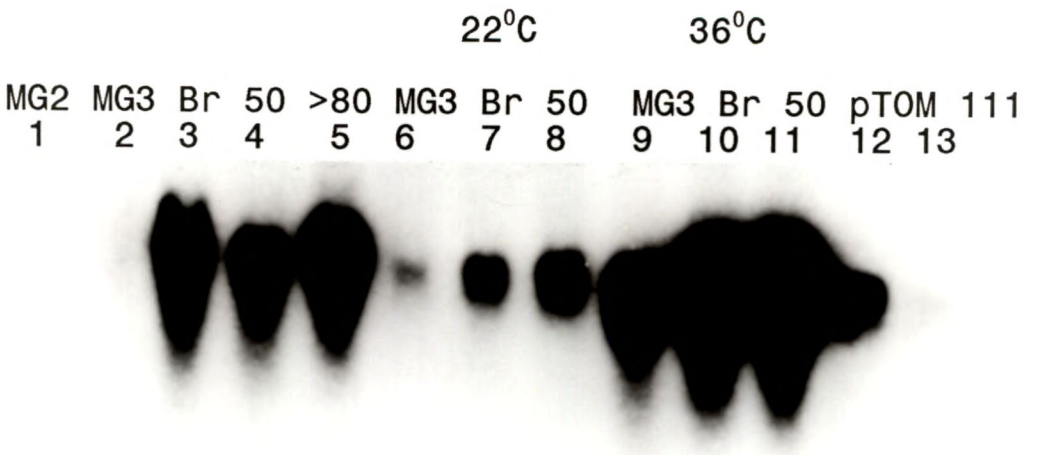


Figure 4-11. The steady state level of RNA for pTOM 111 increases during development in tomato fruit isolated from field grown fruit (lanes 1-5) and fruit harvested at MG and held at 22°C (lanes 6-8). The amount of RNA that is induced during heat stress also increases as fruit ripens (lane 9-11). RNA from mature green 2 (MG2), mature green 3 (MG3), breaker (Br), 20-50% red ripe (50), and more than 80% red ripe (>80) pericarp tissue was separated by electrophoresis on denaturing agarose gels (15 μg total RNA per lane) and blotted onto nylon membrane. Lanes 12 and 13 contain pTOM 111 *in vitro* transcript and a 1:5 dilution respectively for comparison between different blots. The membranes were probed with[^{32}P]-labeled pTOM 111. Films were exposed for 4 hours with 2 intensifying screens.

led to a greater accumulation of this transcript during ripening than if fruit was harvested and ripened in the dark at a uniformly nonheat stress temperature.

Fruit at 3 different developmental stages was subjected to 6 hours of 36^{0}C. The internal temperature of fruit transferred from 22^{0}C to 36^{0}C took about 4 hours to rise above 33^{0}C. Tomatoes held continuously at this temperature were visibly inhibited in carotenoid accumulation. Exposure to heat stress also resulted in increased accumulation of pTOM 111 transcript in all ripening stages (Fig. 4-11, lanes 6 versus 9, 7 versus 10 or 8 versus 11). The MG fruit accumulated about half of the RNA upon heat stress than did more mature fruit. These data show that this gene codes for a message that is associated with ripening. It also shows that the same gene or a highly homologous gene is induced by heat stress.

Characterization of pTOM 41 Transcripts in Vivo

RNA from the same tissue was also probed with pTOM 41. The amount of transcript present in tomato fruit that hybridizes to this cDNA increased as ripening progressed (Fig. 4-12, lane 1-5). A comparison of RNA from tissue held at high and low temperature revealed that a second smaller hybridizing transcript (about 400 bases less) was produced in material that had been exposed to heat stress (Fig. 4-12, lanes 9-11). The RNA isolated from field-grown fruit contained proportionally less of this smaller transcript than fruit held at 36°C. Because of the production of this smaller transcript, heat stressed material contained more total message that hybridized to pTOM 41. Since the discovery of a smaller sized transcript and an

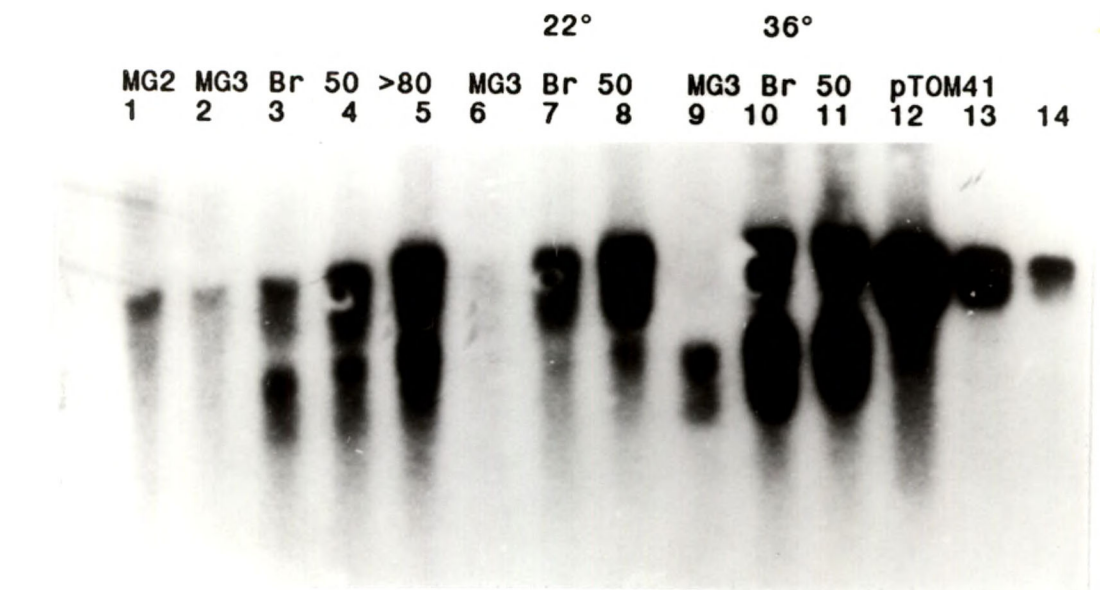


Figure 4-12. The steady state level of RNA for pTOM 41 increases during ripening in tomato fruit isolated from the field (lanes 1-5) and fruit harvested at MG and held at 22°C (lanes 6-8). A second smaller transcript appears, which also increases with developmental stage, when fruit is subjected to heat stress at 36°C for 6 hours (lanes 9-11). RNA from mature green 2 (MG2), mature green 3 (MG3), breaker (Br), 20-50% red ripe (50), and more than 80% red ripe (>80) pericarp tissue was separated by electrophoresis on denaturing agarose gels (15 μg total RNA per lane) and blotted onto nylon membrane. Lanes 12, 13 and 14 contain pTOM 41 in vitro transcript and 1:5 dilutions respectively for comparison between different blots. The membranes were probed with [^{32}P]-labeled pTOM 41. Films were exposed overnight with 2 intensifying screens.

increase in pTOM 41 transcript during heat stress was unexpected, the same samples were hybridized with a sequence of known function. The pTOM 6 cDNA, which codes for the ripening associated enzyme polygalacturonase (PG), was chosen because the level of its message was not expected to change after such a short heat stress. As expected, the transcript for pTOM 6 increased during ripening in all three treatment groups. However, no new sized transcript or dramatic change in the amount of transcript that hybridized to pTOM 6 due to heat stress was observed (data not shown).

Discussion

To begin an examination of the transition of chloroplasts into chromoplasts, we were interested in identifying plastid proteins that are increasingly synthesized during this period. Previous studies have either focused on the decrease in synthesis of photosynthetic proteins (Piechulla et al., 1985, 1986 and 1987; Livine and Gepstein, 1988) or have relied on the identification of specific chromoplast-associated proteins from total plastid extracts (Newman et al., 1989). Because the latter method can only be applied to abundant proteins, we chose to use import assays to identify cDNAs for any transcripts that are ripening enhanced and encode chromoplast-targeted proteins.

The identification of a plastid localized low MW hsp was both gratifying and puzzling. Since the fruit used to clone pTOM 111 was not known to be specifically

heat stressed, the finding of such a protein during ripening was unexpected. Other hsp cDNAs have been isolated from heat stressed material and later the proteins have been localized to the plastid. Consequently, the results from sequence analysis of this cDNA supported the plastid localization identified by the import assay.

The lack of a standard ORF in pTOM 41 could be the result of a cDNA synthesis artifact, or could represent a novel method of regulatory control. Since the first sequenced cDNA from this library for pTOM 13 was also altered in a similar manner (Hamilton et al., 1991), the first explanation cannot be ignored. The fact that pTOM 41 can produce an *in vitro* translation product capable of being imported suggests that the cDNA is, at worst, only slightly changed from its functional sequence.

Translation of the *in vitro* transcript can be explained as frameshifting caused by the presence of numerous consecutive runs of individual bases within the first 180 nucleotides of the pTOM 41 sequence (Fig. 4-6). Such single base repeats have been called "slippery runs" (Atkins et al., 1990) because they can facilitate +/- frameshifts during translation. The best characterized examples of this phenomenon have been documented in the (RF)2 gene of *E. coli* and in retroviruses. These sequences are considered to be a mechanism for translational control because they can alter the efficiency of translation for specific genes. In the retrotransposon Ty1 of yeast a simple sequence CUU AGG C has been shown to cause a +1 frameshift up to 50% of the time (Weiss, 1991). The $tRNA_{leu}$ recognizes the CUU codon; however, it can also utilize the +1 frameshift codon UUA. An additional requirement for efficient

translational frameshift in this sequence is the presence of the rare arginine codon AGG (present 8.1/1000 codons in yeast). Perhaps when this rare AGG codon is encountered, the ribosomes pause while waiting for the minor tRNA, and this allows the resident leucine tRNA to slip. The ribosomes can actually stack up along the transcript because translation effeciency is so compromised. Overexpression of the arginine tRNA, by transformation of its gene on a high copy plasmid, reduces the level of frameshifting. The conservation of this type of translational mechanism is demonstrated by the ability of rabbit reticulocytes to produce the frameshifted (RF)2 protein of *E. coli* (reviewed by Weiss, 1991).

A hypothesis of how a frameshifted pTOM 41 protein could be produced is presented in Fig. 4-13. The first 28 codons of the 2nd and 3rd reading frames are shown. A +1 frameshift could occur at codon 18. Not only is the same codon used in either frame, so that the same tRNA could be utilized, but if the codon preference is examined, which has been tabulated for numerous species (Wada et al.,1992), it is apparent that the following 20th and 21st codons of frame 2 are fairly rare codons. A comparison between codons used in tomato versus rabbit and wheat (Fig. 4-13B) revealed that the 20th and 21st codons of the second reading frame are even less common in these organisms from which the in vitro translation systems have been isolated. Since the successful frameshift in Ty1 of yeast occurred because AGG represented every 8.1/1000 codons (Wada et al., 1992), it seems plausible that the rabbit and wheat systems may be particularly underrepresented for the tRNAs needed at codon 20 and 21 (found 7.3 and 6.4/1000 in rabbit). This suggests that, like

A

CODON#	2	3	4	5	6	7	8	9	10	11	12	13	14
	GCT	TCC	TGT	AAT	ATT	CTT	TGT	AGT	GTC	AAA	CCC	CAA	ATT
FRAME# 2	A	S	C	N	I	L	C	S	V	K	P	Q	I
TOMATO	23.4	8.7	8.5	27.8	31.7	25	8.5	12.1	10.6	30.3	7.4	21.4	31.7

	CTT	CCT	GTA	ATA	TTC	TTT	GTA	GTG	TCA	AAC	CCC	AAA	TTG
FRAME# 3	L	P	V	I	F	F	V	V	S	N	P	K	L
TOMATO	25	17.3	9.9	10.6	19.5	23.7	9.9	17.8	16.7	20.8	7.4	30.3	24.6

CODON#	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	GAT	TCT	TTT	AAA	AAG	AGT	TGT	TTT	TTT	TCA	GTC	ATG	GTC	AAA
FRAME# 2	D	S	F	K	K	S	C	F	F	S	V	M	V	K
TOMATO	33.5	17.4	23.7	30.3	34.4	12.1	8.5	23.7	23.7	16.7	10.6	26.4	10.6	30.3

	ATT	CTT	TTA	AAA	AGA	GTT	GTT	TTT	TTT	CAG	TCA	TGG	TCA	AAT
FRAME# 3	I	L	L	K	R	V	V	F	F	Q	S	W	S	
TOMATO	31.7	25	10.3	30.3	14.6	31.7	31.7	23.7	23.7	11.1	16.7	13.2	16.7	27.8

B

CODON#	17	18	19	20	21
	TTT	AAA	AAG	AGT	TGT
FRAME# 2	F	K	K	S	C
TOMATO	23.7	30.3	34.4	12.1	8.5
RABBIT	16.4	18.4	36.4	7.3	6.4
WHEAT	13.4	5.9	25.1	3.9	4.1

C

```
CODON#    18  19  20  21
FRAME# 2 AAA AAG AGT TGT
           |__||__||__||_
FRAME# 3  AAA  AGA GTT GTT
```

Figure 4-13. Model explaining a possible +1 frameshift in the pTOM 41 transcript. A) Analysis of the codon usage for the second and third forward reading frame of pTOM 41. The 3rd frame is offset from the 2nd frame by a single base. Reading frame 2 appears above reading frame 3. Codon usage was tabulated by Wada et al. (1992) as the number of times the codon appears per 1000 codons. B) Comparison of codon usage in tomato, rabbit, and wheat for the codons appearing in position 17 to 21 in the 2nd frame of the pTOM 41 sequence. C) Model for possible +1 frameshift at codon 18.

the scenario in yeast, a large pTOM 41 protein could occur by a +1 frameshift caused by inefficient translation *in vitro*. Since the import assay involves the translation and import of a single transcript, it is possible that even an inefficiently translated protein could be detected. Perhaps the only other partially import competent protein produced in this reaction is the 28 amino acid peptide found in the second reading frame. A protein of this size is observed in the translation mixture (data not shown). This small protein could be imported and quickly degraded inside of the plastids, since the cleaved transit peptides are also not found inside the organelle. Alternatively, the smaller protein, which does not contain the entire transit peptide, may be outcompeted by the larger pTOM 41 translation product. Until it can be determined that the pTOM 41 cDNA is a functional representative of the gene *in vivo*, attempts to clarify a precise frameshift mechanism seem premature.

In order to determine how pTOM 41 is translated *in vivo* a genomic clone of pTOM 41 should be isolated and sequenced in this region. Hamilton et al. (1991) resolved the error generated in the pTOM 13 cDNA by direct RNA sequencing. However, given the A:T richness of this region in pTOM 41 and the relatively low abundance of this message, direct sequencing is probably not practical.

If additional cloning and sequencing demonstrate that this unusual sequence is normally present *in vivo*, then it would be important to make an antibody to the long ORF produced in *E. coli* in order to detemine that this protein is actually present in ripening tomato fruit. At this point we have only shown that the transcript

is increasingly present during fruit maturation. Additionally, the antisense mutagenesis of tomato with pTOM 41 (see Appendix) is presently underway. This may produce a demonstrable mutant phenotype proving that the transcript is important *in vivo*.

Finally, it is possible that the frameshift outlined above is a mechanism used *in vivo* to control translation of the pTOM 41 transcript. It is possible to sequence radiolabeled translation products, so that a determination can be made about what reading frame has been used to produce the pTOM 41 translation product (Bassham et al., 1991). This technique determines where the radiolabeled amino acid is incorporated in the sequence. For example if [^{3}H]-leucine were used in the sequencing reaction the long unmodified ORF in frame 3 would be labeled at the 1st, 3rd, 15th, 17th and 18th position. In the first 28 amino acids of frame 2 the same reaction would label only the 7th amino acid (Fig. 4-13). Alternatively, lysine could be used to confirm the initial protein sequencing results. In this way, the precise position of the frameshift could be demonstrated.

More information is needed about the function of the *E. coli* ORF before conclusions can be made about what the homology to pTOM 41 means to the understanding of chromoplast development. This ORF, which is referred to as ORF2, is transcribed as a tricistronic message with phosphoribosylpyrophosphate (PRPP) synthetase. PRPP is an intermediate in the synthesis of pyridine nucleotide coenzymes, histidine, tryptophan, and the pyrimidine and purine nucleotides. The tricistronic message only represents about 5% of the total amount of PRPP

synthetase message produced (Post et al., 1992). The rest is synthesized from a promoter within the ORF2 on a monocistronic transcript that is inducible by pyrimidine starvation. In addition, the ORF1 has been shown to complement mutants in δ-aminolevulinic acid (ALA) synthesis (Ikemi et al., 1992). The ALA mutants are deficient in cytochromes, and consequently, result in respiratory deficiencies. These authors have suggested that the ORF1 may synthesize one of the subunits for a tRNA glutamyl NADPH dehydrogenase, which is a step in the 5 carbon pathway of ALA synthesis. It is unclear why the synthesis of PRPP and ALA should be coordinated. The only obvious involvement of PRPP in the ALA pathway may be in the production of the NADPH. In addition, these data do not suggest a function for the ORF2 or pTOM 41. Until a mutant phenotype for the ORF2 or pTOM 41 is produced, or a connection is made between the synthesis of ALA and PRPP, the function of these two ORFs will remain enigmatic.

The second transcript that hybridizes to pTOM 41 may be produced by a second gene or may result from processing of pTOM 41 itself. Normally, heat stress can inhibit the transcription, processing, stability of messages and the translation of constitutively expressed proteins (Gurley and Key, 1991). However, the opposite seems to occur with pTOM 41. In this case, not only is more total pTOM 41 hybridizing message produced during heat stress, but a smaller transcript is formed. Southern analysis of tomato DNA with pTOM 41 suggests that only 1 or 2 genes are present (data not shown). Since more steady-state RNA is present in the heat stressed material, it is unlikely that a stress related transcript degradation is the

predominant factor for the increase in total amount of RNA that hybridizes to pTOM 41 in heat stressed material. The simplest interpretation of these data is that heat stress actually induces the production of the second transcript. Perhaps a second pTOM 41 protein is produced that is not targeted to plastids. If the pTOM 41 transcript did not contain a transit peptide, comparison to the bacterial ORF suggests that the transcript would be approximately 340 bases shorter. This second transcript could be produced by a second gene or result from altered processing of pTOM 41. Recently, Chiu et al.(1992) found that the same gene in yeast produced two transcripts for a histidyl-tRNA synthetase. One of these proteins was targeted to the mitochondria while a shorter transcript coded for a cytoplasmic form of the enzyme. Site directed mutagenesis of the gene and transformation back into yeast confirmed what sequence analysis had suggested. Antisense RNA transformation of pTOM 41 is presently underway (see Appendix). Hopefully the production of an antisense mutant in tomato will help to clarify the role of pTOM41 during chromoplast development.

The results reported in Fig. 4-11 confirm that the pTOM 111 transcript is ripening associated at normal temperatures and also that more transcript is produced in response to heat stress as ripening proceeds. These results are strikingly reminiscent of data shown by Olson et al. (1991) for the accumulation of ACC synthase 1 in tomato fruit during ripening, with and without wounding. The ACC synthase 1 transcript codes for a member of a small multigene family that is involved in the synthesis of ethylene. The induction of this hormone is known to occur during

both ripening and wounding. Fruit wounded at different stages of maturity showed an increasing amount of wound induced ACC synthase 1 transcript as ripening progressed. In other words, the combination of ripening and wounding acts synergistically to affect the steady state level of this transcript. This analogy proposes that the increase in hsp transcript is due to chromoplast development and is enhanced by heat stress.

The role for the low MW heat shock proteins remains obscure (Vierling, 1991). It is generally accepted that these proteins are induced by the presence of unfolded or aberrant proteins (Neumann et al., 1989). Unlike the hsp 60s and 70s, the low MW hsps in plants are usually only observed during times of stress. However, a few exceptions to this have been noted. For example, low MW hsp transcript is present in dry and germinating embryos of wheat (Helm and Abernethy, 1990) and sunflower (Almoguera and Jordano, 1992). It has also been observed during somatic embryo formation in alfalfa (Gyorgyey et al., 1991). However, there have been no previous reports of a developmentally induced plastid-localized low MW hsp. Perhaps this type of evidence will suggest a function for these enigmatic proteins.

The low MW hsps have also been associated with cadmium, arsenite, and osmotic stress in plants in some instances (Czarnecka et al., 1984; Edelman et al.,1988; Gyorgyey et al., 1991; Almoguera and Jordano, 1992). Low MW hsps have also been associated with oxidative stress in *Drosophila* and mammalian cells (Drummond and Steinhardt, 1987; Cajone and Bernelli-Zazzera, 1988; Courgeon et

al., 1988). Both heat and oxidative stress are believed to create aberrant and unfolded proteins (Neumann et al., 1989). Although the response to these stresses is usually lower than the response to heat stress, the fact that the low MW hsps are induced by other stresses does suggest that changes in the environment of ripening fruit should be considered.

The question that immediately arises from these observations is, what role does a plastid localized low MW hsp perform during ripening? Is the production of this transcript due to some massive reprogramming of protein structure during the formation of chromoplasts or does some other aspect of the environment within the ripening fruit induce expression of this hsp?

A recent paper by Romer et al. (1992) found that several free radical scavenging enzymes and compounds increase during the development of chromoplasts in pepper fruit. However, the evidence for oxidative stress during chromoplast formation in tomato fruit is contradictory. Perl-Treves and Galun (1991) found no increase in either of the plastidial free radical scavenging superoxide dismutase (SOD) isozymes during tomato fruit development. Additionally, they found that the single plastidial transcript they followed is low at MG and actually undetectable later in development. Livine and Gepstein (1988) however, found that cross-reacting material to a plastidial SOD peaks at the orange stage. Consequently, it is unclear whether tomato chromoplasts are oxidatively stressed during ripening.

One result of oxidative stress can be a loss in membrane integrity. Examination of the plastid membrane during chromoplast development in pepper

and tomato fruit has shown that no loss in integrity occurs (Legge et al., 1986; Whitaker, 1991). In other words, although there is evidence for an increase in oxidative stress in pepper chromoplasts, there is currently no evidence for damage of plastid membranes due to oxidative stress during chromoplast development. Consequently, even if there is some increase in free radical scavengers during ripening it is uncertain if this changing environment leads to an increase in damaged plastid proteins.

One possible explanation for the induction of pTOM 111 during ripening is that the stress response is a general one caused by changes in the cytoplasm during development. Since pTOM 66 also increases during ripening in the absence of heat stress (Picton and Grierson, 1989), perhaps the induction is dependent on the tissue rather than the compartment in which the protein resides. However, this idea requires that the induction of some hsps during ripening may be superfluous.

The ultimate location within the plastid may help predict the function of the plastid-localized low MW hsps, however, these data have been somewhat conflicting. Kloppstech et al. (1985) have proposed that the plastid hsp protects the photosynthetic membranes from heat stress because they found it associated with the thylakoid membranes. However, later studies revealed that only plastids exposed to heat stress under low light intensities have an hsp attached to this membrane (Glaczinski and Kloppstech, 1988). Work by Chen et al. (1990) and Clark and Crithley (1992), show that this protein accumulates predominantly in the stroma under normal light conditions even in heat stressed chloroplasts. Since Chen et al.

(1990) have found this hsp in heat stressed pea root amyloplasts and our data showed an increasing abundance of the transcript as the thylakoid membranes degrade, a broader role for this protein than simply protecting photosynthetic membranes must be postulated.

An examination of the conserved domains of plastid-localized low MW hsps has shown that the third domain, which is unique to these hsps, is similar to the methionine rich region in SRP54 a protein found in the endoplasmic reticulum and known to be responsible for binding signal sequences and transporting newly translated proteins (Chen and Vierling, 1991). It is tempting to speculate that the hsp accumulates in the stroma and is involved in sequestering unfolded proteins. This role could be important during heat or oxidative stress and during developmental changes as well, since all three of these conditions may cause an accumulation of unfolded proteins.

The breakdown of the photosynthetic membranes during chomoplast development may be an important factor in the induction of pTOM 111. Piechulla et al. (1987) and Livine and Gepstein (1988) have examined the loss of photosynthetic proteins during chromoplast development in tomato fruit. They find that a dramatic loss of LHCP between mature green and the yellow stage. Considering the abundance of this protein in the plastid, this could represent a significant increase in unfolded proteins. Our data show an increase in transcript for the hsp only after MG. If the induction of this protein is correlated with chloroplast breakdown, then increase in this hsp transcript may be expected at this time.

Characterization of the accumulation of this hsp in the tomato mutant *green flesh* might be useful, since the thylakoid membranes persist longer in mutant fruit than in normal fruit. If the plastid hsp is also deterred in this mutant then perhaps an involvement of this hsp in plastid protein breakdown would be suggested. In addition, the antisense mutagenesis of tomato with an antisense pTOM 111 construct is currently underway (see Appendix). Perhaps if this protein is responsible for the ordered dismantling of thylakoid membranes during chromoplast formation then the antisense mutant will be inhibited in breakdown of the photosynthetic membranes, or may result in diminished carotenoid synthesis because the plastid cannot adequately respond to this developmental stress.

An increase in the amount of transcript for two low MW hsps occurs during tomato fruit ripening at nonheat stress temperatures. This suggests that either both cytoplasm and plastid are undergoing massive structural reprogramming or a general increase in stress occurs in this tissue during ripening. A correlation between the breakdown of plastid proteins and the increase in the steady-state level of this transcript may exist. However, it probably is not such a simple correlation, since the transcript remains high after plastid conversion and a cytoplasmic low MW hsp is also induced. Examination of the breakdown of the photosynthetic membranes in the antisense pTOM 111 compared to normal fruit may help to clarify the involvement of this protein in the chloroplast to chromoplast transition.

CHAPTER 5
CONCLUSIONS

Chromoplast development is correlated with an increase in the steady-state level of a subset of transcripts encoding proteins directed to this organelle. We have identified 2 proteins from experiments examining the import of *in vitro* translation products from total poly A^+ RNA, and 3 proteins from the analysis of proteins encoded by cDNAs that are increasingly synthesized during this transition. Since pTOM5 (chapter 3) and PDS (Pecker et al., 1992) are increasingly synthesized as tomato fruit ripens, these data suggest that the deposition of carotenoids is not simply due to a repartitioning of the breakdown products of photosynthesis. In addition, these findings imply that the import apparatus remains functional during this period.

The characterization of a chromoplast-associated low MW hsp brings up more questions then it answers. Does this imply that the chromoplast is under stress during its development? Is ripening tomato fruit generally undergoing some type of stress? Does the transformation of the chloroplast into a chromoplast result in the induction of this hsp? The correlation between the degradation of thylakoid membrane proteins and the induction of the plastid-localized low MW hsp implies that induction of this protein may be caused by an increase in the amount of unfolded

proteins in the chromoplast. However, since induction of the hsp transcript appears to persist after the breakdown of the photosynthetic apparatus, perhaps an increase in oxidative stress during ripening also contributes to the induction of this protein.

The identification of the low MW hsp during chromoplast development was unexpected. It demonstrates the power such an unbiased assay can have on the characterization of a developmental process. Proteins not generally associated with chromoplast development or proteins of low abundance can be identified. In this way, a broader understanding of this transition is possible.

APPENDIX
CONSTRUCTION AND EXPRESSION OF ANTISENSE pTOM 41 AND pTOM 111 PLASMIDS IN TOBACCO

Introduction

A complete examination of the proteins involved in chromoplast development includes a functional analysis of the protein's role in this developmental process. An advantage of the experimental approach described within this dissertation is that once the chromoplast-associated protein has been identified, it has also been cloned. This permits production of plasmids that encode sense and antisense transcripts that can be transformed into tomato plants and hopefully produce a mutant phenotype. Not only can the transition from chloroplast to chromoplast be described, but the loss of function for particular proteins involved in this process can be monitored.

Antisense transformation has been utilized successfully for a number of ripening-associated genes. The expression of the cell wall softening enzymes polygalacturonase (PG) and pectin methylesterase, the ethylene biosynthetic enzymes ACC oxydase and ACC synthetase, and the carotenoid biosynthetic enzyme phytoene synthase have all been successfully inhibited by introduction of plasmids encoding antisense transcripts (Sheehy et al., 1988; Smith et al., 1988; 1990; Hamilton et al., 1990; Bird et al., 1991; Oeller et al., 1991; Tieman et al.,1992).

Antisense mutagenesis has produced dramatic consequences with ACC synthetase, where mutant fruit fail to soften, undergo the respiratory climacteric or produce carotenoids (Oeller et al., 1991). The application of ethylene is required before this fruit will proceed through the processes associated with ripening. PG, for example, is transcribed but it is not expressed. In this manner, the requirement of ethylene for the induction of ripening-associated proteins can be examined.

The results of antisense mutagenesis for PG, however, have been less striking (Sheehy et al., 1988; Smith et al., 1988; 1990). It was initially assumed that, since the transcript was enhanced during fruit ripening, and because this protein was produced in such vast amounts that it could be identified on a Commassie stained gel, inhibition of PG expression during ripening would lead to obvious reductions in cell wall softening. Although the production of the protein can be inhibited by antisense mutagenesis, cell wall softening is almost unaffected. This type of functional analysis was required to clarify the significance of PG during fruit ripening.

In the following section, the construction of a vector for production of antisense transcripts will be described. The ability of this vector to express transcripts was tested in tobacco. The transformation of sense and antisense pTOM 41 and pTOM 111 plasmids into tomato plants is currently underway. Given our current knowledge about these genes, the potential phenotype for successful antisense mutagenesis will be discussed.

Materials and Methods

Construction of Vectors for Production of Senses and Antisense Transcripts and Transformation into *Agrobacterium*

The vector system constructed for production of sense and antisense transcripts depends on three separate plasmids. First, both orientations of the pTOM cDNAs were subcloned into pGEM-4Z (Promega) in order to benefit from the ß-galactosidase selection built into this plasmid (Fig. 1A and B). The cDNAs can then be directionally excised from pGEM-4Z and ligated into a cauliflower mosaic virus (CaMV) 35S promoter/ nopaline synthase (nos) terminator cloning cassette cloned into Peth 3c (Fig 2A) (McCarty et al., 1991). Finally, the cloning cassettes containing the sense and antisense pTOM cDNAs are ligated into an *Agrobacterium* vector pGA472 (An et al., 1985), which has also been modified to contain the gene for ß-glucuronidase (GUS). Details of the vector construction and directional cloning of the pTOM cDNAs are given below.

Initially, the pTOM 111 and pTOM 41 cDNAs were blunt-end ligated into pGEM-4Z (Fig. 1). All molecular methods described in this section were taken from Sambrook et al.(1989) unless indicated otherwise. The pTOM 111 cDNA was isolated from pBluescript (KS) (Stratagene) (see chapter 4) by digestion with *Eco RV and SmaI* and ligated directly into pGEM-4Z at the *SmaI* site. The pTOM 41 cDNA was isolated from pSport 1 (Bethesda Research Laboratories) by digestion with *PstI* and made blunt ended with T4 DNA polymerase (Bethesda Research Laboratories) before it was ligated into the pGEM-4Z *SmaI* site. Plasmids were transformed into the *E. coli* strain TB1 and DNA was isolated by an alkaline lysis miniprep method

of Lee and Rasheed (1990). Sense and antisense orientations of the cDNAs in pGEM-4Z were confirmed by the presence of asymmetrical *Eco RV* sites in pTOM 41 (Fig. 1A) and a *NdeI* site in pTOM 111 (Fig 1B). The pGEM-4Z vector contains *KpnI* and *BamHI* sites on either side of the *SmaI* restriction site used for the initial cloning (Fig. 1A and B).

The plasmids containing the sense and antisense orientations of the pTOM cDNAs were ligated into the vector outlined in Fig. 2A. This vector was constructed in the following manner. The plasmid pBI221 (Clonetech) contains the coding region of GUS between the CaMV 35S promoter and the nos terminator. A polylinker shown in Fig. 2A was created at the ICBR DNA Synthesis Core and ligated into the *XbaI* and *SstI* sites of pBI221. This excised the GUS coding region and placed the polylinker between the CaMV 35S promoter and the nos terminator. The entire cloning cassette, which contained the CaMV 35S promoter, polylinker and nos terminator was then excised by restriction with *HindIII* and *Eco RI* and ligated into Peth 3c. Sense and antisense orientations of the pTOM cDNAs were excised from the pGEM plasmids by restriction with *KpnI* and *BamHI* and ligated into the polylinker of the cloning cassette at the *KpnI* and *BamHI* sites. The pTOM cDNAs were now cloned in both orientations between the CaMV 35S promoter and the nos terminator. In this manner, both sense and antisense transcripts would be produced.

The vector for transformation into *Agrobacterium* and ultimately into plant material was prepared as follows. The vector pGA472 was restricted with *ScaI*. The entire gene for the production of GUS with CaMV promoter and nos terminator,

was excised from pBI221 by restriction with *HindIII* and *EcoRI.* The GUS gene was made blunt ended with T4 DNA polymerase and ligated into the unique *ScaI* site of pGA472 (Fig. 2B). The cloning cassettes, which contained the sense and antisense pTOM cDNAs were excised from Peth 3c with *HindIII* and *BglII* and ligated into pGA472 GUS, which had been restricted with the same enzymes. The *Agrobacterium* strain AGL1 (Lazo et al., 1991) was transformed (An, 1987) with the plasmid DNA.

Successful transformation into AGL1 was confirmed by PCR amplification of bacterial DNA with primers specific for the NPTII and the GUS genes of the modified pGA472. The template for the PCR reaction was prepared by growing the transformed AGL1 at 28°C overnight. A 5 µl aliquot of this culture was diluted in 95 µl of water and boiled for 1 min. The PCR reaction contained 10 µl of this bacterial culture, 10 µl 10X TAQ polymerase buffer (Bethesda Research Laboratories), 200 µM each dNTP, 100 ng each primer, 1.25 µ of TAQ polymerase in 100 µl final volume.

Plant Material

Tobacco plants (*Nicotiana tabacum L.* cv. Kentucky 14) were grown in culture cups in sterile soil.

Transformation of Tobacco

Tobacco transformation were performed according to the procedure outlined by Horsch et al.(1985). I did not personally perform this particular procedure, so details of the protocol will not be outlined here.

Preparation of RNA

Tobacco leaf RNA was isolated as described by Dunsmuir et al. (1988). Briefly, this involved grinding 2 g of leaf tissue in liquid N_2 with a mortar and pestle. A 4.5 ml aliquot of NTS buffer, which contained 0.1M NaCl, 0.01M Tris-HCl pH 7.5, 1 mM EDTA and 1% SDS was added to the finely ground tissue. A 3 ml volume of phenol/chloroform/isoamyl alcohol (25:24:1) (PCI) was added and the tissue was ground until the mixture thawed. This was poured into a 50 ml capped tube and the mortar and pestle was rinsed with an additional 4.5 ml of NTS buffer and 3 ml of PCI. The mixture was vortexed for 5 min and the phases separated by centrifugation at 1000 x g for 10 min. The aqueous layer was saved and the phenol layer was washed with 2 ml of NTS and separated again by centrifugation. The combined aqueous layers were extracted with phenol/chloroform (1:1), reextracted with chloroform and precipitated by incubation overnight at -20°C with 0.1 volumes of 3M sodium acetate and 2 volumes of ethanol. The nucleic acids were collected by centrifugation at 10,000 x g for 10 min. The pellet was washed with 70% ethanol and repelleted. The nucleic acids were resuspended in 2 ml of sterile water and 2 ml of 4 M lithium acetate was added. The RNA was precipitated by incubation for 3 hours at 4°C and pelleted by centrifugation for 10 min at 10,000 x g. The pellet was resuspended in 0.9 ml sterile water, reprecipitated with 0.1 ml 3 M sodium acetate and 2 ml ethanol for 2 hours at -20°C and collected by centrifugation for 10 min at

10,000 x g. The pellet was rinsed with 70% ethanol, dissolved in 400 µl water and stored at -70°C.

Northern Hybridization

Total leaf RNA (10 µg/lane) was loaded onto formaldehyde denaturing gels, blotted, prehybridized, hybridized and washed as described in chapter 4. Probes for pTOM 111 and pTOM 41 were prepared as described in the previous chapter. A portion of the GUS gene from the *Xba1* to the *EcoRV* site of pBI221 was used as a probe and prepared for hybridization as described in the previous chapter.

Results

Agrobacterium transformation plasmids were constructed, as described in the Materials and Methods, that produce sense or antisense transcripts of pTOM 111 and pTOM 41 (Fig.A-1 and A-2). Tobacco leaf pieces were transformed with these plasmids, selected with kanamycin and tested for GUS activity (Jefferson, 1987). I did not perform the tobacco transformation or GUS staining so these technical details will not be discussed at this time.

Total RNA was extracted from leaf tissue of greenhouse grown transgenic tobacco plants, run on 1% agarose formaldehyde denaturing gels, blotted and hybridized as described in the previous chapter. Each blot was probed with either pTOM 111 or pTOM 41 cDNA, stripped and reprobed with a portion of the gus

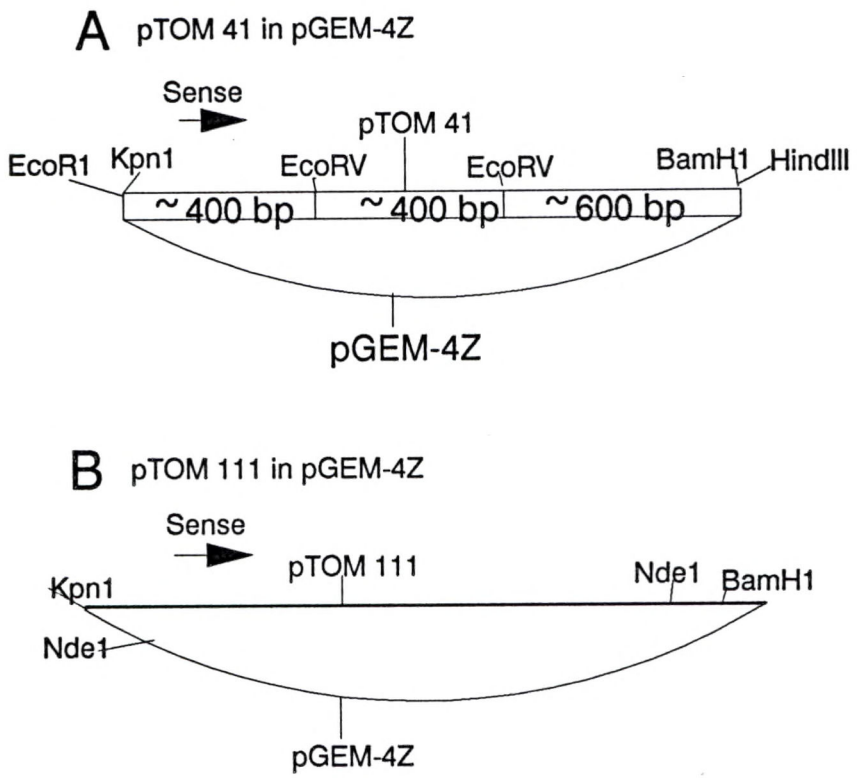


Figure A-1. Cloning of pTOM 41 and pTOM 111 into pGEM-4Z in the sense orientation. A) pTOM 41 contains two *EcoRV* sites. One of these restriction sites is within the first 400 bases of the 5' end of the clone. The pTOM 41 was cloned into pGEM-4Z at the *SmaI* site, which is bounded by *BamHI* and *KpnI*. B) pTOM 111 contains a *NdeI* site 30 bases from the 3' end of the clone. The pGEM-4Z contains an *NdeI* site ~290 bases from the *KpnI* site.

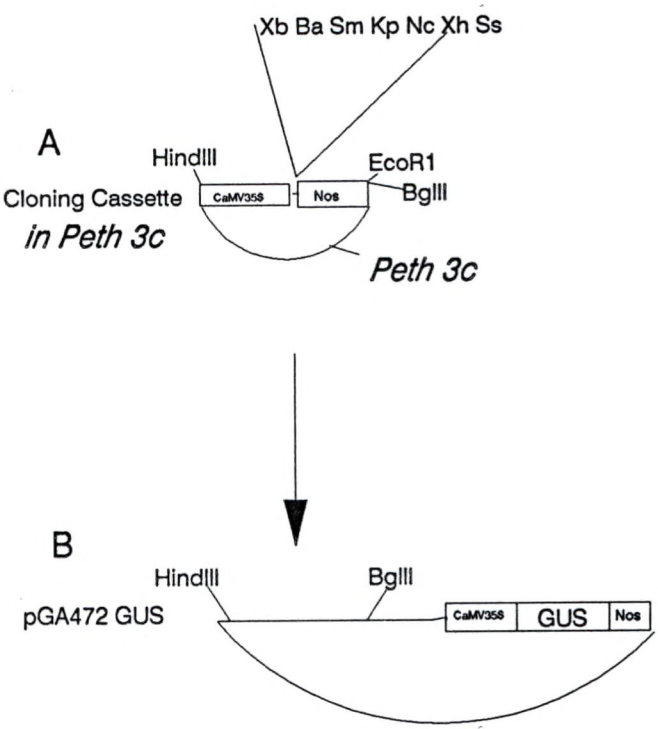


Figure A-2. The vectors used for cloning pTOM 41 and pTOM 111 cDNAs in the sense and antisense orientation. A) The cloning cassette contains a CaMV 35S promoter, polylinker and nos terminator. The polylinker contains an *XbaI, BamHI, SmaI, KpnI, NcoI, XhoI,* and *SstI* restriction sites. The pTOM cDNAs were cloned into this cassette at the *KpnI* and *BamHI* sites of the polylinker. B) The *Agrobacterium* vector pGA472 contains a GUS gene at the *ScaI* site. The cloning cassette is cloned into this vector at the *HindIII* and *BglII* sites.

gene as described in the Materials and Methods. A representative blot is presented for pTOM 111 (Fig. A-3) and for pTOM 41 (Fig. A-4).

A total of 30 separate transgenic plants were tested for the ability to express transcript. Tobacco plants do not normally appear to contain any RNA that hybridizes to pTOM 111 or pTOM 41 cDNA. This was shown by examining plants that have been transformed with a plasmid containing the GUS gene alone (Fig. A-3, lanes 11 and 12; and Fig. A-4, lanes 2 and 8). Most plants transformed with these constructs expressed the GUS gene, although, slight differences in the level of expression were seen in the individual plants. This demonstrates that these newly constructed vectors were capable of being stably transformed and expressed in tobacco (Fig. A-3 and A-4). However, 7 of the 30 putatively transformed plants did not contain RNA that hybridized to the GUS probe. Either the gene or expression of the GUS gene was selectively lost in 6 of these plants, because 6 GUS negative transformants were positive for expression of pTOM transcript. A striking example of this is shown in Fig. A-3, lane 9. This plant is producing copious amounts of pTOM 111 sense transcript, but apparently no GUS message is produced. Partial loss of T-DNA during transformation is not uncommon, nor is inhibition of expression of individual genes following transformation (Jones et al., 1987; Deroles and Gardner, 1988).

The level of expression of the sense and antisense transcripts of the pTOM cDNAs varied dramatically between individual transformants (Fig. A-3 and A-4). In addition, different orientations expressed very different levels of transcript. For

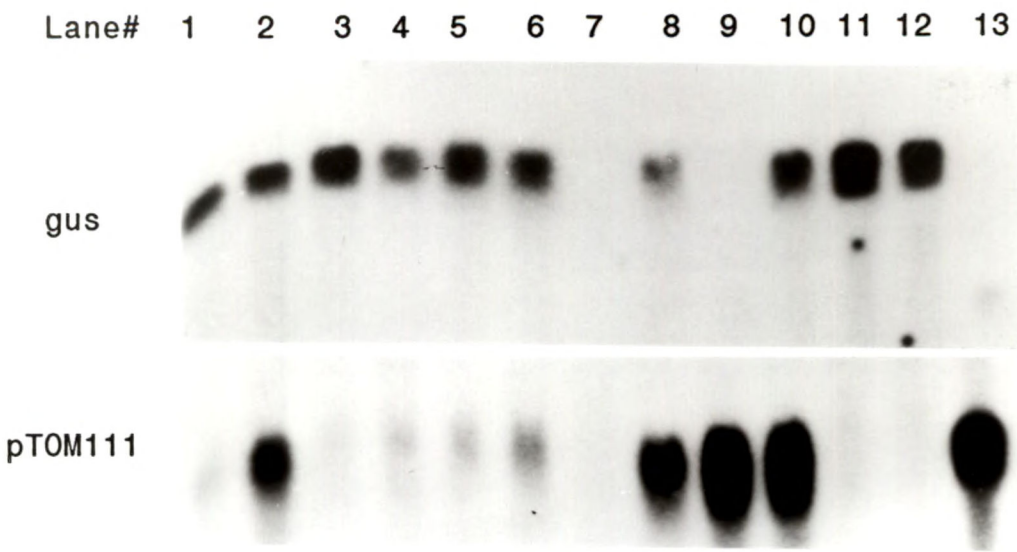


Figure A-3. Individual transgenic tobacco plants were transformed with constructs of the pTOM 111 cDNA that produced either sense or antisense transcripts. RNA was extracted from leaf tissue. Total RNA (10μg/lane) was loaded onto 1% agarose formaldehyde denaturing gels, blotted and probed as described in the Materials and Methods. The bottom panel represents the results obtained when the blot was probed with the entire pTOM 111 cDNA. The blot was stripped and reprobed with an *XbaI* to *EcoRV* fragment of the GUS gene. Lanes 2, 7, 8, 9 and 10 were individual transgenic plants that produced sense transcripts of the pTOM 111 cDNA. Lanes 1, 3, 4, 5, and 6 represent RNA from individual transgenic plants containing constructs that produce antisense transcripts of pTOM 111. Lanes 10 and 11 contain RNA from plants transformed with pGA472 GUS alone. Lane 13 contains 5 μg total RNA from >80% red ripe fruit.

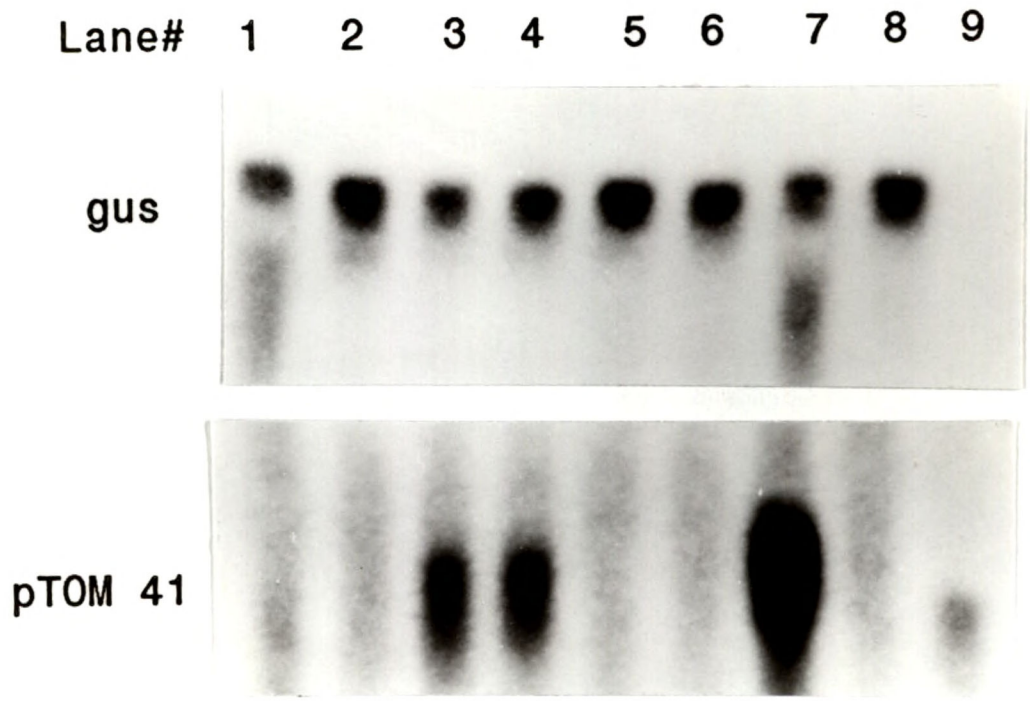


Figure A-4. Individual transgenic tobacco plants transformed with constructs of the pTOM 41 cDNA that produced either sense of antisense transcripts. RNA was extracted from leaf tissue. Total RNA (10 µg/lane) was loaded onto 1% agarose formaldehyde denaturing gels, blotted and probed as described in the Materials and Methods. The bottom panel represents the results obtained when the blot was probed with the entire pTOM 41 cDNA. The blot was stripped and reprobed with an *XbaI* to *EcoRV* fragment of the GUS gene. This is shown in the top panel. Lanes 3, 4, and 7 contain RNA isolated from individual transgenic plants transformed with a plasmid that produces antisense pTOM 41. Lanes 1, 5 and 6 contain RNA from individual transgenic plants transformed with a plasmid that produces sense transcripts of pTOM 41. Lanes 2 and 8 contain RNA from individual transgenic plants that have been transformed with pGA472 GUS alone. Lane 9 contains RNA from 50% red ripe tomato fruit.

example, the sense orientation of pTOM 111 produced large amounts of transcript whereas the antisense orientation did not (Fig. A-3). Plants transformed with the antisense orientation of pTOM 111 often contained hybridizing RNA that produced a low level smear when hybridized with pTOM 111 as a probe. However, this was not a general property of the RNA since hybridization of the same blot with the GUS probe produced a discrete band (Fig. A-3). This appears to be an inherent result of the antisense pTOM 111 construct because this was observed in at least 9 out of 11 individual transformants. In contrast, 4 out of 5 plants transformed with the sense orientation expressed RNA at levels similar to the amount expressed during chromoplast development in tomato fruit. This is shown by comparison of the RNA produced by transformed plants to the RNA from >80% red ripe tomato fruit (Fig. A-3, lanes 2, 8, 9 and 10 versus lane 13). These results confirm that the cloning cassettes containing either the sense or antisense pTOM 111 constructs are capable of expressing transcript.

A similar analysis was performed with tobacco plants transformed with constructs capable of producing sense and antisense transcripts of pTOM 41. Fig. A-4 shows that in this case 3 individual transgenic plants of 3 tested, which contain antisense pTOM 41, produced large amounts of antisense transcript. Comparison of the amount of RNA produced by these plants to the amount normally found in 50% red ripe tomato fruit shows that a large excess of antisense RNA is produced by the transgenic plants (Fig. A-4, lane 9 versus lanes 3, 4, and 7). Unfortunately, no RNA was produced with the sense constructs of pTOM 41 in the 9 individual transgenic

plants tested. It is unclear why the expression of the sense transcript of pTOM 41 is inhibited. These plants produce measurable amounts of GUS transcript; therefore, they have been successfully transformed. Either a portion of the plasmid containing the pTOM 41 cloning cassette has been selectively lost or the expression of this transcript has been selectively inhibited.

No obvious phenotype resulted from the transformation of tobacco with these constructs. However, the plants have not set seed yet so it is too early to tell if embryo development will be affected by the new genes.

Discussion

The purpose of this study was to test whether the constructed plasmids could effectively produce transcripts so that ultimately they could be used in tomato transformation. The constructs were first tested in tobacco, because, although there are reports in the literature on tomato transformation, it is not an entirely straight-forward procedure. We have shown that the GUS gene placed at the *ScaI* site of pGA472 is stably expressed in mature tobacco leaves. The cloning cassette can also be effective in stable expression of large amounts of RNA. These plasmids will have utility beyond this study, since they can be used in other situations and with other species.

The 11 plants transformed with the antisense construct of pTOM 111, however, do not appear to maintain high levels of this transcript. This result may occur for a number of reasons. First, the transformed tobacco plants may selectively

lose expression of the gene during transformation and growth of the transgenic plant. Secondly, the antisense RNA may interact with a tobacco leaf plastid-localized low MW hsp transcript. A loss in antisense RNA was observed when endogenous message to pTOM 13 was induced by wounding antisense pTOM 13 plants (Hamilton et al., 1990). The production of the endogenous transcript caused degradation of both messages. It is unclear how homologous the tobacco hsp gene is to pTOM 111. However, considering the fact that a petunia hsp gene is 81% homologous to the tomato hsp and that successful reduction in tobacco chalcone synthase message occurred with transformation of a petunia antisense construct that was only 86% homologous to the endogenous transcript, the idea that an interaction could occur between the pTOM 111 antisense transcript and the endogenous message seems feasible (van der Krol et al., 1988).

Perhaps the plants should be maintained in conditions that do not lead to selection against expression of the antisense transcript. If an interaction is occurring between the antisense construct and the tobacco plant, then high level expression of a stable transformant in tomato tissue will be difficult. The tomato transformation experiment could be performed with a fruit specific promoter rather than the nonspecific CaMV 35S promoter. This would not be difficult since I have also constructed a cloning vector with the fruit specific promoter E8 (Fischer, personal communication). In this way, even if the endogenous hsp is produced in the leaves it would not be inhibited by the antisense construct. Therefore, the effect of inhibiting hsp induction would be studied only during chromoplast development.

What type of phenotype may be expected by the transformation of antisense transcripts of pTOM 41 into tomato plants? This question is extremely difficult to answer. It is unclear from sequence comparison to the ORF2 from *E. coli* what function pTOM 41 performs during chromoplast development. Since the synthesis of the ORF2 is coordinated with the synthesis of PRPP synthetase and a gene involved in δ-ALA synthesis, perhaps it is involved with the synthesis of NADPH. The stepwise dehydrogenation of phytoene requires pyridine nucleotides to function. Perhaps pTOM 41 is involved in the synthesis of these cofactors. In that case, the inhibition of pTOM 41 could result in loss of carotenoid synthesis.

The loss of pTOM 111 during chromoplast development via antisense mutagenesis may result in a failure in the ordered breakdown of the thylakoid membranes during the chloroplast to chromoplast conversion. This has been suggested by the correlation between the induction of the pTOM 111 transcript with the loss of LHCP during chromoplast development. If the induction of pTOM 111 occurs because of an increase in unfolded proteins during the degradation of photosynthetic proteins, then it is possible that the degradative process will continue. Perhaps the loss of LHCP will be slowed in the mutant plants and this may be demonstrated by comparisons of developmental profiles of proteins from tomato fruit extracts. It is likely that the continued synthesis and deposition of carotenoids could be inhibited by the plastids inability to cope with a buildup in partially degraded proteins. If a function of this protein is uncovered during chromoplast development, perhaps it will suggest a function for these hsps during heat stress.

REFERENCES

Abernethy, R.H., D.S. Thiel, N.S. Petersen, and K. Helm. 1989. Thermotolerance is developmentally dependent in germinating wheat seed. Plant Physiol. 89:596-576

Almoguera, C., and J. Jordana. 1992. Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock protein and Lea mRNAs. Plant Mol. Biol. 19:781-792

Altschul,S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. A basic local alignment search tool. J. Mol. Biol. 215:403-410

An, G. 1987. Binary Ti vectors for plant transformation and promoter analysis. Methods in Enzymol. 153:292-305

An., G., B.D. Watson, S. Stachel, M.P. Gordon, E.W. Nester. 1985. New cloning vehicles for transformation of higher plants. Embo Journal 4:277-284

Armstrong, G.A., M. Alberti, J.E. Hearst. 1990a. Conserved enzymes mediate the early reactions of carotenoid biosynthesis in nonphotosynthetic and photosynthetic prokaryotes. Proc. Natl. Acad. Sci. USA. 87:9975-9979

Armstrong, G.A., A. Schmidt, G. Sandmann, and J.E. Hearst. 1990b. Genetic and biochemical characterization of carotenoid biosynthesis mutants of *Rhodobacter capsulatus*. The J. Biol. Chem. 265(14):8329-8338

Arrigo, A.P. 1987. Cellular localization of hsp23 during *Drosophila* development and following subsequent heat shock. Develop. Biol. 122:39-48

Atkins, J.F., R.B. Weiss, and R.F. Gesteland. 1990. Ribosome gymnastics-Degree of difficulty 9.5, style 10.0. Cell 62:413-423

Barja de Quiroga, G. 1992. Brown fat thermogenesis and exercise: two examples of physiological oxidative stress. Free Radical Biology and Medicine. 13:325-340

Bartley, G.E., P.V. Vitanen, I. Pecker, D. Chambovitz, J. Hirschberg, and P.A. Scolnik. 1991. Molecular cloning and expression in photosynthetic bacteria of a soybean cDNA coding for phytoene desaturase, an enzyme if the carotenoid biosynthesis pathway. Proc. Natl. Acad. Sci. USA. 88:6532-6536

Bartley, G.E., P. V. Vitanen, K. O. Bacot, and P. A. Scolnik. 1992. A tomato gene expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway. Journal of Biological Chemistry 267(8):5036-5039

Bassham, D.C., D. Barling, R.M. Mould, B. Dunbar, P. Weisbeek, R.G. Herrmann, and C. Robinson. 1991. Transport of proteins into chloroplasts. J. of Biol. Chem. 266(35):23606-23610.

Bathgate, B., M.E. Purton, D. Grierson and P.W. Goodenough 1985. Plastid changes during the conversion of chloroplasts to chromoplasts in ripening tomatoes. Planta. 165:197-204

Berry-Lowe, S.L., and G.W. Schmidt. 1991. Chloroplast protein transport. In: L. Bogorad, and I.K. Vasil (eds.) The Molecular Biology of Plastids Cell Culture and Somatic Cell Genetics of Plants, Academic Press Inc. New York. 7A:257-302.

Biggs, M.S., R.W. Harriman and A.K. Handa. 1986. Changes in gene expression during tomato fruit ripening. Plant Physiol. 81:395-403

Bird, C.R., J.A. Ray, J.D. Fletcher, J.M. Boniwell, A.S. Bird, C. Teulieres, I. Blian, P.M. Bramley, and W. Schuch. 1991. Using antisenes RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes. Biotechnology. 9:635-639

Bond, U., and M.J. Schlesinger. 1987. Heat-shock proteins and development. Advances in Genetics. 24:1-29

Bouchard, R.A. 1990. Characterization of expressed meiotic, prophase repeat transcript clones of *Lilium*:meiosis-specific expression, relatedness, and affinities to small heat shock protein genes. Genome 33:68-79

Bowler, C., T. Alliotte, M. De Loose, M. Van Montagu, and D. Inze. 1989. The induction of manganese superoxide dismutase in response to stress in *Nicotiana plumbaginifolia*. The Embo Journal. 8(1):31-38

Bowsher, C.G., B.J.M. Ferrie, S. Ghosh, J. Todd, J.E. Thompson, and S.J. Rothstein. 1992. Purification and partial characterization of a membrane-associated lipoxygenase in tomato fruit. Plant Physiol. 100:1802-1807

Bramley, P., C. Teulieres, I. Blain, C.Bird, and W. Schuch. 1992. Biochemical characterization of transgenic tomato plants in which carotenoid synthesis has been inhibited through the expression of antisense RNA to pTOM5. The Plant Journal. 2(3):343-349

Burke, J.J., Hatfield, J.L., Klein, R.R., and Mullet, J.E. 1985. Accumulation of heat shock proteins in field-grown cotton. Plant Physiol. 78:394-398

Cajone, F., and A. Bernelli-Zazzera. 1988. Oxidative stress induces a subset of heat shock proteins in rat hepatocytes and MH_1C_1 cells. Chem.-Biol. Interactions. 65:235-246

Camara, B. 1984. Terpenoid metabolism in plastids. Sites of phytoene synthetase activity and synthesis in plant cells. Plant Physiol. 74:112-116

Camara, B., and J. Brangeon. 1981. Carotenoid metabolism during chloroplast to chromoplast transformation in *Capsicum annuum* fruit. Planta. 151:359-364

Carde, J.P., B. Camara and C. Cheniclet. 1988. Absence of ribosomes in *Capsicum* chromoplasts. Planta. 173:1-11

Cathala, G., J.-F. Savouret, B. Mendez, B.L. West, M. Karin, J.A. Martial, and J.D. Baxter. 1983. A simple method for isolation of RNA. DNA. 2(4):329-335

Cervantes-Cervantes, M., N. Hadjeb, L.A. Newman and C.A. Price. 1990. ChrA is a carotenoid-binding protein in chromoplasts of *Capsicum annuum*. Plant Physiol. 93:1241-1243

Chen, Q., and E. Vierling. 1991. Analysis of conserved domains identifies a unique structural feature of a chloroplast heat shock protein. Mol. Gen. Genet. 226:425-431

Chen, Q., L.M. Lauzon, A.E. DeRocher, and E. Vierling. 1990. Accumulation, stability, and localization of a major chloroplast heat-shock protein. The J. of Cell Bio. 110:1873-1883

Cheniclet, C., F. Rafia, A. Saint-Guily, A. Verna, and J.P. Carde. 1992. Localization of the enzyme geranylgeranyl-pyrophosphate synthase in *Capsicum* fruits by immunogold cytochemistry after conventional chemical fixation or quick-freezing followed by freeze-substitution. Labelling evolution during ripening. Biol. cell. 75:145-154

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid Guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159

Clarke, A.K., and C. Critchley. 1992. The identification of a heat-shock protein complex in chloroplasts of barley leaves. Plant Physiol. 100:2081-2089

Cline K (1986) Import of proteins into chloroplasts. Membrane integration of a thylakoid precursor protein reconstituted in chloroplast lysates. J Biol Chem 261: 14804-14810

Cline K (1988) Light-harvesting chlorophyll a/b protein. Membrane insertion, proteolytic processing, assembly into LHC II, and localization to appressed membranes occurs in chloroplast lysates. Plant Physiol 86: 1120-1126

Cline K, D.R. Fulsom and P.V. Viitanen 1989. An imported thylakoid protein accumulates in the stroma when insertion into thylakoids is inhibited. J Biol Chem 264:14225-14232

Courgeon, A.M., E. Rollet, J. Becker, C. Maisonhaute, and M. Best-Belpomme. 1988. Hydrogen peroxide (H_2O_2) induces actin and some heat-shock proteins in Drosophila cells. Eur. J. Biochem. 171:163-170

Czarnecka, E., L. Edelman, F. Schoffl, and J.L. Key. 1984. Comparative analysis of physical stress responses in soybean seedlings using cloned heat shock cDNAs. Plant Mol. Biol. 3:45-58

Dalling, M.J. 1987. Proteolytic enzymes and leaf senescence. In: W.W. Thomson, E.A. Nothnagel, and R.C. Huffaker (eds.) Plant senescence: Its biochemistry and physiology, American Society of Plant Physiologists. Rockville, Maryland. pp. 54-70

Davies, J.W., and Cocking, E.C. 1965. Changes in carbohydrates, proteins and nucleic acids during cellular development in tomato fruit locule tissue. Planta. 67:242-253

Davies, K.M. and D. Grierson. 1989. Identification of cDNA clones for tomato (*Lycopersicon esculentum* Mill.) mRNAs that accumulate during fruit ripening and leaf senescence. Planta. 179:73-80

Davies, J.N., and C.E. Hobson. 1981. The constituents of tomato fruit-the influence of environment, nutrition and genotype. CRC Crit. Rev. Food Sci. Nutr. 15:205

deBoer D, F Cremers, R Teertstra, L Smits, J Hille, S Smeekens and P Weisbeek (1988) In vivo import of plastocyanin and a fusion protein into developmentally different plastids of transgenic plants. Embo J 7: 2631-2635

DellaPenna, D., D.C. Alexander, and A.B. Bennett. 1986. Molecular cloning of tomato fruit polygalacturonase: Analysis of polygalacturonase mRNA levels during ripening. Proc. Natl. Acad. Sci. USA. 83:6420-6424

DellaPenna D, JE Lincoln, Rl Fischer and AB Bennett (1989) Transcriptional analysis of polygalacturonase and other ripening associated genes in Rutgers, rin, nor, and Nr tomato fruit. Plant Physiol. 90: 1372-1377

Deroles, S.C., and R.C. Gardner. 1988. Analysis of the T-DNA structure in a large number of transgenic petunias generated by *Agrobacterium*-mediated transformation. Plant Mol. biol. 11:365-377.

Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387-395

Dogbo O, F Bardat, A Laferriere, J Quennemet, J Brangeon and B Camara (1987) Metabolism of plastid terpenoids. I. Biosynthesis in plastid stroma isolated from higher plants. Plant Sci 49: 89-101

Drummond, I.A.S., and R.A. Steinhardt. 1987. The role of oxidative stress in the induction of Drosophila heat-shock proteins. Experimental Cell Research. 173:439-449

Dunsmuir,P., D. Bond, K. Lee, D. Gidoni, J. Townsend. 1988. Stability of introduced genes and stability in expression. Plant Mol. Bio. Manual C1:1-17 Kluwer Academic Publications Dordrecht. Belguim

Edelman, L., E. Czarnecka, and J.L. Key. 1988. Induction and accumulation of heat shock-specific poly (A^+) RNAs and proteins in soybean seedlings during arsenite and cadmium treatments. Plant Physiol. 86:1048-1056

Fischer, R.L., and A.B. Bennett. 1991. Role of cell wall hydrolases in fruit ripening. Ann. Review of Plant Physiol. and Plant Mol. Biol. 42:675-703

Fleming, J.E., I. Reveillaud, and A. Niedzwiecki. 1992. Role of oxidative stress in Drosophila aging. Mutation Research. 275:267-279

Fray, R.G., G.W. Lycett, and D. Grierson. 1990. Nucleotide sequence of a heat-shock and ripening-related cDNA from tomato. Nucleic Acid Research. 18(23):7148

Gepstein, S. 1988. Photosynthesis. In: L.D. Nooden, and A.C. Leopold (eds) Senescence and aging in plants. Academic Press Inc. New York. pp.85-109

Glaczinski, H., and K. Kloppstech. 1988. Temperature-dependent binding to the thylakoid membranes of nuclear-coded chloroplast heat-shock proteins. Eur. J. Biochem. 173:579-583

Goodenough, P.W. 1986. A review of the role of ethylene in biochemical control of ripening in tomato fruit. Plant Growth Regulation. 4:125-137

Goodwin, T.W. 1980. The Biochemistry of the Carotenoids. vol 1 Plants. New York NY. Chapman and Hill

Goodwin, T.W., and L.J. Goad. 1970. Carotenoids and triterpenoids. In:A.C. Hulme (ed) The Biochemistry of Fruits and their Products. Academic Press. London. vol.1

Goodwin, T.W. and, E.I. Mercer. 1983. Introduction to Plant Biochemistry Pergamon Press Oxford and N.Y.

Gray, J.C. 1987. Control of isoprenoid biosynthesis in higher plants. Advances in Botanical Research. 14:25-88

Grierson D, A Slater, J Speirs and GA Tucker (1985) The appearance of polygalacturonase mRNA in tomatoes: one of a series of changes in gene expression during development and ripening. Planta 163: 263-271

Grierson, D. 1986. Gene expression in ripening tomato fruit. CRC Crit Rev in Plant Science. 3(2):113-132

Gruissem, W., X.W. Deng and, J.Tonkyn. 1989. Control of chloroplast gene expression during plant development and environmental changes. Photosynthesis 269-284

Guedon, G.F., G.J.P. Gilson, J.P. Ebel, N.M-T. Befort, and P.M. Remy. 1986. Lack of correlation between extensive accumulation of bisnucleoside polyphospates and the heat-shock response in eucaryotic cells. The J. of Biol. Chem. 261(35):16459-16465

Guiamet, J.J., E. Schwartz, E. Pichersky, And L.D. Nooden. 1991. Characterization of cytoplasmic and nuclear mutations affecting chlorophyll and chlorophyll-binding proteins during senescence in soybean. Plant Physiol. 96:227-231

Gurley, W.B., and J.L. Key. 1991. Transcriptional regulation of the heat-shock response: A plant perspective. Biochemistry 30(1):1-11

Gyorgyey, J., A. Gartner, K. Nemeth, Z. Magyar, H. Hirt, E. Heberle-Bors, and D. Dudits. 1991. Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol. Biol. 16:999-1007

Hadjeb, N., I. Gounaris and C.A. Price. 1988. Chromoplast-specific proteins in *Capsicum annuum*. Plant Physiol. 88:42-45

Hamilton, A.J., G.W. Lycett, and D. Grierson. 1990. Antisense gene inhibits synthesis of the hormone ethylene in transgenic plants. Nature. 346:284-287

Harman, D. 1992. Free radical theory of aging. Mutation Research. 275:257-266

Harriman, R.W., D.M. Tieman, and A.K. Handa. 1991. Molecular cloning of tomato pectin methylesterase gene and its expression in Rutgers, Ripening Inhibitor, Nonripening, and Never Ripe tomato fruits. Plant Physiol. 97:-80-87

Harris, W.M., and A.R. Spurr. 1969. Chromoplasts of tomato fruits. II. The red tomato. Amer. J. Bot. 56(4):380-389

Hatanaka, A., T. Kajiwara, K. Matsui, and A. Kitamura. 1992. Expression of lipoxygenase and hydroperoxide lyase activities in tomato fruits. Z. Naturforsch. 47c:369-374

Helm, K.W., and R.H. Abernethy. 1990. Heat shock proteins and their mRNAs in dry and early imbibing embryos of wheat. Plant Physiol. 93:1626-1633

Helm, K.W., N.S. Petersen, and R.H. Abernethy. 1989. Heat shock response of germinating embryos of wheat: effects of imbibition time and seed vigor. Plant Physiol. 90:598-605

High, S., and B. Dobberstein. 1991. The signal sequence interacts with the methionine-rich domain of the 54-kD protein of signal recognition particle. J. Cell Biol. 113:229-233

Holdsworth, M.J., C.R. Bird, J. Ray, W. Schuch, and D. Grierson. 1987. Structure and expression of an ethylene-related mRNA from tomato. Nucleic Acid Research. 15(2):731-739

Horsch, R.B., J.E. Fry, N.L. Hoffmann, D. Eichholtz, S.G. Rogers, R.T. Fraley. 1985. A simple and general method for transferring genes into plants. Science 227:1229-1231.

Hrazdina, G., and R.A. Jensen. 1992. Spatial organization of enzymes in plant metabolic pathways. In: W.R. Briggs (ed.) Annual review of Plant Physiol. and Plant Mol. Bio. 43 Annual Reviews Inc. Palo Alto, Ca. pp.241-267

Hugueney, P., S. Romer, M. Kuntz, and B. Camara. 1992. Characterization and molecular cloning of a flavoprotein catalyzing the synthesis of phytofluene and gamma-carotene in *Capsicum* chromoplasts. Eur. J. of Biochem. 209:399-407

Hunt C.M., R.C. Hardison, and C.D. Boyer 1986. Restriction enzyme analysis of tomato chloroplast and chromoplast DNA. Plant Physiol 82: 1145-1147

Hurtado-Hernandez, H., and P.G. Smith. 1985. Inheritance of mature fruit color in *Capsicum annuum* L. The J. of Heredity. 76:211-213

Ikemi, M., K. Murakami, M. Hashimoto, and Y. Murooka. 1992. Cloning and characterization of genes involved in the synthesis of δ-aminolevulinic acid in *Escherichia coli*. Gene. 121:127-132

Issels, R.D., S. Bourier, B. Boning, G.C. Li, J.J. Mak and W. Wilmanns. 1987. Influence of oxidative stress induced by cysteamine upon the induction and development of thermotolerance in chinese hamster ovary cells. Cancer Research. 47:2268-2274

Iwatsuki, N., R. Moriyama and T. Asahi. 1984. Isolation and properties of intact chromoplasts from tomato fruit. Plant and Cell Physiol. 25(5):763-768

Jaattela ,M., and D. Wissing. 1992. Emerging role of heat shock proteins in biology and medicine. Annals of Medicine. 24:249-258

Jefferson, R.A. 1987. Assaying chimeric genes in plants:the GUS gene fusion system. Plant Mol. Biol. Rep.5:387-405

Jeffery, D., P.W. Goodenough, and P.D.J. Weitzman. 1986. Enzyme activities in mitochondria isolated from ripening tomato fruit. Planta. 168:390-394

Jones, J.D.G., D.E. Gilbert, K.L. Grady, and R.A. Jorgensen. 1987. T-DNA structure and gene expression in petunia plants transformed by *Agrobacterium tumefaciens* C58 derivatives. Mol. Gen. Genet. 207:478-485.

Kader, A.A., and L.L Morris. 1976. Correlating subjective and objective measurements of maturation and ripeness of tomatoes. Proc. 2nd Tomato Quality Workshop. University of California. Davis p57-62

Keegstra K 1989. Transport and routing of proteins into chloroplasts. Cell 56: 247-253

Keegstra, K., L.J. Olsen and S.M. Theg. 1989. Chloroplastic precursors and their transport across the envelope membranes. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:471-501

Khudairi, A.K. 1972. The ripening of tomatoes. American Scientist. 60:696-707

Kimpel, J.A., and J.L. Key. 1985. Presence of heat shock mRNAs in field grown soybeans. Plant Physiol. 79:672-678

Kimpel, J.A., R.T. Nagao, V. Goekjian, and J.L. Key. 1990. Regulation of the heat shock response in soybean seedlings. Plant Physiol. 94:988-995

Klee, H.J., M.B. Hayford, K.A. Kretzmer, G.F. Barry and G.M. Kishore. 1991. Control of ethylene biosynthesis by expression of a bacterial enzyme in transgenic tomato plants. The Plant Cell. 3:1187-1193

Kloppstech, K. G. Meyer, G. Schuster, and I. Ohad. 1985. Synthesis, transport and localization of a nuclear coded 22-kD heat-shock protein in the chloroplast membranes of peas and *Chamydomonas reinhardi*. The Embo J. 4(8):1901-1909

Klosgen R.B., H. Saedler, and J.-H. Weil 1989. The amyloplast-targeting transit peptide of the waxy protein of maize also mediates protein transport in vitro into chloroplasts. Mol Gen Genet 217: 155-161

Kobayashi H. 1991. Differentiation of amyloplasts and chromoplasts. In: L Bogorad, and IK Vasil (eds) The photosynthetic apparatus:molecular biology and operation. Academic Press, San Diego CA. pp. 395-415

Kobayashi, H., J. Ngernprasirtsiri and T. Akazawa. 1990. Transcriptional regulation and DNA methylation in plastids during transitional conversion of chloroplasts to chromoplasts. Embo J. 9(2):307-313

Kozak, M. 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. Journal of Biol. Chem. 266(30):19867-19870

Kuntz, M., J.L. Evrard, A. d'Harlingue, J.H. Weil and, B. Camara. 1989. Expression of plastid and nuclear genes during chromoplast differentiation in bell pepper (*Capsicum annum*) and sunflower (*Helianthus annus*). Mol. Gen. Genet. 216:156-163

Kuntz, M., G. Romer, C. Suire, P.H. Hugueney, J.H. Weil, R. Schantz, and B. Camara. 1992. Identification of a cDNA for the plastid-located geranylgeranyl pyrophosphate synthase from *Capsicum annum*-correlative increase in enzyme activity and transcript level during fruit ripening. Plant J. 2:25-34

Kushwaha, S.C., C. Subbarayan, D.A. Beeler, and J.W. Porter. 1969. The conversion of lycopene-15-15'-^{3}H to cyclic carotenes by soluble extracts of higher plant plastids. The J. of Biol. Chem. 244(13):3635-3642

Kuswaha, S.C., G. Suzue, C. Subbarayan, and J.W. Porter. 1970. The conversion of phytoene-^{14}C to acyclic, monocyclic, and bicyclic carotenes and the conversion of

lycopene-15,15'-^{3}H to mono- and dicyclic carotenes by soluble enzyme systems obtained from plastids of tomato fruit. The J. of Biol. Chem. 245(18):4708-4717

Lazo, G.R., P.A. Stein, and R. A. Ludwig. 1991. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. Biotechnology 9:963-967

Lee, P.C., B.R. Bochner, and B.N. Ames. 1983. AppppA, heat-shock stress, and cell oxidation. Proc. Natl. Acad. Sci. USA. 80:7496-7500

Lee S., and S. Rasheed 1990. A simple procedure for maximum yield of high quality plasmid DNA. Biotechniques 9: 676-679

Legge, R.L., K. Cheng, J.R. Lepock, and J.E. Thompson. 1986. Differential effects of senescence on the molecular organization of membranes in ripening tomato fruit. Plant Physiol. 81:954-959

Leshem, Y. 1988. Plant senescence processes and free radicals. Free Radical Biology and Medicine. 5:39-49

Lincoln, J.E., S. Cordes, E. Read, and R.L. Fischer. 1987. Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit development. Proc. Natl. Acad. Sci. USA. 84:2793-2797

Lincoln J.E., and R.L. Fischer 1988. Diverse mechanisms for the regulation of ethylene-inducible gene expression. Mol Gen Genet 212: 71-75

Linden, H., N. Misawa, D. Chamovitz, I. Pecker, J. Hirschberg, and, G. Sandmann. 1991. Functional complementation in *Escherichia coli* of different phytoene desaturase genes and analysis of accumulated carotenes. Z. Naturforsch. 46c:1045-1051

Lindquist, S., and E.A. Craig. 1988. The heat-shock proteins. Annu. Rev. Genet. 22:631-677

Livine, G. and S. Gepstein. 1988. Abundance of the major chloroplast polypeptides during development and ripening of tomato fruits. Plant Physiol. 87:239-243

Longhurst, T.J., H.F. Tung, and C.J. Brady. 1990. Developmental regulation of the expression of alcohol dehydrogenase in ripening tomato fruits. J. of Food Biochem. 14:421-433

Loven, D.P. 1988. A role for reduced oxygen species in heat induced cell killing and the induction of thermotolerance. Medical Hypotheses. 26:39-50

Lutcke, H., S. High, K. Romisch, A.J. Ashford, and B. Dobberstein. 1992. The methionine-rich domain of the 54 kD subunit of signal recognition particle is sufficient for the interaction with signal sequences. The Embo Journal. 11(4):1543-1551

Lutke-Brinkhaus F, B Liedvogel, K Kreuz and H Klenig (1982) Phytoene synthase and phytoene dehydrogenase associated with envelope membranes from spinach chloroplasts. Planta 156: 176-180

Mannsson, P.E., D. Hsu and D. Stalker. 1985. Characterization of fruit specific cDNAs from tomato. Mol. Gen. Genet. 200:356-361

Marano, M.R., and N. Carillo. 1991. Chromoplast formation during tomato fruit ripening. No evidence for plastid DNA methylation. Plant Molecular Biology. 16:11-19

Matile, P., T. Duggelin, M. Schellenberg, D. Rentsch, K. Bortlik, C. Peisker, and H. Thomas. 1989. How and why is chlorophyll broken down in senescent leaves. Plant. Physiol. Biochem. 27(4):595-604

Matoo, A.K., and Aharoni, N. 1988. Ethylene and plant senescence. In: L.D. Nooden, and A.C. Leopold (eds) Senescence and aging in plants. Academic Press Inc. New York. pp. 241-280

Maunders M.J., M.J. Holdsworth, A. Slater, J.E. Knapp, C.R. Bird, W. Schuch and D. Grierson 1987. Ethylene stimulates the accumulation of ripening-related mRNAs in tomatoes. Plant Cell and Environ 10: 177-184

Mayer, M. P., V. Nievelstein, and P. Beyer. 1992. Purification and characterization of a NADPH dependent oxidoreductase from chromoplasts of *Narcissus pseudonarcissus*: a redox-mediator possibly involved in carotene desaturation. Plant Physiol. Biochem. 30(4):389-398

Mayfield, S.P., T. Nelson, W.C. Taylor and, R. Malkin. 1986. Carotenoid synthesis and pleiotropic effects in carotenoid-deficient seedlings of maize. Planta. 169:23-32

McCarty D.R. 1986. A simple method for extraction of RNA from maize tissues. Maize Genetics Coop Newsletter 60-61

McCarty, D.R., T. Hattori, C.B. Carson, V. Vasil, M. Lazar, and I.K. Vasil. 1991. The Viviparous-1 developmental gene of maize encodes a novel transcriptional activator. Cell 66:895-905.

McGarvey, D.J., R. Sirevag and R.E. Christoffersen. 1992. Ripening-related gene from avocado fruit. 1. Ethylene-inducible expression of the mRNA and polypeptide. Plant Physiol. 98:554-559

Mehta, R.A., T.W. Fawcett, D. Porath, and A.K. Matoo. 1992. Oxidative stress causes rapid membrane translocation and in vivo degradation of ribulose-1-5-bisphosphate carboxylase/oxygenase. The J, of Biol. Chem. 267(4):2810-2816

Misawa, N., M. Nakagawa, K. Kobayashi, S. Yamano, Y. Izawa, K. Nakamura, and K. Harashima. 1990. Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. J. of Bacteriology. 172(12):6704-6712

Misawa, N., S. Yamano, and H. Ikenaga. 1991. Production of beta-carotene in *Zymomonas mobilis* and *Agrobacterium tumefaciens* by introduction of the biosynthetic genes from *Erwinia uredovora*. Applied and Environ. Micro. 57(6):1847-1849

Nakagawa, M., and N. Misawa. 1991. Analysis of carotenoid glycosides produced in gram-negative bacteria by introduction of the *Erwinia uredovora* carotenoid biosynthetic genes. Agri. Biol. Chem. 55(8):2147-2148

Nieto-Sotelo, J., E. Vierling and T.-H.D. Ho. 1990. Cloning, sequence analysis and expression of a cDNA encoding a plastid-localized heat shock protein in maize. Plant Physiol. 93:1321-1328

Neumann, D., L. Nover, B. Parthier, R. Rieger, K.D. Scharf, R. Wollgiehn, and U.Z. Nieden. 1989. Heat shock and other stress response systems of plants. Biol. Zentralbl. 108:1-156

Newman, L.A., N. Hadleb and C.A. Price. 1989. Synthesis of two chromoplast-specific proteins during fruit development in *Capsicum annuum*. Plant Physiol. 91:455-458

Ngernprasirtsiri, J., H. Kobayashi and T. Akazawa. 1988. DNA methylation occurred around lowly expressed genes of plastid DNA during tomato fruit development. Plant Physiol. 88:16-20

Nock, L.P., L.J. Rogers, and H. Thomas. 1992. Metabolism of protein and chlorophyll in leaf tissue of *Festuca pratenesis* during chloroplast assembly and senescence. Phytochemistry 31(5):1465-1470

Oeller, P.W., L. Min-Wong, L.P. Taylor, D.A. Pike, and A. Theologis. 1991. Reversible inhibition of tomato fruit senescence by antisense RNA. Science. 254:437-439

Olson, D.C., J.A. White, L. Edelman, r.N. Harkins and H, Kende. 1991. Differential expression of two genes for 1-aminocyclopropane-1-carboxylate synthase in tomato fruits. Proc. Natl. Acad. Sci. USA. 88:5340-5344

Papastephanou, C., F.J. Barnes, A.V. Briedis, and J.W. Porter. 1973. Enzymatic synthesis of carotenes by cell-free preparations of fruit of several genetic selections of tomatoes. Archives of Biochem. 157:415-425

Pecker, I., D. Chamovitz, H. Linden, G. Sandmann, and J. Hirshberg. 1992. A single polypeptide catalyzing the conversion of phytoene to gamma-carotene is transcriptionally regulated during tomato fruit ripening. Proc. Natl. Acad. Sci. USA. 89:4962-4966

Penarrubia, L. and J. Moreno. 1990. Increased susceptibility of ribulose-1,5-Bisphosphate carboxylase/oxygenase to proteolytic degradation caused by oxidative treatments. Archives of Biochem. and Biophys. 281(2):319-323

Perl-Treves, R., and E. Galun. 1991. The tomato Cu, Zn superoxide dismutase genes are developmentally regulated and respond to light and stress. Plant Molecular Biology. 17:745-760

Picton, S., and D. Grierson. 1988. Inhibition of expression of tomato-ripening genes at high temperature. Plant, Cell and Environment 11:265-272

Piechulla, B., K.R. Chonoles I. and W. Gruissem. 1985. Plastid gene expression during fruit ripening in tomato. Plant Molecular Biology. 5:373-384

Piechulla, B., E. Pichersky, A.R. Cashmore and W. Gruissem 1986. Expression of nuclear and plastid genes for photosynthesis-specific proteins during tomato fruit development and ripening. Plant Molecular Biology. 7:367-376

Piechulla, B., R.E. Glick, H. Bahl, A. Melis, and W. Gruissem. 1987. Changes in photosynthetic capacity and photosynthetic protein pattern during tomato fruit ripening. Plant Physiol. 84:911-917

Post, D.A., B. Hove-Jensen, and R.L. Switzer. 1992. Characterization of the hemA-prs region of the *Escherichia coli* and *Salmonella typhimurium* chromosomes. Identification of two open reading frames and implications for prs expression. Journal of Gen. Micro.

Qureshi, A.A., A.G. Andrewes, N. Qureshi, and J.W. Porter. 1974. The enzymatic conversion of cis-[^{14}C]Phytofluene, and trans-zeta-[^{14}C]Carotene to more unsaturated acyclic, monocyclic and dicyclic carotenes by a cell-free preparation of red tomato fruits. Archives of Biochem. and Biophy. 162:93-107

Rabinowitch, H.D., D. Skian, and P. Budowski. 1982. Photo-oxidative damage in the ripening tomato fruit: Protective role of superoxide dismutase. Physiol. Plant. 54:369-374

Rattanapanone, N., J. Speirs and D. Grierson. 1978. Evidence for changes in messenger RNA content related to tomato fruit ripening. Phytochemistry. 17:1485-1486

Ray, J., M. Maunders, D. Grierson, and W. Schuch. 1987. Sequence of pTOM5, a ripening related cDNA from tomato. Nucl. Acids. Res. 15(24):10587

Richards, C.M., S.B. Hinman, C.D. Boyer, and R.C. Hardison. 1991. Survey of plastid RNA abundance during tomato fruit ripening: the amounts of RNA from the ORF 2280 region increase in chromoplasts. Plant Mol. Biol. 17;1179-1188

Roberts, D.R., J.E. Thompson, E.B. Dumbroff, S. Gepstein, and A.K. Matoo. 1987. Differential changes in the synthesis and steady-state levels of thylakoid proteins during bean leaf senescence. Plant Mol. Biol. 9:343-353

Robinson, N.L., J.D. Hewitt, and A.B. Bennett. 1988. Sink metabolism in tomato fruit 1. Developmental changes in carbohydrate metabolizing enzymes. Plant Physiol. 87:727-730

Romer, S., A. d'Harlingue, B. Camara, R. Schantz, and M. Kuntz. 1992. Cysteine synthase from *Capsicum annuum* chromoplasts characterization and cDNA cloning and up-regulated enzyme during fruit development. J. of Biological Chemistry. 267(25):17966-17970

Rosso, S.W. 1968. The ultrastructure of chromoplast development in red tomatoes. J. Ultrastructure Science. 25:307-322

Rothan, C., and J. Nicolas. 1989. Changes in acidic and basic peroxidase activities during tomato fruit ripening. Hortscience. 24(2):340-432

Rottmann, W.H., G.F. Peter, P.W. Oeller, J.A. Keller, N.F. Shen, B. P. Nagy, L.P. Taylor, A.D. Campbell and A. Theologis. 1991. 1-aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. J. Mol. Biol. 222:937-961

Roy, H. 1989. Rubisco assembly: A model system for studying the mechanism of chaperonin action. The Plant Cell. 1:1035-1042

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning a Laboratory Manual. 2nd edition Cold Spring Harbor Laboratory Press. NY

Scandalios, J.G. 1993. Oxygen stress and superoxide dismutases. Plant Physiol. 101:7-12

Sheehy, R.E., M. Kramer, and W.R. Hiatt. 1988. Reduction of polygalacturonase activity in tomato fruit by antisense RNA. Proc. Natl. Acad. Sci. USA. 85:8805-8809

Sitte, P., H. Falk and B. Liedvogel. 1980. Chromoplasts. In: Czygan, F.C. (ed.) Pigments in Plants. Fischer Stuttgart pp.117-148

Slater, A., M.J. Maunders, K. Edwards, W. Schuch and D. Grierson. 1985. Isolation and characterization of cDNA clones for tomato polygalacturonase and other ripening-related proteins. Plant Molecular Biology. 5:137-147

Smith, C.J.S., C.F. Watson, J.Ray, C.R.Bird, P.C. Morris, W. Schuch, and D. Grierson. 1988. Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. Nature. 334(25):724-726

Smith, C.J.S., C.F. Watson, P.C. Morris, C.R. Bird, G.B. Seymour, J.E. Gray, C. Arnold, G.A. Tucker, W. Schuch, S. Harding, and D. Grierson. 1990. Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. Plant Mol. Biol. 14:369-379

Solomos, T. 1988. Respiration in senescing plant organs: Its nature, regulation, and physiological significance. In: L.D. Nooden, and A.C. Leopold (eds) Senescence and aging in plants. Academic Press Inc. New York. pp.111-145

Speirs, J., C.J. Brady, D. Grierson and E. Lee. 1984. Changes in ribosome organization and messenger RNA abundance in ripening tomato fruits. Aust. J. Plant Physiol. 11:225-33

Thayer, S.S., H.T. Choe, A. Tang, and R.C. Huffaker. 1987. Protein turnover during senescence. In: W.W. Thomson, E.A. Nothnagel, and R.C. Huffaker (eds.) Plant senescence: Its biochemistry and physiology, American Society of Plant Physiologists. Rockville, Maryland. pp. 71-80

Thelander, M., J.O. Narita, and W. Gruissem. 1986. Plastid differentiation and pigment biosynthesis during tomato fruit ripening. Current Topics in Plant Biochemistry and Physiology 5:128-141

Theologis, A., T.I. Zarembinski, P.W. Oeller, X. Liang, and S. Abel. 1992. Modification of fruit ripening by suppressing gene expression. Plant Physiol. 100:549-551

Thomas, H., and P. Hilditch. 1987. Metabolism of thylakoid membrane proteins during foliar senescence. In: W.W. Thomson, E.A. Nothnagel, and R.C. Huffaker (eds). Plant senescence: Its biochemistry and physiology, American Society of Plant Physiologists. Rockville, Maryland. pp. 114-122

Thomas, H., K. Bortlik, D. Rentsch, M. Schellenberg, and P. Matile. 1989. Catabolism of chlorophyll in vivo:significance of polar chlorophyll catabolites in a non-yellowing senescence mutant of *Festuca pratensis* Huds. New Phytol. 111, 3-8

Thompson, J.E., G. Paliyath, J.H. Brown, and C.L. Duxbury. 1987. The involvement of activated oxygen in membrane deterioration during senescence. In: W.W. Thomson, E.A. Nothnagel, and R.C. Huffaker (eds.). Plant senescence: Its biochemistry and physiology, American Society of Plant Physiologists. Rockville, Maryland. pp. 146-155

Thomson, W.W., and K.A. Platt-Aloia. 1987. Ultrastructural changes associated with senescence. In: W.W. Thomson, E.A. Nothnagel, and R.C. Huffaker (eds.) Plant senescence: Its biochemistry and physiology, American Society of Plant Physiologists. Rockville, Maryland. pp. 20-30

Tieman, D.M., R.W. Harriman, G. Ramamohan, A.K. Handa. 1992. An antisense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit, The Plant Cell. 4:667-679

Tomato Genetic Cooperative Reports. 1987. Classical Map 37:linkage maps 6. USDA-ARS Cornell University, Ithaca, N.Y.

Tsang, E.W.T., C. Bowler, D. Herouart, W. Van Camp, R. Villaroel, C. Genetello, M. Van Montagu, and D. Inze. 1991. Differential regulation of superoxide dismutases in plants exposed to environmental stress. The Plant Cell. 3:783-792

van der Krol, A. R., N.M. Mol, and A.R. Stuitje. 1988. Antisense genes in plants: an overview. Gene 72:45-50

Van Der Straeten, D., R.A. Rodrigues Pousada, J. Gielen, and M. Van Montagu. 1991. Tomato alcohol dehydrogenase expression during fruit ripening and under hypoxic conditions. Febs Letters. 295:39-42

Vierling, E., R.T. Nagao, A.E. DeRocher, and L.M. Harris. 1988. A heat shock protein localized to chloroplasts is a member of a eukaryotic superfamily of heat shock proteins. The Embo J. 7(3): 575-581

Vierling, E. 1991. The role of heat shock proteins in plants. Annu. Rev. Plant Physiology Plant Mol. Biol. 42:579-620

Wada, K.-N., Y. Wada, F. Ishiashi, T. Gojobori, and T. Ikemura. 1992. Codon usage tabulated from the GenBank genetic sequence data. Nucl. Acid. Res. 20, Suppl.:2111-2118

Wang, H., and W.R. Woodson. 1991. A flower senescence-related mRNA from carnation shares sequence similarity with fruit ripening-related mRNAs involved in ethylene biosynthesis. Plant. physiol. 96:1000-1001

Weber, L.A. 1992. Relationship of heat shock proteins and induced thermal resistance. Cell Prolif. 25:101-113

Weiss, R.B. Ribosomal frameshifting, jumping and readthrough. 1991. Curr. Opin. in Cell Biol. 3:1051-1055

Whitaker, B.D. 1991. Growth conditions and ripening influence plastid and microsomal membrane lipid composition in bell pepper fruit. J. Amer. Soc. Hort. Sci. 116(3):528-533

Woolhouse, H.W. 1984. The biochemistry and regulation of senescence in chloroplasts. Can. J. Bot. 62:2934-2942

Wrench, P.M., M. Olive, R.G. Hiller, C.J. Brady, and J. Speirs. 1987. Changes in plastid proteins during ripening of tomato fruits. J. Plant Physiol. 129:89-122

Yelle, S., J.D. Hewitt, N.L. Robinson, S. Damon, and A.B. Bennett. 1988. Sink metabolism in tomato fruit III. Analysis of carbohydrate assimilation in a wild species. Plant Physiol. 87:737-740

Yip, W-K., J-G. Dong, J.W. Kenney, G.A. Thompson, And S.F. Yang. 1990. Characterization and sequencing of the active site of 1-aminocyclopropane-1-carboxylate synthase. Proc. Natl. Acad. Sci. USA. 87:7930-7934

BIOGRAPHICAL SKETCH

I grew up in Minnesota thinking 3 months of tough sledding was a normal way of life. My family moved to the Smoky Mountains of Asheville, North Carolina, where I spent the last two years of high school. I graduated from a very small experimental school, in a graduating class of 16 students. The experience gave me a fairly utopian perspective of education, which was augmented by my undergraduate education. I pursued a Bachelor of Sciences degree from Beloit College, a small liberal arts college in southern Wisconsin.

Upon graduation from college, I took a research position in the Department of Veterinary Sciences at the University of Wisconsin for a year and at McCardle Labs for Cancer Research the following three years. I decided to go back to school in plant molecular biology. This was not really a change in direction, since my undergraduate education specialized in botany.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Don Huber
Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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