# SOURCE-SINK RELATIONSHIP

# DURING PAPAYA FRUIT DEVELOPMENT AND RIPENING

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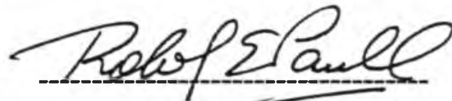

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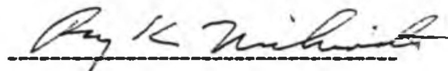

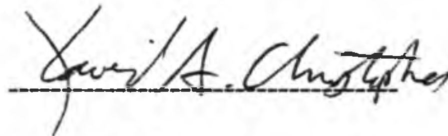

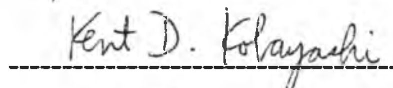

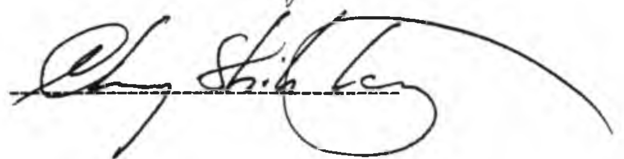

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# ABSTRACT

The source sink relationship during papaya fruit development and ripening was investigated. The source size and sink strength were modified by single or continual defoliation, and fruit thinning, respectively. The relationship between fruit growth, respiration, sugar accumulation and the activity of sucrose phosphate synthase (SPS), sucrose synthase (SS), and acid invertase were determined in fruit from 14 days after anthesis (DAA) to 140 DAA (harvest maturity) and in response to defoliation and fruit removal. A putative complete invertase gene and a SS gene fragment were isolated and characterized from nearly mature green papaya fruit. Single defoliation significantly reduced new flower and fruit set, and ripe fruit total soluble solids (TSS) but did not reduce fruit production, average fruit mass, percentage fruit flesh and seed, seed mass ratio and seed dry mass during a six weeks period. Continual defoliation in addition reduced fruit size, sugar and invertase enzyme activity and fruit production. The responses of defoliation and fruit thinning varied between different cultivars, weather conditions, defoliation time, degree and method. The pattern of gene expression during fruit development was compared with invertase extracted enzyme activity in the presence and absence of sodium chloride (NaCl) and by western blot analysis. The papaya invertase sequence had an open reading frame that encoded a polypeptide chain of 582 residues and calculated molecular weight of 65, 684 Da. The protein was highly homologous to known plant cell wall invertase and 67% identical at the amino acid level to carrot cell wall invertase. The cloned 720 bp SS fragment was highly homologous to *A. glutinosa* (X92378) and SS genes from other species. Invertase gene was expressed at a higher level during late fruit development stage than in young fruit and other tissues of papaya plant. SS gene expression was higher in young fruit and petiole tissues than in other tissues. The data demonstrated that SS enzyme was a major enzyme in fruit sink establishment and maintenance. Apoplastic invertase had an important function in phloem unloading during papaya fruit sugar accumulation and the activity was regulated at both transcriptional and translational levels.

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## LIST OF ABBREVIATION AND SYMBOLS

| | |
|---|---|
| bp | base pair |
| BSA | Bovine Serum Albumin |
| DAA | Days after anthesis |
| cDNA | Complimentary DNA |
| DNA | Deoxyribonucleic acid |
| dNTP | deoxyribinucleotide triphosphate |
| *E.coli* | *Escherichia coli* |
| EDTA | (Ethylendinitrilo)-Tetracetic Acid |
| DTT | Dithiothreitol |
| FW | fresh weight |
| IPTG | Isopropyl β-D-thigalactopyranoside |
| kDa | kilodalton |
| mRNA | messenger RNA |
| MW | molecular weight |
| RNA | Ribonucleic acid |
| PAGE | polyacrylamide gel electrophoresis |
| Pfu | plaque formation unit |
| PI | isoelectric point |
| PMSF | Phenylmethylsulfonyl Fluoride |
| Poly (A) | poly-adenylate |
| PCR | Polymerase chain reaction |
| RT-PCR | Reverse transcriptase-PCR |
| Rnase | Ribonuclease |
| SDS | sodium dodecyl sulfate |
| SPS | sucrose phosphate synthase |
| SS | sucrose synthase |

| | |
|---|---|
| TEMED | N,N,N=,N=-tetramethylethylenediamine |
| Tris | Tris[hydroxymethyl]aminomethane |
| TSS | total soluble solids |
| UDP | Uridine diphosphate |
| UDPG | Uridine diphosphate glucose |
| X-Gla | 5-Bromo-4-chloro-3-indolyl-β-D-galactoside |

# CHAPTER 1

# INTRODUCTION

Papaya (*Carica papaya* L.) is cultivated throughout the tropics for its fruit (Purseglove, 1968). The world papaya production in 1990 was 4.4 million tons, 42% of which was from the South American countries, 34% from Asia and the rest from Africa and North America (Yon, 1994). In 1994, fresh papaya fruit production in Hawaii totaled about 62,000,000 lb., and more than 60% was shipped to the US mainland and Japan. The value of papaya production was $13,831,000 (Hawaii Agricultural Statistic Service, 1995). It has great potential in the markets of Europe, Japan, the Middle East and USA (Radi et al., 1994).

Sugars play an important role in the flavor characteristics of the papaya and are also a commercial measure of fresh fruit quality. The refractometric sugar value or "total soluble solids" (TSS) in the juice extracted from four equal cores taken at right angles to the longitudinal axis at the four quarters is used as a grade standard. The State of Hawaii Wholesale and Consumer Standard for papaya requires that in a given lot of fruit, the TSS of the edible pulp juice average not less than 11.5, and not more than 5% by count of the fruit in the lot may have TSS less than 10.5% (Hawaii Administrative Rules, 1986).

Papaya sometimes has low TSS (<10%) and poor flesh color when ripe (Paull et al., 1997). Affected fruit cannot be visually culled, as there are no obvious external signs. Four causes have been suggested for the low sugars: I) harvesting green fruit without a trace of skin yellowing; ii) diseases such as a virus or a pathogenic mycoplasma-like organism (MLO), iii) reduced photosynthesis and loss of active photosynthetic area due to strong winds, drought, disease, insect feeding or other environmental stress; or, iv) high self-shading and low temperatures. Papaya fruit has no stored starch reserves in the flesh tissue, and sugars move into the fruit during late fruit growth. Harvested green fruit has inherently lower sugars. No MLO pathogenic organisms have been detected nor demonstrated as a causal agent.

This project considers the third possibility by studying the processes affecting the source-sink relationship during papaya fruit development. The factors affecting fruit sugar level are

complex, and include genotype, crop deterioration, foliage damage by hurricane, insects, disease, soil nutrition deficiency and environmental factors. Generally, once a genotype is selected, the environmental conditions and foliage damage influence the photosynthetic capacity of the plant and, as a result, influence fruit carbohydrate accumulation. Papaya is a perennial herbaceous dicotyledonous plant with a single stem and a crown of large palmately lobed leaves (Nakasone and Paull, 1998). Once initial flowering occurs, the papaya tree flowers and fruits continuously. Papaya fruit needs 5-6 months from pollination to ripening in Hawaii. Fruit of all stages of development are present on a single plant. This creates a significant demand for an adequate supply of photosynthate. However, no information is available on the fruit growth requirements, effect of defoliation on sugar metabolism and source-sink relationships of papaya. Many cultivated plants compensate for partial defoliation by increasing the photosynthetic capacity of the remaining leaf area (Boucher et al., 1987; Flore and Irwin, 1983; Layne et al., 1992). However, once the damage or defoliation is significant, leaf capacity is unable to compensate for the loss, and carbohydrate accumulation will be reduced. It is reported that papaya foliage injury can occur by insects (e.g, Broad mite), diseases such as powdery mildew, papaya mosaic virus and papaya ring spot virus (Decker and Tio, 1958; Marler et al., 1993; Nakasone, 1986). The papaya plant, with its large lobed leaves and long petioles, is easily damaged by high trade winds in Hawaii. It is necessary to investigate how this type of damage influences fruit growth, fruit sugar accumulation and the rate of recovery from this loss to photosynthetic area.

This project focused on source (leaf)-sink (fruit) manipulation on fruit growth and development, carbohydrate assimilation and relative activity of key enzymes of carbohydrate metabolism during fruit development. In this project, photosynthetic area (source) in papaya was altered by defoliation and sink strength altered by fruit thinning. Key enzyme activities were measured during these treatments. The critical enzymes necessary for sugar accumulation were identified and their genes cloned. The next step would be to genetically modify sink strength using biotechnology so as to improve fruit development and fruit quality.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Papaya fruit development and ripening

### 2.1.1 Introduction

#### 2.1.1.1 Botany and fruit morphology

The papaya (*Carica papaya* L.) is a member of the family of *Caricaceae* and has 2n=18 chromosomes (Nakasone and Pauli, 1998). The genomic size was reported as 0.39 pg per copy (Arumuganathan and Earle, 1991). All *Carica* species are native to tropical America (Morishidi, 1996), but only *C. papaya* L., *C. candamarcensis* Hook and *C. monoica* have horticultural importance (Muthukrishnan and Irulappan, 1985). Papaya (*C. papaya* L.) is the most important economic species in *Carica*. Common names include papaya, papaw or pawpaw, papayer (French), melonbaum (German), lechosa (Spanish), mamao, mamoeiro (Portuguese) and mugua (Chinese) (Nakasone and Paull, 1998).

Papaya is a herbaceous, dicotyledonous plant with a single main stem that can attain heights up to 9 m, terminating with a crown of large palmately lobed leaves (Nakasone, 1986). Most cultivars have flowers borne in modified cymose inflorescence that appear at the leaf axis just below the growing point (Nakasone, 1986). The plant can be dioecious, monoecious or gynodioecious. The cultivated *C. papaya* is dioecious or gynodioecious (Yon, 1994). There are three major sexual flower types in *C. papaya*: pistillate, hermaphrodite and staminate (Nakasone, 1986).

Papaya fruit resembles a melon, being spherical, pyriform, oval or elongated in shape and varies with flower types and cultivars (Nakasone and Paull, 1998). The fruit is normally composed of five longitudinal carpels united laterally to form large central cavity where numerous seeds are attached to the placenta in the parietal position (Nakasone 1986). The skin of the fruit is thin, usually smooth, green when immature, and yellow to orange-yellow when ripe. Upon ripening the flesh color turns from white to yellow or orange-yellow, to salmon-pink or red, depending upon cultivar (Nakasone 1986; Yon 1994). Fruit size range from less than 250 g to 10

kg (Chan and Tang, 1979) depending upon cultivar. In Hawaii, a small (454 g) fruit is desired (Nakasone 1986) and referred to as 'Solo' type, major varieties belonging to 'Solo' type are 'Kapoho', 'Sunrise', 'Sunset' and 'Waimanalo' (Nakasone and Paull, 1998). The new transgenic ringspot virus resistant cultivars are 'Rainbow' and 'Sunup' (Nakasone and Paull, 1998).

#### 2.1.1.2 Importance

Papaya is one of Hawaii's major agricultural export crops. In 1994, fresh papaya fruit production in Hawaii totaled about 62,000,000 lb., and more than 60% was shipped to the US mainland and Japan. The world papaya production in 1990 was 4.4 million tons, 42% from the South American countries, 34% from Asia and the rest from Africa and North America (Yon, 1994).

Papaya fruit is delicious and an excellent source of provitamin A and ascorbic acid (Wenkam, 1990). Papaya fruit is consumed as breakfast, desserts and juice. Immature fruit is consumed as salad and vegetables. Papaya is also processed into various forms such as dry fruit slices, chunks and slices for tropical fruit salads and cocktails, or processed to purée for juices and nectar base, usually frozen and canned nectar, mixed drinks and jams (Nakasone and Paull, 1998). The value of papaya as a medical plant has been reviewed (Quisumbing, 1951; Chopra, 1958). Papain is a proteolytic enzyme that digests proteins and is used as meat tenderizer, as digestive medicine in the pharmaceutical industry, textile and brewing, and tanning industries (Singh, 1980; Muthukrishnan et al., 1985; Nakasone and Paull, 1998).

### 2.1.2 Fruit development and ripening

#### 2.1.2.1 Fruit set and growth pattern

Fruit set is about 76%, with flower abortion occurring soon after anthesis and most fruit are dropped when small (<6 cm) (Ong, 1983). A positive correlation exists between seed number and fruit weight (Allan, 1969; Chittiraichelvan and Shanmugavelu, 1978).

Fruit development from pollination to maturation varies widely due to factors such as cultivar, age of bearing trees (maturity delayed as trees age), time of year, and the stage selected as an index of maturity (Nakasone, 1986). Fruit growth and development and the timing of different tissue

development in the papaya has been determined for several Asian cultivars (Ong, 1983; Selvaraj et al., 1982a; Muda et al., 1994) and Hawaii (Qui et al., 1995). Fruit length and fruit mass show a double sigmoid growth (Ong, 1983; Selvaraj et al., 1982a). In 'Sunset' papaya, there are two major peaks in mass and volume growth. The first period of rapid growth occurs 75 days after anthesis, the second 90 to 105 days post antheses (Qiu, et al., 1995). Fruit change in volume, parallels the increase in fruit length and mass (Ong, 1983).

Rate of fruit development is significantly affected by field temperature. Low temperatures (<15 $^{o}$C) during the early phase of growth significantly delay growth and reduce fruit size (Allan et al., 1987). In areas without low winter temperatures, fruit development from pollination to ripeness takes approximately 5 to 6 months. Fruit maturity is delayed during the winter months in Hawaii by approximately 2 to 3 weeks (Nakasone, 1986). The variation in days from fruit set to ripeness ranges from 173 days when grown under 30 $^{o}$C day/20 $^{o}$C night to 282 days at 24 $^{o}$C day/12 $^{o}$C night (Allan et al., 1987; Kuhne and Allan, 1970).

2.1.2.2 Respiration and ethylene

Papaya is a climacteric fruit (Akamine, 1966; Selvaraj et al., 1982a). Fruit harvested at the color-turning stage have already passed the preclimacteric minimum. Fruit respiration climacteric peak occurrs between 4 to 5 months after anthesis in four cultivars in India (Selvaraj et al.,1982a). The increase in ethylene production parallels the respiration rise and reaches a maximum at the same time as the respiratory climacteric (Pauli and Chen, 1983).

2.1.2.3 Color and texture

During papaya fruit development, the most obvious changes are in the skin, flesh and seed colors and start about fifteen weeks after anthesis in 'Eksotika' and 'Batu Arang' (Muda et al., 1994). The flesh and the seeds are both initially white. As the fruit matures, the skin color changes to yellow and the flesh becomes yellow orange or red and the seed change to black (Muda et al., 1994). Chlorophyll degradation in 'Kapoho' papaya follows a linear pattern, decreases to about 0.01 mg $g^{-1}$ flesh mass when ripened at 25$^{o}$C (Ali et al., 1994). In 'Eksotika' papaya, the L, a and b values of the peel increase during ripening (Muda et al., 1994).

The total flesh carotenoids level also increases 14 fold during ripening (Ali et al., 1994; Selvaraj et al., 1982a). Yellow and red fleshed papaya differ in the level and type of pigments (Yamamoto, 1964). Yellow fruit contains more than 4 times the δ-carotene content of the red-fleshed fruit, while red fleshed fruit has about 65.3% lycopene that is absent in yellow-fleshed fruit. These carotenoids are good source of vitamin A (Wenkam, 1990).

As the fruit matures and ripens the firmness decreases (Muda et al., 1994; Qui et al., 1995). In young fruit (30-110 days after anthesis), the firmness is about 95 N and decreases to 84.9 N and 77.5 N when fruit attained the mature green and 25% yellow stage, respectively (Muda et al., 1994). Papaya soft to an edible stage in 6 to 12 days when harvested at the color break stage (Paull 1993). Cell wall degrading enzyme, xylanase (EC 3.2.1.32) and polygalacturonase (EC 3.2.1.15) activity peak occur when the fruit has 40-60% skin yellowing (Paull and Chen, 1983). Pectin methyl esterase (EC 3.1.1.15) and CMC-cellulase both continue to increase as the fruit ripen, only declining as the fruit becomes over ripe (Paull, 1993). β-galactosidase (E C 3.2.1.23) activity doubles during ripening (Lazan et al., 1991).

2.1.2.4 Sugars and volatiles

The concentration of sugar, organic acids, and levels of volatile compound as well as phenolic compounds contribute to fruit taste and flavor. The edible portion of papaya is composed mostly of water (86.8%), and carbohydrate (12.2%) (Wenkam, 1990). Sucrose, glucose and fructose are the three major soluble sugars in papaya fruit (Chan et al., 1979; Selvaraj et al., 1982a). A very low starch content (about 0.1%) is detected in the late fruit developmental stage and is mainly associated with the skin (Chan et al., 1979; Selvaraj et al., 1982a). Early in fruit development, glucose is the major fruit sugar and glucose and fructose slowly increase from 110 day to 135 days after anthesis but as a percentages of the total sugar they decrease (Chan et al., 1979; Selvaraj et al., 1982). Sucrose remains low until 110 days after anthesis and then rapidly increases to 80% of the sugars (Chan et al., 1979).

TSS usually is used as a simple index of sugar content especially in fruit such as papaya with low acidity. The Hawaii grade standard requires fruit to have 11.5% TSS (Hawaii

Administrative Rules, 1986), the color break stage normally meets this standard (Akamine and Goo, 1971). Papaya is notably low in organic acids but a good source of ascorbic acid (Selvaraj et al., 1982 a; Lazan et al., 1990). A 106 volatile compounds have been identified in papaya fruit (Katayue and Kirch, 1965; Chan et al., 1979a). The major volatile compounds are linalool, linalool oxides, ethylacetate, phenyl acetonitrile and benzyl isothiocyanate (Paull, 1993).

Sucrose phosphate synthase (SPS), sucrose synthase (SS) and □-fructosidase (invertase) are the three major enzymes involved in sucrose metabolism. SPS synthesizes sucrose from UDPG and fructose-6-phosphate, and invertase catalyzes the cleavage of sucrose into glucose and fructose (McCollum et al., 1988). SS can function in both directions of sucrose synthesis and cleavage. There is a very high invertase enzyme activity in ripe papaya fruit (Chan et al., 1976; Hubbard et al., 1991; Selvaraj et al., 1982). This high invertase activity explains that earlier reported values for the sugar composition of papaya (Chan et al., 1979). Low SPS and SS activities were reported in ripe fruit (Hubbard et al., 1991). Nevertheless, the relationship between fruit development, sugar accumulation and the relative carbohydrate metabolic enzyme activity has not been determined.

**2.2 Preharvest factors affecting quality on papaya and other fruit**

Variety and ripening stage of fruit at harvest are two factors that influence final sugar composition of papaya (Akamine et al., 1971; Chan et al., 1979; Imunyi et al., 1990; Selvaraj et al., 1982b; Yon 1994). Akamine and Goo (1971) suggested that to meet the minimum TSS of 11.5% required by Hawaiian Grade Standards for marketable papayas, the fruit should have at least 6 % skin yellowing.

Once initial flowering occurs, the papaya tree flowers and sets fruit continuously. Therefore, a continuous supply of carbohydrates for fruit growth and development is required. Experience has shown that fruit TSS is higher during the warmer long day period than during the cooler, shorter day length periods of the year, or when rainfall is higher and longer periods of cloudy weather occur (Nakasone et al., 1974). Lower TSS content has been observed in

tomatoes grown at higher summer temperatures (Alban et al., 1948). Differences in carbohydrate synthesis rate between seasons appears to be affected by moisture and sunlight conditions, assuming that nutrients and temperatures are not limiting (Nakasone, 1986).

Cultural practices such as plant spacing (density), irrigation, mulching, pruning, thinning and fertilizers can influence fruit quality (Pantastico, 1975). Closer tree planting having less sweet fruit (cf. Pantastico, 1975). High irradiation can increase numbers of cucumber fruit per plant, and individual fruit growth rate (Marcelis, 1993). However, Ong and Kwok (1983) did not find any effect of hours of sunshine on papaya yield in Malaysia. Wet conditions can decrease papaya fruit sugar content, increase disease problems, larger fruit size, and a greater tendency for carpellody (Awada and Ikeda, 1957). Excessive irrigation decreases TSS content of tomato fruit (Pantastico, 1975). While irrigation increases both the number and size of marketable papaya fruit (Yon 1994), flooding of muskmelon (*Cucumis melo* L) reduces fruit sugar content but not leaf carbon exchange rate (Kroen et al., 1991). Low irrigation rate increases papaya petiole and fruit sugar (Awada et al., 1957) as found in tomato (Pantastico, 1975). However, lack of moisture generally retards papaya plant growth and causes flower and fruitlets abortion leading to sterile phases or 'skips' period with no fruit production (Yon, 1994). Light duration, intensity and quality can affect citrus, mango and other fruit trees quality at harvest (Pantastico, 1975). Fruit thinning is considered an important practice to regulate papaya production and improve the percentage of uniform size marketable fruit (Yon, 1994).

Potassium fertilizer significantly increases the TSS in carrot (Abrahamson et al., 1998) and is associated with increased fruit size and TSS in papaya (Nakasone, 1986; Purohit, 1977). Nitrogen, iron and zinc fertilization have been reported to influence fruit quality (Bahadur et al.,1998; Costa et al., 1997; Fallahi et al., 1997; Sanz et al., 1997). High nitrogen fertilizer rates increase apple fruit weight but decrease fruit quality and have no influence on fruit total production (Raese and Drake 1997). Peach fruit size and abnormal ripeness is found to be associated with iron deficiency (Sanz et al., 1997). Soil application of zinc sulphate (0.5kg $tree^{-1}$) significantly increases fruit TSS in mango (Costa et al., 1997).

Gibberellic acid (GA), influences the steady-state level of SPS and its activity in soybean and spinach plants (Cheikh et al., 1992) and GA at 200 ppm increases the TSS and acidity of 'Coorg Honey Dew' papaya, 1.8% and 0.19%, respectively (Shanmugavelu, et al., 1973). Ethephon, Alar and Phosfon D at 250 ppm, increase the total and reducing sugar content of papaya (Shanmugavelu et al., 1973). However, Modlibowska and Wickenden (1982) found that TSS of cherries decreased following GA or GA plus auxin treatments.

Welles and Buitelaar (1988) suggested that muskmelon fruit, having a low growth rate, e.g. a long maturation period, and grown under a low night temperatures had highest TSS. Hence plants with high leaf area, harvesting ripe fruit and selecting slow-maturation cultivars may contribute to fruit with high TSS (Welles et al., 1988). In tomato, fruit size and sugar concentration is influenced by potential capacity to import assimilates and competition for assimilates within the plant (Bangerth and Ho, 1984). These factors may also be important in determining papaya TSS content, though no reports are available.

**2. 3 Source-Sink relationship during fruit development**

Leaves that provide photosynthetic assimilates are referred to as the source. Young leaves, flowers, fruit, stems and roots, which import photosynthetic assimilates, are defined as sinks. Source limitation, naturally or artificially induced, has been shown to affect the reproductive biology (Awada 1967; Spears et al., 1988), fruit size and quality, and vegetative senescence in many plants (Bertin 1995; Chen et al., 1979; Hubbard et al., 1990; Hunter 1991; Koblet et al., 1994; Pavel et al., 1993). Source limitation reduces flower production and increases flower and fruit abortion rates in several species of plants (Bertin, 1995; Chamont, 1992; Stephenson, 1981;). Organ initiation often decreases and organ abortion increases as source strength decrease (Wardlaw, 1990). Sexual expression is also altered by source limitation in many hermaphroditic species (Wilson, 1983; Spears et al., 1988). Source-sink competition results in postharvest leaf blackening in inflorescence of protea (Dai and Paull, 1995), reduces grain yield

and the chemical content of corn kernels (Chen et al., 1978), and causes poor muskmelon fruit quality (Hubbard et al., 1990b).

2.3.1 Carbohydrate metabolism in ripening fruit as affected by leaf area

Developing fruit are very strong sinks that depend on translocatable carbohydrates from the leaf canopy (Hubbard et al., 1990b). During fruit development, carbohydrates are accumulated generally in the form of starch, sucrose or hexose sugars. Fruits that store starch, such as banana and apple, sweeten during ripening as a result of starch degradation and subsequent conversion to soluble sugars (Beaudry et al., 1989; Hubbard et al., 1990b; Tucker and Grierson, 1987). Fruit that lack stored carbohydrate reserve, such as muskmelon and papaya, must remain attached to the plant to allow accumulation of soluble sugars (Chan et al., 1979; Hubbard et al., 1990a; Tucker and Grierson, 1987; Hubbard et al., 1991).

Plants with a large leaf area have, in general, an increased photosynthetic capacity and at a given fruit load can lead to higher fruit TSS level (Welles and Buitelaar, 1988; Hubbard et al., 1990a). The optimum leaf number and area required for the development of individual fruit has been determined for several fruit trees (Antognozzi et al., 1992; Chacko and Reddyaud et al., 1982; Famiani 1997; Fishier et al., 1983; Palmer et al., 1991; Roper et al., 1987; Snelgar et al., 1997). In mango, more than 30 leaves are required if a mango fruit is dependent on current photo-assimilates for growth (Chacko et al., 1982). Kiwi fruit formed "early" (in a flowering cycle) and "late" (30 days later) after heavy pruning, had reduced total yield and poorer fruit quality (Galliano et al., 1990). The fruit/leaf-ratio also has an important influence on growth and composition of apples (Hansen, 1982), plums (Toldam-Anderson et al., 1993), grape (Koblet et al., 1994) and muskmelon (Bartolo and Schweissing, 1998).

Hubbard et al. (1990b) tested the response of a sweet and a non-sweet muskmelon genotype to leaf area reduction. When the leaf area is reduced in the sweet genotype, fruit sucrose accumulation declined. A 50% reduction the leaf area of the sweet genotype, 8 days before fruit initiation, had fruit with a similar sucrose accumulation to that of the non-sweet type. The normal increase in fruit sucrose phosphate synthase (SPS) activity during fruit maturation is

reduced by leaf removal. The greater the reduction in canopy photosynthetic capacity, the lower the fruit SPS activity. Acid invertase activity is lower during muskmelon fruit maturation and did not change between the control and 50% leaf removal treatment (Hubbard et al., 1990b).

Foliage injury can result from various biotic and abiotic factors including: mites, leaf hoppers, pathogenic fungi, bacteria, or viruses; pesticide phytotoxicity; wind or hail and storm damage; and air pollution. As result of injury, parts of individual leaves may become photosynthetically nonfunctional. The extent and timing of injury may reduce the carbon assimilation potential of a tree (Layme and Flore 1992). Papaya plants are easily damaged or suffer lodging when exposed to strong winds (Raveendranathan, 1989). Typhoons can lead to uprooting, trees being blown down, trunk breaking, leaf shedding and flower abscission (Yon, 1994). Papaya ringspot virus reduces papaya leaf photosynthesis and increases leaf respiration rate (Decker and Tio, 1958; Marler et al ., 1993). In muskmelon, the decreased soluble solids content was found correlated to increased severity of *Alternaria* leaf blight epidemics (Latin, R. et al., 1994)

2.3.2 Defoliation and source-sink manipulation

Fruits represent strong sinks within a tree and can compete successfully for assimilates with vegetative organs. High crop loads reduces citrus shoots, leaves, roots growth and the crop can account for 50% of the total dry matter production of a tree at harvest (Pavel et al., 1993). In cucumber, the pattern of assimilate distribution shows a sink hierarchy switched from fruit > flowers > axis to fruit > axis > flowers (Chamont, 1992). A high level of photosynthate competition can induce flower and fruit abortion (Chamont, 1992; Stephenson 1981; Wardlaw 1990). Cucumber fruit growth rate is greatly increased by increasing assimilate supply, but the fruit growth period is not noticeably affected. Low assimilate supply reduces both cell number and cell size (Marcelis 1993b). The assimilates import rate into individual tomato fruit during the early fruit development is crucial for setting the fruit's growth potential (Ho 1984). Fruit yield is determined by the balance between source and sink strengths of the plant, sugar content is determined by the transport and metabolism of sugars within the fruit (Ho 1996).

Many cultivated plants can compensate for partial defoliation by increasing the photosynthetic capacity of the remaining leaf area (Boucher et al., 1987; Flore and Irwin, 1983; Hodgkinson, 1974; Layne et al., 1992; Layne et al., 1995; Shaw and Samborski, 1956; von Caemmerer and Farquhar, 1984; Wareing et al., 1968). Removal of 25% of the leaf area of tomato (Stacey, 1983) and cucumber plants (Ramirez et al., 1988) did not significantly affect fruit yield or whole plant dry matter accumulation. Removing 50% of the leaf area of potted apple trees reduced dry weight accumulation 40% (Maggs, 1964). Flore and Irwin (1983) did not observe a significant reduction in total plant fresh mass until 20% or more of the leaf area of the whole plant is removed, demonstrated compensation may occur simultaneously. Layne et al. (1992) found that photosynthetic compensation occurs when 20% of the sour cherry leaf area is removed. Photosynthetic compensation for leaf area reduction is most likely due to enhancement both of carboxylating efficiency and RuBP regeneration capacity (Layne et al., 1995). Within 24 hours of partial defoliation, net assimilation rate of most recently expanded source leaves of defoliated plants is significantly higher than the control plant throughout the diurnal photoperiod. Between two and seven days after defoliation, assimilate is 30 to 50% higher and stomatal conductance rate 50-100% higher than the controls. In contrast, continuous lighting reduces the assimilate by two to three fold and the carboxylating efficiencies four-fold (Layne et al., 1995). Under shaded conditions, papaya plants have reduced plant height, leaf area, stomatal density, palisade mesophyll cell length, specific leaf weight, and leaf thickness and higher chlorophyll concentration (Buisson and Lee, 1993). In response to drought, papaya shed their oldest leaves (Marler et al., 1994). Leaf pruning of papaya, leaving 15 functional leaves, however, does not affect fruit TSS (Ito, 1976). Thinning papaya to one fruit per node increases fruit size and has no effect on fruit sugars (Martinez, 1988). These results may indicate that source-sink balance (fruit/leaf) adjust in response to long term leaf pruning and fruit thinning. Papaya varieties differ in photosynthetic efficiency, and their photosynthetic rates correlates with fruit TSS, but not fruit yield (Salazar, 1978).

Three days following defoliating of white clover plants, in which only one mature leaf is left on the main stolon and no leaves on the branches, resulted in: increased net photosynthetic rate in all the remaining leaves, increased percent export of fixed carbon from one of the four leaves; and an increase in the export to the main stolon apex from all except the eldest leaf and export to branches from three of the four leaves; and a decrease in the export to the stolon and roots from all leaves (Chapman et al., 1991). These responses seem to ensure the fastest possible replacement of lost leaf area and thus restoration of homoeostatic growth. The observed pattern of carbon -assimilation and distribution in both non-defoliated and defoliated white clover plants is consistent with the general rules of source-sink theory; the distance between sources and competing sinks and their relative sink strength, emerge as the most important inter-plant factors governing carbon movement. These results emphasize the need to consider plant morphology and the modular nature of plant growth when interpreting patterns of resource allocation as plants respond to stresses such as partial defoliation.

The effect of defoliation and fruit thinning on plant growth and development depends on the time of the defoliation and number of leaf, flower, or fruit removal (Mulas, 1997). In corn and sorghum, defoliation during silking or anthesis reduces grain yield primarily due to a decrease in kernel number. Later defoliation close to the mature stage has less effect on yield, though kernel weight declined. Leaf removal decreases yield and results in a slight increase in sorghum seed sugar and crude protein but a decrease in starch content. In contrast, partial ear (sink) removal from sorghum results in an increase in seed weight and starch content of the sorghum grain (Chen et al., 1978). The effect of ear removal on the grain yield of corn differs with ear position. First ear removal induces a doubling in kernel number and grain weight of the second ear, but there is no significant effect on first ear grain weight when the second ear is removed (Chen et al., 1979). Prioul and Schwebel-Dugue (1992) found that corn ear removal led to dry matter accumulation in stalks, leaves sheaths, and blades, and accelerated vegetative senescence. This response is referred to as feedback inhibition (Foyer, 1987). In contrast, the leaf excision has little impact on the remaining leaf and stalk, but drastically reduces the number of developing grains.

The rate of dry matter accumulation in those developed grains is nearly identical to that in control plants.

In peach, individual fruit size increases with declining tree crop load. The difference in growth between fruit on thinned and un-thinned trees remains nearly constant during the mid growth stage. Later in the season, at the beginning of fruit growth stage III, fruit on thinned trees grew more rapidly than fruit on un-thinned trees of the three cultivars tested (Pavel et al., 1993). Reducing fruit load in small fruited sour cherry increases fruit growth slightly when the concentration of total and soluble dry matter is not affected (Hansen, 1993). On rabbit eye blueberry (Lyrene, 1992), premature defoliation reduced flower formation and the effect depends on defoliation time. Defoliation had a greater impact on total marketable yield and yield of individual marketable class when it occurred near its onset of bulbing in onion (Bartolo et al., 1994).

Several methods of artificial defoliation have been used to simulate pest damage and establish crop damage-yield relationships for various crops. Leaf area reduction using a cork borer or paper hole-punch (Boucher et al., 1987; Flore and Irwin, 1983), leaf injury by cutting the midrib or pricking the lamina (Li and Proctor, 1984), and leaf removal (Stacey, 1983) have all been used to simulate pest damage. Poston et al. (1976) noted that reducing leaf area with a cork borer adequately simulated painted lady caterpillar and green clover-worm defoliation of soybean.

### 2.3.3 Sink strength and relative enzyme activities during fruit development

Many studies have been conducted to explain the control of assimilate partitioning between sinks competing for a limited supply of assimilates (Bangerth and Ho, 1984; Chamont, 1992; Ho, 1984; Marcelis, 1996; Pavel et al., 1993; ). Sinks such as fruit, change their competitive ability as they grow, leading to diversion towards the "stronger sinks" (Ho, 1980; Wright, 1989). The term sink strength can be defined as the competitive ability of an organ to attract assimilates. However, there is much debate and confusion about the term sink strength because this term is not clearly defined (Marcelis, 1996). Generally, sink strength can be described as the product of sink size and sink activity. Sink size is a physical restraint that includes cell number and size e.g.

the total weight of the sink tissue. However, in cucumber cell number is not a suitable measure of sink size (Marcelis, 1996). Sink activity is a physiological restraint that includes multiple factors and key enzymes involved in carbohydrate utilization and storage (Ho, 1984). Distal fruit are known to have a lower sink strength than proximal tomato fruit (Bangerth and Ho, 1984). A model for dry matter partitioning into generative plant parts, based on organs sink strength is described by Marcelis (1996). The potential growth rate has been shown to be an important parameter that quantitatively reflects the sink strength of an organ. The potential growth rate of plant's organs is not static but changes dynamically with its age and temperature.

It has been postulated that an accumulation of assimilates in altering either the size or activity of the sink results in changes in either, or both, transport patterns or rate of carbohydrate flow into a sink. Flow rate is dependent upon supply, presence of other sinks, and the resistance to flow in the transport pathway, hence it is not a property of the sink alone, but the entire system (Minchin et al., 1996). Reducing the photosynthate supply by shading barley seedling shoots, reduces the partitioning of recently fixed photosynthate to the root within about 20 min (Minchin et al., 1996). Clearly, source supply influences partitioning between the root and shoot. Minchin et al. (1993) proposed a simple mechanistic model of phloem transport between a single source and multiple sinks. This model describes bulk flow through resistive conduits (sieve tubes), driven by an osmotically generated pressure gradient, and saturable unloading, described by Michaelis-Menten kinetics. This model defines a sink in terms of its Michaelis-Menten parameters, Vmax and Km. The maximum possible flow into a sink is given by Vmax, and corresponds to Wareing and Patrick's (1975) potential capacity with un-limited supply. When the remaining other sinks are eliminated, competition for available supply is suggested as a means of measuring potential capacity or equivalently Vmax. If sink demand for sucrose is high, sucrose synthesis is increased at the expense of starch synthesis and vice versa (Ho, 1986). Black (1993) proposed an additional term, sink capacity duration, such as sucrose synthase activity through part or all of the sink development period.

Three enzymes are thought to be principally involved in sugar metabolism during fruit development and ripening. Sucrose phosphate synthase (UDPG: D-fructose 6-2-glucosyl-transferase E.C. 2.4.1.14) (SPS) is involved in sucrose synthesis (Brunean et al. 1991). Sucrose synthase (SS), can function in sucrose synthesis or cleavage (UDPG: D-fructose 2-glucosyl-transferase, E.C.2.4.1.13) (Sung et al., 1988; Wang et al., 1994). Acid and neutral invertases (β-fructofuranosidase, E.C.3.2.1.26) catalyzes the hydrolysis of sucrose to glucose and fructose (McCollum et al., 1988).

2.3.3.1 Sucrose Phosphate Synthase

Sucrose phosphate synthase (SPS), a key enzyme for sucrose biosynthesis in plants, is regulated at two levels: (1) a metabolic fine control and, (2) a coarse control (Bruneau et al., 1991; Chan and Kwok, 1976; Cheikh and Brenner, 1992; Galtier et al. 1993; Hubbard et al., 1989; 1991; Lingle and Dunlap 1987: Klein, 1993; McCollum, et al., 1988; Moriguchi and Yamaki, 1988). Fine control of enzyme activity is exerted by metabolic effects that instantaneously activate or inhibit catalysis. Coarse control refers to slower changes in extractable activity of an enzyme caused by covalent modification or changes in the rate of either or both enzyme synthesis and degradation (Klein et al., 1993). The mechanism underlying covalent modification of SPS is protein phosphorylation by SPS-kinase. Phospho-SPS is dephosphorylated/activated by a type 2A protein phosphatasee (SPS-PP). SPS-kinase is inhibited by glucose-6-P and SPS-PP is inhibited by Pi (Huber, 1992). Coarse control involves changes in the activity of SPS in response to light / dark transitions (Bruneau et al., 1991; Huber et al., 1987; 1989; Kromer et al., 1988; Rufty et al., 1983), source-sink manipulations (Rufty and Huber, 1983), stage of tissue development (Bruneau et al., 1991; Giaquinta, 1978; Huber and Israel, 1982; Huber et al., 1987;), and adaptation to low temperature (Guy et al., 1992). Extractable activity also responds to certain environmental and physiological changes, e.g. water stress (Castrillo, 1992), and plant growth regulators (Cheikh and Brenmer 1992).

Prioul et al. (1992) reported that at the grain-filling stage of maize, SPS activity more accurately reflected assimilate demand than did metabolite level or enzyme activity for carbon fixation. The importance of SPS in the regulation of carbon partitioning in leaves has been recently confirmed using recombinant DNA technology. Transgenic tomato plants expressing high level of maize SPS have lower levels of leaf starch and increased concentration of sucrose in the leaf (Worrell et al., 1991). In general, the results indicate that plant growth and yield are sometimes enhanced even though the effect on photosynthesis is small. Fruit dry mass and soluble solids are increased when leaf SPS activity is enhanced (Laporte et al., 1997).

During periods of rapid growth, the high sucrose storing sugarcane exhibits slower rates of sucrose accumulation than during periods of slow growth. This response suggests that SPS activity may sometimes impact assimilate partitioning (Moore, 1993). In most cases, higher leaf SPS activity reflects higher assimilation of sucrose in the sink tissue. Tomato plants expressing maize SPS have increased leaf SPS activity and also increased fruit soluble solids and yield under certain conditions (Laporte et al., 1997). Reimholz et al. (1994) found that SPS from non-photosynthetic tissues (potato tubers) is regulated by metabolites and protein phosphorylation in an analogous manner to the leaf enzyme. However, differences in sink activity are not paralleled by differences in source supply. Some wild relatives of sugarcane store less than 2% of the fresh weight as sucrose while some commercial sugarcane cultivars store sucrose in excess of 62% of the dry weight or 25% of the fresh weight. The photosynthetic rates of the former (*S. spontaneum*) is nearly twice that of the latter (*S. officinarum*) (cf. Moore, 1993). On this basis, Moore (1993) hypothesized that the differences in sucrose storage appear to be regulated at the level of sink or within the translocation system between the source and sink, however, the mechanism is unclear.

Recent reports have suggested that SPS is not only a key enzyme in sucrose biosynthesis in photosynthetic "source" tissue, but may also be important in some sucrose accumulating "sink" tissues (Dali et al., 1992; Hubbard et al., 1991; Hesse et al., 1995;). Genotypes that accumulate different amounts of sucrose have similar acid invertase activity and

different SPS activity (Hubbard et al., 1991). Sucrose accumulation *in situ* in peach, strawberry, kiwi, mango (Hubbard et al., 1991; MacRae et al., 1992), and banana (Hubbard et al., 1991; Cordenunsi and Lajolo 1995) is related to an increase in SPS activity in the fruit. In banana, the accumulation of sucrose is correlated to starch degradation and happens 4 day after SPS mRNA and activity reached their maxima (Nascimento et al., 1997).

2.3.3.2 Sucrose synthase

Sucrose synthase (SS) appears to play a major role in tomato fruit sink establishment and maintenance by cleaving imported sucrose and providing UDP-glucose for biosynthetic reactions (Wang et al. 1993a; Wang et al 1994). Its activity is also involved in sugar accumulation in Asian pear fruit (Moriguchi et al. 1992), cucumber (Gross and Pharr, 1982; Schaffer et al., 1987) peach (Moriguchi et al., 1988), sugar beet (Hesse and willmitzer,1996), and *Vicia faba* seed coat (Heim et al., 1993). The increase in sucrose concentration in strawberry is associated with an increase in SS activity and neutral invertase (Hubbard et al., 1991). Déjardin et al., (1996) found that SS could cleave and produce sucrose in the pea seed coat. A labeling experiment on seed coats has shown that SS activity is reversible *in vivo* and can produce 37% of newly synthesized sucrose in the seed coat cells (Déjardin et al., 1997). In addition, the SS gene in maize is sugar modulated (Koch et al., 1996). The responsiveness of SS genes to carbohydrate availability can exert transcriptional influence at the first step of imported sucrose (Koch et al., 1996). Several authors have therefore suggested that SS activity could be used as a biochemical marker for sink strength (Claussen et al. 1986; Sung et al., 1989).

2.3.3.3 Invertase

Plant invertases (β-fructosidases, E.C. 3.2.1.26) cleave sucrose and related sugars into hexoses and have been extensively studied (c.f. Sturm et al., 1990). Most tissues analyzed contain multiple forms of invertase that are characterized by different pH optima and isoelectric points. Soluble invertase ranging in their pH optima from slightly alkaline (pH 7.5) to acidic (pH 4.5) have been described. Soluble invertases are intracellular, located in either the vacuole (acid optima) (Giaquinta et al., 1983; Leigh et al., 1979) or cytosol (neutral or alkaline) (Fahrendorf et

al., 1990; Karappiah et al., 1989). Insoluble invertase, with a pH optimum between pH 4.0 and pH 5.3, is ionically bound to the cell walls and can be solublized by extracting cell walls with high salt (Fahrendorf and Beck 1990).

Soluble invertase activity is often found in young seedlings, tuberous roots, and mature fruit (cf. Sturm 1990). It has been proposed that the soluble invertase participates in the regulation of the hexose level in mature tissues and in the utilization of sucrose stored in vacuoles. High extracelluar invertase activity is usually found in rapidly growing tissues having a high demand for hexoses, such as expansion zone of root tips and elongating internodes, at sites of emerging secondary roots, in developing tap roots and leaves. When cell growth declines and finally stops, extracellular invertase plays a role in phloem unloading by maintaining a steep sucrose concentration gradient between source and sink regions of a plant (cf. Sturm 1990). Recent studies have also suggested participation of vacuolar invertase in sink-strength regulation (Arai et al., 1991; Klann et al., 1996; Morris and Arthur, 1985). The relationship between soluble invertase activity and import is particularly clear during the earliest phase of bean pod enlargement and maize kernel development (Geiger et al., 1996). Timing of the maize kernel development is also closely related to expression of the sugar responsive maize invertase genes (Geiger et al., 1996).

Sucrose accumulation is controlled in developing tomato fruit by a single recessive gene and is associated with low levels of acid invertase protein (Klann et al., 1993). Variable invertase activities in different species of tomato are due to invertase gene transcriptional silencing or different mRNA levels at different development stages (Elliott et al., 1993; Klann et al., 1993). The lack of acid invertase activity in sucrose accumulating fruit was correlated with inheritance of the *L. chemielewskii* acid invertase gene and the absence of acid invertase mRNA in developing fruit (Klann et al., 1993).

Acid invertase activity has been associated with fruit ripening in tomato (Wang et al., 1993) and papaya (Chan et al. 1976; Chan and tang, 1979; Hubbard et al., 1991). Yelle et al. (1991) have demonstrated that sucrose accumulation is associated with greatly reduced levels of

acid invertase, though normal levels of SS. However, higher invertase activity is associated with higher hexose sugar and rapid tomato growth ( Bucheli et al., 1994; Johnson et al., 1988). Higher hexose content in ripe tomato fruit of *L. Pimpinellifolium* is correlated with earlier vacuolar invertase mRNA than in the species of *L. esculentum* during fruit development. Invertase activity in *L. pimpinellifolium* green fruit may create a stronger sink earlier in fruit development than in *L. esculentum* (Elliott et al., 1993).

A highly significant positive correlation is found between sucrose accumulation and SS but a negative correlation with acid invertase activity (Isla et al., 1995). During the initial growth phase soluble acid invertase activity is relatively high, declining concomitantly with sucrose accumulation in sugarcane (Hatch and Glasziou, 1963), sugar beet (Giaquinta, 1979), sweet melon (Hubbard et al., 1989; Lingle and Dunlap, 1987; McCollum et al. 1988; Schaffer et al. 1987), carrot (Ricardo and Rees, 1970), citrus (Kato and Kubota, 1978), mango (Castrillo et al., 1992), tomato (Miron and Schaffer, 1991) and grapefruit (Lowell et al., 1989). In two peppers' genotype, the increasing hexose sugar concentration is associated with an increase in acid and neutral invertase activity (Hubbard et al., 1992). In strawberry, soluble acid invertase activity increases in parallel with the accumulation of hexose sugars (Ranwala et al., 1992). In cold treated potato tubers, extractable invertases are involved in the regulation of the ratio of hexose to sucrose (Zrenner et al., 1996). Nevertheless, acid invertase is not always an indication of sink strength (Miron et al., 1988). Tomato fruit sink strength measured as the rate of assimilate import may be more related to the routes of sugar transport into the sink cells during fruit development (Ho, 1996). The different effects of invertase in different fruit tissue may indicate that there are multiple factors in controlling of sugar accumulation in fruit sinks.

Vacuolar invertase activity may determine the sugar composition of mature fruit, but may not affect the overall dry matter accumulation of tomato fruit (Ho 1996). Immuno-localization experiment indicated that the invertase protein, associated with the cell wall, remains in the mature sucrose accumulating genotypes while the vacuolar invertase protein is lost (Miron et al., 1996). However, further experiments illustrated that sucrose is taken up intact by sucrose-

accumulating-tomato fruit. In addition, sucrose is not particularly compartmentalized in the vacuole in sucrose-accumulating-fruit but rather sucrose concentrations increase simultaneously in the apoplast, cytoplasm and vacuole, through development (Miron et al., 1996). In Asian pear, the relationships between soluble invertase activity and sucrose content are not significant (Moriguchi et al., 1992). Expression of a cytosolic yeast invertase in potato tuber leads to a decrease in yield and starch content and an accumulation of glucose but not fructose, whereas expression of a apoplastic yeast improves tuber growth (Sonnewald et al., 1994; 1997). Preharvest application of elevated $CO_2$ throughout the tomato fruit growing period significantly increases reducing sugar and acid invertase activity at harvest (Isla et al.,1995).These results suggest that sucrose hydrolysis might determine sink strength.

Invertase activity can be modulated by an invertase inhibitor, substrate (sucrose) and product (fructose) coreesth several divalent metal ions (Weil et al., 1994), protein (Isla et al., 1995) and DTT (Weil and Rausch, 1990). The inhibitor peptides described so far have molecular weights ranging from 17 to 23 kDa (Weil., et al., 1994). A heat stable invertase inhibitor was reported in tomato fruit with a MW 18 kD, and inhibition dependent on pH (Pressey, 1994). The inhibitor separates with invertase at pH 6.5. and maximum inhibition was found at pH 5.0. Tomato inhibitor also inhibits potato tuber invertase activity but not yeast invertase. The presence of the inhibitor may explain why invertase activity *in vitro* is well in excess of the requirement of sucrose hydrolysis to regulate the unloading process (Johnson et al., 1988). Total invertase activity (i.e. assayed after destroying the endogenous invertase inhibitor present in the extract generally reflects sugar changes more closely than did basal activity (i.e. assayed with the inhibitor present) (Richardson et al., 1990). While low levels of invertase in sucrose accumulating fruit due to low levels of invertase rather than the presence of an invertase inhibitor (Yelle et al., 1991). A tobacco apoplasmic invertase inhibitor protein has been isolated (Weil and Rausch, 1994) and the gene cloned and characterized (Greiner et al., 1998). The inhibition of cell wall invertase by invertase inhibitor can be protected by sucrose concentration, $Ca^{2+}$, $Mg^{2+}$, and $Zn^{2+}$.(Weil et al., 1994). Inhibition of invertase by fructose can occur in a simple or a complex competitive fashion (Isla et

al., 1995). BSA and DTT were reported to increase invertase activity *in vitro* (Lopez et al., 1988;Weil and Rausch 1994), the mechanism of latter probably due to its reducing agent nature in destroying invertase inhibitor disulfide bridge (Weil and Rausch 1994).

2.3.3.4 Enzymes and sink strength

Sink strength is determined in other cases by more than one enzyme during sink development. Snap bean pod elongation is associated with acid invertase while SS is associated with fruit dry matter accumulation (Sung et al., 1994). All neutral invertase activities during pod and seed development are too low to have a role in sucrose cleavage. In potato, during stages of tuber development, soluble invertase is the predominant sucrose metabolizing activity. Later in development, when the accumulation of storage compounds like starch and proteins occurs, invertase activity declines and SS is most likely responsible for the entire sucrose cleavage (Hajirezaei et al., 1996). Sucrose accumulation in *Cucumis* is characterized by a metabolic complex that includes low acid invertase activity together with relatively high activity of SPS, SS and alkaline invertase. Final sucrose content, however, is primarily a function of the length of the sucrose accumulation period that is genetically determined (Burger et al.,1996). Sucrose accumulation in sugarcane is controlled by the difference between SPS and invertase enzyme activity (Zhu et al., 1997).

In carrot, sink strength was thought by the common action of SS and vacuolar invertase and, especially in the case of apoplastic unloading, by the directed and active transport of sucrose across membrane (Sturm, 1996). Because the activity of the vacuolar invertase is inhibited by millimolar concentrations of fructose (Isla et al., 1991; Lopez et al., 1988; Sampietro et al., 1980). Sucrose cleavage does not go to completion and the sugar stored in mature tap roots are a mixture of fructose, glucose and sucrose (Sturm 1996). However, the factor that transgenic plant expressed with antisense of carrot cell wall invertase abolished development of carrot tap root and antisense of vacuolar invertase reduced growth of tap root demonstrate the importance of cell call invertase in carrot root development (Sturm, 1998). Zamski and Barnea (1996) found that the genotype associated with higher sucrose content also exhibited a higher

SPS, SS and invertase activity. However, blueberry fruit development does not appear to be limited by either or both sucrose metabolism enzyme activity or the ability to accumulate sugars in either $GA_3$-treated or pollinated fruit (Cano-Medrano et al., 1997).

Hubbard et al. (1991) hypothesis that sucrose and invertase may be present in either or both different intracellular locations or different cells within the papaya mesocarp tissues sampled in the study. These results also imply that the sucrose is unloaded from the phloem into the apoplast and suggests the existence of a hexose transporter. Since papaya ripen from the inside outward, it could be anticipated that a spacial difference in different enzymatic activities could exist at an early ripening stage. Nevertheless, no SPS, SS invertase enzyme activity in green fruit has been reported. The relationship between papaya fruit development, sugar accumulation and the relative carbohydrate metabolic enzyme activity has not been determined.

The above results suggest that in different species, different tissues and at different times, particular enzymes have greater or lesser importance in determining sink strength and size. Papaya have fruit at various ages on the fruit column, it is possible that different sink related enzymes may be expressed at different stages of development with the early stage enzymes being associated with fruit growth and later associated with sugar accumulation.

### 2.3.4 Sugar unloading pathway at sinks

Sink unloading, such as in fruit, is much less studied than loading, as it varies between different species (Formmer and Sonnewald, 1995). There is also a close spatial relationship in fruit between phloem unloading and storage (Ruan and Patrick, 1995). Two models exist (Sonnewald et al., 1995): the first involves unloading along a concentration gradient and the second has sucrose unloading into the apoplast. In the first, the gradient is maintained via sugars being stored in an insoluble form such as starch, making "direct" symplastic unloading feasible. The second model would be expected to have either a sucrose transporter or invertase and hexose transporter (Eschrich, 1980; Godt and Roitsch, 1997). Extracellular invertase and hexose transporters are not only functionally linked but also are coordinately regulated (Godt and Roitsch, 1997). Hexose transporter has been isolated from *Arabidopsis* and tobacco, some of

which are specifically expressed in sink tissues (Sauer et al., 1990; Sauer and Stadler, 1993). The glucose carriers are homologous to sugar transporters from bacteria, fungi, blue/green algae, and mammalian organisms. Multiple genes are found in a species and the genes are differentially expressed (Sauer and Stadler, 1993). Using symplastic and apoplastic tracers, Ruan and Patrick (1995) showed that the post-phloem cellular pathway in tomato fruit shifts from the symplast during starch accumulation (13 to 14 days after anthesis (DAA)), to apoplast during the rapid hexose accumulation (23 to 25 DAA) stage. An energy-coupled plasma membrane carrier is expressed in the latter stage of fruit development (Ruan and Patrick, 1995). Hexose levels in the fruit pericarp are controlled by intrinsic factors within the storage parenchyma cells. Estimates of *in vitro* and *in vivo* sugar flux into the metabolic and storage pools within the fruit pericarp demonstrated that membrane transport rather than metabolism plays a major role in the control of the hexose levels within storage parenchyma (Ruan et al., 1996). The invertase / hexose transporter model with cycling is also reported for sugarcane internodes (Moore, 1995).

Hexose transporters have been described for plant plasma membranes and tonoplasts (Rausch, 1991; Shiratake et al., 1997). The tobacco cell plasma membrane hexose transporter is insensitive to p-chloromecuribenzene sulfonic acid (PCMBS) and N-ethylmaleimide (NEM), while the tonoplast hexose transporter is inhibited (Verstappen et al., 1991). A genomic sequence for a hexose transporter has been isolated from *Arabidopsis* using a previously isolated *Chlorella* cDNA (Sauer et al., 1990). Hexose transporter activity has been measured in tomato fruit pericarp tissue using the uptake rate of $C^{14}$-glucose and $C^{14}$-fructose (Ruan and Patrick, 1995). Lee et al. (1996) suggested that IAA stimulates both the activation of acid invertase and the uptake of sugars, and an increase concentration of sucrose stimulates activity of SS. The sink activity increased in these ways thus trigger fruit growth. Further experiments indicated that changes in activity of cell wall bound acid invertase and sucrose synthase were not clearly associated with fruit growth, while the increase in activity in soluble acid invertase is accompanied by an increase in endogenous IAA content (Lee et al., 1997).

2.3.5 Structure and function of invertase enzymes and genes among plant species

The higher enzyme activity of cell wall bound extracellular invertase in the presence of 100 mM glucose and sucrose is paralleled by an increased expression of the corresponding gene. The activity of both neutral and acidic intracellular invertases is not affected by pre-incubation of autotrophic cultures with sugars, nor do they show a tissue specific distribution in *Chenopodium rubrum* plant. while in maize, two soluble invertase genes have been shown to be sugar enhanced and starvation tolerant, respectively, and have different distribution in tissues (Koch et al., 1996; Xu et al., 1996). Additional data from *Arabidopsis, Chenopodium*, bean and other species indicates that these species may have corresponding differential sugar-responsive classes of the invertase genes. Analysis of invertase expression in carrot plants indicate that changes in invertase gene expression can result from modification of source-sink relations, and in turn, have the potential to affect specific aspects of sink import (cf. Koch et al., 1996; Ehness et al., 1997).

The invertase protein has been purified and characterized from a number of dicot and monocot plants (cf. Unger et al., 1992; Weil and Rausch 1994). The molecular masses determined by gel-filtration chromatography vary considerably (50-9000 kDa), as do the molecular masses determined by SDS/PAGE (11-73 kDa) (Unger et al., 1992). The existence of multiple bands during invertase purification have been reported in tomato (52, 30, 22 kDa) (Yelle et al., 1991), mung bean (70, 30, 38 kDa) (Arai et al., 1992), potato (60, 2 bands at 30kDa) (Bracho and Whitaker, 1990), melon (70, 50, 24 kDa), (Iwatsubo et al., 1992), carrot (68, 43, 25 kDa) (Unger et al., 1992), castor bean (78,000, 7subunits of 11 kDa) (Bracho and Whitaker, 1990), date (130, 70 kDa, cf. Bracho and Whitaker, 1990). Most have been proofed as small molecular band as a subunit or degradation of product of the mature protein.

Most plant vacuole and cell wall invertase are glycoproteins (Berges et al.,1993; Koch et al., 1996; Isla et al., 1995; Lauriere et al., 1988; Rojo et al., 1994; Weil and Rausch, 1990; 1994). The glycosylation is the principal chemical modification to most plasma membrane and secretory

proteins (Lodish et al., 1996). It is believed that glycosylation functions in protein folding and increasing protein solubility, the carbohydrates generally play no role in the catalytic function of these membrane associated enzymes (Lodish et al., 1996). Deglycosylation of the native tobacco cell wall invertase led to partial removal of glucan without severely affecting enzyme activity. However, glycosylation did increase resistance towards further protease degradation *in situ* (Weil and Rausch, 1994). The effect of glycosylation inhibitor reflects a decreased stability of the nonglycosylated enzyme after secretion (Weil and Rausch, 1990). Glycosylation could occurred in O-linked or N-linked amino acid. O-linked sugars, N-acetylgalactosamine are invariably linked to serine or threonine and, in collagens, galactose is linked to hydroxylysine. In all N-linked oligosaccharides, N-acetylglucosamine is linked to asparagine (Lodish et al., 1996). The proposed N-link site of protein sequence is N X S/T (Arai et al., 1992). Among six potential N-glycosylation site in the carrot ceil wall invertase cDNA sequence, three of them are N-giycolysated, including one high-mannose glycans and two complex glycans (Sturm and Chrispeels, 1990). Two high-mannose and two complex glycans were found in tobacco crown-gall cell wall invertase protein (Weil and Rausch, 1994). Tobacco cell wall invertase strongly cross-react with an antiserum directed against deglycosylated carrot cell wall invertase only after denaturing the enzyme (Krausgrill et al., 1996).

Invertase genes have been cloned from many plant species; carrot (Sturm et al., 1990; Unger et al., 1994), chenopodium (Roitsch et al., 1995), potato (Hedley et al., 1994; Zhou 1994), tomato (Elliott et al., 1993; Klann et al., 1992; Ohyama et al., 1992; Okio et al., 1994), maize (Koch et al., 1996), mung bean (Aria et al., 1992; Weber et al., 1995), tobacco (Greiner 1995), Asparagus (Dwyer et al., 1997), grape (Davis et al., 1996), and arabidopsis (Mercier and Gogarten, 1995). Two vacuolar and two cell wall invertase genomic gene have been identified in *Arabidopsis thaliana* plant (Haouazine-Takvorian et al., 1997). Seven exons and six introns exist with an identical organization in the two vacuolar genes. A short exon skipping is induced by cold stress in potato invertase gene transcript (Bournay et al., 1996). Multiple invertase genes also have been found in tomato (Elliott et al., 1993; Godt and Roitsch, 1997; Klann et al., 1992;

Ohyama et al., 1992; Okio et al., 1994;), potato (Hedley et al., 1994; Zhou 1994), maize (Koch et al., 1996), *Chenopodium* (Roitsch et al., 1995), and carrot (Sturm 1996).

The four extracellular invertase genes from tomato do not cross-react, although they are 75% to 79% identical in the nucleic acid levels. The specific regulation of the four tomato invertase gene suggests an important function of apoplastic cleavage of sucrose by cell wall-bound invertase in establishing and maintaining sink metabolism (Godt and Roitsch, 1997). Extracellular invertase gene expression is increased by wounding (Sturm et al., 1990; Zhang et al., 1996), bacteria infection (Sturm et al., 1996) and gravistimulation (Wu et al., 1993).
The acid invertase appears as a pro-protein with signal peptides and N-terminal pro-peptides. A comparison of the amino acid sequences of leader peptides of different carrot isoenzymes shows no homology with vacuolar invertase that is acidic, while cell wall protein is basic. The vacuolar proteins share some regions of homology with the cell wall enzymes but other parts are quite different. This difference explains the marked differences in their isoelectric points (Sturm 1996). The cDNA derived amino acid sequences of the vacuolar invertase also contains short C-terminal extensions, most likely containing the information for vacuolar targeting (Unger et al., 1994). A greater distance is evident between soluble and insoluble invertase of the same species than there is between soluble invertases of different species (Koch 1996). In carrots, the soluble and insoluble (cell wall) invertase show weak immunogenic cross-reactivity (Lauriere et al., 1988). The localization of the invertase, and the site of increased hexose production can have profound effects on tuber and fruit physiology of potato and tomato, respectively. Transgenic potato plants that expressed a yeast invertase gene in the cytosol of tubers gave rise to a reduction in tuber size and an increase in tuber number per plant. In contrast, Whereas apoplastic targeting led to an increase in tuber size and decrease in tuber number per plant (Sonnewald et al., 1997). A link between soluble sugar levels and fruit size in transgenic plants has been demonstrated in tomato. Antisense inhibition of an intracellular acid invertase activity, probably localized in the vacuole, led to significantly altered soluble sugar composition and a reduction in fruit size of up to 30% (Klann et al., 1996).

In transgenetic *Arabidopsis* expressing apoplastic antisense invertase I stimulate apoplastic iso-invertase II at both the transcriptional and translational level. This phenomena further confirms that invertase gene control sugar partitioning and gene expression (Chaivisuthangkura et al., 1998). In somatic embryos expressing antisense mRNA for cell wall invertase and soluble invertase, carrot plant development was interacted differentially. If the plantlets were provided with glucose and fructose instead of sucrose, the transgenic plantlets looked more or less normal. When plantlets from hexose-containing media are transferred to soil, mature plant expressing cell wall invertase antisense mRNA have a bushy appearance and the tap root development is markedly delayed and reduced. On average, plants expressing antisense mRNA for soluble invertase change the leaf to root ratio, develop tap roots normally but remained smaller (Tang and Sturm, 1998). These data indicate that invertase have multiple roles and functions.

Acid invertase activity in ripe papaya fruits is high (Chan and Kwok 1976, Chan and Tang 1979; Hubbard et al., 1991), while SPS and SS activity is low (Hubbard et al., 1991). Buffer soluble invertase from mature green and ripe papaya fruit has been partial purified (Lopez et al., 1988; Chan and Kwok 1976). It has a pH optimum of 4.5, and an optimum temperature of 40°C (Chan and Kwok, 1976; Lopez et al., 1988). The Km is ca. 4.2 mM (Lopez et al., 1988). The apparent molecular weight as determined by gel filtration is 275kDa (Chan and Kwok, 1976) but only 52 kDa by gel electrophoresis (Lopez et al., 1988). The much higher value obtained by gel filtration is possibly due to aggregation at iow salt concentration. Papaya invertase is inhibited by fructose (Lopez et ai., 1988) and idoacetamide (Chan and Kwok, 1976); 90% of the invertase activity is lost after exposure of the enzyme preparation to 60°C for two minutes (Chan and Kwok, 1976). The fact that more than 50% sugar was sucrose in mature papaya fruit when invertase was inactivated by heating before extraction suggested that either invertase was inhibited by fructose, an invertase inhibitor is present *in vivo* or a physical separation occurs between substrate and invertase enzyme. No information is available in invertase protein localization, activity and gene regulation, invertase protein sequence and gene sequence in papaya fruit.

## 2.4 Conclusion

Fruit quality and production is influenced by varieties, maturation, environmental factors and source-sink balance. SPS, SS, and acid invertase activity in fruit could represent sink activities in different fruit type and at different stages of development. There is little information on the relationship between fruit development and carbohydrate accumulation and SPS, SS and invertase enzyme activities in papaya. Source-sink manipulation of carbohydrate assimilation and the relative enzymes in indeterminate fruiting types such as developing papaya have not been investigated. The function of these enzymes in the accumulation of sugars in papaya fruit is poorly understood. Elucidation the functions may come from biochemical studies of each enzyme and substrates during fruit development. Also, the effects of different cultivars, seasonal changes and defoliation on papaya fruit development and sugar accumulation are unclear and a significant genetic component may influence final sugar content in fruit. Hence, a biochemical understanding of partitioning and accumulation of carbohydrates could lead to an improvement in papaya fruit sugar content.

# CHAPTER 3

# HYPOTHESIS AND OBJECTIVES

## 3.1 Hypothesis

(1) Foliage injury, caused by insects, disease or hurricane, are major factors influencing the leaf photosynthesis capacity and plant carbohydrates availability and could subsequently, reduced sugar and dry matter accumulation in both the plant and the fruit.

(2) Papaya have fruit at various ages on the fruit column, and that different sink related enzymes are expressed at different stages of development with early stage enzymes being associated with fruit growth and later stage enzymes associated with sugar accumulation.

(3) High invertase activity and low SS and SPS activity in ripe papaya fruit indicate that invertase enzyme is a key enzyme in regulating sugar accumulation during the late stage of papaya fruit development and is the major enzyme determining fruit sink strength.

## 3.2 Objectives

The broad goal of this project was to understand the mechanism of carbohydrate partitioning and accumulation during papaya fruit development and determine the factors and their relative importance in influencing fruit growth and sugar accumulation. The four specific objectives relative to the above hypothesis were:

I. Determine the time course between the increase in fruit flesh weight, seed development, flesh color development, sugar accumulation and related enzyme activities.

II. Quantify the effects of defoliation on sugar accumulation in fruit, final fruit quality and the key enzymes of sugar synthesis and metabolism.

III. Identify the critical enzymes (sucrose phosphate synthase (SPS), sucrose synthase (SS), and acid invertase) that regulate papaya carbohydrate metabolism during fruit development and in response to defoliation.

IV. Isolate the genes for the key enzymes identified in step III controlling sugar accumulation in papaya fruit.

# CHAPTER 4

# THE RELATIONSHIP OF PAPAYA FRUIT GROWTH, RESPIRATION, SUGAR ACCUMULATION AND THE ACTIVITY OF SPS, SS, INVERTASE ENZYME DURING PAPAYA FRUIT DEVELOPMENT AND RIPENING


**Abstract**

Developing papaya (*Carica papaya* L.) fruit were strong sinks that continually import carbohydrates from leaf until harvest. This study used 'Sunset' papaya to determine the relationship between fruit flesh and seed growth, color development, fruit respiration, sugar accumulation and the activity of sucrose phosphate synthase (SPS), sucrose synthase (SS), and acid invertase with fruit development from 14 days after anthesis (DAA) to 140 DAA (harvest maturity). Fruit sugar levels and SPS, SS and acid invertase activities were also compared to 'Kapoho', the major Hawaii's cultivar, and UH801, a low sugar line during fruit late development (one month before harvest) and in five available cultivars during postharvest ripening, respectively. The relationship between fruit fresh mass and fruit length and diameter was expressed as a linear regression after log transformation (Log fruit mass = 3.1253 X (log fruit length) - 0.9525, $r^2 = 0.97$; Log fruit mass = 2.583 X (log fruit diameter) + 0.2513, $r^2 = 0.99$, respectively, $p < 0.0001$). Fruit flesh dry mass percentage decreased from 14 to 56 DAA, then remained constant from 56 to 112 DAA, then rapidly increased one month before harvest. Fruit flesh sugar accumulation and dry matter growth rate increased after seed maturation. Fruit respiration rate decreased from 48 ml $CO_2$ $h^{-1}$ $Kg^{-1}$ in fruit 14 DAA to 12 ml $CO_2$ $h^{-1}$ $Kg^{-1}$ at 70 DAA, then remained constant until fruit skin showed color break. Fruit sugar began to increase about one month before harvest, with 40 to 50% of the total sugars as sucrose. SPS activity remained very low throughout fruit development and increased slightly before harvest. SS activity was very high in 14 DAA fruit and decreased to less than one third within 42 to 56 days, and remained constant in the late stage of fruit development. Acid invertase activity was very low in the young fruit and increased more than 10 fold 42 to 14 days before maturation. The

increase in fruit flesh dry mass percentage coincided with fruit flesh color (CIE 'L *a *b') while fruit skin color 'a' value was only partially correlated with dry mass accumulation during fruit development. A regression model (CIE a = $-0.2767 \times (TSS)^2 + 7.49144 \times (TSS) - 32.708$, p= 0.0001, $r^2 = 0.67$, n=213) between fruit flesh 'a' color and total soluble solids in full ripe fruit was also obtained. Correlation analysis indicated that SS was highly correlated with respiration ($r^2$ = 0.9511, p=0.00003) and invertase was associated with sugar accumulation ($r^2$= 0.738, $p< 0.05$) during the last phase of fruit development. The relationship between enzyme activity and calculated fruit flesh carbon import rate suggested that SS and acid invertase were the two major enzymes that determined papaya fruit sink strength in the early and late development phase, respectively. Comparison of low sugar accumulation fruit type (UH801) with the commercial Solo types in sugar levels and enzyme activities demonstrated that the differences in invertase activities could account for the difference in final fruit sugar levels. SPS, SS enzyme activities decreased and acid invertase activity dramatically increased during postharvest ripening of papaya. The relationship between SPS, SS and acid invertase enzyme activities and sugar partitioning within the fruit during postharvest ripening was not clear.

### 4.1 Introduction

Papaya (*Carica papaya* L.) is an important tropical fruit crop. In 1994, fresh papaya fruit production in Hawaii totaled about 62,000,000 lb., and more than 60% was shipped to the US mainland and Japan. The fruit has potential markets in Europe, Japan, the Middle East and USA (Radi et al., 1994).

Papaya fruit increases in fruit size, volume and fresh mass has been previously reported (Muda et al., 1994; Qui et al., 1995). The changes in physical properties during maturation occur simultaneously with the changes in the chemical component of the fruit. The most obvious changes occur in the skin, flesh and seed color (Muda et al., 1994). While the most important biochemical changes during maturation and ripening of papaya is the substantial increase in soluble sugar (Chan et al., 1979; Selvaraj et al., b 1982). The total soluble solids (TSS) is used in

the commercial assessment of papaya fruit quality (Hawaii Administrative Rules, 1986) and correlates to fruit maturation and skin color (Akamine and Goo, 1971). However, there is no quantitative data or model available to illustrate the relationship between fruit fresh mass, volume (length, diameter and flesh thickness), as well as skin and flesh, seed color development, dry mass accumulation and fruit final sugar content.

Sugars play an important role in the flavor characteristics of papaya and is also a commercial measure of fresh fruit quality (Hawaii Administrative Rules, 1986). An understanding of sugar metabolism during fruit development is a pre-requisite to being able to improve this aspect of fruit quality. Developmental studies have shown that total sugar, especially sucrose increased rapidly in papaya fruit approximately 20 to 30 days before harvest (Chan et al., 1979; Selvaraj et al., 1982).

Sucrose phosphate synthase (SPS), sucrose synthase (SS), and acid invertase are the three major enzymes influencing sugar accumulation in developing fruit (Hubbard et al., 1989; 1990a; 1991; Klann et al., 1993; Lingle et al., 1987; Miron et al., 1991; Moriguchi et al., 1988; 1992; Yamaki, 1995; Yelle et al., 1991). SPS (UDPG: D-fructose 6-2-glucosyltransferase E.E.2.4.1.14) is involved in sucrose synthesis (Brunean et al., 1991). SS has a function either in sucrose synthesis or cleavage (UDPG: D-fructose 2-glucosyltransferase, E.C.2.4.1.13) (Dejardin et al., 1997; Huber et al., 1986; Sung et al., 1988; Wang et al., 1994;). Acid and neutral invertases (β-fructofuranosidase, E.C.3.2.1.26) catalyze the hydrolysis of sucrose to fructose and glucose (McCollum et al., 1988). Different pathways for sucrose accumulation among species have been summarized as acid invertase type, SPS type, SS type and SS/SPS type (Yamaki 1995). Different enzymes are more or less important during fruit development and sugar accumulation in different species, different tissues and at different times (Hubbard et al., 1989; Klann et al., 1993; Lingle et al., 1987; Lowell et al., 1989; McCollum et al., 1988; Miron et al., 1991; Moriguchi et al., 1988; 1992; Schaffer et al., 1987; Sun et al., 1994; Wang et al., 1993; Yamaki 1995; Yelle et al., 1991; Zhu et al., 1997). High soluble acid invertase activity (Pal et al., 1987; Chan et al, 1976; Chan and tang, 1979; Hubbard et al., 1991) and low SPS and SS

activities have been found in ripe papaya fruit (Hubbard et al., 1991), and these activities need to be reconsidered with the high sucrose composition of ripe papaya. How these enzymes change during papaya fruit development and the enzyme most involved in fruit development and sugar accumulation have not been determined.

The objective of current study was to determine the time course and the relationship between fruit fresh weight, size (length and diameter), skin and flesh color development and dry sugar, mass accumulation, and the activities of sucrose phosphate synthase, sucrose synthase and acid invertase during papaya fruit development and ripening. The patterns of enzyme activities during development were compared between a low sugar line and the normal commercial cultivars.

### 4. 2 Material and Methods

#### 4.2.1 Plant material

Plants of *Carica papaya* L. (cv. Sunset) were grown at the Poamoho Experimental Station on the island of Oahu, Honolulu. Flowers of papaya on selected plants were tagged weekly at anthesis. When the first tagged fruit reach harvest maturity (color break to 20% yellow), fruit aged 14 to 140 days, at 14 day intervals were harvested in the subsequent two weeks. Therefore, ten developmental stages (treatments) within 8 plants (replicates) at two harvest dates (subsamples), two to four fruit per stages per harvest date (samples) were used in the experiment. All data presented was the mean of two harvest subsamples within each plant unless otherwise indicated.

Plants of *Carica papaya* L. 'X-77' (Waimanalo), and 'UH801' were grown at the Poamoho Experimental Station, on the island of Oahu. The fruit of 'Line 8' and 'Kapoho' were obtained from Dole Fresh Fruit Company fields, in central Oahu. Fruit developmental stages of 'Kapoho' were determined by tagging flowers at anthesis. The fruit from several developmental stages before maturity were used as experimental material. The fruit developmental stages for UH 801 and other cultivars were estimated by fruit skin and flesh color (Akamine and Goo, 1971). For

comparison of fruit sugar and SPS, SS, and acid invertase activities at above five cultivars during postharvest ripening period, fruit were harvested from color break to 30% skin yellow stage and, sampled 1 day, 3 day and 7 days after storage at 23°C.

4.2.2 Chemicals

All chemicals were reagent grade or better, and purchased from Sigma and Fisher Scientific unless otherwise noted.

4.2.3 Observations

Fruit mass (g fruit$^{-1}$), total soluble solids (TSS), fruit length (cm), width (cm) and flesh thickness (cm), fruit skin and flesh color, fruit respiration were measured from all fruit harvested 14 to 140 days after anthesis (DAA) from eight plants. Fruit sugar (g kg$^{-1}$ fresh mass), flesh, seed fresh mass (g fruit$^{-1}$), dry mass and SPS SS invertase enzyme activity were determined on fruit from the ten developmental stages on the same plant. Skin and flesh color were determined with a Minolta Chromameter (CR:110, Minolta, Ramsey, N. J.) and expressed as CIE "L* a* b". TSS was determined by using refractive index, two measurement were made in the middle of each fruit.

4.2.4 Sugar assay

Flesh sugar was determined as previously described (Paull et al., 1984). Two grams of tissue was heated 10 min in a boiling water bath (unless otherwise stated) before extraction with 90% ethanol, 5 ml of the supernatant solution was dried and dissolved in 2.5 to 5 ml deionized water (for young fruit and mature fruit, respectively). Sucrose, fructose and glucose were separated and quantified by HPLC from retention times and peak area under known standard. The amount of the three sugars was summed and regarded as the total.

4.2.5 Dry mass assay

Dry mass percentage for each DAA was determined by drying ten gram of fresh tissue or seed sample (n=3) at 60°C for 6 days (Qiu et al.,1995). Fruit flesh and seed dry mass accumulation were calculated from dry mass percentage times average fruit fresh mass at the

same ages. Flesh and seed dry mass growth rate (g $day^{-1}$ $fruit^{-1}$ dry mass) were calculated as $GR=(dw\ dt^{-1})$.

4.2.6 Fruit respiration rate

Fruit of 10 different ages were harvested from eight 'Sunset ' plants and used for determination of fruit respiration rate during fruit development. Fruit were sealed individually for one hour in a 970 ml jar and 1 ml headspace gas samples taken for $CO_2$ measurement. An infrared $CO_2$ gas analyzer was used to determine $CO_2$ concentration (Clegg et al., 1978).

4.2.7 Carbon import rate by fruit flesh

Carbon import rate was calculated from the sum of carbon consumed by respiration per fruit per day and carbon accumulated per fruit per day as dry mass growth rate multiplied by 0.47 (Huang et al., 1992).

4.2.8 Enzyme extraction

SPS, SS, and acid invertase enzymes were extracted according to the method of Hubbard et al. (1989), with slight modification. Fruit mesocarp tissue was sampled, frozen in liquid $N_2$, and stored at -80°C until use. Frozen fruit tissue was ground in liquid $N_2$ in a chilled mortar and pestle. Three grams of powder was transferred into a centrifuge tube that contained 12 ml of extraction buffer (100 mM MOPS-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 0.5 mg $ml^{-1}$ BSA, 2% v/v glycerol, 1 mM PMSF, 0.05% Triton X-100) and homogenized for 30 sec. to 1 min at high speed in a ULTRA TURRAX homogenizer. After centrifugation at 10,000xg, 5 ml of supernatant was desalted and concentrated by Centriflo membrane cones (Amicon CF-25) by three additions of desalting buffer (50 mM MOPs-NaOH pH7.5, 5 mM $MgCl_2$, 2.5 mM DTT, 0.5 mg $ml^{-1}$ BSA, 1 mM PMSF). The final volume of solution was measured and the concentration factor calculated.

4.2.9 SS and SPS assay

The reaction mixture (70μl) used to determine SPS activity contained 50 mM MOPS-NaOH (pH 8.0), 15 mM $MgCl_2$, 10 mM fructose 6-P, 30 mM glucose 6-P, 20 mM UDPG, and 45 μl desalted enzyme extract. Reaction mixtures were incubated at 37°C with shaking and the

reaction terminated at 0 and 30 min with 70 μl 30% KOH and placing the tube in boiling water bath for 10 min. After cooling, 1 ml of 0.14% (w/v) anthrone in 14 M (v/v) $H_2SO_4$ was added (Hubbard et al., 1989) and incubated at 40 °C for 20 min. After cooling, color development was measured at 620 nm. SS activity assay (in the sucrose direction) was identical to that of SPS except that the reaction mixtures contained 40 mM fructose and did not contain fructose 6-P and glucose 6-P (Hubbard et al., 1989).

4.2.10 Invertase assay

Invertase activity was assayed in 60 μl of 0.1 M $K_2HPO_4$ - 0.1 M citrate buffer (pH 5.0), 20 μl 0.1 M sucrose, and 20 μl of concentrated or diluted enzyme extract at 23°C. The reaction was stopped by adding 1 ml borate buffer (pH 9.0) and reducing sugar determined by adding 0.2 ml 1% (W/V) cyanoacetamide and boiling the mixture for 10 min, reading the absorbency at 276 nm, and using glucose and fructose as standard (Gross, 1982).

4.2.11 Data analysis

Statistical analysis was performed using Excel spreadsheet and SAS general linear models and correlation program. Log transformations of the fruit mass and length, diameter, flesh thickness were performed. These transformations gave a linear relationship between fruit mass and the other parameters.

## 4.3 Results

4.3 1 Color development

Sunset papaya matured 140 DAA during the warm season. The most obvious changes during papaya fruit development occurred in the skin, flesh and seed colors. Papaya fruit skin color changes from light green to dark green, then turns to light green and yellow during maturation and ripening, respectively. Skin CIE 'L' (lightness) and 'b' (blue to yellow) values were initially high, and gradually decreased from 15 to 112 DAA, then increased during maturation and ripening, especially the 'b' value (Figure 4.1A). The large variation between replicates in harvest maturity indicated that color changes were rapid. The 'a' value remained low and increased

slightly during development (Figure 4.1A). The 'a' and 'b' value rapidly increased during ripening when the fruit turned yellow (e.g. 'a' from below 0 increased to 6, 'b' from 25 increased to 50). Lightness remained high (ca. 80) while the flesh was still white until about one month before harvest (Figure 4.1B), then significantly decreased (ca. 50) at harvest. In contrast, flesh value 'a' and 'b' increased in parallel to the development of the red-orange flesh. Papaya seed color changed from white to brown approximately one month before harvest, then turned black about 14 days before harvest.

4.3.2 Fruit growth curve and respiration rate

The growth of 'Sunset' papaya showed a double sigmoid growth curve in terms of the increase in fruit mass, length and diameter (Figure 4.2). There was a large variation of fruit mass among fruit aged 100 to 126 DAA (Figure 4.2A). This variation also occurred in fruit diameter (Figure 4.2C). Fruit length changed rapidly during early development, then slowly during the late stages of fruit development (Figure 4.2B). Fruit flesh thickness, from two separate subsamples, had greater variation between different fruit at the same stage (Figure 4.2D).

There was an almost linear increase in both flesh and seed fresh mass 70 to 112 DAA (Figure 4.3A). However, dry mass percentage pattern differed between the flesh and the seed (Figure 4.3B). Fruit flesh dry mass percentage slightly decreased from 14 to 56 DAA, then remained constant from 70 to 112 DAA, and increased approximately one month before harvest. While the seed dry mass percentage rapid increase from 70 to 126 DAA, then decreased before harvest. As seed dry mass growth rate increased, (Figure 4.3C), flesh dry mass growth decreased or slightly increased. Fruit dry matter accumulation exhibited a double-sigmoid pattern with the first fast growth phase from 14 to 42 DAA, followed by a second period of slow increase in dry mass and a final phase characterized by rapid fruit dry mass increase (Figure 4.4). In contrast, seed dry mass increased slowly from 14 to 70 DAA followed by a gradual increase from 84 to 126 DAA, then the mass remained constant (Figure 4.4A). Fruit respiration rate (Figure 4.4B) decreased from 48 ml $CO_2$ $h^{-1}$ $kg^{-1}$ in fruit 14 DAA to 12 ml $CO_2$ $h^{-1}$ $kg^{-1}$ at 70 DAA, then remained constant until fruit color break stage.

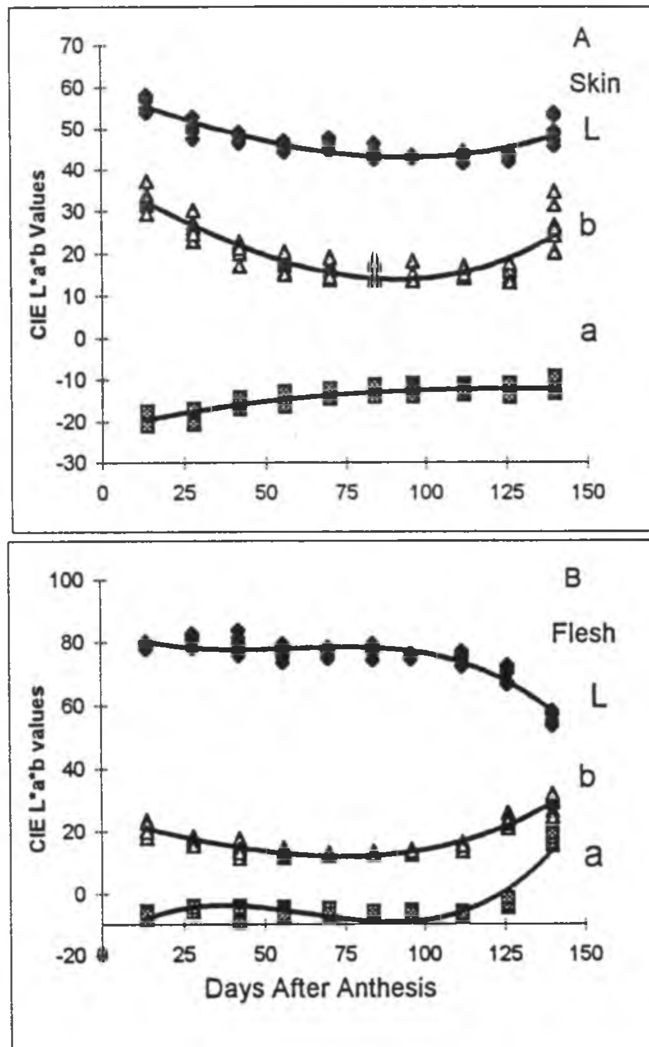



Figure 4.1. Sunset papaya fruit skin (A) and flesh color (B) changes expressed as CIE L *a *b color space during fruit development as days after anthesis. Papaya skin lightness L, $Y = 0.002X^2 - 0.375X + 60.55$, $r^2 = 0.8029$, $p = 0.0001$ ($n = 60$). Papaya skin value a, $Y = -0.0006X^2 + 0.154X - 21.35$, $r^2 = 0.8314$, $p = 0.0001$. Papaya skin value b, $Y = 0.0034X^2 - 0.598X + 40.48$, $r^2 = 0.7903$, $p = 0.0001$. Papaya flesh lightness L, $Y = -0.0023X^2 + 0.214X + 75.09$, $r^2 = 0.7997$, $p = 0.0001$. Papaya flesh value a, $Y = 7E\text{-}05X^3 - 0.014X^2 + 0.724X - 15.37$, $r^2 = 0.8673$, $p = 0.0001$. Papaya flesh value b, $Y = 0.0033X^2 - 0.46X + 27.63$, $r^2 = 0.8977$, $p = 0.0001$.

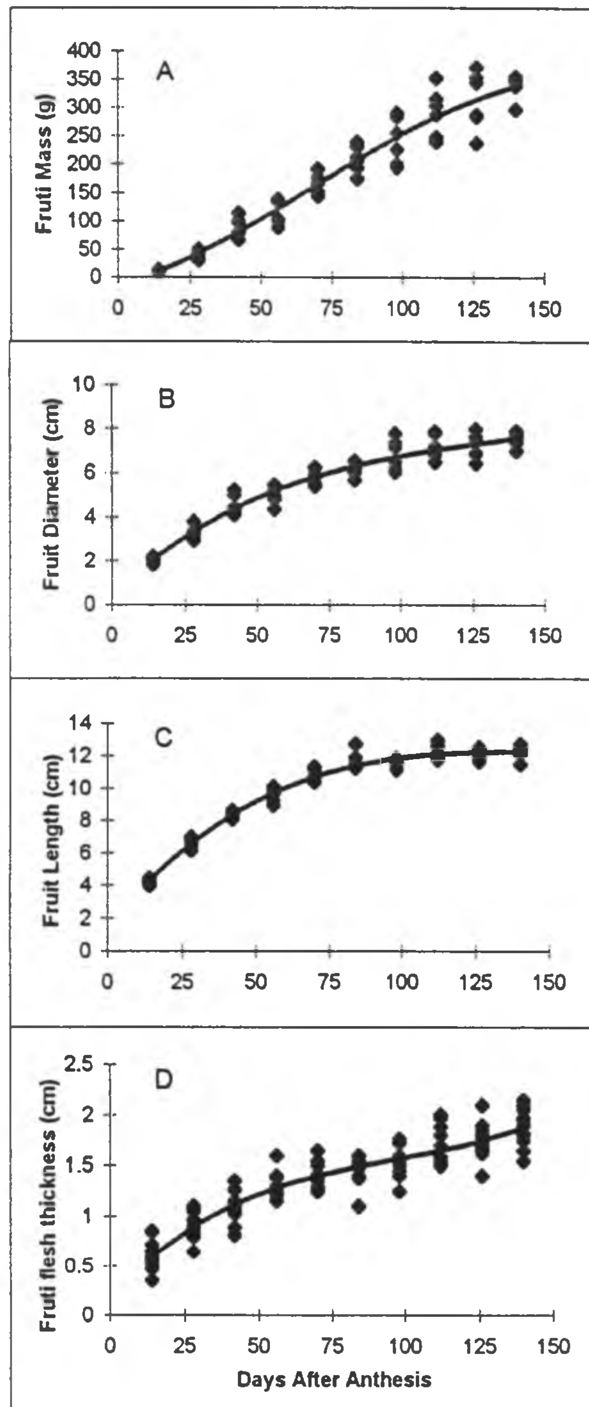



Figure 4.2. Papaya fruit growth (fresh mass (A), length (B), width (C) and flesh thickness (flesh) (D)) curves in summer grown 'Sunset' as days after anthesis. Fruit mass: $Y = -0.0001X^3 + 0.00224X^2 + 1.5492X - 15.434$, $r^2 = 0.9462$, p = 0.0001. Fruit length: $Y = 3E\text{-}06X^3 - 0.0015X^2 + 0.2208X + 1.4827$, $r^2 = 0.9789$ p = 0.0001. Fruit diameter: $Y = 2E\text{-}06X^3 - 0.0008X^2 + 0.1212X + 0.5106$, $r^2 = 0.9478$, p = 0.0001 Fruit flesh thickness: $Y = 9E\text{-}07X^3 - 0.0003X^2 + 0.0311X + 0.2123$, $r^2 = 0.8784$, p = 0.0001. The means of two subsamples were used for fruit length and diameter, individual subsample data were used in fruit flesh thickness.

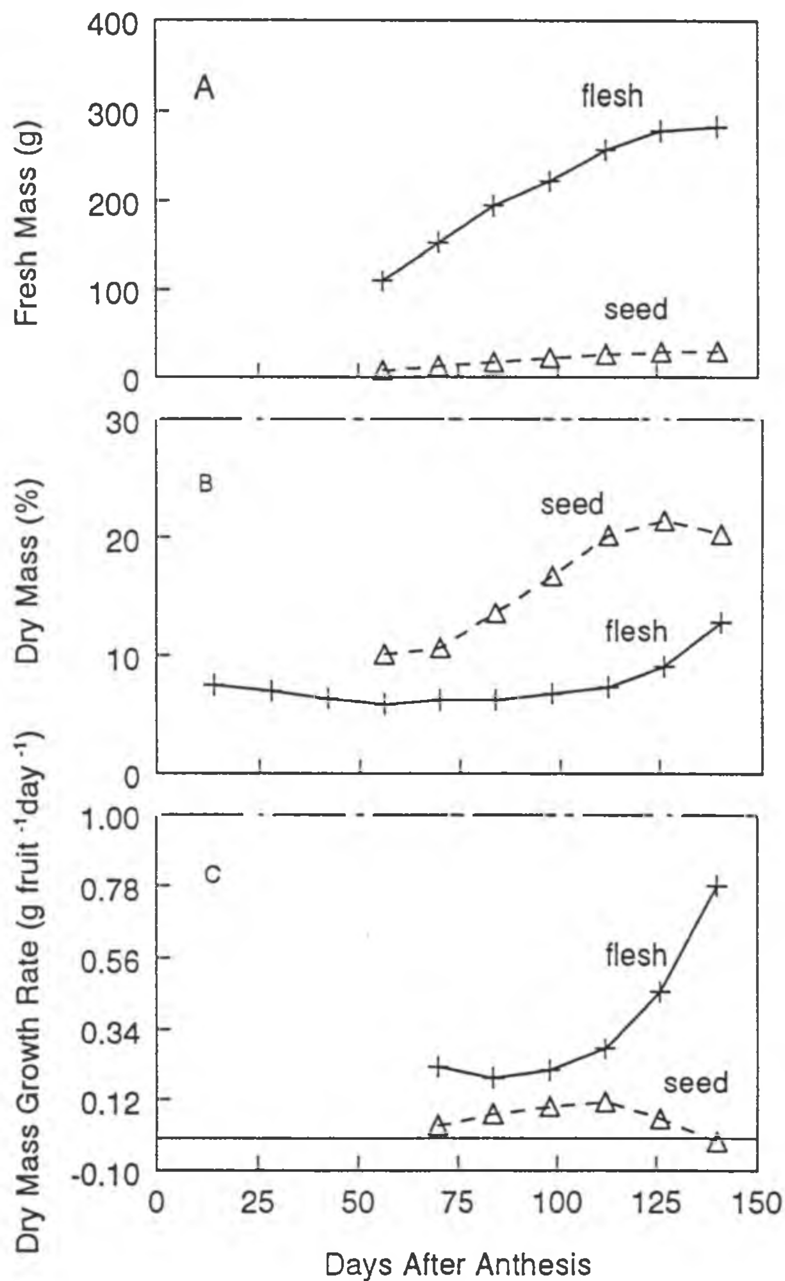



Figure 4.3. Changes in papaya fruit flesh, seed fresh mass (A) and dry mass percentage (B), flesh and seed dry mass growth rate (C)(g dry mass fruit$^{-1}$ day$^{-1}$) during fruit development as days after anthesis. Fruit flesh fresh growth: $Y = -0.0002X^3 + 0.0277X^2 + 1.2749X - 20.601$, $r^2 = 0.9982$, $p = 0.0001$. Seed fresh growth: $Y = -3E\text{-}05X^3 + 0.007X^2 - 0.223X + 3.2524$, $r^2 = 0.9999$, $p = 0.0001$.

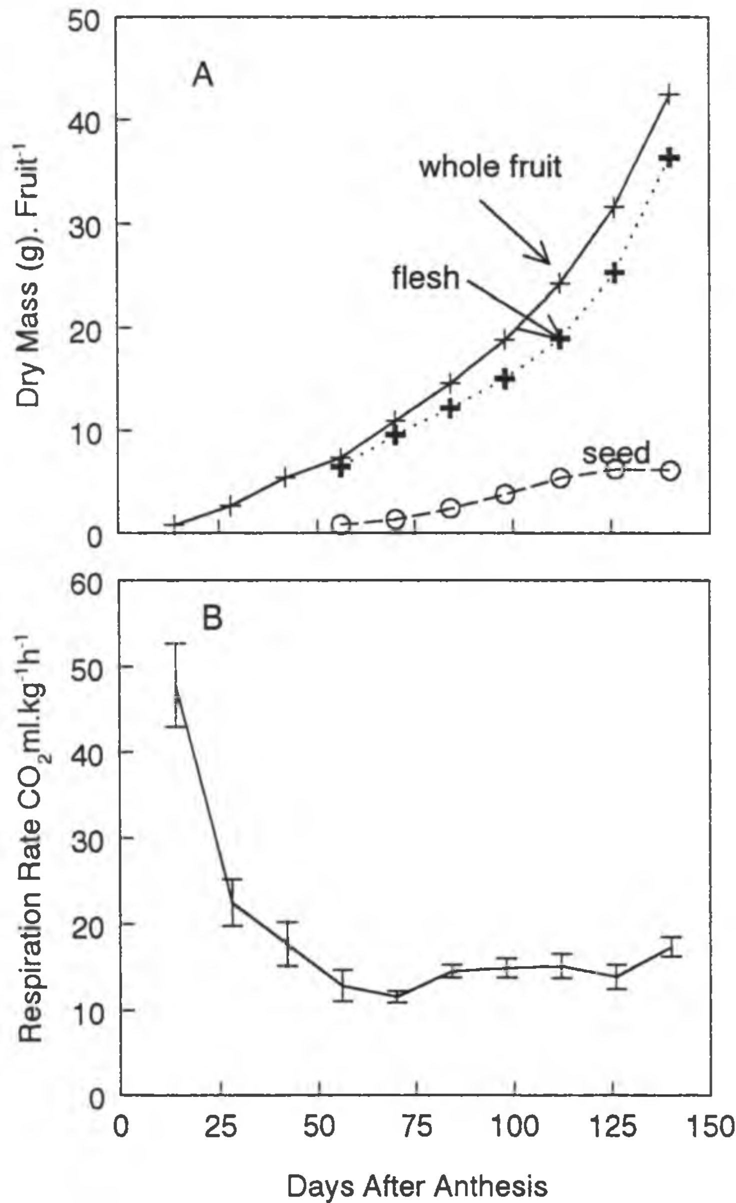



Figure. 4.4. Papaya fruit dry mass growth (A) and respiration rate (B) during fruit development expressed by day after anthesis. For dry mass growth, each data point = dry mass percentage (n=3) X fresh mass (n = 8 (plant) X 2(sample date) x fruits, 1 to 3 approximately). Dry mass growth for whole fruit: $Y=2E\text{-}05X^3-0.0023X^2 + 0.2556X - 2.6805$, $r^2 = 0.999$, $p = 0.001$; flesh: $Y=7E\text{-}05X^3 - 0.0181X^2 + 1.6362X - 41.551$, $r^2 = 0.9997$, $p = 0.001$. Seed: $Y= -3E\text{-}05X^3 + 0.0074X^2 - 0.5906X + 15.401$, $r^2 = 0.9989$, $p = 0.001$. For respiration rate, each data point represents at least 16 fruit. $Y= 0.0522X^4 - 1.3806X^3 + 13.138X^2 - 52.687X + 88.18$, $r^2 = 0.9863$, $p= 0.001$.

4.3.3 Fruit sugars

4.3.3 fruit sugars

Fructose, glucose and sucrose were detected via HPLC after invertase was heat inactivated prior to extraction. Fruit sugars (glucose, fructose and sucrose) in the flesh did not significantly increase until about 112 DAA (Figure 4.5A). Sucrose rapidly increased one month before maturation and accounted for 40 to 50 % of total sugar in mature fruit.

4.3.4 SPS, SS, and acid invertase enzyme activities

SPS remained low throughout fruit development and increased only slightly in mature fruit (Figure 4.5C). The difference in SPS activity between each developmental stage was not significant. SS activity was assayed in the synthesis direction at pH 8.0, which gave a much higher activity than at pH 7.5. SS activity was very high (>40 $\mu$moles $g^{-1}$ FW) in young fruit (14 DAA) and declined to 12 $\mu$moles $g^{-1}$ FW 56 DAA and then remained relatively low during the rest of fruit development (Figure 4.5B). There was about a 30 % increase of SS activity 14 days before maturation in a later experiment (Table 4.1). Acid invertase activity was very low in the young fruit and increased more than 10 fold, 42 to 14 days before maturation (Figure 4.5B), and invertase activity paralleled sugar accumulation. Invertase activity varied greatly 7 to 14 days before maturation with invertase being higher in the outer white flesh tissue (14 days before maturation) than in the riper inner flesh tissue (Table 4.1). No neutral or alkaline invertase was detected in both young and ripe papaya fruit.

4.3.5 The relationship between fruit mass, and length, diameter, and flesh thickness

The relationships between fruit mass, diameter (Figure 4.6A), length (Figure 4.6B), and flesh thickness (Figure 4.6C) after log transformation, were linear (Figure 4.6D, E, F). Fruit diameter was more closely related to fruit mass ($r^2$ = 0.99, p<0.0001) than fruit length. Flesh thickness and fruit weight were highly correlated. However, the practical application of the relationship was limited, as it required fruit detachment and there was large variability in flesh thickness at different cutting sites. The regression equations between fruit diameter, length and flesh fruit mass were consistent between fruit diameter and length estimated and observed fruit

Table 4.1 Activity of acid invertase and sucrose synthase (SS), sucrose phosphate synthase (SPS), and invertase enzymes in winter grown 'Sunset' papaya. Each data represents the means of three replications and ± SD.

| Fruit tissue | Enzyme activity (μmoles sucrose.$g^{-1}$ $h^{-1}$ FW) | | |
|---|---|---|---|
| | Acid invertase | SS | SPS |
| 4 weeks before maturity | 31.1± 2.5 | 4.2 ± 0.2 | 1.1± 0.08 |
| 2 weeks before maturity (outer mesocarp) | 74.7 ± 3.5 | 6.2 ± 0.3 | 1.6 ± 1.55 |
| 2 weeks before maturity (inner mesocarp) | 46.9 ± 3.3 | 4.3 ± 0.3 | 1.6 ± 0.14 |
| mature fruit | 46.3 ± 5.1 | 4.6 ± 0.3 | 1.6 ± 0.13 |

mass and gave a $p < 0.0001$, $r^2 = 0.99$ and 0.97. Fruit growth and development, especially fruit size, varied between different plants and different fruit on the same plant.

4.3.6 The relationship between fruit skin, flesh color and dry mass accumulation

Skin, flesh and seed colors changes indicate fruit maturity. However, only flesh color was a more accurate predictor of fruit dry mass percentage during fruit development (Figure 4.7A). Fruit skin color 'a', was only partially correlated ($r^2 = 0.91$) to flesh dry mass accumulation (Figure 4.7B).

4.3.7 The relationship between flesh color and TSS in full ripe fruit

Papaya fruit skin color changed rapidly during postharvest ripening while flesh color and fruit sugar remaining constant. A quantitative relationship was obtained for flesh 'a' value against fruit TSS $Y = -0.2767X^2 + 7.9144X - 32.708$ (Figure 4.8). Fruit flesh color (red) rapidly increased as fruit TSS increased, however, once fruit TSS was 10% or greater, the flesh color showed little change.

4.3.8.Correlation between respiration, sugar content and enzyme activity

Correlation analysis data indicated that SS was positively correlated ($r^2 = 0.9511$, p= 0.00004) with fruit respiration (Figure 4.9A) and acid invertase activity was correlated ($r^2$=0.738,

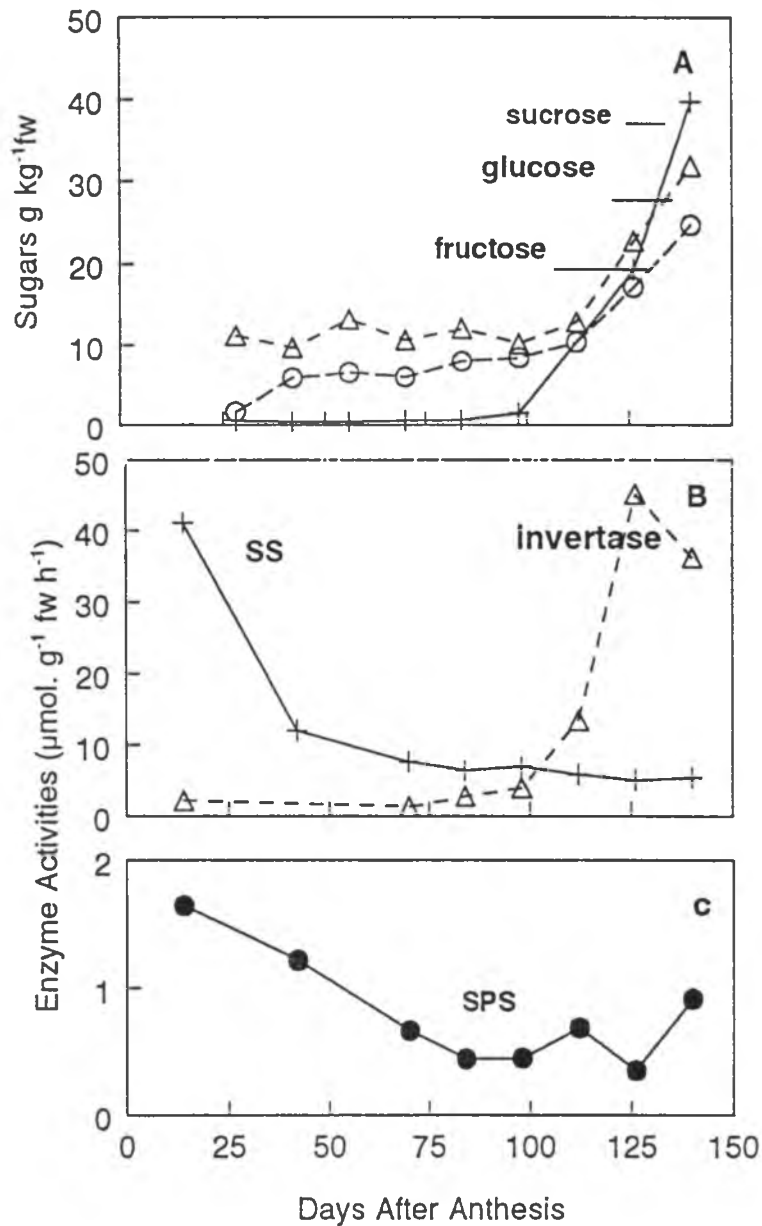



Figure 4.5. Time course of sugar accumulation (A) and activities of SS, invertase (B) and SPS (C) during papaya fruit development expressed as day after anthesis. Each data point represents at least 3 or 2 extraction for sugar and enzymes activities, respectively.

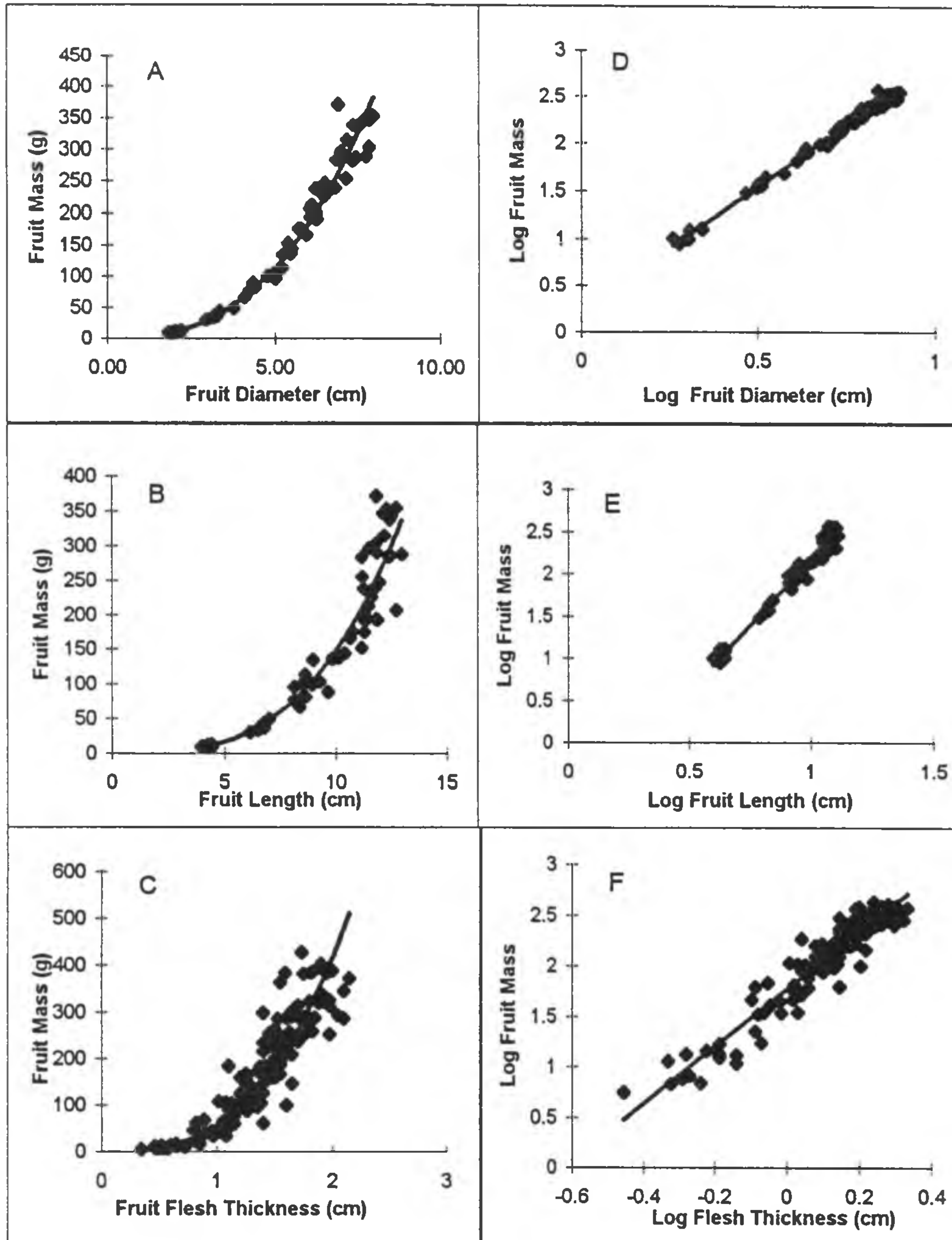



Figure 4.6. The relationship between fruit diameter (A, D), length (B, E) and flesh thickness (C, F) and fruit fresh mass. For original data (A, C, E) and transformed data (B, D, F). Using the same data set from Figure 4.2. Log fruit mass = 2.583 X (log fruit diameter) + 0.2513, $r^2$ = 0.9924, p= 0.00001. Log fruit mass = 3.1253 X (log fruit length) - 0.9525, $r^2$ = 0.9739, p = 0.0001. Log fruit mass = 2.8296 X ( log flesh thickness) + 1.7676. $r^2$ = 0.9104, p = 0.0001.

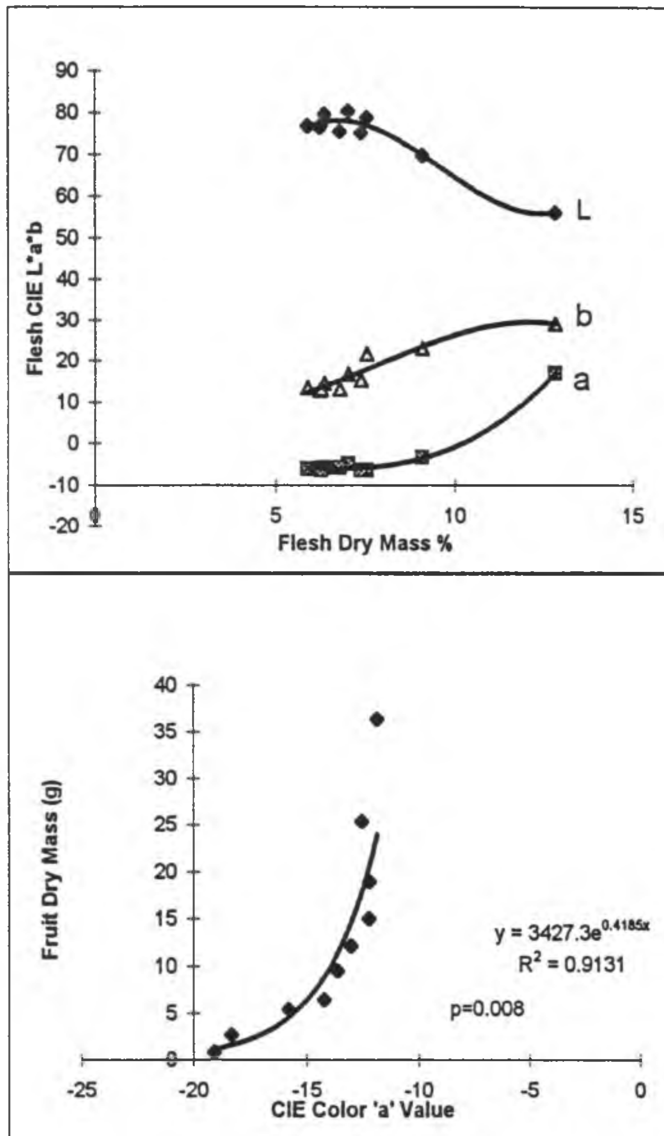



Figure 4.7. The relationship between fruit flesh color development and fruit flesh dry mass percentage (A) and fruit skin color a value and fruit dry mass accumulation (B). Each data point represents the mean of each developmental stage. Color L = Lightness: $Y = 0.2397X^3-6.8982X^2 + 60.33X - 88.542$, $r^2 = 0.9488$, $p = 0.0001$. Color A = green to red: $Y = 0.0496X^3 - 0.6878 X^2 + 2.5838X - 7.4876$, $r^2 = 0.9952$, $p = 0.0001$. Color B = blue(-) to yellow(+), $Y = -0.0651X^3 + 1.5126X^2 - 8.031X + 20.447$, $r^2 = 0.906$, $p = 0.0001$.

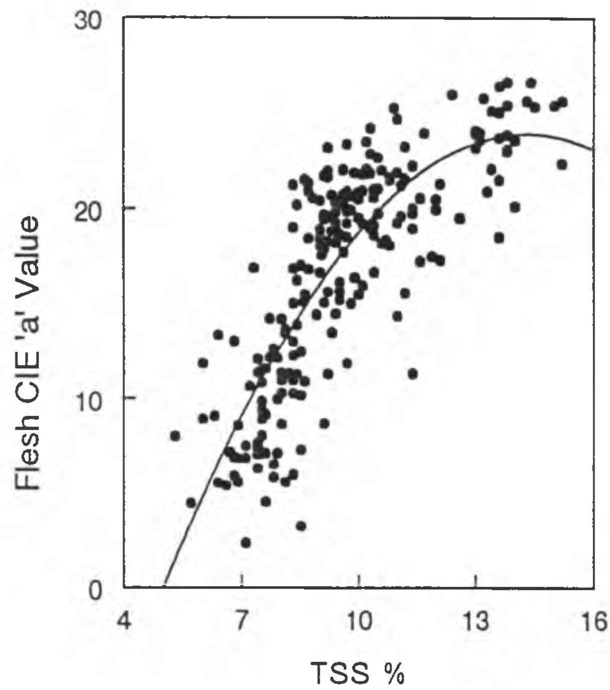



Figure 4.8. The relationship between fruit flesh color and TSS levels in ripe 'Sunset' fruit. Fruit were harvested at color break to 30% yellowing, stored at 23°C for about 7 to 10 days until the fruit reached full skin yellow and soft. Each data point is the average of two measurements for each fruit. $Y = -0.2767X^2 + 7.9144X - 32.708$, $r^2 = 0.6742$, $p = 0.0001$. $n = 213$.

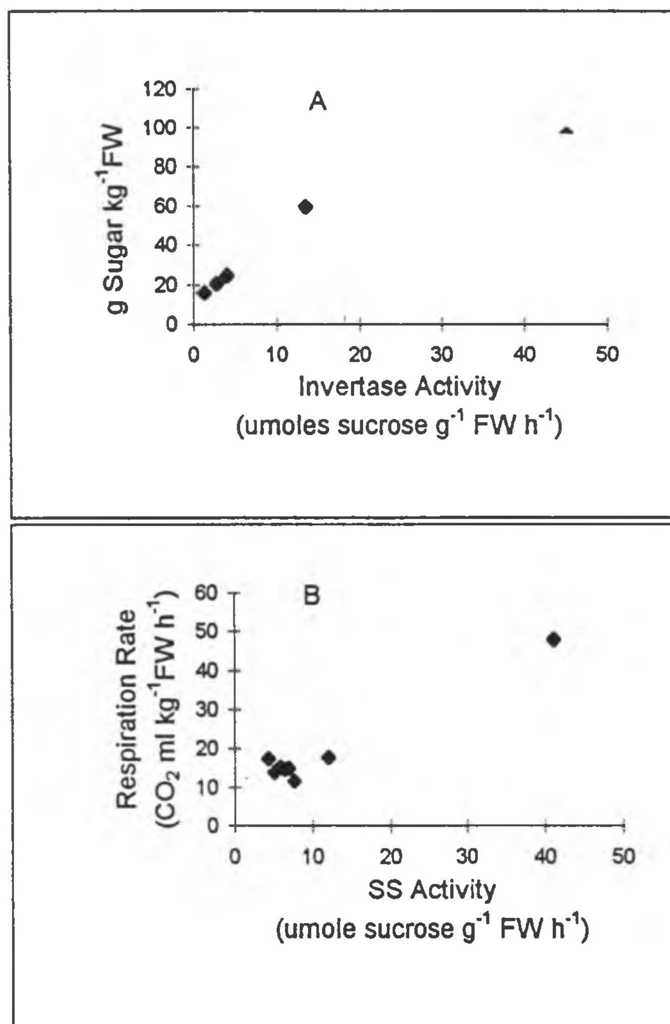



Figure 4.9. The relationship between sugar accumulation and invertase activity (A), fruit SS activity and respiration rate (B) during papaya fruit development in day after anthesis. One stage earlier invertase enzyme activity (from 84 to 126 DAA) was used against next stage sugar level (98 to 140 DAA). The correlation between fruit sugar and invertase activity, $r^2$ = 0.9326, p = 0.0076. SS and respiration: $r^2$ = 0.9511, p = 0.00004.

p = 0.05) with fruit sugar accumulation. If invertase activity in fruit 14 days earlier than the fruit sugar sampling date for correlation analysis during the sugar accumulate period, a higher linear correlation coefficient ($r^2 = 0.9326$, p = 0.0076) was obtained (Figure 4.9B).

4.3.9 Carbon import and enzyme activity in fruit flesh tissue

The carbohydrate imported from leaf to fruit ware used either for respiration, or carbohydrate storage. When sucrose metabolizing enzyme activity were plotted against the carbon import per fruit per day (sugar plus structural carbon in flesh tissue dry mass and total carbon consumed by whole fruit respiration) the relationships between fruit carbon import and SS or invertase enzyme activity were complex (Figure 4.10). It suggests that neither SS nor invertase alone can be regarded as major factors regulating fruit growth and sugar accumulation. On a whole fruit basis, SS activity increased rapidly during early fruit development and remained constant while carbon import rapidly increased during fruit maturation (Figure 4.10B). Invertase was initially low and did not increase until carbon import rate significantly increased during the late fruit development stage (Figure 4.10A). Though a linear correlation existed between SPS and fruit carbon import (Figure 4.10C), the role of SPS in carbon import in papaya flesh was questioned, as SPS activity was less than one tenth that of SS and invertase activities during papaya fruit development.

4.3.10 Sugar accumulation and enzyme activity in 'Kapoho' and 'UH 801' cultivars during fruit late developmental stage

To further verify the relationship between fruit sugar accumulation and enzyme activity, a low sugar line cultivar 'UH 801' was compared the major commercial cultivar 'Kapoho' at the late fruit developmental stage. 'Kapoho' and 'Sunset' showed a similar sugar accumulation pattern and enzyme activity as UH801 (Figure 4.11). Invertase activity significantly increased 14 days before maturation then slightly decreased in mature fruit. SS remained constant and SPS was low and increased in mature fruit. Lower sugar level in 'UH801' was apparently associated with lower invertase, and lower SPS activities but had similar levels of SS (Figure 4.11A, C, D, E).

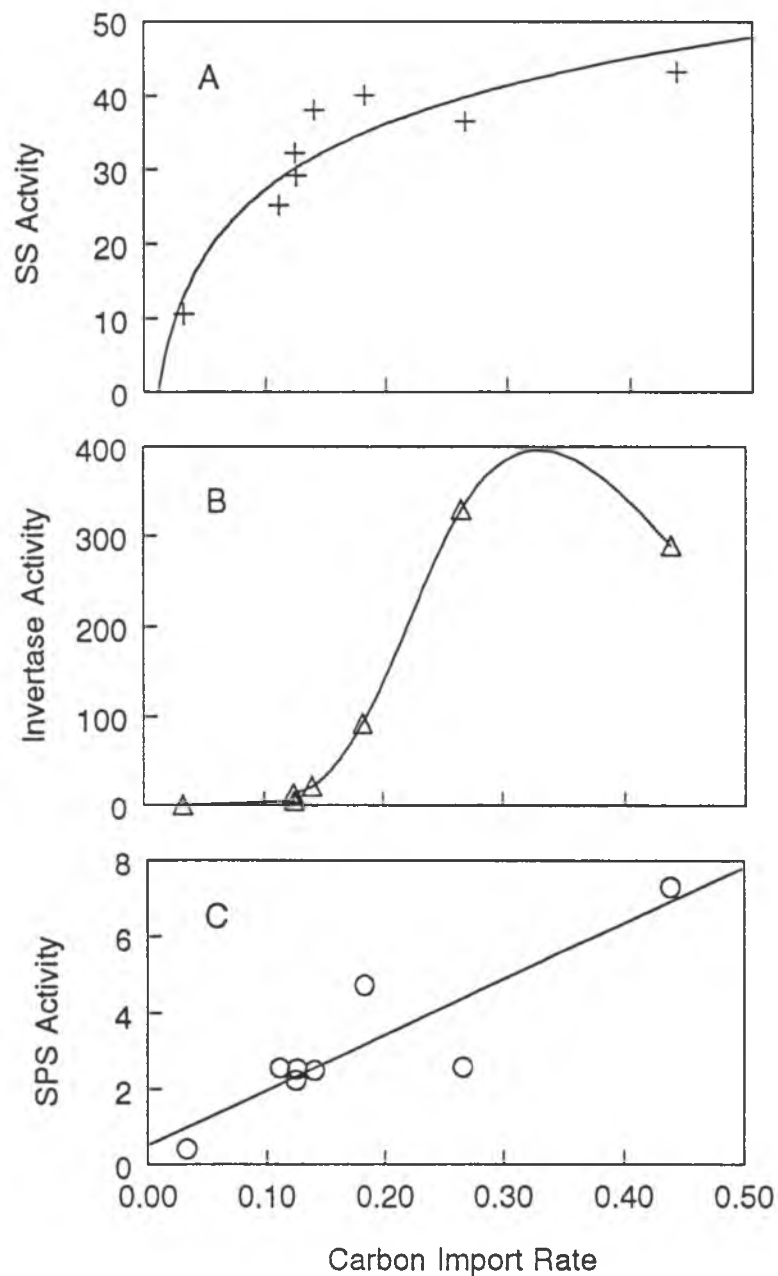



Figure 4.10. The relationship between fruit flesh carbon import rate (carbon consumed by respiration and dry mass accumulate per fruit per day) and SS (A), invertase (B), SPS (C) enzyme activities during papaya fruit development in day after anthesis. Regression equation for carbon import and SS: Y (Ln carbon import rate) = 0.067X (SS) - 4.09, $r^2$ = 0.858, p = 0.0009; for carbon import and invertase: Y (carbon import rate) = -6.2 E-08 $X^3$ +2.34E-05 $X^2$ - 0.00049X +0.1018, $r^2$ = 0.918, p = 0.039.

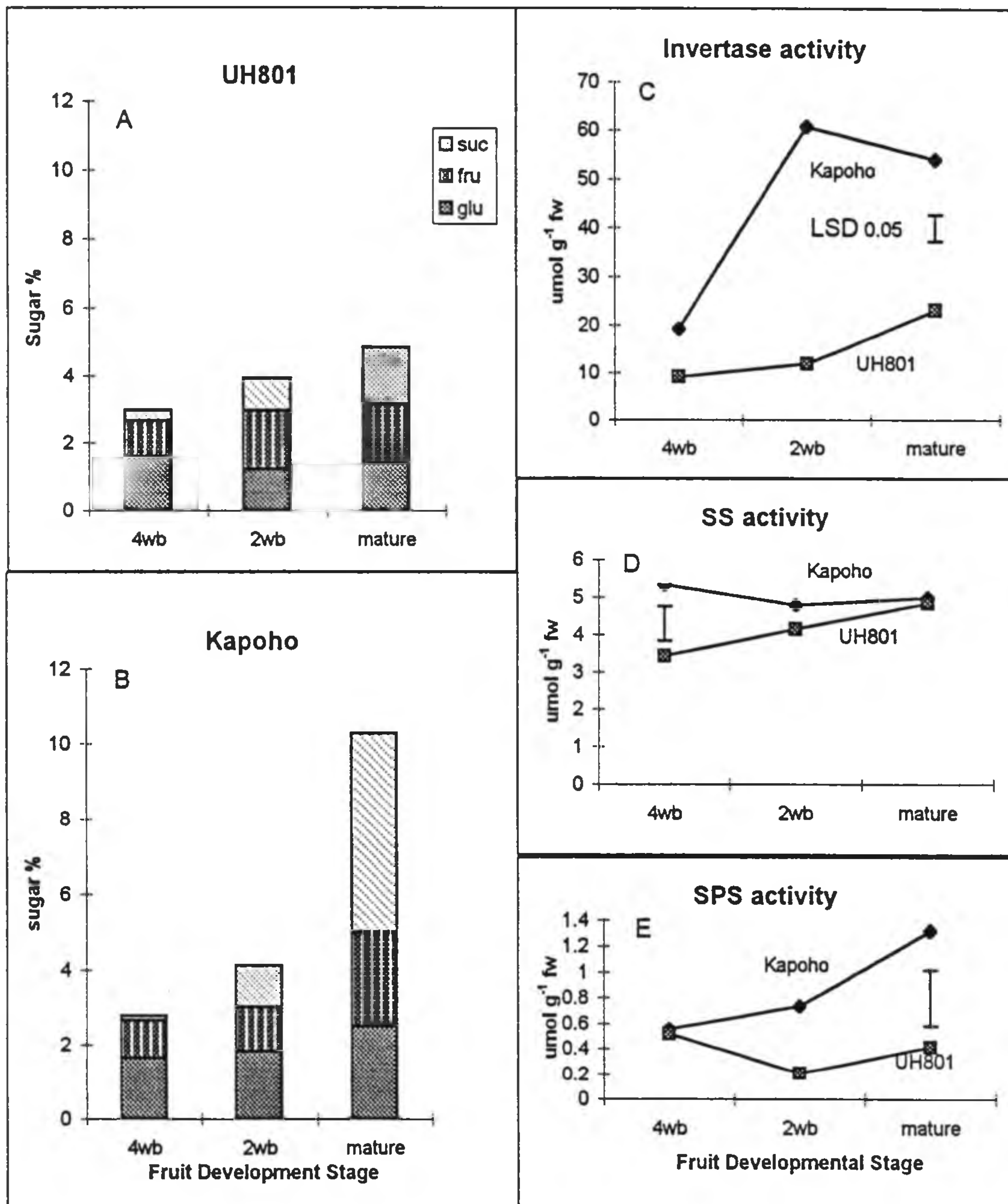



Figure 4.11. Comparison of sugar accumulation and SPS, SS, invertase enzyme activities in 'kapoho' and 'UH 801' cultivars during fruit late development stage; 4 weeks (4wb), 2 weeks (2wb) before harvest and mature. A: 'UH801' sugar, B: 'Kapoho' sugar, C: invertase activity, D: SS activtiy, E: SPS activity. Vertical bar indicate LSD (p=0.05).

4.3 11 Sugar levels and enzyme activities during papaya postharvest ripening of five cultivars

Papaya ripening was characterized by a large increase in invertase activity, a decrease in SS activity and a low SPS activity (Table 4.2). 'Sunset', 'Kapoho', 'Line-8' and 'X-77' had similar sugar levels in ripe fruit. The variability in sugar levels within these four cultivars at the three sampling times was probably due to fruit samples being taken from different location on different vigorous plants when fruit were available. The purpose of this experiment was to compare these cultivars with 'UH801', a line reported to have lower sugar level. Total sugar levels and sucrose percentage in the five cultivars from one day after harvest to seven days after harvest varied widely (Table 4.2). Average sucrose percentage, was significantly higher in 'Kapoho' than in other cultivars (Table 4.2). SPS activities remained low in all five cultivars at the three sampling times and there was no significant difference between cultivars and between sample times. SPS activity in 'X-77' and 'UH801' cultivars was significantly higher than in other cultivars three days and seven days after harvest, respectively. SS activity was significantly lower in 'X-77' and higher in 'UH801' than in the other cultivars three days and seven days after harvest, respectively. UH801 had significant higher SS activity than the other four cultivars. All cultivars showed the same postharvest trend in invertase activity, though 'UH801' had a lower invertase activity than the other four cultivars. The much higher invertase and lower SS activity in 'Line-8' cultivar than in other cultivars one day after harvest paralleled earlier fruit flesh ripening before color break. The average SS activity was higher and invertase lower in the low sugar line 'UH801' than in other four cultivars. These results indicated that neither SS, invertase nor SPS activity was correlated with total sugar or sucrose in ripe papaya fruit. The difference in the trends of SPS, and the higher level of SS and lower invertase in 'UH801' (big fruit, lower sugar) suggested that the difference between these three enzymatic activities contributed to the different cultivar sugar level.

Table 4.2 Papaya fruit sugar content and sucrose percentage changes during postharvest ripening of five cultivars. Means in the same column with same letter were not significant different at 5% level. n=3

| | Total sugar (g 100 g-1 FW) | | | |
|---|---|---|---|---|
| Cultivars | day 1 | day 3 | day 7 | average |
| Sunset | 10.6 | 13.7 | 12.3 | 12.2 a |
| Kapoho | 11.7 | 12.2 | 11.5 | 11.8 b |
| Line-8 | 8.6 | 9.9 | 12.3 | 10.3 c |
| X-77 | 11.3 | 11.8 | 12.4 | 11.9 ab |
| UH 801 | 5.9 | 5.8 | 5.3 | 5.7 d |

| | Sucrose (%) | | | |
|---|---|---|---|---|
| Cultivars | day 1 | day 3 | day 7 | average |
| Sunset | 46 | 51 | 33 | 44 c |
| Kapoho | 59 | 56 | 62 | 59 a |
| Line-8 | 53 | 54 | 50 | 52 b |
| X-77 | 54 | 34 | 45 | 44 c |
| UH 801 | 44 | 38 | 38 | 40 c |

Table 4.3. Sucrose phosphate synthase (SPS), sucrose synthase (SS), invertase enzyme activities during postharvest ripening of five cultivars. Means in the same column with same letter were not significant different at 5% level. n=2.

| | SPS (µmoles sucrose $g^{-1}h^{-1}$ FW) | | | |
|---|---|---|---|---|
| Cultivar | day 1 | day 3 | day 7 | average |
| Sunset | 1.1 a | 0.8 b | 0.5 b | 0.8 |
| Kapoho | 1.3 a | 0.7 b | 0.5 b | 0.9 |
| Line-8 | 1.1 a | 1.0 b | 0.8 b | 1.0 |
| X-77 | 1.1 a | 1.6 a | 0.6 b | 1.1 |
| UH 801 | 0.5 a | 1.1 b | 1.7 a | 1.1 |

| | SS (µmoles sucrose $g^{-1}h^{-1}$ FW) | | | |
|---|---|---|---|---|
| Cultivars | day 1 | day 3 | day7 | average |
| Sunset | 3.6 b | 2.2 b | 0.8 b | 2.2 b |
| Kapoho | 5.0 a | 1.6 c | 0.2 c | 2.3 b |
| Line-8 | 1.5 c | 1.3 c | 0.3 c | 1.0 d |
| X-77 | 3.4 b | 0.6 d | 0.4 bc | 1.4 c |
| UH 801 | 5.3 a | 2.8 a | 1.3 a | 3.1 a |

| | Invertase (µmoles sucrose $g^{-1}$ $h^{-1}$ FW) | | | |
|---|---|---|---|---|
| Cultivars | day 1 | day 3 | day 7 | average |
| Sunset | 47 bc | 285 ab | 328 ab | 220 ab |
| Kapoho | 54 b | 203 b | 421 a | 226 ab |
| Line-8 | 147 a | 238 b | 358 a | 248 a |
| X-77 | 52 b | 301 a | 240 b | 198 b |
| UH 801 | 19 c | 97 c | 208 bc | 109 c |

### 4. 4 Discussion

Papaya fruit growth and development from pollination to maturation varies widely due to factors such as cultivar, age of bearing trees (maturity delayed as trees age), time of the year, and the stage selected as an index of maturity and environment factors (Nakasone, 1986). 'Sunset' papaya fruit growth varied from 140 days to 180 days in the same plant for the first years' fruit and the second years' fruit (flowered at June or December), respectively. The variation was probably due to fruit growth and development being slower in the combined condition of temperature, tree ages and fruit competition. Unlike other fruit species, papaya plant flower and fruit continually, and only about 3 to 6 flowers opened a week in each plant. Different aged fruit in the same plant probably have different growth rate due to their positions in the plant column. Fruit size varied from 100 to 130 DAA fruit between different plant in this experiment (Figure 4.2A). The regression models between papaya fruit mass and diameter or length from our research could provide a useful tool to non-destructively investigate fruit growth rate under field conditions and reduce the variance between different fruit. Similar models have been developed for tomato (Wang, 19930, peach (Pavel and DeJong, 1993), and have shown great practical convenience (Bertin, 1993).

The pattern of sugar accumulation during fruit development in 'Sunset' was similar to that observed by others in different varieties (Chan et al., 1979; Selvaraj et al., 1982). Papaya dry mass percentage (Figure 4.3B) increased mainly due to soluble sugar increase (Figure 4.5A) in the late fruit development stage. Fruit flesh dry mass growth rate increased as seed dry mass growth rate decreased (Figure 4.3C), suggesting that papaya seed development was favored over flesh dry mass accumulation.

Many studies have been conducted to explain the control of assimilate partitioning between sinks competing for a limited supply of assimilates (Bangerth et al., 1984; Bertin 1995; DeJong et al., 1989; Demnitz-King et al., 1997; Ho, 1980; Pavel et al., 1993; Wright, 1989). Sink strength has been described as the product of sink size and sink activity (Ho, 1984). SS and acid invertase are both involved in the breakdown of translocated sucrose to and in the sink tissue.

The activities of SS and acid invertase are proposed as indicators of active sink strength for different crops (Wang et al., 1993; Sung et al., 1994). SS is positively correlated with tomato relative growth rate and the starch content of the mesocarp tissue (Wang et al., 1993). Acid invertase is associated with snap bean pod elongation while SS is associated with fruit dry matter accumulation (Sung et al., 1994). There is no information available in papaya fruit sink strength.

SS and acid invertase activities were indicators of sink activity in papaya fruit during the early and late development stages, respectively (Figure 4.5). SS apparently played a major role in papaya fruit sink establishment and maintenance by cleaving imported sucrose and providing UDP-glucose for biosynthetic reactions (Figure 4.5B). SS was very high in 14 DAA fruit, and as fruit growth occurs, SS declined to about 15% 70 DAA fruit, then remained relatively constant during the rest of fruit development. When SS activity was calculated on the fruit basis, SS increased from 14 DAA to 98 DAA then remained constant throughout the remaining period. This period corresponded to the carbon import into the mesocarp tissue during early fruit development stage ($r^2 = 0.858$, $P = 0.0009$, $Ln(Y) = 0.067X - 4.09$).

Acid invertase activity increased significantly during the last phase of fruit growth (Figure 4.5B and 4.11C) and was associated with an increased carbon import rate ($r^2 = 0.918$, $P = 0.039$, Y (carbon import rate) $= -6.2E{-}08\ X^3 + 2.34E{-}05\ X^2 - 0.00049\ X + 0.1018$). A relationship between carbon import rate and fruit sucrose levels in tomato fruit has also been observed (Walker et al., 1978). In papaya, final fruit sugar relied on continuing sucrose import, rather than starch degradation as occurred in other fruit (Chan et al., 1979). The constant level of SS activity and the rapidly increased acid invertase activity in late fruit development stage of papaya led to a strong sink to compete for sugar unloading at the fruit mesocarp tissue. SPS activity was low throughout development, and may only play a minor role in papaya fruit sink strength.

The increase in mesocarp sugar paralleled the increase in acid invertase activity during the last phase of fruit development suggested a central role for this enzyme in sugar accumulation. Acid invertase enzyme activity increased before the sugar increased, and was correlated with sugar level ($r^2 = 0.738$, $p = 0.05$), then declined before harvest (Figure 4.5A). If

enzyme activity 14 days earlier was compared with the sugar level, the linear correlation coefficient was higher ($r^2$ = 0.9326, p=0.0076). No correlation was found between sugar and SPS or SS activity during fruit development. The loss of soluble invertase activity and the high SPS are regarded as preventing sucrose hydrolysis and allowing the accumulation of sucrose in muskmelon and tomato (Hubbard et al., 1989; McCollum et al., 1988; Miron et al., 1991; Yelle et al., 1991). Acid invertase has negatively correlation with sucrose content in apple (Beurter, 1985; Yamaki et al., 1986; grape (Hawler, 1969), tomato (Klann et al., 1993; Manning et al., 1975; Miron 1991; Yelle et al., 1991), citrus (Kato et al., 1978), muskmelon (Lingle et al., 1987), and cucumber (Schafter et al., 1987). In some tissues, reducing sugar content has shown a positive relationship with invertase activity (Manning et al., 1975; Ranwala et al., 1992; Walker et al., 1976). However, low acid invertase does not necessarily lead to the accumulation of sucrose in some tomato genotypes (Miron et al., 1991) and no correlation is found between sucrose content and acid invertase activities in 23 pear varieties (Moriguchi et al., 1992). In papaya, invertase activity increased before sugar accumulation and reached a peak two weeks before maturity then declined 20 to 40% in mature fruit (Figure 4.10 and 4.11). The reduced invertase activity together with an increased SPS activity could contribute to sucrose accumulation in the vacuole. An increase in SPS activity was observed during late fruit development, but was not coincident with maximum rates of sucrose accumulation.

Ripening of papaya fruit paralleled the loss of SS activity and dramatically increased acid invertase enzyme activity in all five tested cultivars (Table 4.3). Nevertheless, the sucrose content in postharvest ripening papaya (Table 4.2) was relatively stable. The relationship between SPS, SS and invertase activity and sugar composition was not clear in ripe papaya. The observed relationship can be explained by the high invertase activities detected *in vitro* may not occurred *in vivo*, or the enzyme activity and sucrose may not be in the same compartment of the fruit cell (Hubbard et al., 1991). The optimal acid invertase assay pH and the high solubility in extraction buffer suggested it is a vacuolar invertase (Chan and Kwok 1976; Lopez et al., 1988). Loss of sucrose during homogenization and extraction without heat inhibition of invertase (Chan

et al., 1976) demonstrate that sucrose and invertase could be either physically separated or the invertase is inhibited *in vivo*. The solubility of invertase has been shown to be different in different buffers, pH and extraction method (chapter 6). The possibility of invertase being located in the papaya ripe fruit flesh cell wall could not be excluded though only buffer soluble activity was measured in the present study. Sugars located apoplastically in the free space increased with fruit maturation and ripening in pears (Yamaki et al., 1993). The sugar content in free space of pear accounted for about 40% of the total sugars. Ripe papaya contains about 40 to 60% sucrose (Chen et al., 1964; Chan et al., 1979), and was similar to pears (Yamaki et al., 1993). Lower invertase activity in 'UH801' during postharvest ripening was consistent with lower sugar accumulation before harvest. However, it was not clear if the same invertase isoforms existed before and after harvest or during ripening and if different cultivars had different isoforms or how invertase enzyme activity was regulated in papaya fruit *in vivo*.

# CHAPTER 5

## SOURCE (LEAF)-SINK (FRUIT) MANIPULATIONS

### Effect of Defoliation and Fruit Removal on Papaya Fruit Production, Sugar Accumulation and SPS, SS, Acid invertase Enzyme Activities

**Abstract**

The source-sink relationship during papaya fruit development and ripening was investigated. The source size and sink strength was modified by single defoliation or continual defoliation, and fruit thinning, respectively. Three defoliation levels (0, 50%, 75%) and two defoliation methods (75% leaf removal from the oldest to the youngest leaf vs 75% leaves removed in a spiral from the oldest leaf) were compared to fruit thinning on 'Sunset' papaya during Hawaii's warm season. There was no significant difference between 0% and 50% defoliation in new fruit set or ripe fruit TSS. Removal of 75% of the leaves significantly reduced new flower and fruit set, and decreased ripe fruit TSS. Removal of leaves in a spiral reduced the number of new flower and fruit set more than the defoliation from the bottom to top. Defoliation of the 'Kamiya' cultivar significantly reduced ripe fruit TSS and sugar level, and dry mass percentage two weeks after defoliation. There was no significant difference between defoliated and non-defoliated treatment in fruit production, average fruit mass, and percentage of fruit flesh and seed, seed dry mass and seed mass ratio during the test period. Fruit thinning increased new fruit set and ripe fruit TSS level in 'Line-8', and 'Sunset' but not 'Kapoho'. Fruit thinning of 'Line-8' also increased young fruit TSS and sugar levels on the remaining fruit on plants compared to same aged fruit on the control and defoliated plants. There was no effect on 'Kapoho' young fruit TSS and sugar levels after defoliation. However, fruit thinning increased average ripe fruit mass in 'Kapoho' but not 'Sunset' and 'Line-8' varieties. Ripe fruit TSS varied with weather conditions, plant growth conditions and cultivars.

Continual defoliation resulted in lower new fruit set (25% of control), smaller fruit size (77% of control), and lower TSS (85% of control) in the 168 days experimental period. In

contrast, there were 52% and 100% more new fruit on the fruit removal treatment plant than on the control plant within the first 56 days and 168 days, respectively. Larger fruit size, faster fruit development, lower respiration and higher sugar contents were observed in the immature fruit (140, 154, and 175 DAA) picked from the fruit removal treatment. No significant difference was found in TSS level of color break fruit between fruit removal and control plant. Fruit removal plus defoliation gave the same number and mass of new fruit as the control and slightly lower TSS in mature fruit than in control. Source-sink balance was critical for fruit set, development and sugar accumulation in papaya. Correlation analysis between fruit sugar and the sugar enzyme activities further demonstrated that invertase enzyme is possibly involved in fruit sugar unloading and accumulation during late fruit development stage.

### 5.1 Introduction

Source limitation, naturally or artificially induced, has been shown to affect reproductive biology in papaya and other fruit (Awada 1967; Spears et al., 1988), fruit size and quality, vegetative senescence in many plants (Bertin 1995; Chen et al., 1979; Hubbard et al., 1990; Hunter 1991; Koblet et al., 1994; Pavel et al., 1993). Source limitation reduces flower production, organ initiation, increases flower or fruit abortion rates in several species of plants (Bertin, 1995; Chamont, 1992; Lyrene 1992; Stephenson, 1981; Wardlaw, 1990). Sexual expression is also altered by source limitation in many hermaphroditic species (Spears et al., 1988; Wilson, 1983). Source-sink competition results in postharvest leaf blackening of Protea flower (Dai and Paull, 1995), reduced grain yield and chemical content of corn kernel (Chen et al., 1978), and poor fruit quality in muskmelon (Hubbard et al., 1990b).

Plants with a large leaf area have, in general, an increased photosynthetic capacity and at a given fruit load can lead to higher fruit TSS level (Hubbard et al., 1990b; Welles and Buitelaar, 1988;). The optimum leaf number and area required for the development of individual fruit has been determined for several fruit trees (Antognozzi et al., 1992; Chacko et al., 1982; Famiani 1997; Fishler et al., 1983; Palmer et al., 1991; Reddy 1996; Roper et al., 1987;

Samanci, 1997; Snelgar et al., 1997). Kiwi fruit formed "early" (in a flowering cycle) and "late" (30 days later) after heavy pruning, had reduced total yield and poorer fruit quality (Galliano et al., 1990). The fruit/leaf-ratio also has an important influence on growth and composition of apples (Hansen, 1982), and plums (Toldam-Anderson et al., 1993).

Papaya (*Carica papaya* L.) plants flower and fruit continuously after initial flower initiation. Knowledge of the impact of altered source size on fruit development and quality is of practical significance. Papaya foliage injury can occur by insects, (e.g. Broad mite), disease such as powdery mildew, papaya mosaic virus and papaya ring spot virus (Decker and Ito, 1958; Marler et al., 1993; Nakasone 1986) and strong winds (Raveendranathan, 1989). Uneven fruit production, abnormal fruit size and poor fruit quality (low sugar) can occur in papaya commercial production (Camp, 1994). Our hypothesis was that source-sink relationship and the regulation of carbohydrate partitioning among sink was essential in predicting papaya fruit production and quality. Previous reports indicated that papaya leaf pruning to 15 functional leaves does not affect fruit production or total soluble solids (TSS) of the fruit (Ito, 1976). Thinning papaya to one fruit per node leads to a increase in fruit size and has no effect on fruit sugar (Martinez, 1988). Defoliation and deflowering altered papaya flower form, trunk growth, leaf dry weight, height elongation (Awada, 1967). However, it is not clear how papaya fruit growth and quality varied in response to different fruit/leaf ratio and in different cultivars. The time from loss of source leaves on its impact of fruit size and quality and the time required for recovery are essential to enable prediction of future production, fruit size distribution and quality.

The objectives of current research were: (1) to investigate papaya fruit set, growth and ripe fruit quality in response to different fruit/leaf ratio (defoliation and fruit thinning) and defoliation method. (2) To elucidate the response of fruit development and sugar accumulation of papaya to defoliation and fruit thinning of different cultivars and in different seasons. And (3) to evaluate the fruit physiology (fruit set, fruit development), sugar accumulation and relative levels of enzymes (SPS, SS, invertase) activities in response to fruit removal and continual defoliation in 'Sunset' papaya.

## 5. 2. Materials and Methods

### 5.2.1 Plant material

'Sunset', and 'UH801' papaya were grown at the Poamoho Experimental Station on the island of Oahu. The plants of 'Line8' and 'Kapoho' were grown at Dole Fresh Fruit Company fields, in central Oahu. The plants of 'Kamiya' were grown at a private farm, on the north shore of Oahu.

### 5.2.2 Single defoliation and fruit thinning experiment in 'Sunset' cultivator

Three defoliation levels (0%= control, 50% and 75%) and two defoliation methods (75% of the leaves cut off from the oldest to the youngest vs 75% removed in a spiral from the oldest) were compared with fruit thinning during Hawaii's warm season. The leaves and fruit in different treatments were removed at the beginning of experiment. Mature fruit were harvested weekly. The fruit mass and TSS were determined after fruit were held at 23°C for 7 days. New flower and fruit set were counted from the first week of the experiment to six weeks (42 days) after treatment. Fruit abortion was counted where there is no fruit at a leaf axil. Five plants (replicates) were used for each treatment.

### 5.2.3 Defoliation on 'Kaminya' cultivar

Defoliation experiment (about 75% defoliation, from bottom to top) compared with control was conducted in the spring of 1995. Fruit were harvested at color break to 30% yellow. Fruit fresh mass was determined on the day of harvest. Fruit TSS and sugar were sampled at the day of harvest and 6 days after harvest. Fruit flesh and seed fresh mass, dry mass was determined during a six weeks period. Sugar enzyme activities (SPS, SS, acid invertase) were determined on the day of harvest and after 6 days at 23°C.

### 5.2.4 Defoliation and fruit thinning experiment in 'Kapoho' and 'Line-8' cultivars

Flowers were tagged at anthesis from June to November, 1996. Defoliation was conducted in December, 1996 when the first tagged fruit reached color break. Four treatments were initially installed 1. Defoliation (about 65% leaves removed, in a spiral), 2. Fruit Thinning (one third of the fruit removed), 3. Fruit thinning plus defoliation, and 4. Control. Ripe fruit TSS

and mass were determined weekly. Young fruit at different ages were harvested three weeks after defoliation. Fruit flesh and seed fresh mass, dry mass, flesh TSS, sugar level and SPS, SS, invertase enzyme activity were determined.

### 5.2.5 Fruit removal and continual defoliation of 'Sunset' papaya

Sixteen papaya (*Carica Papaya* L. cv: Sunset) trees were divided into four treatments installed randomly when the first fruit on the plant reached maturity. I. Continual defoliation (DL): 12 to 14 of the green leaves (from the bottom of plant) were cut off on Dec. 6, 1995, leaving about 9 leaves (petiole longer than the axil of the young leaf) on the plant. Two or three additional leaves were removed weekly, maintaining the same leaf number during the experimental period. II. Fruit removal (DF): Fruit set before November was removed at the beginning of experiment with no additional fruit being removed until the end of experiment. III. Fruit removal plus continual defoliation (DF +DL). VI. Control: No leaves or immature fruit removed during the experimental period.

### 5.2.6 Observations for fruit removal and continual defoliation

Flowers were tagged weekly at anthesis, with new flower and fruit set being counted for the first 8 weeks. Total new fruit set was counted from the first tagged fruit to the youngest fruit at the end of experiment. Mature fruit were harvested weekly. Fruit mass and TSS was taken on the harvested fruit. Fruit mass, respiration, skin, flesh and seed color and seed dry mass, flesh sugars (sucrose, glucose and fructose), SPS, SS and invertase enzyme activities were measured on fruit of different ages harvested on May 29, 1996.

### 5.2.7 Fruit respiration rate

Fruit at four different ages from the four different treatments were harvested on May 29, 1996 and used for determination of fruit respiration rate. Fruit respiration rate was expressed as $CO_2$ ml $Kg^{-1}$ FW $h^{-1}$ as described previously (Chapter 4).

5.2.8 Samples for SPS, SS, invertase assay in ripe fruit of 'Sunset' in single defoliation experiment

For the control and defoliation treatment, fruit were harvested at the 10 to 20% yellow stages and stored at 23°C for seven days prior to enzyme activity assay. In the fruit thinning treatment, fruit of the same age were picked at 10 to 20% yellow stage and ripened at 23°C or picked 7 days later after ripening on the plant.

5.2.9 Enzyme extraction and assay

SPS, SS and invertase enzymes were extracted and assayed as previously described (Chapter 4).

5.2 10 Sugar and dry mass measurement

Flesh sugar assay was performed as previously described (Paull et al., 1984).Two grams of tissue was heated in the microwave for 1.5 min (unless otherwise stated) before extraction with 90% ethanol, 5 ml of the supernatant solution was dried and dissolved in 2.5 to 5 ml (for young fruit and mature fruit, respectively) deionized water. Sucrose, fructose and glucose were separated and quantified by HPLC from retention times and peak area under known standard. The sum of the three sugars was regarded as the total.

Dry mass percentage for each DAA was determined by drying three replication of 10 gram each of fresh tissue or seed at 60°C for 6 days. Fruit flesh and seed dry mass accumulation was calculated from dry mass percentage multiplied by the average fruit fresh mass in the same sample.

5.2.11 Data analysis

Statistical analysis was performed using SAS general linear models. The correlation coefficients between fruit sugar and SS and acid invertase enzyme activity at four developmental stages and four treatments were obtained by using the enzyme and sugar data of the same aged fruit or at one stage earlier enzymatic activity with the next stage sugar level.

## 5.3 Results

5.3.1 New flower and fruit set

Papaya flower and fruit set was reduced or increased by a single defoliation and fruit thinning, respectively. Node abortion and new fruit set reduction were observed in the 75% defoliation of 'Sunset' plant compared to the control plant (Table 5.1). There was also a significant difference between two defoliation methods. Plants in which the leaves were removed in a spiral manner had lower fruit set (47%) and higher fruitlet abortion (2.4 fold) than plants defoliated from the bottom to the top (Table 5.1). Fruit thinning increased fruit set by increasing fruit number per node (Table 5.1). There was no significant difference between 0% and 50% defoliation in the node abortion and new fruit set.

Continual defoliation and fruit removal significantly altered new flower and fruit set. Six weeks (42 days) after treatment, plants with fruit removed had the highest flower set, fruit removal plus defoliated plant ranked second in flower set, control plants were third and the defoliated plant gave the lowest flower set (Table 5.2). Two weeks later, the difference in new fruit set between fruit removal and fruit removal plus defoliation treatment was not significant. Defoliated plants produced less than 1/3 of fruit of the control, and 1/4 of the fruit than the fruit removal treatments, respectively. However, at the end of experiment, fruit removal plants had double the fruit set of the control plants. Continual defoliation only had 1/4 of fruit set of the control plants (Table 5.2). Continual defoliation on the fruit removed plant had a similar source sink balance as control plant. No significant difference was found between fruit removal plus continual defoliation and control plant in the total fruit set during the entile experimental period.

5.3.2 Ripe fruit TSS and fruit mass

Ripe papaya fruit TSS varied with weather conditions, plant growth and fruit loading conditions and cultivars. Removal of 70-75% of the leaves significantly decreased TSS levels within 14 to 21 days after defoliation in ripe fruit of 'Sunset' and 'Kamiya' in Hawaii warm season respectively (Figure 5.1A and 5.2A). Fruit TSS recovered about 42 days after defoliation. However, defoliation effects were not consistent in 'Kapoho' and 'Line-8' cultivars during rainy

season (Table 5.3). Defoliation significant lowered fruit TSS in 'Kapoho' but not in 'Line-8' during the 42 days period after treatment.

Defoliation significantly lowered ripe fruit mass on 'Line-8', but not in 'Kamiya', 'Sunset' and 'Kapoho' (Figure 5.1B 5.2B and Table 5.3). Fruit thinning increased ripe fruit mass in 'Kapoho' and reduced fruit mass in 'Line-8' (Table 5.3) and had no significant effect on 'Sunset' (Figure 5.2B). Defoliation plus fruit thinning gave a fruit mass and TSS value between the control and defoliation treatment on 'Kapoho' (Table 5.3).

Continual defoliation significantly reduced ripe fruit TSS and fruit mass during the 168 day experimental period by 85%, and 77% of control, respectively, (Table 5.2). Monthly data showed that final fruit TSS on the control plant increased from February to May, while fruit TSS on the continual defoliation plant reduced from January to May (Figure 5.3). Defoliation did not immediately lower mature fruit TSS indicating that the aged fruit was stronger sinks than new fruit for a limited assimilate. Unlike the fruit thinning experiment, fruit removal increased mature fruit mass but not TSS compared to the control (Table 5.4). Fruit removal occurred at the early fruit development stage while fruit thinning was performed at the late fruit development stage. Five months later, when young fruit on the fruit removed plant enter the sugar accumulation period, plants had similar or more fruit number as the control plant, mature fruit TSS was not increased. Fruit removal plus defoliation had the similar fruit mass but lower TSS level than the control (Table 5.4). Continual defoliation on the fruit removed plant had similar fruit/leaf balance at the early period of experiment. When more new fruit developed and defoliation was continued, final fruit TSS was reduced. These results suggested that fruit mass was readily affected during the early development stage, while final fruit TSS was determined by the source sink balance before harvest.

5.3.3 Fruit development

Papaya fruit growth was faster on the plant in which the older fruit was removed than in the control plant (Table 5.5). Higher CIE 'a' value or visible color and higher fruit TSS level on the fruit removed plant suggested that those fruit matured earlier than the ones on the control

Table 5.1. Effect of defoliation and fruit thinning on new fruit set and the number aborted nodes in 'Sunset' papaya.

| Treatment* | Control | 50% defoliated | 75% defoliated Bottom upward | 75% defoliated Spiral | Fruit thinned at beginning |
|---|---|---|---|---|---|
| Fruit set* | 18.4B | 19.2 B | 13.6C | 7.4 D | 22.5 A |
| Nodes aborted | 0.4 C | 0.4 C | 3.6 B | 8.8 A | 0 C |

*Means in the same row with same letter were not significantly different at 1% level. n = 5.

Table 5.2. Effect of continual defoliation on flower set, fruit set and ripe fruit number TSS and mass (12/6/95-5/22/96)

| | Number plant$^{-1}$ | | | | | |
|---|---|---|---|---|---|---|
| Parameter | Flower set | Fruit set | | Ripe fruit | | |
| Treatment | first 6 wk | first 8 wk | 24 wk | Number | TSS(%) | Mass (g) |
| Control | 11 b | 15 b | 28 b | 54 a | 12.2 a | 303 a |
| Defoliated | 4 c | 4 c | 7 c | 47 a | 10.4 b | 234 b |
| Fruit removed | 20 a | 23 a | 61 a | 5 b | | |
| Fruit removed +defoliated | 16 a | 21 a | 29 b | 4 b | | |

Means in the same column with same letter were not significantly different at 5% level.

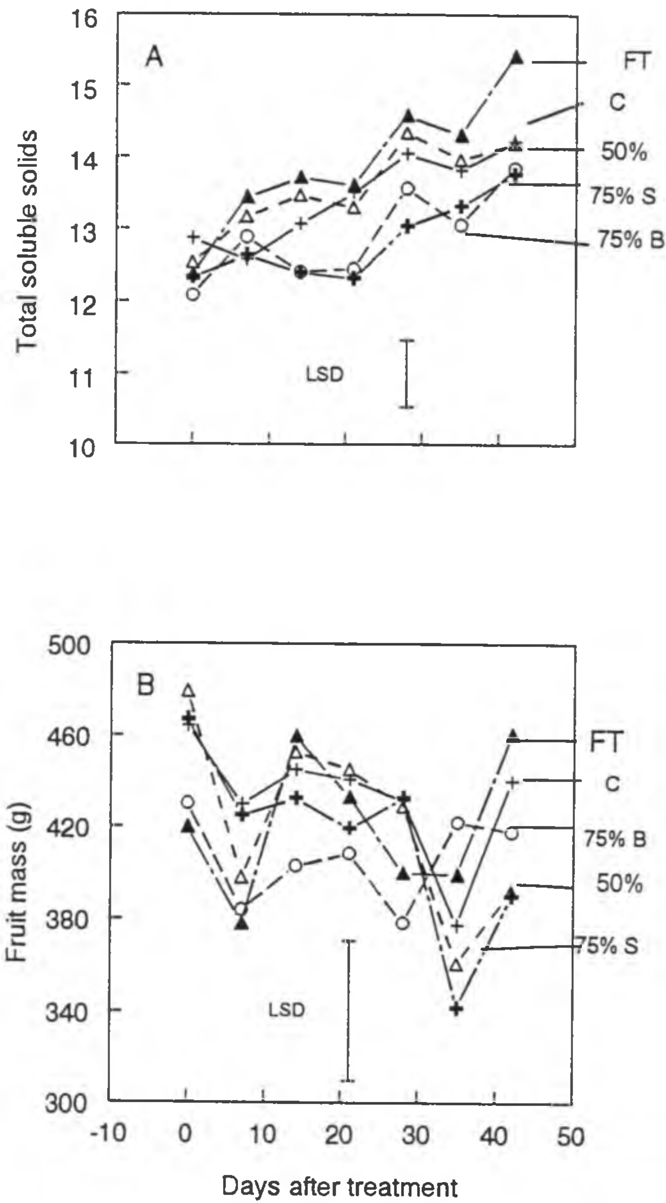



Figure 5.1 Fruit total soluble solids (A) and fruit mass (g fruit $^{-1}$) (B) in response to defoliation and fruit thinning treatment in 'Sunset' cultivar. C: control, FT. fruit thinned, about 40 fruit were removed, 50%: 50% defoliation, 75% S: 75% defoliation in a spiral, 75% B: 75% defoliation from bottom to top. Fruit were harvested at color break stage and allowed to ripen at 23°C before evaluation.

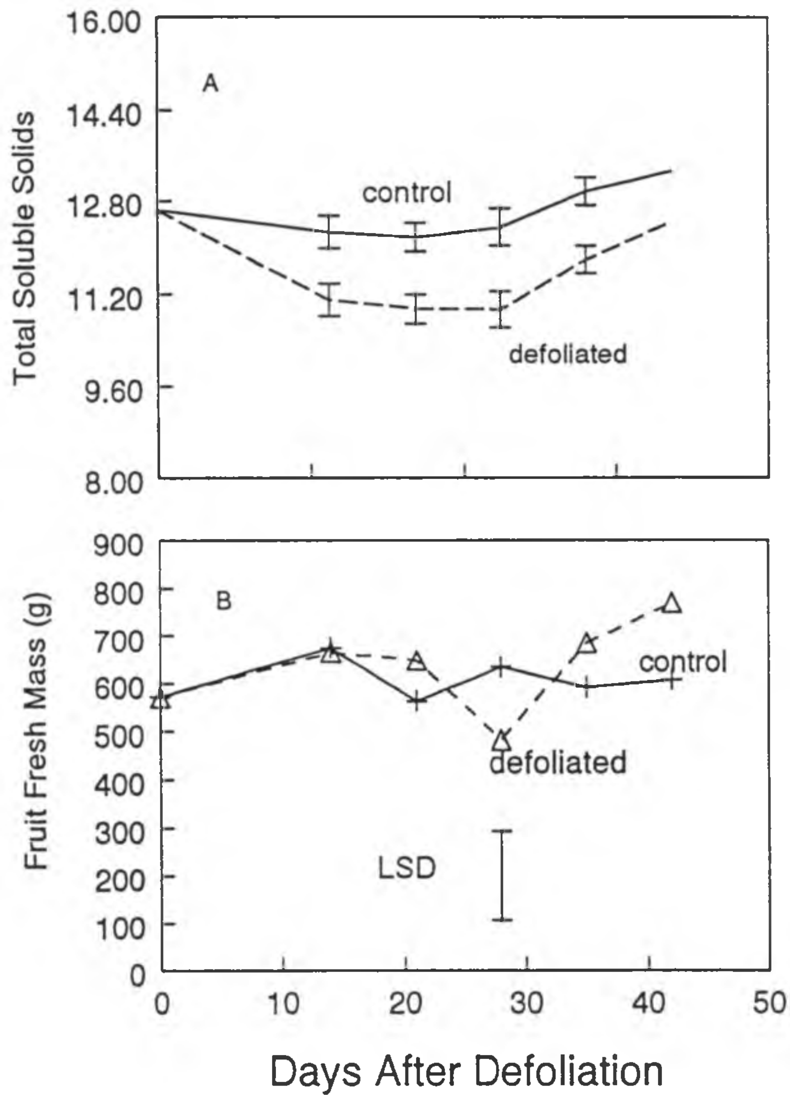



Figure 5.2 Fruit total soluble solids (A), fruit mass (g $fruit^{-1}$) (B) in response to defoliation in 'Kamiya' cultivar. Fruit were harvested at color break stage and allowed to ripen at 23°C before evaluation. Vertical bars in graph A indicated standard deviation in each week within treatment.

Table 5.3. Effect of defoliation and fruit thinning on fruit mass and TSS of 'Kapoho', and 'Line-8' cultivar.

| Treatment* | Control | Defoliated | Fruit thinned | Defoliated + fruit thinned |
|---|---|---|---|---|
| Fruit mass (g) | | | | |
| Kapoho | 353 b | 344 b | 380 a | 356 a b |
| Line-8 | 460 a | 410 b | 404 b | |
| TSS (%) | | | | |
| Kapoho | 11.2 a b | 10.9 c | 11.4 a | 11.1 b |
| Line-8 | 11.4 b | 11.0 b | 11.8 a | |

Means in the same row with same letter were not significantly different at 5% level. (Data pool from 0, 7, 21,28, 42 days after treatment, each treatment had at least five plants. n = 406 and 394, for fruit mass and TSS, respectively).

Table 5.4. Comparison of ripe papaya fruit mass, TSS among four treatment (5/8/96-5/29/96)

| Treatment | *Fruit number | Fruit mass (g) | TSS (%) |
|---|---|---|---|
| Control | 38 | 270 b | 13.2 a |
| Defoliated | 29 | 173 c | 10.4 c |
| Fruit removal | 38 | 347 a | 13.1 a |
| Defoliate + Fruit removal | 31 | 261..b | 11.9 b |

* Total tested fruit per treatment. Means in the same column with same letter were not significantly different at 5% level.

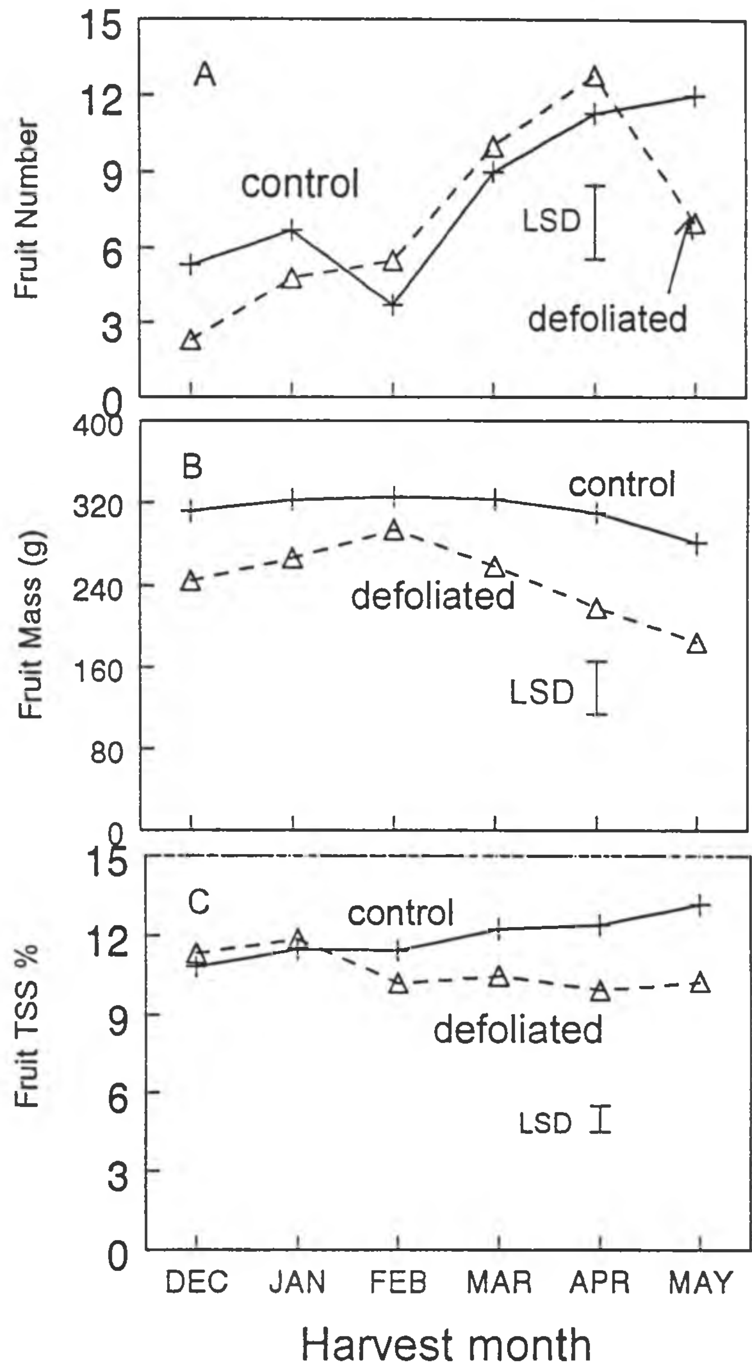



Figure 5.3 Average mature fruit number per plant (A), individual fruit mass (B), and fruit TSS (C) between Dec. 95 and May 96.

plant. Defoliation delayed fruit growth but no significant difference was obtained between continual defoliation and the control in 175 DAA fruit (Table 5.5).

There was no significant difference in percentage of fruit flesh and seed, seed mass ratio and seed dry mass in ripe fruit (Figure 5.4) between defoliated and non-defoliated treatment during the experimental period. However, fruit flesh dry mass was affected and the result was consistent with TSS data (Figure 5.4). Young fruit growth was delayed as indicated as lower seed dry mass percentage (Figure 5.5), 21 days after defoliation compared to the control. Fruit thinning also increased young fruit sugar levels compared to the control in 'Line-8' cultivar (Table 5.6).

### 5.3.4 SPS SS and acid invertase activity in single defoliation

SPS, SS and acid invertase enzyme activity of fruit flesh were assayed in ripe fruit from the defoliation treatments. There was a decreased SPS and SS activities and an increased invertase enzyme activity from the day of harvest to 6 days after harvest. Defoliation significantly increased acid invertase enzyme activity in the fruit of one day after harvest but not in the 6 days after harvest about 28 days after defoliation of the 'Kamiya' cultivar. There was also no significant difference in 'Kapoho' and 'Line-8' cultivar between treatment. However, fruit thinning significantly increased SS activity of ripe fruit than in control and defoliated treatment (Table 5.7). The trends of higher invertase in defoliated and lower invertase in the fruit thinned treatment were observed but was not significantly different due to high variation (Table 5.7).

### 5.3.5 Respiration, sugar and enzyme activities in continual defoliation

The time course of fruit respiration, sugar accumulation, sucrose synthase, and acid invertase enzyme activities during late fruit development stage were similar as reported earlier (Chapter 4). The trends in respiration in this experiment agreed with previous observations, 140 and 154 DAA fruit had higher respiration rate than 175 DAA fruit that entered the lowest respiration stage before the climacteric peak. The reason of lower respiration in the fruit removal treatment was not clear. One possibility was that fruit size was bigger in the fruit removed plant than fruit from the other treatments.

Table 5.5. Comparison of papaya fruit maturity among treatment with same age fruit (175 DAA)

| Treatment | Skin color | Flesh color | Flesh CIE 'a' | Seed color | Mass (g) | TSS (%) |
|---|---|---|---|---|---|---|
| Control | green | 3/10 red | -4.08 | black | 281 b | 6.6 b |
| Fruit removal | light green | 7/10 red | 5.6 | black | 369 a | 10 a |
| DF+DL | light green | 5/10 red | 2.18 | black | 290 b | 9.7 a |
| DL | green | 3/10 red | -2.09 | black | 128 c | 5.9 b |

*Means in the same column with same letter were not significantly different at 5% level.

Table 5.6. Effect of defoliation and fruit thinning on fruit sugar (g. $100g^{-1}$ FW) of 'Line8' cultivar.

| | Fruit sugar g 100g | |
|---|---|---|
| Treatment | 1 week before harvest | 3 weeks before harvest |
| Control | 6.2 a | 2.6 b |
| Fruit thinned | 6.8 a | 3.0 a |
| Defoliated | 5.4 b | 2.6 b |

*Means in the same column with same letter were not significantly different at 5% level. n = 3.

Fruit removal resulted in higher sugar level in the 140, 154 and 175 DAA fruit compared to the control (Figure 5.6B). However, there was no significant difference in the color break fruit between fruit removal and the control treatments that had similar TSS (Table 5.4). Mature and 154 DAA fruit from continual defoliation had a less sugar content than the control plant, but no difference was detected between 140 and 175 DAA fruit. Fruit sugar from plants receiving continual defoliation plus initial fruit removed was similar as continual defoliation alone except for the 175 DAA fruit. As mentioned earlier, higher sugar content in the immature fruit indicated a more rapid maturation processing in the same aged fruit. Therefore, fruit picked at harvest maturity may have variable fruit ages among the different treatment plant.

Enzymatic activities (SPS, SS, acid invertase) in the fruit picked from control plants were similar to previous results (Chapter 4). SPS was low and increased from 140 DAA to 175 DAA but no significant difference was obtained among treatments (Figure 5.6C). SS activity was relatively stable during the late fruit development stage (Figure 5.6D) when sugars were accumulating (Figure 5.6B). 154, 175 DAA and mature fruit from continual defoliation and fruit removal plus continual defoliated plants had lower SS activities than those fruit from the control plant. No difference of SS activity was found in all four stages of fruit between the fruit removal and the control. Invertase enzyme activity increased earlier in 140 DAA fruit from fruit removed plant than those in other treatments (Figure 5.6 E). No significant difference was detected in fruits at the other three stages between fruit removal and control. Invertase activity was decreased 154 DAA fruit but increased in mature fruit in the continual defoliated plant compared to control. There was a positive correlation between SS with sugar content among the treatments within the same aged fruit but no correlation was found in the four treatments of four development stages. Acid invertase was correlated to glucose content in the four treatment of the four development stages ($r=0.511$, $p=0.05$, Table 5.8). When the enzymatic activity one stage earlier was compared to sugar content, invertase activities were highly significantly correlated to sugar levels in four treatment three stages: 140, 154, 175 DAA enzyme vs 154, 175

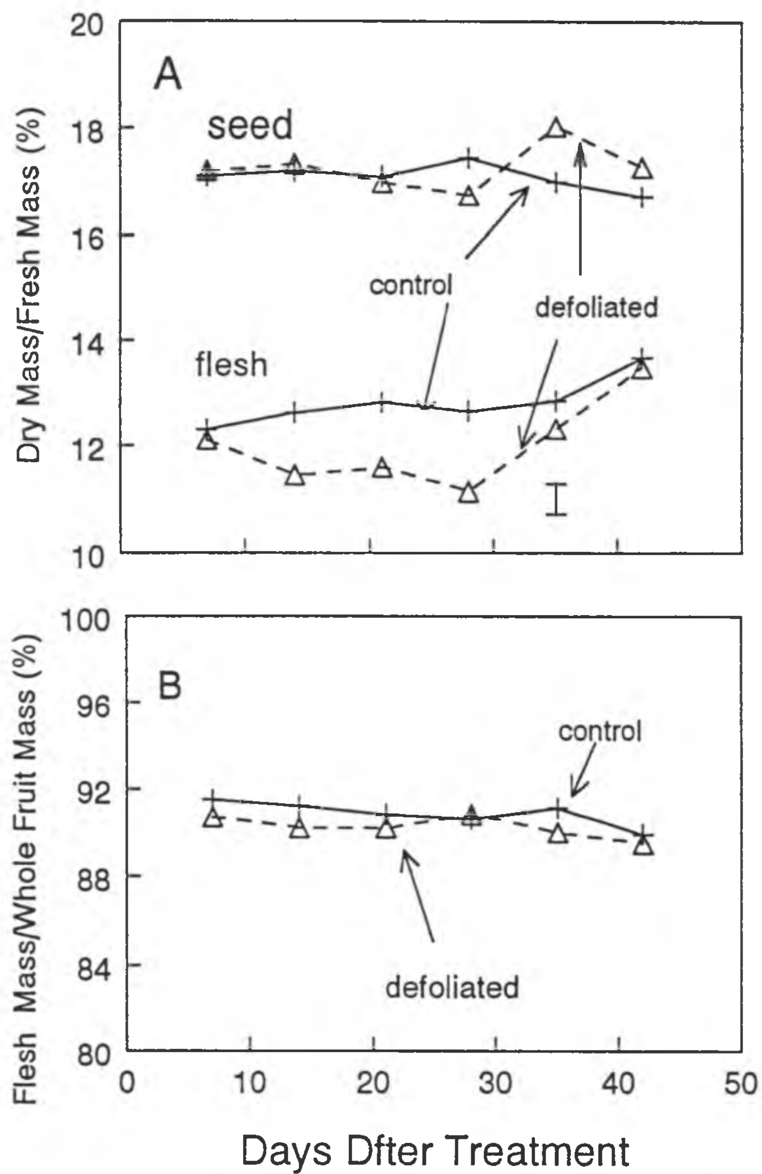



Figure 5.4 Effect of defoliation on ripe papaya fruit seed and flesh dry mass percentage (A) and flesh fresh mass percentage in whole fruit fresh mass (B) (cv: kamiya).

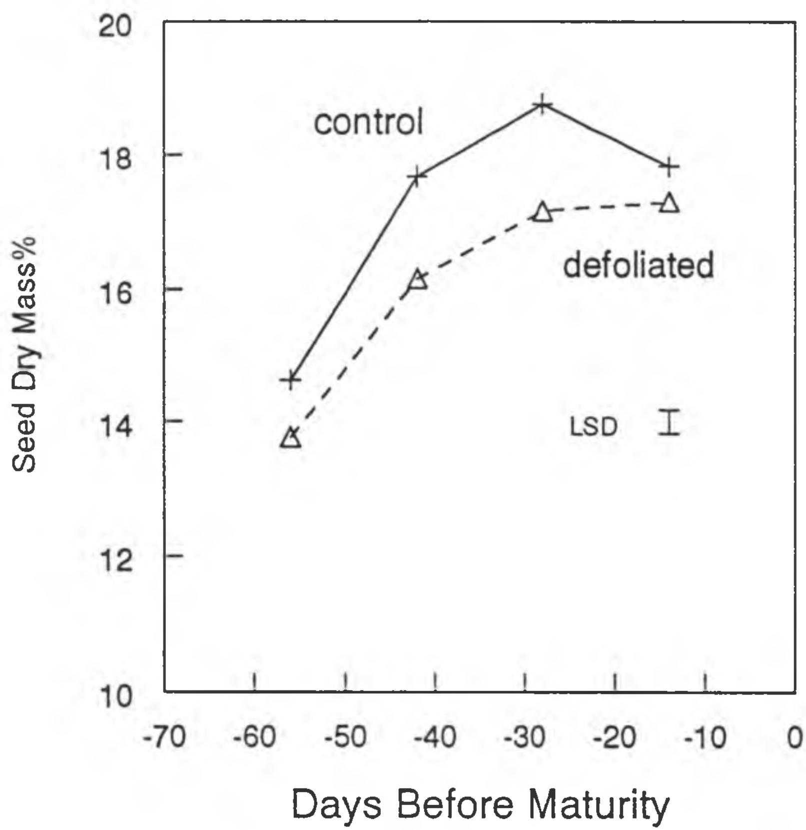



Figure 5.5 Effect of defoliation on young fruit seed dry mass percentage three weeks after defoliation in 'Kapoho' cultivar.

Table 5.7. Effect of defoliation and fruit thinning on sucrose synthase (SS), acid invertase enzyme activity (μmoles sucrose $g^{-1}$ $h^{-1}$ FW) in ripe papaya fruit.

| Treatment | Control | Defoliated | Fruit thinned |
|---|---|---|---|
| SS* | 1.1 b | 1.0 b | 2.1 a |
| *Acid invertase | 619 (22) | 739 (186) | 407 (228) |

* Means in the same row with same letter were not significantly different at 5% level. n = 3. Data in the parentheses indicates the standard deviation of three samples.

Table 5.8. Correlation coefficients between fruit sugar and SPS, SS, invertase enzyme activities at four developmental stages and four treatments.

| Enzyme | Sugar stage | Sucrose | Glucose | fructose | Total |
|---|---|---|---|---|---|
| Inverrtase | same | 0.44 NS | 0.51* | 0.38 NS | 0.44 NS |
| | one stage earlier | 0.80** | 0.80** | 0.80** | 0.83*** |
| SS | same | -0.36 NS | 0.21 NS | -0.25 NS | 0.29 NS |
| | one stage earlier | -0.04 NS | 0.31 NS | 0.11 NS | 0.10 NS |

NS: not significantly different at 0.05, *, **, ***, significantly at p= 0.05, 0.01, 0.001 levels, respectively.

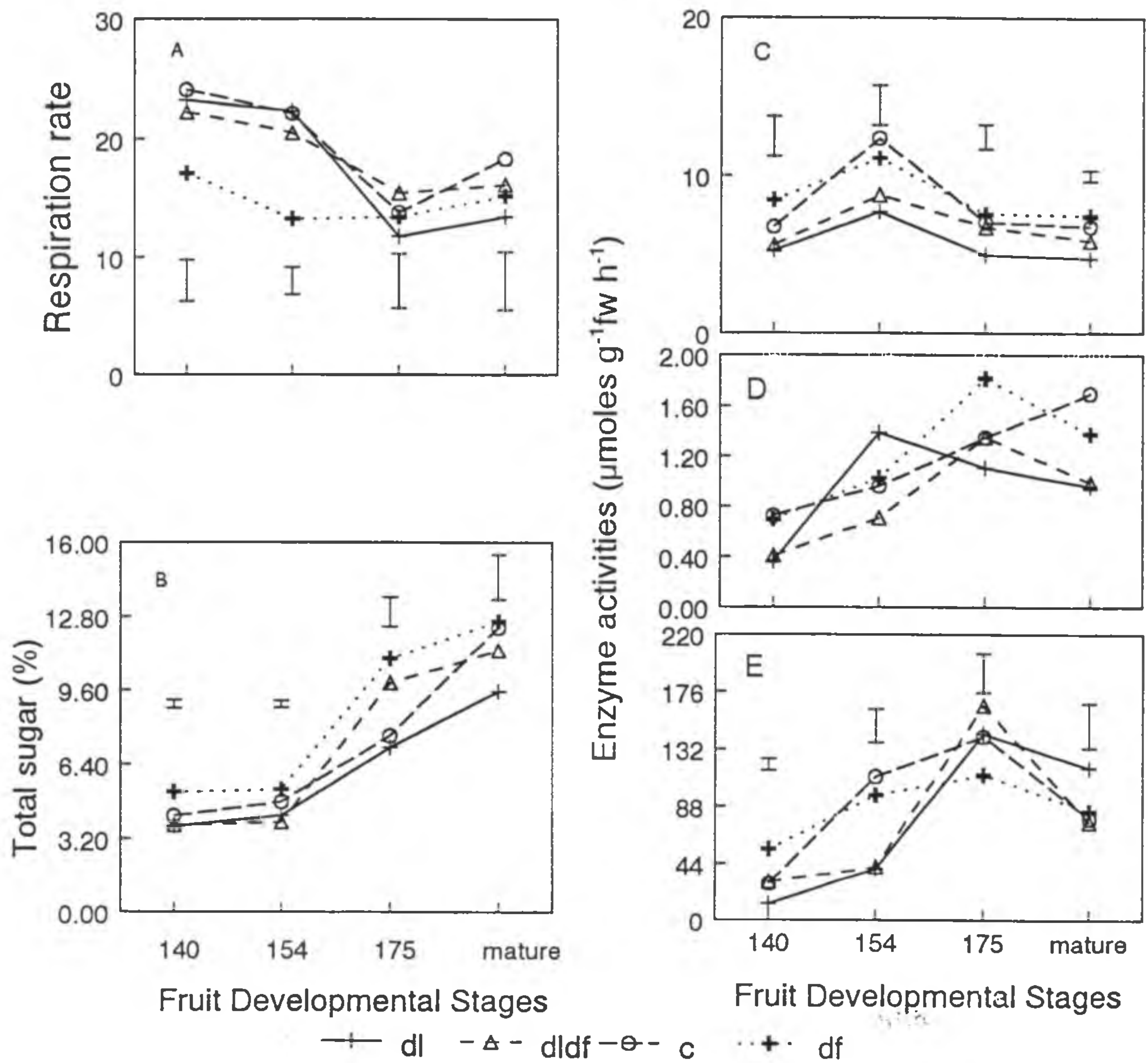



Figure 5.6 Comparisons of fruit respiration rate (A), fruit flesh sugar content (B), SS (C), SPS (D) and acid invertase (E) enzyme activities in different ages and treatments (control (C), fruit removal (DF), continual defoliation (DL), fruit removal + continual defoliation (DF+DL). * Fruits those picked in mature stage in different treatment may have different ages. Vertical bars in each graph indicated LSD (0.05) in each development stage for four treatments. There is no significant difference between treatments in SPS (C).

DAA and mature fruit sugar, respectively, invertase vs sucrose: (r =0.7996, p = 0.01); invertase vs glucose: (r = 0.8017, p = 0.01); invertase vs fructose: (r = 0.8025, p = 0.01); invertase vs total sugar: (r = 0.833, p = 0.001).

### 5.4 Discussion

The source-sink ratio was a critical factor in controlling papaya fruit set, fruit growth, development, and final quality of ripe fruit. New flower and fruit set was an index of plant assimilate supply. When leaf (source) assimilate capacity was larger than sink demand, new flower and fruit were set continuously. When assimilate was limited, plant flower development was arrested. Fruit set increased 22% than in the control plant after 1/3 of fruit were removed from each node during 42 days period. 82% more new flower than on control plant in 80% of the old fruit removed plant in the first 42 days and 52% more fruit than control in the first 56 days of the experiment, respectively (Table 5.2). In the contrast, 75 % defoliation reduced new fruit set 60% than control plant during first 42 days period. Continual defoliation produced new fruit less than 1/3 of control in the first 56 days and less than ¼ of control in the 168 days period, respectively (Table 5.2). Continual defoliation on the fruit removed plant had similar new fruit set as on the fruit removal alone during the first 56 days, as defoliation continued, new fruit set was reduced. By the end of experiment, fruit removal plus continual defoliation had less than half the new fruit set compared to fruit removal alone, but was similar to the control plant and 3 fold more than continual defoliation alone. Since wound injury effect was mimicked between defoliation and defoliation plus fruit removal, Hormone effect may have been minimized between these treatments. The results demonstrated that tassimilate availability was a major factor in controlling papaya new fruit set. Any environmental stress that reduced plant leaf photosynthetic capacity probably could influence papaya plant potential production. Papaya ringspot virus reduces papaya leaf photosynthesis and increases leaf respiration rate (Decker and Tio, 1958; Marler et al., 1993). If source assimilate still do not meet the demand of reduced sink size (fewer and smaller fruit), final fruit TSS (sugar) would be affected. From current research, we suggest

that the phenomena of 'summer sterility in papaya thought to be due to high temperature or dry weather may be imbalance of the source-sink ratio, caused by high temperature and water deficit on flower reproductive capacity. This is supported by the failure to arrest all new flower and fruit set in the same field.

Many cultivated plants compensate for partial defoliation by increasing the photosynthetic capacity of the remaining leaf area (Boucher et al., 1987; Flore and Irwin, 1983; Hodgkinson, 1977; Shaw and Samborski, 1956; von Caemmerer and Farquhar, 1984; Wareing et al., 1968; Layne et al., 1992; Layne et al., 1995). Removal of 25% of the leaf area of tomato (Stacey, 1983) and cucumber plants (Ramirez et al., 1988) did not significantly affect fruit yield or whole plant dry matter accumulation. Removing 50% of the leaf area of potted apple trees reduced dry weight accumulation by 40% (Maggs, 1964). Papaya is an indeterminate plant, that develops new leaves and fruits continuously simultaneously, and fruit at all stages of development are present on a single plant. Therefore competition exists between vegetative and reproductive sinks, between new fruit and aged fruit sinks. In papaya, 50% defoliation did not significantly affect fruit TSS or new fruit set rate (Figure 5.1A, Table 5.1) indicating that the photosynthetic compensation may occur in the papaya plant. 60-75% defoliation did not immediately influence mature fruit TSS or sugar level, TSS was reduced 14 to 21 days after defoliation (Figure 5.1A and 5.2A).

The effect of defoliation and fruit thinning on plant growth and development depends on the time of the defoliation and the number of leaf, flower, or fruit removal (Lyrene, 1992; Mulas 1997; Pavel et al., 1993). When defoliation occurred was important to affect on fruit mass and TSS in papaya. When plant had a full fruit load, two weeks after defoliation, the fruit TSS declined significantly then recovered in about four weeks as new leaves developed and less new fruit were produced (Figure 5.1 and 5.2). When plants were defoliated before the plant column was fully loaded, defoliation did not lower TSS in first two months' of ripe fruit (Figure. 5.5). Fruit number and size were smaller in the first two month on the defoliated plant than the control plant (Figure 5.3A, B). Continual defoliation reduced 'source' supply and produced smaller fruit with

lower TSS (Table 5.2). Fruit removal plus defoliation balanced source and sink size at the beginning of experiment, resulting in similar fruit set and fruit size to the control (Table 5.2). When more fruit were set and defoliation continued, source supply was lower than sink demand and mature fruit TSS was affected (Figure. 5.3C). Mature fruit TSS was not higher on the fruit removal treatment as the plant had adjusted to the fruit load and fruit had reach the maximum sugar accumulation for that stage of develop (Table 5.4). A desirable ratio of fruit number to photosynthetic leaf area was essential for final fruit sugar content as the fruit entered the maturation stage. This experiment supports and expands on the previous observations with papaya (Awarda, 1967; Martinez, 1988).

The different responses to defoliation method demonstrated that leaf position was more important than leaf area. Loss of photosynthetic capacity from the old leaves or the leaf position effect with young leaves providing assimilate to new flower and fruit, and old leaves supporting other sink growth such as the trunk and root. Another possibility was that young leaves produce more plant growth regulators that stimulated new fruit set.

Reduced papaya fruit set and delayed young fruit growth after leaf loss subsequently reduced papaya production in the next harvest season, though defoliation and fruit thinning did not significantly influenced papaya fruit production during 6 weeks experimental period. Failure to set fruit in every papaya leaf axil was due to a combination of flower and fruit abortion and change in flower type from hermaphrodite to male. Similar observation have been previously reported for papaya (Awada, 1967) and other hermaphroditic species (Spears et al., 1988; Wilson, 1983).

The different cultivar responses to defoliation and fruit thinning in different seasons indicated that cultivar characteristic and weather were important factors influencing papaya fruit growth and sugar accumulation (Figure 5.1, 5.2 and Table 5.3). These differences can be explained as related to source-sink balance. Defoliation of 'Sunset' and 'Kaminya' were performed in the sunny season (from May to July) and the plants were full loaded with fruit of different ages. Ripe fruit TSS was affected two to three weeks after defoliation. While in 'Kapoho'

and 'Line-8' defoliation experiment, fruit set was reduced prior to artificial defoliation and fruit thinning due to rainy weather. In addition, the photosynthesis was probably reduced under rainy and cloudy weather, that reduced assimilate supply between non-defoliated and defoliated plants. The differential response to fruit thinning between 'Line-8 and 'Kapoho' can relative to their growth features and source sink relationship. 'Line-8' cultivar usually produced about 3 to 4 fruit per node, and fruit softening occurred before fruit skin turns full yellow. While 'Kapoho' grown at Oahu island produced usually only one fruit per node, and fruit maturation was slower than 'Line-8' cultivar. These differences may lead the different impacts to fruit size and TSS level in fruit thinned plant (Table 5.3).

Fruit growth and the time to maturity of papaya was affected by source-sink balance as well as temperature. Nakasone (1986) mentioned that papaya growth period was prolonged about two weeks in the Hawaii cold season. We found that 'Sunset' papaya fruit set in June reach color break stage within 140 days. While fruit set in October on the same plant needs about 180 days to the color break while average temperature declined from 84 F to 76 F (from June to December 1996). This slowing of fruit development due to lower temperatures and plant age (Nakasone, 1986). Our data suggested that assimilate availability could altered fruit maturation time in addition to temperature and plant ages. Comparison of fruit size, fruit flesh color and sugar level, seed color, seed dry mass percentage, in different treatment and ages fruit indicated that fruit grow rapidly and mature earlier on plants in which older fruit were previously removed than in control and defoliated plants (Table 5.5 for 175 DAA fruit). However, the suddenly loss of photosynthetic capacity usually led to old fruit shading and delayed young fruit growth. Under long term source limitation papaya fruit size was reduced and sugar content tend to be lower but the fruit growth period was not significantly increased compared to the control (Table 5.5).

Acid invertase enzyme activity was associated with sugar accumulation during the late fruit development stage, through enzymatic activity developmental pattern and the comparison of a lower sugar line cultivar and the commercial cultivar of Hawaii (Chapter 4). Fruit removal

and continual defoliation reduced invertase activity and sugar level in young fruit, providing additional evidence to support the role of invertase. This finding was consistent with the finding from tomato plants that have lower vacuolar invertase activity, that had smaller fruit (Klann et al., 1996) and lower sugar content (Bucheli et al., 1994). Expression of a cytosolic yeast invertase in potato tuber leads to a decrease in yield and starch content and an accumulation of glucose but not fructose, whereas expression of a apoplastic yeast improves tuber growth (Sonnewald et al, 1994; 1997).

Source-sink balance can be used to predict and adjust fruit production and quality. Fruit mass was smaller than the commercial requirement in 'Sunset' plant continual defoliated due to poor water and fertilizer management. In this case, fruit thinning was necessary to ensure good fruit size. Fruit abortion occurred in the first few weeks after anthesis, fruit longer than 6 cm in length usually do not drop until ripen (Ong, 1983). Fruit shape, and potential fruit size in papaya is usually determined in early in development. Once fruit growth is abnormal in the early stage, that never developed to normal commercial product. In addition, a plant fully loaded with fruit early in plant development resulted in poor fruit quality and less fruit production in the next season, especially when the plant was exposed to a subsequent stress. Adjustment of fruit number to leaf number could result in desirable fruit size and even fruit production.

# CHAPTER 6

# GENETIC AND BIOCHEMICAL MECHANISM OF SUGAR ACCUMULATION IN PAPAYA FRUIT

## Cloning and expression of the genes encoding cell wall invertase and sucrose synthase in papaya fruit


### Abstract

We have previously shown that the enzyme invertase is a major enzyme contributing to sugar accumulation in papaya fruit during the last stage of development. To understand the biochemical regulation of sugar accumulation during fruit development, a cDNA library from immature green papaya fruit was constructed and a putative complete invertase gene and a SS gene fragment was isolated and characterized. The relationship between sugar accumulation in papaya fruit and the expression of papaya invertase and SS gene was investigated. The pattern of gene expression during fruit development was compared with invertase enzyme activity extracted in the presence and absence of sodium chloride (NaCl). The complete deduced amino acid sequence of papaya invertase had an open reading frame that encoded a polypeptide chain of 582 residues and calculated molecular weight of 65,684 Da. The protein was 67% identical at the amino acid level with carrot cell wall invertase and similarly homologous to invertase from other plants. The cloned 720 bp SS fragment was highly homologous to SS gene of *A. glutinosa* (X92378, 81% identical) and SS gene of many other species. The invertase gene was expressed at a higher level in the late stage of fruit development than in other papaya plant tissues. The pattern of increased mRNA expression during late fruit development paralleled the increase in invertase protein level and *in vitro* enzyme activity. However, the fold-increase in enzyme activity wad much higher than the increase in mRNA level and protein. SS gene expression was higher in young fruit and petiole tissues, and lower in the stem, flower and root tissue, but significantly higher than in the developing fruit flesh tissue and seed. The results indicate that invertase and SS genes are differentially expressed during plant and fruit development and, had

different roles in papaya plant development. Southern blot analysis indicated that both invertase and SS genes were encoded by a low copy number gene. The data confirmed that apoplastic invertase has an important function in phloem unloading during the period of sugar accumulation in papaya fruit. Invertase activity may be regulated at transcriptional, translational and post-translational levels.

### 6.1 Introduction

The timing of expression and the location of sucrose-cleaving enzyme activities are important for sink development and the control of carbon entry into metabolism. Sucrose synthase (SS) and invertase are the two enzymes that convert sucrose into hexose in plant sink tissue. SS (UDPG: D-fructose 2-glucosyl-transferase, E.C.2.4.1.13) is a glycosyl transferase, that catalyzes a reversible reaction that degrades sucrose into UDP-glucose and fructose (Huber et al., 1986; Sung et al., 1988; Chourey et al., 1991; Déjardin et al., 1997; Wang et al., 1994). In several fruit tissue, SS has been found associated with fruit establishment and maintenance (Wang et al., 1993a; 1994) and sugar accumulation (Moriguchi et al., 1988; 1992; Schaffer et al., 1987; Gross and Pharr, 1982; Hesse et al., 1996; Heim et al., 1993; Hubbard et al., 1991). Several investigators have suggested that SS activity could be used as a biochemical marker for sink strength (Clanssen et al., 1986; Sung et al., 1989). In monocot plants, two isoforms of SS are known (Chourey and Nelson, 1976; Chourey, 1981), with similar protein sequences (Huang et al., 1994), but their genes are regulated differentially (Chourey et al., 1986). In most dicot plants, only one gene and one SS polypeptide have been found (Sturm, 1996).

Invertase (β-fructosidase; EC 3.2.1.26) catalyzes the hydrolysis of sucrose into glucose and fructose. Numerous forms of plant invertases are characterized by solubility, different pH optima, iso-electric points and subcellular localization (Sturm et al., 1990). Soluble invertases range in their pH optima from slightly alkaline (pH 7.5) to acidic (pH4.5). Soluble invertases are intracellular, located in either the vacuole (acid optima) (Leigh et al., 1979; Giaquinta et al., 1983) or cytosol (Karappiah et al., 1989; Fahrendorf et al., 1990). Insoluble invertase, with a pH

optimum between pH 4.0 and pH 5.3, is ionically bound to the cell walls and can be solublized by extracting cell walls with high salt (Fahrendorf et al., 1990).

Developing papaya fruit accumulates soluble sugars during the last stage of fruit growth (Chan et al., 1979; Zhou et al., 1997). Sucrose synthase contributes to papaya fruit sink establishment and maintenance, and a buffer soluble acid invertase activity is correlated with sugar accumulation during the last phase of fruit development (Chapter 4). Fruit removal and defoliation alters sugar levles and invertase enzyme activity in papaya fruit (chapter 5) and suggests that invertase enzyme is involved in sugar unloading during sugar accumulation. Papaya invertase has been partially purified (Chan and Kwok, 1976; Lopez et al., 1988), but not separated from other proteins. There is no information as to which invertase forms are present in papaya fruit and if they are the same isoforms present in immature fruit or during postharvest ripening.

The molecular mechanism of controlling SS and invertase enzyme activities during fruit development is not clear. We have used a molecular approach to investigate the relationship between sugar accumulation and enzyme activities in papaya fruit and the expression of invertase and SS genes. A cDNA library from immature green papaya fruit was constructed and a putative complete invertase cDNA and a SS cDNA fragment were isolated and characterized. Invertase and SS in the papaya plant and fruit were differentially expressed. Analysis of the DNA sequence of the invertase cDNA indicated that the encoded for protein was likely to be localized to the cell wall. The levels of mRNAs encoding invertase during fruit development were compared with invertase activity extracted in the presence and absence of sodium chloride (NaCl).

## 6.2 Materials and methods

### 6.2.1 Plant tissue

Papaya (*Carica papaya* L. cv 'Sunset') fruit grown at the University of Hawaii Poamoho Experimental Station, Hawaii were used as experimental material. Fruit at different invertase

Y1 Y2 Y3 2WB mature full ripe

Figure 6.1. Fruit at different developmental stages (cv.Sunset) used as experimental material. Label: Y1, young fruit stage1, seeds white and small; Y2, stage2, seeds still white, almost full size; Y3, stage3, seed starting to turn brown, sugar level starting to increase in fruit mesocarp about 4 weeks before harvest; 2WB, immature green fruit, ca. 2 weeks before harvest, a cDNA library was construct from this stage. Mature, harvest stage, color break to 30% yellow; Full ripe, one week after harvest, fruit at eating stage.

developmental stages (Figure 6.1) were used for the extraction of total protein, the assays of invertase activity and the extraction of total RNA for Northern blot analysis. Fruit development stages and maturity were estimated by skin, flesh and seed color based on previous research (Chapter 4). After harvest from the plant, fruit tissue was sampled and frozen in liquid $N_2$ and store at -70°C until used for extraction.

6.2.2 Invertase enzyme extraction and assay

Invertase enzyme extraction and assays were performed during several independent experiments. The solubility of invertase protein in immature green fruit was examined using different buffer components, and modifying the pH of extraction buffer and the salt concentration. For the time course, buffer soluble invertase and total invertase were extracted in the absence or presence of 1M NaCl during different stages of papaya fruit development. The 1 M NaCl extraction is able to extract cell wall bound invertase (Fahrendorf et al., 1990). The extraction buffers contained the following compounds:

| Extraction buffer (pH7.5) (buffer 1) | Desalt buffer (pH 7.5) (buffer 2) |
|---|---|
| 100 mM Mops-NaOH, | 50 mM MOPS-NaOH |
| 5 mM $MgCl_2$ | 5 mM $MgCl_2$ |
| 1 mM EDTA | |
| 2.5 mM DTT | 2.5 mM DTT |
| 0.5 mg $ml^{-1}$ BSA | 0.5 mg $ml^{-1}$ BSA |
| 2% v/v glycerol | |
| 1 mM PMSF | 1 mM PMSF |
| 0.05% triton x-100 | |

The extraction procedure and desalt method were the same as described in chapter 4 unless otherwise stated.

| Extraction buffer (pH 5.0) | Invertase assay mixture (total 100 μl) |
|---|---|
| 0.1 M Citrate Acid | 60 mM Citrate Acid |
| 0.1M $K_2H\ PO_4$ | 60 mM $K_2H\ PO_4$ |
| 2.5 mM DTT | 20 mM sucrose |
| 0.5 mg $ml^{-1}$ BSA | 20 μl extracts |
| 1 mM PMSF | |

6.2.3 Protein gel electrophoresis and Immunoblotting

Proteins from different fruit developmental stages were extracted as the enzyme activity assay, except BSA was absent in the extraction buffer to enable quantification of protein content (Lowry et al., 1951), and 1 mM dethiodipyridine and 10 μm E-64 was added to inactivate papain activity during extraction. Total proteins were separated on 10% SDS -PAGE gel and either stained with Coomaasie blue or electrophoretically transferred onto 0.45 μm nitrocellulose membrane for immuno-analysis. Rabbit polyclonal antibodies to carrot invertase (Lauriere et al., 1988; Unger et al., 1992) kindly provided by Dr. Strum, was used and cross reactive polypeptides were visualized use goat anti-rabbit IgG conjugated to alkaline phosphatase. Western blot and immuno-detection were performed following the ECL Western Blotting Protocols (Amersham International Plc. 1991).

6.2.4 RNA isolation

Total RNA was isolated according to the method of Lopez-Gomez and Gomez-Lim (1992) and Ikoma et al.(1995) with modifications. Twenty grams tissue was ground to a powder with a cold mortar and peste in liquid nitrogen, then vortexed in 2 volumes of lysis buffer (2% SDS, 1% β-mercaptoethanol, 50 mM EDTA,150 mM Tris base adjusted to pH 7.5 with boric acid) plus 3 volumes of water-saturated phenol (pH 6.5). Before centrifugation, 60 ml chloroform was added and mixed well. After centrifugation, the aqueous phase was transferred into a new bottle that contained the same volume of acid phenol and the phases were mixed thoroughly. An equal amount of chloroform was added, mixed and the mixture was centrifuged at 4000xg for 10 min at room temperature. The aqueous phase was quickly vortexed with 0.25 volume of ethanol and

0.11 volume of 5 M potassium acetate plus 30ml of phenol and 30 ml chloroform, then allowed to stand for 1 hour on ice, the aqueous phase was recovered by centrifugation at 15,000xg for 10 min. The aqueous phase was re-extracted with chloroform:isoamyl alcohol (49:1) and the aqueous phase was recovered by centrifugation. Total RNA was precipitated by adding LiCl to a final concentration of 3 M and stored at -20°C overnight. After centrifugation at 20,000xg for 30 min, the RNA pellet was dissolved in sterile water and precipitated again with 3M LiCl. The pellet was then suspended in sterile water, and precipitated with 2.5 volume of ethanol and 0.3 M sodium acetate. RNA was dissolved in sterile $H_2O$ and quantified by measuring the absorbance at 260 nm (1 optical density = 40 μg/ml). RNA quality was analyzed by electrophoresis on 5% formaldehyde/1.0% agarose gels (16 mM Mops, 4 mM sodium acetate, and 1 mM EDTA, pH 7.0) (Hoffer and Christopher, 1997). Total RNA isolated using this method was directly used for RT-PCR and Northern blotting or poly $A^+$ mRNA isolation for cDNA library construction.

6.2.5 Reverse Transcriptase PCR (RT-PCR)

RT PCR was performed to isolate an invertase cDNA fragment from immature green fruit tissue (about 2 weeks before harvest) and to study gene expression. The first strand cDNA was synthesized from 5 μg of total RNA using Oligo dT or Random Hexamer primer following the instruction of Superscript™ Pre-amplification System for the first strand cDNA synthesis kit (Gibco BRL, Life Technologies, Cat. no. 18089-011). Primer I 5'-AAG(A)AAT(C)TGGATG AAT(C)GAT(C)CC (upstream), and primer II 5'-AAG(A)TCIG(A)C(G)G(A)CATTCCCACATICC (downstream), were from highly conserved region of known cell wall and vacuolar invertase genes. Primer III 5'-GGA(GT)ATIA(C)DT(C)TGAT(C)ICT(C)IGCC (downstream), was from a cell wall invertase gene. These primenrs were used to isolate invertase cDNA fragments from RT PCR. Ten percent of first strand cDNA (2 μl) was subjected to the following PCR reaction by using Taq DNA polymerase (Promega):

| Component | Volume (μl) | |
|---|---|---|
| Total | 50 | 100 |
| 10X PCR buffer | 5 | 10 |
| 25 mM $MgCl_2$ | 3 | 6 |
| 10mM dNTP mix | 1 | 2 |
| primer I (~0.1 μg/μl) | 1 | 2 |
| primer II (~0.1 μg/μl) | 1 | 2 |
| Taq DNA polymerase | 0.5 | 1.0 |
| cDNA | 2 | 2 |
| sterile, distilled water | 36.5 | 75 |

The thermocycling regime used (Coy Library, MI) was as follows: 94 °C for 6 min, followed by 94 °C for 1 min, 42 °C for 1 min , 72 °C for 1.5 min (for 40 cycles), followed by a final extension period of 72 °C, for 7 min. Two PCR products of 558 and 889 base pairs in size were purified from a 1% agarose (1X TAE) gel using the freeze-thaw method.

6.2.6 Cloning of invertase gene fragment

DNA sequencing was used to confirm that the PCR product was from a cell wall invertase gene. Then the 558 bp and 889 bp fragments were cloned into pGEM 7Z and pBluescript-SK (-) vector, respectively (Figure 6.2A). For cloning purposes, the restriction enzyme cutting sites Eco*R* I and X*ba I* were added upstream and downstream, respectively at the end of the primers. The PCR products were gel purified and restriction digested with Eco*R* I and X*ba I* restriction enzymes followed by Phenol: chloroform extraction and ethanol precipitation. After ligated to the vector at the same cutting sites, the recombinant DNAs were transferred into E *coli*. (XL-1) Blue strain.

6.2.7 Cloning of SS gene fragment

The SS gene fragment was obtained by PCR using a cDNA library made from immature green fruit. Four primers were designed to conserved regions found within various plant SS protein sequences. Forward primer, 5'-CCTGAC/TACC(T)GGTGGA(C/T)CAGGT-3' and reverse

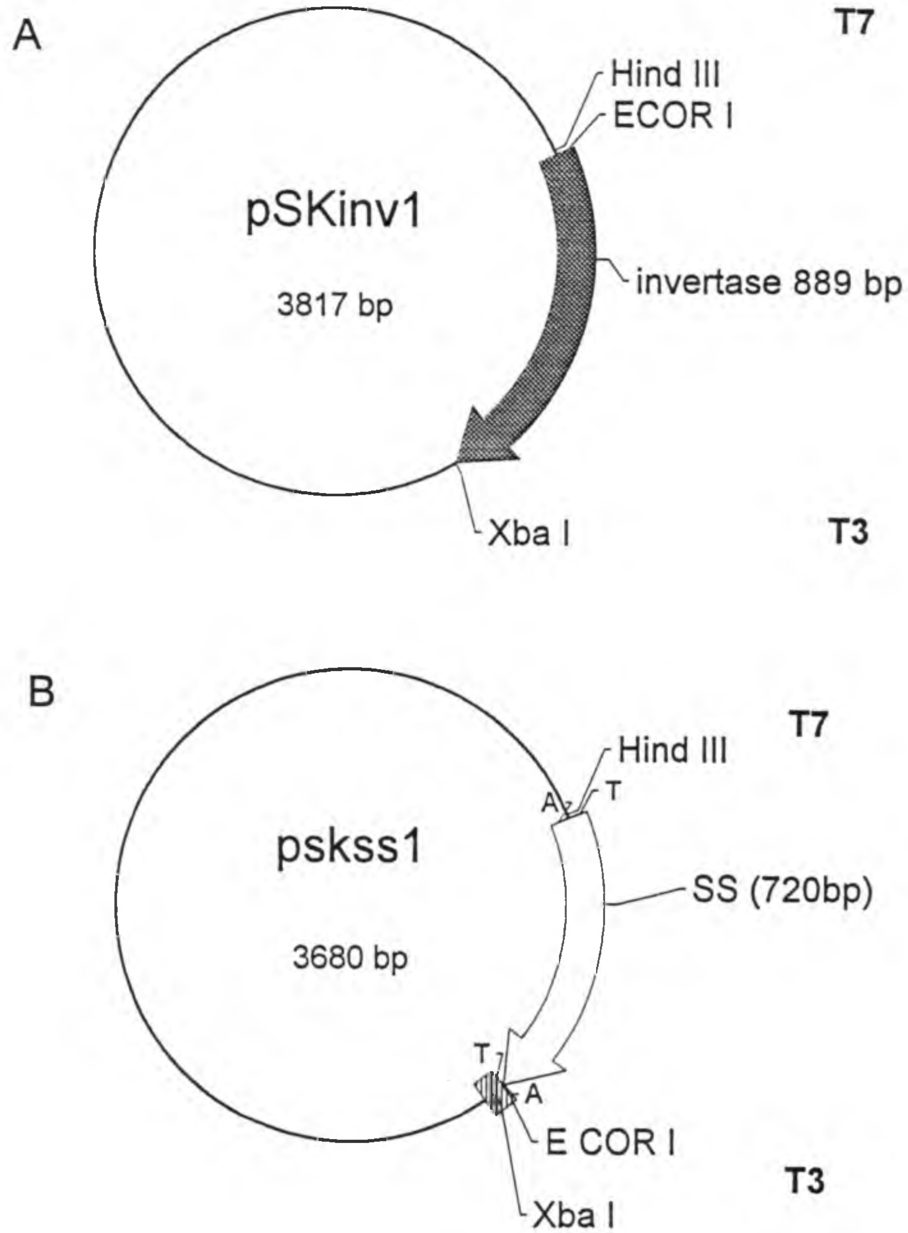



Figure 6.2. The diagrams of recombinant invertase (A) and SS (B) gene fragments in the pBluescript Phagemid SK (-) vector with the map of some restriction enzyme sites.

primer, 5'-TCA(G)GA(T)GTAA(T)GGA(G)AAA(G)TAA(G/T)A-3' gave two bands on a 1% agarose TAE gel. One was about 1kb, the another was about 720 bp. The PCR product 720 bp was close to anticipated size. Direct sequencing of the two purified bands confirmed that the second band was homologous to known SS gene sequences. Fresh PCR product was cloned into the SK (-) phagemid E*coR* V site using the T tail cloning technique (Figure 6.2B). The T tail vector was prepared according to (Marchuk et al., 1991) as follows: one μg of pBluescript SK vector was digested using E*coR*V, the T tail was added using PCR reaction buffer, 2mm dTTP and Taq polymerase enzyme incubating at 72°C for 6 hours. Additional 0.5 μl Taq enzyme was added after three hours incubation. The PCR product was ligated at 14°C overnight to the T tail vector, followed by transformation of E *coli*. (DH 5α strain). The PCR product was ligated at 14 °C overnight to the T-tailed vector, followed by transformation of E *coli*. (DH5α strain) White colonies were selected for further analysis. The positive plasmid conformed by PCR were purified and restriction digested. One μg of plasmid was used for sequence analysis from both ends using T3 and T7 primers.

6.2.8 Northern blot analysis

Total RNA from different papaya tissue: young and mature leaves, flowers, young stem, mature leaf petiole, 6 month seedling root, immature seed, and young fruit (14DAA) and five developmental stages of fruit flesh tissue were used for Northern blot analysis. Total RNA (10 μg) was denatured and fractionated on 1% agarose-5% formaldehyde gel in 16mM mops, 4mM NaOAc and 1mM EDTA (pH 7.0) (Fourney et al., 1988, Hoffer and Christopher, 1997). After electrophoresis, the RNA gel was soaked in 10X SSC (1.5 M NaCl, 0.15 M sodium citrate) for twenty minutes. The RNA was transferred from the gel to nitrocellulose membrane overnight by capillary action in 10X SSC (Sambrook et al., 1989). The RNA blot was labeled and RNA was cross-linked to the blot using UV. The blot was wrapped with plastic film and stored at room temperature.

6.2.8.1 Radioactive probe preparation

Gene specific antisense RNA probes were synthesized and radiolabeled with [$\alpha$-$^{32}$P] UTP (>3000 Ci/mM, ICN Pharmaceuticals, Inc., Costa Mesa, CA) using T3 RNA polymerase according to the manual of 'Riboprobe in vitro transcription systems' (Promega, CA). For the invertase probe, one $\mu$g of phagemid DNA containing the 889 bp papaya cell wall invertase fragment linearized using 5 units of E*coR* I enzyme. For the SS probe, one $\mu$g of phagemid containing the SS fragment was linearized using 5 units of HindIII enzyme (New England Biolabs). The following components were added to each reaction mixture in order at room temperature: 4$\mu$l 5X transcription buffer, 2 $\mu$l 100 mM DTT, 1.0 $\mu$l RNase inhibitor, 1.5 $\mu$l each of 10 mM ATP, GTP CTP, 0.5$\mu$l 10mM cold UTP, 1 $\mu$g linearized DNA template, 6 $\mu$l $\alpha$-$^{32}$P UTP (3000 Cli/mM) and 2 units T3 RNA polymerase with a total volume of 20 $\mu$l. Mixed reaction tube was incubated at 37 °C for 90 min, followed by adding 20 $\mu$l deionized water plus 1 $\mu$l RQ-DNase (RNAase free) and incubating for 15 min at 37°C. The reaction mixture was purified using phoenol:chloroform (1:1, v/v) once and followed by chloroform once. The sense RNA using the same method but T7 RNA polymerase and linearizing at the opposite end of the insert using X *bal* for invertase or E *coR* I for SS was synthesized as a positive control.

6.2.8.2 Hybridization and detection

The blots were prehybridized with 10 ml of hybridization buffer (0.75 M NaCl, 0.075 M sodium acetate, 50% v/v Formamide, 2X Denhardt's solution, 0.25 mM monobasic sodium phosphate, 0.25 mM dibasic sodium phosphate, 1.5% SDS w/v, 5 mg samen DNA) at 55°C for 4 hours (Hoffer and Christopher, 1997). 20 $\mu$l of radioactive probe (2.5 x $10^6$ mcp/$\mu$l) was denatured in 500 $\mu$l prehybridization buffer for 10 min at 80°C and the blot hybridized overnight at 55°C. The blots were washed three times (first 10 min, then for 30 min each at 65°C) in 50 ml of 2X SSPE buffer, 1% SDS then with 0.2X SSPE, 1% SDS for another 30 to 60 minutes. The blot was autoradiographed with X-ray film.

6.2 9 Genomic DNA isolation and southern blot analysis

Genomic DNA isolation from 'Sunset' leaf was performed according to Doyle and Doyel (1987). One gram of leaf tissue was ground in liquid $N_2$ with the mortar and pestle. The frozen powder was transferred into 8 ml 2X CTAB isolation buffer (100mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% Hexadecyltrimethylammonium bromide (CTAB), 0.2% 2-mercaptoethanol) and incubated at 60°C for 30 min with gentle swirling. Before centrifugation, 10 ml of chloroform-isoamyl alcohol (24:1) was added and mixed gently but thoroughly. After centrifugation at 4,500xg for 10 min, the top aqueous phase was transferred into a new tube, and 7 ml cold isopropanol was added and mixed gently. The DNA precipitant was pelleted by centrifugation and then resuspended in 10 ml of wash buffer (70% EtOH, 10mM ammonium acetate). After 5 min centrifugation, the buffer was removed and the pellet was dried briefly and resuspended in 500 μl TE.

The DNA ca.10μg was digested with E*coR* V, Hind III, and X*ba* I (Promega Co., WI) and fractionated on a 0.8% (w/v) agarose gel in 1X TAE buffer (40mM Tris-HCl, pH 7.4, 20 mM sodium acetate, 1 mM EDTA). The DNA was transferred to nitrocellulose by capillary transfer in 20X SSC solution. Prehybridization and hybridization was performed same as Northern blot except hybridization temperature was at 45 °C and washed at 60 °C.

6.2.10 Construction of a cDNA library from immature green papaya fruit

Total cell RNA was isolated from 250 gram of 'Sunset' papaya fruit flesh approximately two weeks before harvest as described earlier. Messenger RNA (poly $^+$ RNA) was isolated using the poly$^+$ Tract mRNA isolation systems (Promega), 10 μg of Poly$^+$ RNA was used for synthesizing the cDNA library according to the manufacture's instructions (ZAP-cDNA synthesis kit, #200400, Stratagene, CA, 1997), except no radioactive dATP was added for synthesizing the second strand of cDNA. PCR reaction using invertase primer FI and primer RI were performed at the each stage of cDNA library construction to monitor the quality of cDNA. The primary library was amplified and then stored at 4 °C or in 7 % (v/v) DMSF at -70 °C.

6.2.11 cDNA library screening

Twenty petri plates (150 $cm^2$ ) with approximately 1.2 X $10^6$ phages were screened by plaque-hybridization onto Magna nylon membranes. After growing the phage for 8 hours, the nylon filters (Micron Separation Inc. MA) were placed on the petri dishes for 2 min. The filters were removed, denatured for 2 min (0.5 M NaOH containing 1.5 M NaCl), and neutralized for 5 min (0.5 M Tris-HCl pH 8.0, 1.5 M NaCl), and wash in 2X SSC containing 0.2 Tris-HCl for 30 seconds. After briefly drying on the Whatman 3 MM papers, the membranes were baked at 80 $^oC$ for 2 hour in vacuum oven. Probe preparation and hybridization were performed as described for Northern blotting (specific activity. 1X $10^9$ cpm/μg).

Positive plaques from the first screen were removed from the top agar and dissolved in 500 μl SM buffer, 1 μl of the aqueous phage from the screened plaques was used as the PCR template. PCR conditions were performed as described earlier, except a total volume of 15 μl was used. Four positive plaques with the correct PCR product size were used for the second screen. More phages were subjected to an additional first screen. A total of five positive clones were converted to P Bluescript SK (-) phagemid using the ExAssist/Solor system according to the manufacturer's instruction (Stratagene, CA).

6.2.12 Analysis of invertase clones

Five putative invertase clones were partially sequenced from both ends using T3 and T7 primers. Three clones were confirmed to encode cell wall invertase by comparison with other published invertase sequences. Two clones that contained identical coding sequences from the 5' end and 3' end were fully sequenced. One clone had a short deletion in the middle of 5' untranslated region.

All sequencing was performed using an automated DNA sequencer, 373A (Perkin Ellmer Applied Biosystems, CA) at the University of Hawaii. A series of primers were synthesized based on the previous sequence data and used for subsequent sequencing.

The cDNA sequence data from each DNA fragment was configured into a whole length sequence and mapped using the Genetic Computer Group (GCG) program (University of

Wisconsin, Madison). The nucleotide sequences available in the National Center for Biotechnology Information (Bethesda, MD) were accessed via the internet using Blast and Entrez search engines provided by the National Center for Biotechnology Information.

### 6.3 Results

6.3.1 Reverse transcriptase PCR (RT-PCR)

Two RT-PCR products based on anticipated size (558 bp and 889 bp, respectively) were obtained by using the same forward primer and different reverse primers (Figure 6.3). The DNA fragments were purified from the agarose gel and were sequenced directly. The products have identical nucleotide sequence in the overlapping region. The nucleotide sequence and deduced amino acid sequence were highly homologous to the sequences from other cell wall invertase gene. The nucleotide sequences of RT-PCR products obtained from two different fruit developmental stages (two weeks before harvest and full ripe fruit, respectively) were also identical. The purified DNA bands were cloned into E*coR* I and X*ba* I sites of pGEM 7z and pBluescript SK (-) vector (558 and 889 bp, respectively). The recombinant clones were confirmed by restriction digestion, PCR and DNA sequence analysis.

6.3.2 Cloning of a cell wall invertase cDNA from immature green papaya fruit

A riboprobe homologous to the 889 bp invertase fragment was used for screening the cDNA library made from immature green papaya fruit. Five positive invertase clones were isolated from the screening procedure and they were sequenced from both ends using T3 and T7 primers. Three clones were confirmed to encode invertase and had the same amino acid sequence, but had varied length in the 5' UTR and 3' poly A (Figure 6.4). Two clones containing different lengths at the 5'UTR were completely sequenced. The sequences of the two clones were identical except at the 5' UTR. The exist of heterogeneous 5' UTR'S from individual genes agreed with the results obtained by directly sequencing of PCR products previously amplified from the cDNA library.

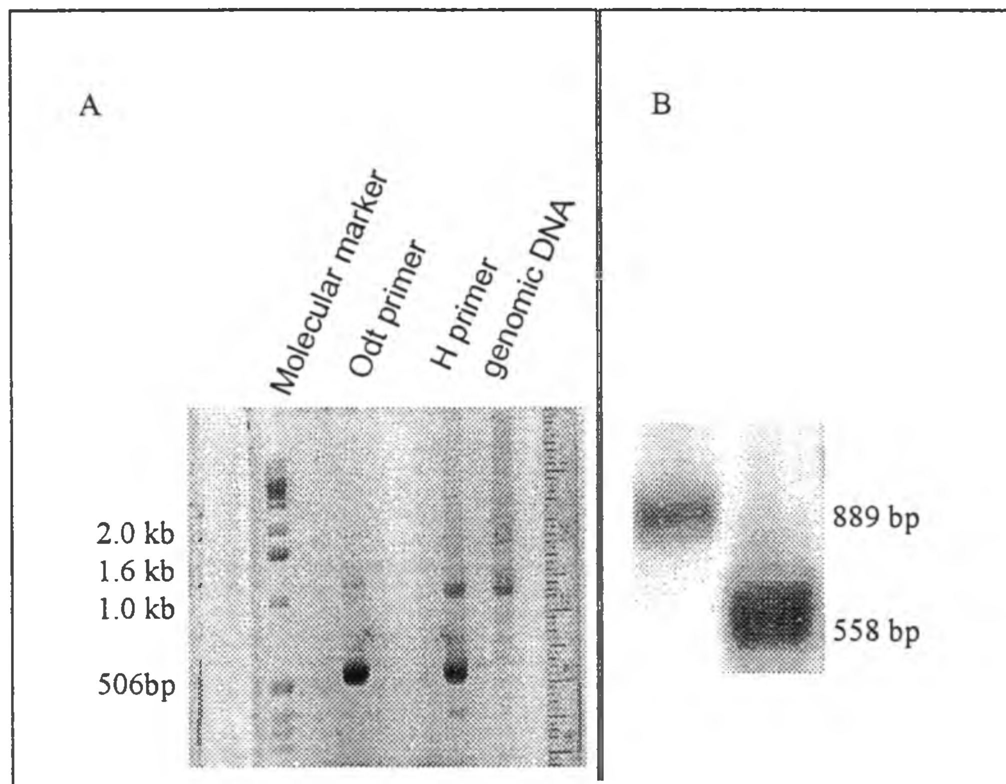



Figure 6.3. Electrophoresis (1% agarose-TAE gel) of the invertase gene fragments. A: amplified cDNA fragments using conserved gene-specific FI and RI primers. Molecular marker= 1 kb DNA ladder; OdT primer = DNA template synthesized using oligo-dT primer; H primer=DNA template synthesized using random hexamers primer; Genomic DNA= 50 ng papaya genomic DNA as positive control. B: Two PCR products of 558 (FI+RI) and 889 (FI+RII) bp in size were sequenced and cloned in to pGEM 7z and Bluescript-SK (-) vectors, respectively.

```
1.GCACGAGATTTCTTATAAATGCTTATCTTTTAAGTGCTTTTCCTTTTAAGTGCTTTGTTTGGTAAGGAAA
2.     GAGATTTCTTATAAATGCTTATCTTTTAAGTGCTTTTCCTTTTAAGTGCTTTGTTTGGTAAGGAAA
3.GCACGAG                                        CTTTTAAGTGC    TTTGGTAAGGAAA

1.AAGATG
2.AAGATG
3.AAGATG
```

Figure 6.4. Comparison of partial cDNA sequence from the 5'UTR of three invertase clones from papaya immature green fruit cDNA library. #1 and #3 clones were sequenced for their full length, the rest of region was identical for the two clones.

The complete deduced amino acid sequence of papaya invertase gene (Figure 6.5) had an open reading frame that encode a polypeptide chain of 582 residues and had a calculated molecular weight of 65,684 Da. In addition to the open reading frame, the cDNA also contained 38 bp or 73 bp 5' untranslated regions (5' UTR) and a 135 bp 3' untranslated regions (3' UTR) with a poly (A) tail. The calculated iso-electric point was 6.94. The sequence of the deduced amino acid was compared with other cell wall invertases and with the conserved region for soluble invertase (Figure 6.6).

6.3.3 Cloning of SS cDNA fragment from Papaya fruit cDNA library

Several primers complementary to the conserved SS sequences were used for PCR to amplify a SS cDNA fragment from the papaya cDNA library. The forward primer, SS 880F and the reverse primer SS 1580R, gave two bands on a 1% agarose (1x TAE) gel (Figure 6.7). Direct sequence analysis of the purified DNA fragment indicated the 720 bp PCR product was homologous to the SS gene. The fresh PCR product was purified and cloned onto the E*coR* V site of the pBleuscript SK (-) vector where a "T tail' was added at the cutting site. The recombinant clone containing the SS gene insert was confirmed by restriction digestion, PCR and DNA sequencing. The nucleic acid sequence (Figure 6.8) was highly homologous to *A. glutinosa* mRNA (X92378, 82% identical), *V. Faba* mRNA (X69773, 81% identical) and SS genes of many other species. The deduced amino acid sequence was about 85% to 90% identical and positive (Figure 6.9), respectively. Some regions were homologous to the SPS gene.

6.3.4 Cell wall invertase enzyme activity during papaya fruit development

Invertase activities from papaya fruit varied in different extraction buffers, depending upon pH, buffer components and extraction method (Table 6.1 &2 &3). No satisfactory method was available to separate vacuole or cell wall invertase by simple extraction. Much higher invertase activity was obtained when 1 M NaCl was included in the extraction buffer (Table 6.3) in immature green fruit tissue. Therefore, total and buffer-soluble invertase activities were determined on extracts made in the presence and absence of 1 M NaCl from mesocarp tissue at different fruit stages of development (Figure 6.10). Both buffer-soluble (absence of NaCl) and

```
      gcacgagcttttaagtgctttgtttggtaaggaaaagatgagtactgcgtcgaagtttt
   1 ---------+---------+---------+---------+---------+---------+ 60
                                         M  S  T  A  S  K  F  Y -
     attcagttttgacgtcgacgttgttttgttatcttgctgtgataacattcatcggaaccg
  61 ---------+---------+---------+---------+---------+---------+ 120
       S  V  L  T  S  T  L  F  C  Y  L  A  V  I  T  F  I  G  T  A -
     ccattaatggcgtcgaagcttctcacaggatttatccgcagtttcagtctctgtctgtcg
 121 ---------+---------+---------+---------+---------+---------+ 180
       I  N  G  V  E  A  S  H  R  I  Y  P  Q  F  Q  S  L  S  V  D -
     acatcgtcgaccaaatccacagaactgcttaccattttcagcctcctaaacactggatta
 181 ---------+---------+---------+---------+---------+---------+ 240
       I  V  D  Q  I  H  R  T  A  Y  H  F  Q  P  P  K  H  W  I  N -
     acgacccaaatgctccaatgtactacaatggcgtgtaccatctcttctaccaatacaacc
 241 ---------+---------+---------+---------+---------+---------+ 300
       D  P  N  A  P  M  Y  Y  N  G  V  Y  H  L  F  Y  Q  Y  N  P -
     caaagggtgccgtgtggggcaacatcgtgtgggcccactcagtttcaacggacttgatca
 301 ---------+---------+---------+---------+---------+---------+ 360
       K  G  A  V  W  G  N  I  V  W  A  H  S  V  S  T  D  L  I  N -
     actggatacctctcaagccagcgatcgttccatctgagccgttcgatatcaaaggctgct
 361 ---------+---------+---------+---------+---------+---------+ 420
       W  I  P  L  K  P  A  I  V  P  S  E  P  F  D  I  K  G  C  W -
     ggtccggatcggctacagtcctacccaacaacatccccatcatcctctacactggtctcg
 421 ---------+---------+---------+---------+---------+---------+ 480
       S  G  S  A  T  V  L  P  N  N  I  P  I  I  L  Y  T  G  L  D -
    actccaacgaaacccaactacaaaactacgccgttccggctaacatttccgatccgcatc
 481 ---------+---------+---------+---------+---------+---------+ 540
       S  N  E  T  Q  L  Q  N  Y  A  V  P  A  N  I  S  D  P  H  L -
     tcgaaaattggataaaacccgccaacaatcccttggtcgcacccgaccataccgtcaacc
 541 ---------+---------+---------+---------+---------+---------+ 600
       E  N  W  I  K  P  A  N  N  P  L  V  A  P  D  H  T  V  N  R -
     gaaccgcattccgtgacccgacaaccgcctggttaggctcagacgggtggtggaaaatgc
 601 ---------+---------+---------+---------+---------+---------+ 660
       T  A  F  R  D  P  T  T  A  W  L  G  S  D  G  W  W  K  M  L -
     tggtgggtaataaaaataaacgtaggggcattgcgcatttatacaagagcaaggatttca
 661 ---------+---------+---------+---------+---------+---------+ 720
       V  G  N  K  N  K  R  R  G  I  A  H  L  Y  K  S  K  D  F  M -
     tgaactgggtcaaggctaaacacccgatccattccagacccgatacgggtatgtgggaat
 721 ---------+---------+---------+---------+---------+---------+ 780
       N  W  V  K  A  K  H  P  I  H  S  R  P  D  T  G  M  W  E  C -
     gcccagatttttcccggttccgaaatcgggtgaaaacggattggatgtgggaattaccg
 781 ---------+---------+---------+---------+---------+---------+ 840
       P  D  F  F  P  V  P  K  S  G  E  N  G  L  D  V  G  I  T  G -
     gtcgagatgttcgacatgtgttgaaagtgagcttggatttaacaagatatgagtactaca
 841 ---------+---------+---------+---------+---------+---------+ 900
       R  D  V  R  H  V  L  K  V  S  L  D  L  T  R  Y  E  Y  Y  T -
     ccatcggtagatattatccggagattgataggtacattccttatgatacattagttgatg
 901 ---------+---------+---------+---------+---------+---------+ 960
       I  G  R  Y  Y  P  E  I  D  R  Y  I  P  Y  D  T  L  V  D  G -
```

Figure 6.5. Nucleic acid and deduced amino acid sequences of the papaya invertase cDNA from immature green papaya (about two weeks before harvest) fruit tissue. The conserved amino acids that were used to make degenerate PCR primers are in underlined in bold.

```
      ggtgggcggggctccgacccgattatggaaattttatgcttccaagtcgtttttgatc
 961  ---------+---------+---------+---------+---------+---------+ 1020
       W  A  G  L  R  P  D  Y  G  N  F  Y  A  S  K  S  F  F  D  P
      ccaagacgaataggaggatactctggggttgggccaatgagtcggattcaagacaagatg
1021  ---------+---------+---------+---------+---------+---------+ 1080
       K  T  N  R  R  I  L  W  G  W  A  N  E  S  D  S  R  Q  D  D
      atgtcgacaagggttgggctggaattcagacaattccaaggaaagtgtggcttgacccaa
1081  ---------+---------+---------+---------+---------+---------+ 1140
       V  D  K  G  W  A  G  I  Q  T  I  P  R  K  V  W  L  D  P  S
      gtgggaagcagctcaggctgtggcctgttgaagaagtagagaagctgagaaaggatcctg
1141  ---------+---------+---------+---------+---------+---------+ 1200
       G  K  Q  L  R  L  W  P  V  E  E  V  E  K  L  R  K  D  P  V
      ttttgatggagaacacggctgttgaactgggtcagcatgttgaggtcaccggagtaactg
1201  ---------+---------+---------+---------+---------+---------+ 1260
       L  M  E  N  T  A  V  E  L  G  Q  H  V  E  V  T  G  V  T  A
      ctgcccagtgtgatgtggaggtagtttcacaattccaagcttggagaaagcagagtccgt
1261  ---------+---------+---------+---------+---------+---------+ 1320
       A  Q  C  D  V  E  V  V  S  Q  F  Q  A  W  R  K  Q  S  P  F
      ttgatccagagtgggtcaatgcacaagacctatgtgctccaatgggtgcaaagaaacagg
1321  ---------+---------+---------+---------+---------+---------+ 1380
       D  P  E  W  V  N  A  Q  D  L  C  A  P  M  G  A  K  K  Q  G
      gtggggttggaccatttgggctcttgacattagcctctgaagacttggaggaagcaactc
1381  ---------+---------+---------+---------+---------+---------+ 1440
       G  V  G  P  F  G  L  L  T  L  A  S  E  D  L  E  E  A  T  P
      ctgtcttcttcagagtcttcaaagctgataccaaatacgtagtcctcatgtgctctgatg
1441  ---------+---------+---------+---------+---------+---------+ 1500
       V  F  F  R  V  F  K  A  D  T  K  Y  V  V  L  M  C  S  D  A
      cttcaagttcctctttgaaggaaggtctttacaagccatcatttgctgggtttgtaaatg
1501  ---------+---------+---------+---------+---------+---------+ 1560
       S  S  S  S  L  K  E  G  L  Y  K  P  S  F  A  G  F  V  N  V
      tagatatagaagcagagaaaaggatctctcttaggagtttgattgatcattcagtcgttg
1561  ---------+---------+---------+---------+---------+---------+ 1620
       D  I  E  A  E  K  R  I  S  L  R  S  L  I  D  H  S  V  V  E
      aaagctttggagctggagggaaaacttgcataacttctagggtttaccctacaaaagcag
1621  ---------+---------+---------+---------+---------+---------+ 1680
       S  F  G  A  G  G  K  T  C  I  T  S  R  V  Y  P  T  K  A  V
      tggatggggaagctcacttgttcgtgttcaacaatgggactgaggctgtccacgtggaga
1681  ---------+---------+---------+---------+---------+---------+ 1740
       D  G  E  A  H  L  F  V  F  N  N  G  T  E  A  V  H  V  E  K
      agctcagtgcctggagcatgaacagaccactgaggatgaacaactgaagataattaagag
1741  ---------+---------+---------+---------+---------+---------+ 1800
       L  S  A  W  S  M  N  R  P  L  R  M  N  N  *
      aaatatcaagaggaggaaattaagattttagtacttcgtacgtcagtagtttgatcatc
1801  ---------+---------+---------+---------+---------+---------+ 1860
      ttgtttgtcgttgtagtttctgtagttttaatgaattaaatgctcctttagatttcact
1861  ---------+---------+---------+---------+---------+---------+ 1920
      aaaaaaaaaaaaaaaaaaaaa
1921  -------    +- 1941
```

Figure 6.5. (continued) Nucleic acid and deduced amino acid sequences of the papaya invertase cDNA from immature green papaya (about two weeks before harvest) fruit tissue. The putative poly (A) signal sequence is shown in *Italics* (aattaaa).

```
carrot1       MART.KILVF SSDSSLFLLS IFSFIF.... .LNINGVDST HRVFPELQS.
carrot2       MGVTIRNRNY DHGSLPFLQS LLAILLVTTT TLHINGVEAF HEIHYNLQS.
potato ....     ..MEIL RRSSSLWVLP ILLLCFF..I NNGVFVDAS. HKVYMHLQS.
Arabidopsis   .......... ..MSAPKFGY VLLLIV..LI NISNNGVDAF HKVFKKLQSK
PAPAYA             MSTAS KFYSVLTSTL FCYLAVITFI GTAINGVEAS HRIYPQFQ..

carrot1 .      ISAVDVKLV HRTGYHFQPQ KHWINDPNGP MFYKGYYHLF YQYNPKGSVW
carrot2       .VGAENVKQV HRTGYHFQPK QNWINDPNGP MYYKGVYHLF YQYNPKGAVW
potato        TTSHVDVSKV HRTGYHFQPP KNWINDPNGP MYYNGVYHLF YQYNPKGAIW
arabidopsis   STSLESVSPL HRTAYHFQPP RHWINDPNAP MLYKGVYHLF YQYNPKGAVW
PAPAYA        SLSVDIVDQI HRTAYHFQPP KHWINDPNAP MYYNGVYHLF YQYNPKGAVW
vacuolar                            KNWMNDPNG

carrot1       GNIVWAHSVS KDLINWIALE PAIFPSKPFD QYGCWSGSAT ILPGNKPVIL
carrot2       GNIVWAHSVS TDLINWTPLE PAIFPSKPFD KYGCWSGSAT ILPGNKPVIL
potato        GNIVWAHSVS KDLINWIPLE PAIYPSKVFD KYGTWSGSAT ILPGNKPVIL
arabidopsis   GNIVWAHSVS KDLINWEALE PAIYPSKWFD INGTWSGSAT HVPGKGPVIL
PAPAYA        GNIVWAHSVS TDLINWIPLK PAIVPSEPFD IKGCWSGSAT VLPNNIPIIL

carrot1       YTGIVSPDPE NAQVQNYAVP ANYSDPFLRE WVKPDNNPL. VGVHTENPSA
carrot2       YTGIVEGPPK NVQVQNYAIP ANLSDPYLRK WIKPDNNPLV VANNGENATA
potato        YTGIV..DAN KTQVQNYAIP ANMSDPYLRK WIKPDNNPLI VADKTINKSQ
arabidopsis   YTGITE...N QTQIQNYAIP QDLSDPYLKT WIKPDDNPIV KPDNGENGSA
PAPAYA        YTGLDS...N ETQLQNYAVP ANISDPHLEN WIKPANNPLV APDHTVNRTA

carrot1       FRDPTTAWFD ..GGHWKMLV GSSRKHRGIA YLYRS.KDFK KWKRSPHPIH
carrot2       FRDPTTAWLD .KSGHWKMLV GSKRNRRGIA YLYRS.KDFI KWTKAKHPIH
potato        FRDPTTAWMG .RDGNWRILV GSVRNHRGKV IMYKSNKNFM KWTKAKHPLH
arabidopsis   FRDPTTAWFN KKDGYWRMLV GSKRKNRGIA YMYKS.RDFK KWVKSKRPIH
PAPAYA        FRDPTTAWLG .SDGWWKMLV GNKNKRRGIA HLYKS.KDFM NWVKAKHPIH

carrot1       TKAETGMWEC PDFYPVSPRS EDG.LDNSKM GRGIKHVLKV SLNSTRYEYY
carrot2       SQANTGMWEC PDFFPVSLKG LNG.LDTSVT GESVKHVLKV SLDLTRYEYY
potato        SAPGTGNWEC PDFFPVSLKN KDG.LDTSYN GKDIKHVLKV SFDVTRFDHY
arabidopsis   SRKKTGMWEC PDFFPVSVTD KKNRLDFSYD GPNAKHVLKV SLDLTRYEYY
PAPAYA        SRPDTGMWEC PDFFPVPKSG  ENGLDVGIT GRDVRHVLKV SLDLTRYEYY
vacuolar       VPGTGMWEC VD

carrot1       TIGRYNRVRD FYVPDNTSVD GWAGLRYDYG NFYASKTFYD PIKKRRILWG
carrot2       TVGTYLTDKD RYIPDNTSVD GWAGLRYDYG NFYASKTFFD PSKNRRILWG
potato        TIGTYDTKKD KYFPDNTSID GWKGLRLDYG NYYASKTFFD SGKNRRILLG
arabidopsis   TLGTYDTKKD RYRPDGYTPD GWDGLRFDYG NYYASKTFFD DKTNRRILWG
PAPAYA        TIGRYYPEID RYIPYDTLVD GWAGLRPDYG NFYASKSFFD PKTNRRILWG
```

Figure 6.6. Multiple alignment of translated invertase protein N terminal region and conserved motif in different plant species. The amino acids (**M, V**) from vacuolar invertase were substituted by (**I, P**) in cell wall invertase, respectively. The vacuolar invertase conserved region was underlined (Davies et al., 1996). Cell wall invertase sequence alignment was done by the author. The data base accession numbers for cell wall invertase sequence are: *Arabidopsis*, U11033 (Mercier and Gogarten, 1995); carrot, GI 18324 (Sturm et al., 1990); potato, Z22645 (Hedley et al., 1994). The papaya cell wall invertase sequence is from the current work.

```
carrot1      WANESDSQID DVQKGWAGIQ LIPRRIWLDP SGRQLVQWPI EEVEGLRGSE
carrot2      WANESDSTAH DVAKGWAGIQ LIPRTLWLDP SGKQLMQWPI EELETLRGSK
potato       WANESDTVDN DVRKGWAGVH PIPRKIWLDP SGKQLVQWPV QELETLRKKK
arabidopsis  WANESDTVQD DTVKGWAGIQ LIPRTILLDS SGKQLVFWPI EEIESLRGKN
PAPAYA       WANESDSRQD DVDKGWAGIQ TIPRKVWLDP SGKQLRLWPV EEVEKLRKDP

carrot1      LHM.RNQKLD MGVHVEVTGI TAAQADVDAT FSFKSLDKAE SFDPEWINLD
carrot2      VKFSRKQDLS KGILVEVKGI TAAQADVEVT FSFKSLAKRE PFDPKWLEYD
potato       VQLN.NKKLN KGEKVEIKGI TVAQADVEVI FSFTSLDKAE PFDPSWADLY
arabidopsis  VQMT.NQKME MGQRFEVQGI TPAQVDVDVT FNVGNLEKAE KFDESFAT.K
PAPAYA       VLME.NTAVE LGQHVEVTGV TAAQCDVEVV SQFQAWRKQS PFDPEWVNAQ

carrot1      AQDVCDSMGS TIQGGLGPFG LLTLASKDLE EYTPVFFRIF K.AEDQKLKV
carrot2      AEKICSLKGS TVQGGVGPFG LLTLASEKLE EYTPVFFRVF K.VQN.THKV
potato       AQDVCAIKGS TVQGGLGPFG LLTLASKNLE EYTPVFFRIF K.AHD.KYKV
arabidopsis  PLELCNLKGS NVNGGVGPFG LITLATSDLE EYTPVFFRVF KDAASNKPKV
PAPAYA       ..DLCAPMGA KKQGGVGPFG LLTLASEDLE EATPVFFRVF KADT..KYVV

carrot1      LMCSDAKRSS LAEG...... ..LYKPSFRG FVDVDLS.DK KISLRSLIDN
carrot2      LMCSDATRSS LKEG...... ..LYRPSFAG FVDVDLATDK KISLRSLIDN
potato       LMCSDASRSS LKNETT.... ..MYKPSFAG YVDVDLA.DK KLSLRSLIDH
arabidopsis  LMCSDAKPSS LKKDTGTDAK ERMYKPSFAG FVDVGL.LDG KISLRSLIDH
PAPAYA       LMCSDASSSS LKEG...... ..LYKPSFAG FVNVDIEAE  KISLRSLIDH

carrot1      SVVESFGAQR KNLISSRVYP TLAIYNNAHL FVFNNGTEPI TVDNLDAWSM
carrot2      SVVESFGAKG KTCISSRVYP TLAVYENAHL YVFNNGSETI TVENLDAWSM
potato       SVVESFGAGG KTCITSRVYP TLAIFDKAHL FAFNNGAERI TIETLNAWSM
arabidopsis  SVVESFGAKG KTVITSRVYP TKAVGEKAHL FVFNNGSQPV TVESLNAWNM
PAPAYA       SVVESFGAGG KTCITSRVYP TKAVDGEAHL FVFNNGTEAV HVEKLSAWSM

carrot1      NSPSEMN....
carrot2      KKPLRMN....
potato           ANAKLH
arabidopsis  QKPLKMNQGA K
PAPAYA       NRPLRMNN
```

Figure 6.6. (Continued) Multiple alignment of translated invertase protein N terminal region and conserved motif in different plant species. The amino acids (**M, V**) from vacuolar invertase were substituted by (**I, P**) in cell wall invertase, respectively. The vacuolar invertase conserved region was underlined (Davies et al., 1996). Cell wall invertase sequence alignment was done by the author. The data base accession numbers for cell wall invertase sequence are: *Arabidopsis*, U11033 (Mercier and Gogarten, 1995); carrot, GI 18324 (Sturm et al., 1990); potato, Z22645 (Hedley et al., 1994). The papaya cell wall invertase sequence is from the current work.

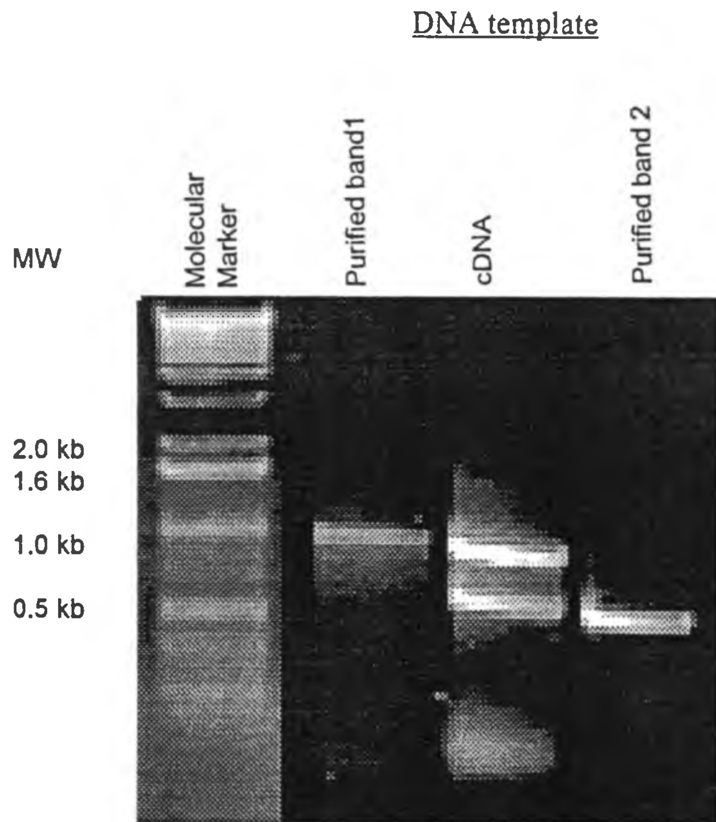



Figure 6.7. PCR-generated SS DNA fragments using purified PCR products or a cDNA library from immature green papaya fruit as templates.

```
      tcctgatactggtggacaggttgtctacatcttggatcaagtccgtgcattggaaactga
  1 ---------+---------+---------+---------+---------+---------+ 60
       P  D  T  G  G  Q  V  V  Y  I  L  D  Q  V  R  A  L  E  T  E  -
      gatgcttcaacgtataaagcagcaaggccttaatatcactccacgaatcctcattattac
 61 ---------+---------+---------+---------+---------+---------+ 120
       M  L  Q  R  I  K  Q  Q  G  L  N  I  T  P  R  I  L  I  I  T  -
      ccggttgctccctgatgcggtggggactacttgtggccagcgtatggagaaagtatatgg
121 ---------+---------+---------+---------+---------+---------+ 180
       R  L  L  P  D  A  V  G  T  T  C  G  Q  R  M  E  K  V  Y  G  -
      caccgagtattcagatattcttcgtgttccctttagaaccgagaagggaattgttcgaca
181 ---------+---------+---------+---------+---------+---------+ 240
       T  E  Y  S  D  I  L  R  V  P  F  R  T  E  K  G  I  V  R  Q  -
      atggatctcacgatttgaagtctggccttacctagagacctccactgaggatgttgcaac
241 ---------+---------+---------+---------+---------+---------+ 300
       W  I  S  R  F  E  V  W  P  Y  L  E  T  S  T  E  D  V  A  T  -
      cgaaatttctaaagagttacagggcaagcctgaccttatcattgggaactacagtgatgg
301 ---------+---------+---------+---------+---------+---------+ 360
       E  I  S  K  E  L  Q  G  K  P  D  L  I  I  G  N  Y  S  D  G  -
      aaacattgttgcctctttattggctcataaactgggggtcactcagtgtaccatcgctca
361 ---------+---------+---------+---------+---------+---------+ 420
       N  I  V  A  S  L  L  A  H  K  L  G  V  T  Q  C  T  I  A  H  -
      cgcccttgagaagaccaagtatcctgattcagatatttactggaaaaagcttgaggacaa
421 ---------+---------+---------+---------+---------+---------+ 480
       A  L  E  K  T  K  Y  P  D  S  D  I  Y  W  K  K  L  E  D  K  -
      ataccatttctcttgccagttcacagcggatcttatagccatgaaccacacagattttat
481 ---------+---------+---------+---------+---------+---------+ 540
       Y  H  F  S  C  Q  F  T  A  D  L  I  A  M  N  H  T  D  F  I  -
      tattacaagtacttaccaagaaattgccggaagcaaggacactgttggtcagtatgagag
541 ---------+---------+---------+---------+---------+---------+ 600
       I  T  S  T  Y  Q  E  I  A  G  S  K  D  T  V  G  Q  Y  E  S  -
      tcactcggctttcactcttcctggactctaccgtgtcgttcatgggattgacgtgtttga
601 ---------+---------+---------+---------+---------+---------+ 660
       H  S  A  F  T  L  P  G  L  Y  R  V  V  H  G  I  D  V  F  D  -
      ccccaagttcaacattgtgtcccccggtgctgatatgagcatctacttcccatacaccga
661 ---------+---------+---------+---------+---------+---------+ 720
       P  K  F  N  I  V  S  P  G  A  D  M  S  I  Y  F  P  Y  T     -
```

Figure 6.8. Nucleotide sequence of cloned SS cDNA fragment from immaturure green papaya fruit.

total (presence of NaCl) Invertase activities were very low two months before harvest and increased in parallel 6 weeks before harvest fruit. However, buffer soluble-invertase activity increased one month to two weeks before color break, decreased then rapidly increased again during ripening. The total invertase activity increased steadily from the pre-maturation to full ripen stage. The total invertase activity was 4.7-fold higher than buffer soluble invertase activity in the fruit at one month before harvest and 18-fold higher at color break. Total invertase activity was only 6% higher than soluble activity in fully ripe fruit because soluble invertase activity increased 26-fold when total invertase activity increased 1.5-fold during 7 to 10 days postharvest period. Increased soluble invertase activity during postharvest ripening did not correlate with the sugar content (Chapter 4).

6.3.5 Western blot analysis of invertase protein levels during papaya fruit development

Invertase protein levels in papaya fruit at different development stages were determined by Western blots analysis using antisera raised against carrot cell wall invertase and soluble

Table 6.1. The effect of 1M NaCl and EDTA on the extraction of invertase enzyme*.

| Resuspension buffer | Relative pellet activity |
|---|---|
| Desalt buffer + 1 M NaCl | 60 |
| Desalt buffer +10 mM EDTA | 0 |
| Desalt buffer + 1 M NaCl + 10 mM EDTA | 100 |

* Desalt buffer components (refers to Material and Methods). The pellet was washed and centrifuged twice (8ml + 12 ml, for 3 g tissue) using desalt buffer after decanting the first supernatant (15 ml), then it was resuspended in 9 ml desalt buffer and steriled at 1 $^{o}$C over night. The extracted pellet was desalted the next day in a similar manner as for the supernatant. Fruit maturity: about two weeks before color break. Each data point represents the means of three replications.

Table 6.2. The effect of extraction buffer components* on invertase enzyme activity.

| Experiment 1 | Relative invertase activity | | |
|---|---|---|---|
| | Supernatant | Pellet (+2X washes) | Supernatant plus Pellet |
| Buffer 1 | 100 | 9.6 | 109.6 |
| Buffer 2 | 78.2 | 24.6 | 102.8 |
| **Experiment 2** | Invertase activity (µmol. $h^{-1}$ $g^{-1}$ FW) | | |
| | Supernatant | Pellet (No additional wash) | Total |
| Buffer 1 | 92.645 | 21.779 | 114.424 |
| Buffer 2 | 49.629 | 21.83 | 71.459 |
| | Supernatant plus Two Washes | Pellet (+2X washes) | Total |
| Buffer 1 | 117.395 | 1.218 | 118.61 |
| Buffer 2 | 79.199 | 7.163 | 86.362 |

*Buffer 1= regular extraction buffer, (refer to Material and Methods), Buffer 2 = desalting buffer. The pellets from each treatment were resuspended in buffer 1 plus 1 M NaCl, stirred at 1°C over night and desalted before activity has assayed. Cultivar: 'Sunset", maturity: immature green, about one week before harvest. Each data point represents the means of three replications.

Table 6.3 Comparison of acid invertase enzyme activity using different extraction buffers (pH 5.0 vs pH 7.5, -NaCl vs +NaCl)*.

| | Invertase activity (μmol. sucrose $g^{-1}$ $h^{-1}$ FW) | |
|---|---|---|
| pH | -NaCl | +NaCl |
| 5.0 | 46.15 ± 10.0 | 119.495 ± 14.496 |
| 7.5 | 12.009 ± 6.976 | 327.915 ± 21.423 |

* All extractions sit at 1°C for 0.5 hour before filteration and centrifugation. Fruit maturity: immature green, about two weeks before color break. The data represents means of three replications ± standard deviation.

```
PAPAYA     PDTGGQVVYI LDQVRALETE MLQRIKQQGL NITPRILIIT RLLPDAVGTT
SOYBN 90%  PDTGGQVVYI LDQVRALENE MLHRIKQQGL DIVPRILIIT RLLPDAVGTT
ARA   90%  PDTGGQVVYI LDQVRALEIE MLQRIKQQGL NIKPRILILT RLLPDAVGTT
PHAAU 90%  PDTGGQVVYI LDQVRALENE MLHRIKQQGL DIVPRILIIT RLLPDAVGTT
VICFA 90%  PDTGGQVVYI LDQVRALESE MLNRIKKQGL DIVPRILIIT RLLPDAVGTT
MAIZE 85%  PDTGGQVVYI LDQVRALENE MLLRIKQQGL DITPKILIVT RLLPDAAGTT


PAPAYA     CGQRMEKVYG TEYSDILRVP FRTEKGIVRQ WISRFEVWPY LETSTEDVAT
SOY BEAN   CGQRLEKVFG TEHSHILRVP FRTEKGIVRK WISRFEVWPY LETYTEDVAT
ARA        CGERLERVYD SEYCDILRVP FRTEKGIVRK WISRFEVWPY LETYTEDAAV
PHAAU      CGERLEKVFG TEHSHILRVP FRTENGIVRK WISRFEVWPY LETYTEDAAH
VICFA      CGQRLEKVYG TEHCHILRVP FRDQKGIVRK WISRFEVWPY LETYTEDVAT
MAIZE      CGQRLEKVIG TEHTDIIRVP FRNENGILRK WISRFDVWPY LETYTEDVSS

PAPAYA     EISKELQGKP DLIIGNYSDG NIVASLLAHK LGVTQ CTIAH ALEKTKYPDS
SOY BEAN   ELAKELQGKP DLIVGNYSDG NIVASLLAHK LGVTQ CTIAH ALEKTKYPES
ARA        ELSKELDGKP DLIIGNYSDG NLVASLLAHK LGVTQQCTIAH ALEKTKYPDS
PHAAU      ELAKELQGKP DLIVGNYSDG NIVASLLAHK LGVTQ CTIAH ALEKTKYPES
VICFA      ELAKELQGKP DLIVGNYSDG NIVASLLAHK LGVTQ CTIAH ALEKTKYPES
MAIZE      EIMKEMQAKP DLIIGNYSDG NLVATLLAHK LGVTQ CTIAH ALEKTKYPNS


PAPAYA     DIYWKKLEDK YHFSCQFTAD LIAMNHTDFI ITSTYQEIAG SKDTVGQYES
SOY BEAN   DIYWKKLEER YHFSCQFTAD LFAMNHTDFI ITSTFQEIAG SKDTVGQYES
ARA        DIYWKKLDDK YHFSCQFTAD IFAMNHTDFI ITSTFQEIAG SKETVGQYES
PHAAU      DIYWKKLEER YHFSCQFTAD LFAMNHTDFI ITSTFQEIAG SKDTVGQYES
VICFA      DIYWKKFEDK YHFSCQFTAD LFAMNHTDFI ITSTFQEIAG SKDTVGQYES
MAIZE      DIYLDKFDSQ YHFSCQFTAD LIAMNHTDFI ITSTFQEIAG SKDTVGQYES


PAPAYA     HSAFTLPGLY RVVHGIDVFD PKFNIVSPGA DMSIYFPYT
SOY BEAN   HTAFTLPGLY RVVHGIDVFD PKFNIVSPGA DQTIYFPHT
ARA        HTAFTLPGLY RVVHGIDVFD PKFNIVSPGA DMSIYFPYT
PHAAU      HSAFTLPGLY RVVHGIDVFD PKFNIVSPGA DQTIYFPYT
VICFA      HTAFTLPGLY RVVHGIDVFD PKFNIVSPGA DQTIYFPYT
MAIZE      HIAFTLPGLY RVVHGIDVFD PKFNIVSPGA DMSVYYPYT
```

Figure 6.9. Amino acid sequence of putative SS fragment aligned with SS from other plant species. The database accession numbers for the sequnces used were: soy bean (SOYBN), sp p13708 (Zhang, and Chollet, 1997); thale cress (ARA), sp p49040 (Martin et al., 1993); mung bean (PHAAU), q01390 (Arai, et al., 1992); fava bean (VICFA), p31926 (Kuster, et a., 1993); maize, p04712 (Werr, et al., 1995).

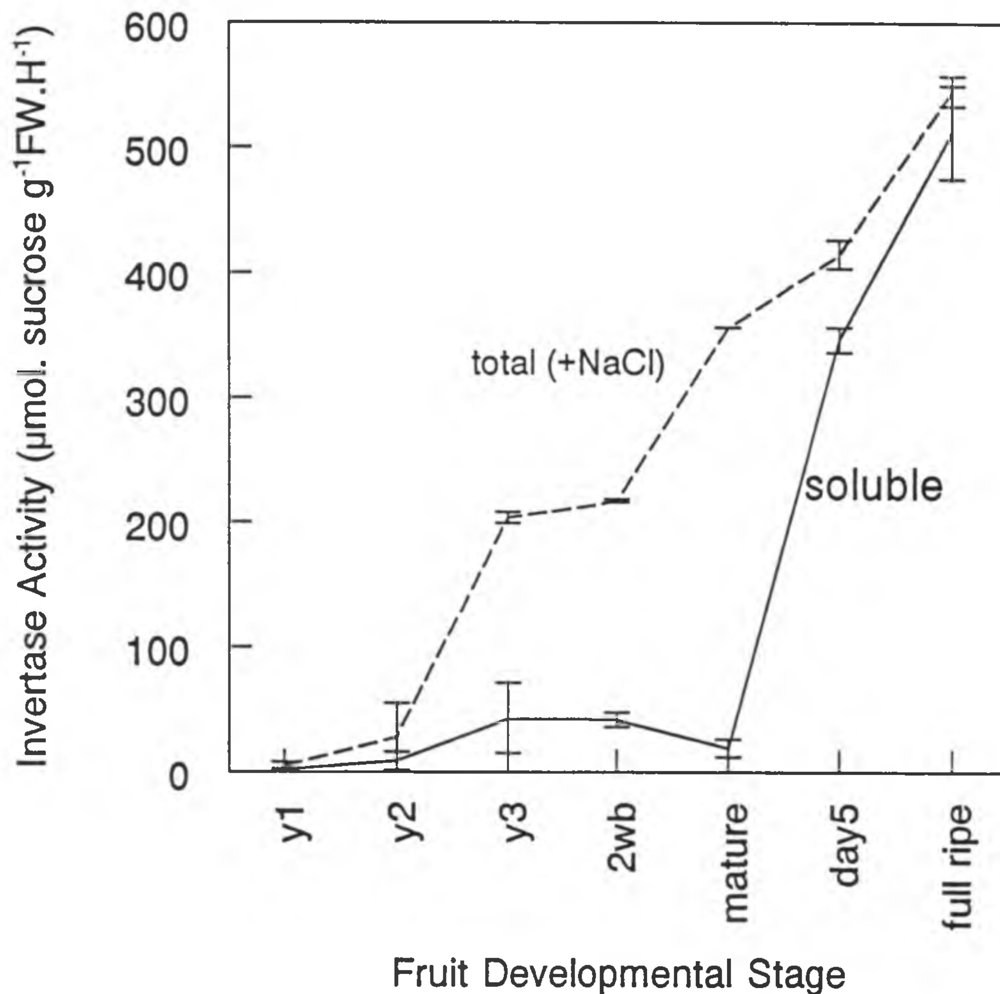



Figure 6.10. Invertase activities (reducing sugars $\mu$mol $g^{-1}$fresh weight $h^{-1}$, pH 5.0, 23°C) during fruit development (refer to Figure 6.1). Total protein was extracted from different fruit developmental stages in the presence (+NaCl, total invertase) and the absence (-NaCl, buffer soluble invertase) of 1M NaCl. Each data point represents at least two individual extractions. Vertical bars indicate standard deviations.

invertases (Figure 6.11). The results indicate that increased invertase enzyme activity *in vitro* paralleled invertase protein level *in vivo* and the major invertase in papaya fruit was the cell wall isoform. The low invertase activity in young fruit was associated with low invertase protein. Antiserium against the carrot soluble invertase 43 kDa and 25 kDa subunits cross-reacted with papaya invertase protein only when hybridization was carried out at 30 °C. A much stronger signal was detected using carrot cell wall invertase antibody in the same hybridization conditions or at room temperature. The strongest band recognized by the carrot cell wall invertase antibody was at about 73 kDa (Figure 6.11). Two weak bands observed at about 53 kDa and 30 KDa could be minor invertase isoforms or invertase degradation products.

### 6.3.6 Northern blot and RT-PCR analysis of invertase mRNA levels during fruit development

The abundance of invertase mRNA (Figure 6.12 & 6.13) increase as the invertase activity increased, however, the activity increase to a greater extend than mRNA abundance (Figure 6.10). The increased invertase protein levels and enzyme activity paralleled the increased mRNA levels from young fruit to the immature green stage (two weeks before harvest fruit) and in mature fruit (0-30% skin yellowing) (Figure 6.12A). The finding was consistent with RT-PCR results (Figure 6.13). After 9 separate experiment of RT-PCR amplification, there was a greater possibility of amplifying a invertase fragment from the fruit two weeks before harvest than from green fruit (about 100 DAA) and full ripe fruit.

### 6.3.7 Northern blot analysis of SS mRNA levels during fruit development

In contrast to the results of invertase mRNAs, SS mRNA levels declined from the young fruit to mature fruit (Figure 12B) and correlated with thchanges in the enzyme activity. However, SS mRNA declined to a very low level in fruit about 100 DAA, but the enzyme activity remained fairly constant suggested that a post-translational mechanism must exist in controlling SS activity.

### 6.3.8 Northern blot analysis of invertase and SS mRNA levels in different tissues in 'Sunset' papaya plant

Invertase gene mRNA abundance was higher in young leaves, and flowers than in thepetiole, root and seed tissue (Figure 6.12A). However, SS mRNA abundance was highest occurred in young fruit and petioles, but was lower in the stem, flower and root and other tissues, especially fruit (Figure 6.12B). The smaller mRNA observed in the leaf sample, was probably either a degradation or a non specific mRNA, since some RNA molecular marker bands were also detected by the probe. The differential expression of the invertase and SS genes in papaya sink tissue indicated that they play different roles in papaya plant growth, fruit development and sugar accumulation.

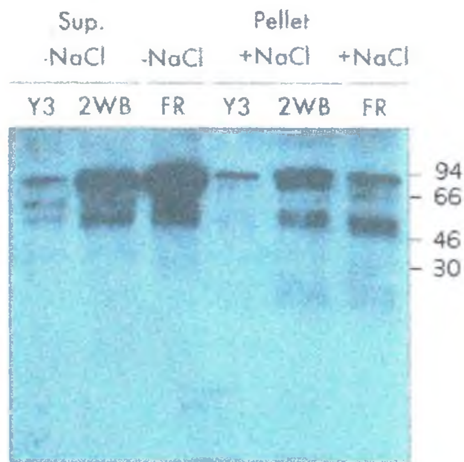
### 6.3.9 Southern blot analysis

Southern blot analysis using restriction digested DNA from 'Sunset' papaya leaves and antisense riboprobes for invertase and SS gene fragments, was undertaken (Figure 6.14). The weak signal and single bands indicated that both SS and invertase was coded by a low copy number or single copy gene.

## 6.4 Discussion

PCR was used to obtain gene-specific cDNA fragments that were used to screen a cDNA library from immature green papaya fruit. Only one invertase cDNA was isolated using this method. This suggested that one cell wall invertase gene was expressed in the developing papaya fruit. Several lines of evidence demonstrated that the putative invertase gene was localized to the cell wall. The protein sequence derived from the cDNA had higher homology to cloned extracellular invertase than to intracellular invertases. and is 67% identical at the amino acid level (from 19 to 581) with carrot cell wall invertase (Sturm et al., 1990. GI 18324). A leader sequence, specific sequence elements, and a high PI have been proposed as characteristic properties of extracellular invertase (Roitsch, et al., 1995). The papaya invertase cDNA sequence isolated in this study contained all the necessary elements for extracellular invertase

A.




B.

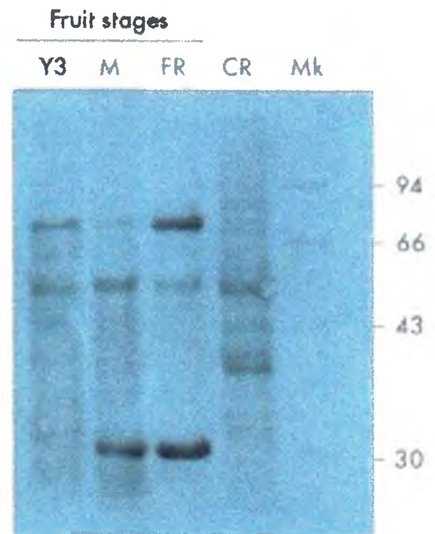



Figure 6.11. Western blot analysis of invertase protein levels during papaya fruit development. A: Carrot cell wall invertase antibody. Protein loaded was 50 μg/lane. Y3, about four weeks before harvest. 2WB, two weeks before harvest. FR, full ripe fruit. + NaCl FR, 1M NaCl was added into extraction buffer. B: Carrot soluble invertase antibody. 40 μg/lane for papaya protein loaded. M, color break, harvest maturity. CR, protein extracted from carrot root, 20 μg/lane.

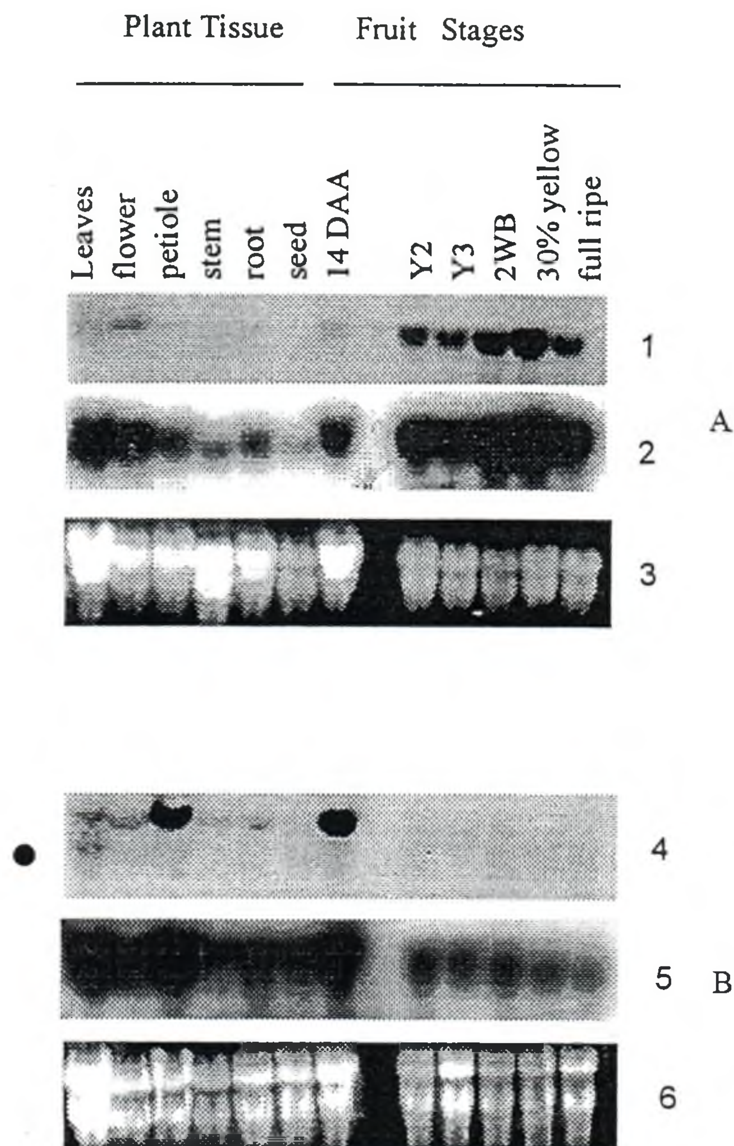



Figure 6.12. Northern analysis of cell wall invertase and SS gene expression in different plant tissues and fruit developmental stages. Gene specific probes for invertase (A) and SS (B) were hybridized to 10 μg total cell RNA separated by electrophoresis on denaturing formaldehyde 1% agarose gels. 1, 4: mRNA band was detect from different tissue samples. 2, 5: longer exposure. 3, 6: the ethidium bromide-stained rRNA included as mRNA references. • = non specific hybridization or degradation product.

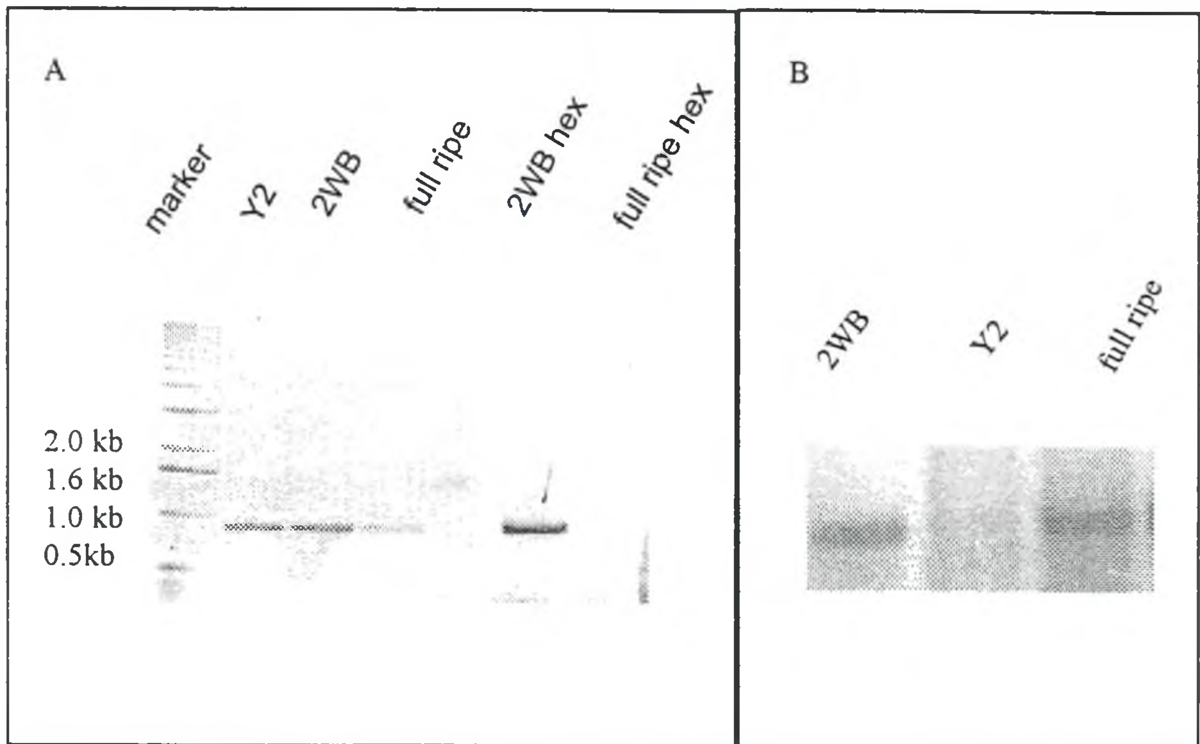



Figure 6.13. Analysis of invertase mRNA abundance using TR-PCR. Electrophoresis on 1% agarose gel of RT-PCR invertase gene expression results. A: lane 1, molecular weight markers. Lanes 2 to 4, first strand cDNA synthesized using Oligo dT primer from total RNA extracted from stages immature (Y2), 2 weeks before harvest (2WB) and full ripe papaya fruit. Lane 5 and 6, first strand cDNA synthesized using random hexamer primer from total cell RNA prepared from fruit mesocarp 2 weeks before harvest and full ripe papaya. B: Repeat of PCR results using the same first stand cDNA in panel A lanes 2 to 4, indicating that Y2 and full ripe stage fruit contain less invertase mRNA than the 2 weeks before harvest stage.

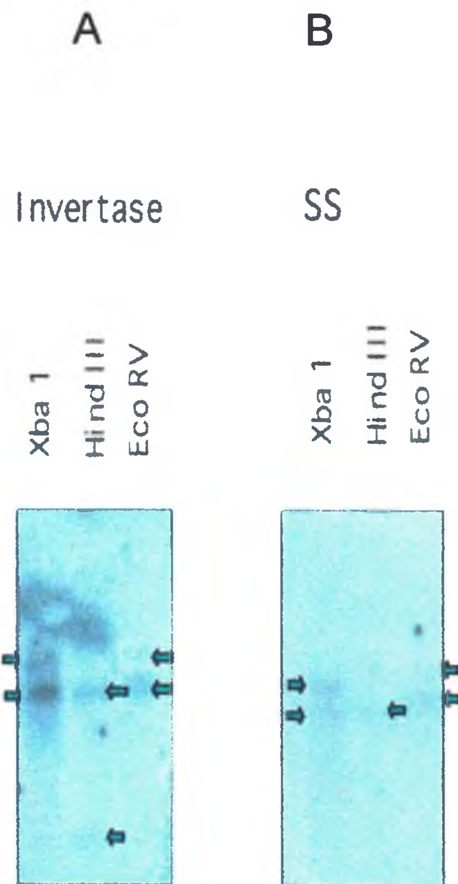



Figure6.14. Genomic Southern blot analysis of papaya cell wall invertase and SS gene. Genomic DNA (10μg) from 'Sunset' leaves was digested with either X *baI* (1), Hind III (lane 2), or E *coR* V (lane 3). The DNA were fractionated by electrophoresises in 0.8% agarose (TAE) gel, transferred to nitrocellular memebrane and probed with a $\alpha$ $^{32}P$ -labelled invertase (A) or SS (B) DNA fragment, respectively.

(Figure 6.5 & 6.6). The calculated isoelectric point of papaya invertase was 6.94, and was lower (0.49~3.22) than known extracellular invertase but higher (0.8~1.49) than known putative vacuolar invertase (Davies et al., 1996). Ehness and Roitsch (1997) also found a putative extracellular invertase gene that has a calculated isoelectric point of 6.2.

Multiple invertase genes have been found in tomato (Klann et al., 1992; Ohyama et al., 1992; Elliott et al., 1993; Okio et al., 1994; Godt and Roitsch, 1997), potato (Hedley et al., 1994; Zhou 1994), maize (Koch et al., 1996), *Chenopodium* (Roitsch et al., 1995, Ehness and Roitsch, 1997) and carrot (Sturm 1996), grape (Davies et al., 1996), and Arabidopsis (Mercier and Gogarten, 1995; Haouszine-Takvorian et al., 1997). A single band in Northern and Southern analysis suggested that the invertase gene was present as a low or single copy number gene in the papaya genome (Figure 6.13). Tomato has the same coding sequence, a slightly different promoter sequence, and is missing some repetitive sequences and 3' untranslated sequence from other species (Elliott et al., 1993). The deletion of the mRNA 5' UTR region in one papaya invertase clone may also indicate the existence of different control mechanisms or different mature proteins, which also occurs for yeast invertase (Carlson and Botstein 1982). It was not clear whether the deleted sequences in one clone was the result of RNA splicing or to multiple promoters.

Antisera (generously provided by Dr. Sturm) immuno-specific for the cell wall and soluble invertase isoforms from carrot were used to distinguish between their papaya homologous. No difference in protein size was detected between immature and full ripe fruit stage, which suggested that they probably were the same invertase protein forms and the increased solubility during fruit ripening was probably associated with the cell wall degradation rather than changes of isoform or subcellular location. A 6-fold increase in water- pectin soluble (cell wall material) have been found during papaya fruit ripening (Qiu, 1992). The result was consistent with the fact that soluble invertase activity increased about 26-fold during ripening, but soluble sugar composition was not affected. However, the presence of vacuolar invertase or neutral invertase in the ripe papaya tissue cannot be excluded, since the enzyme could be

inhibited *in vivo* either by fructose or by an another inhibitor. There was no direct evidence regarding the presence of a papaya invertase inhibitor, though invertase inhibitors have been previously reported from other plants (Pressey, 1994; Weil et al., 1994; Weil and Rausch, 1994; Greiner et al., 1998).

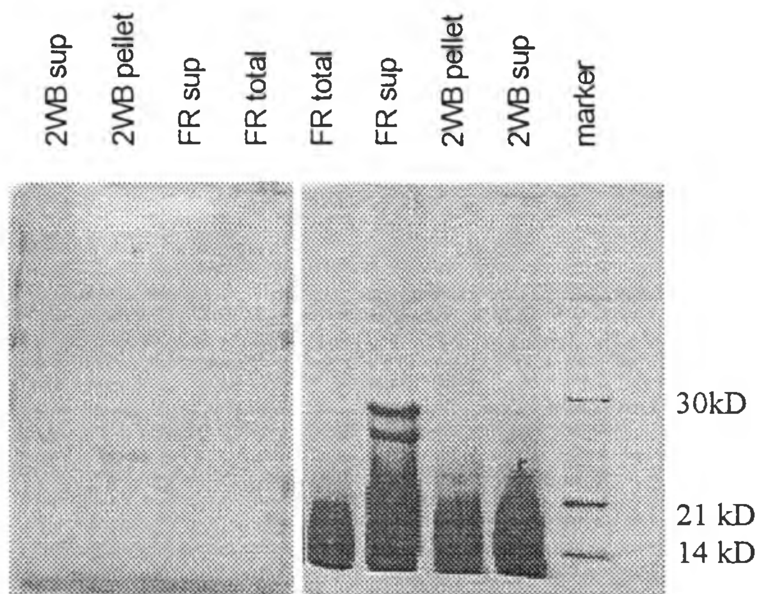
The fast migrating proteins detected by the carrot invertase antibody suggested that papaya invertase was composed of two subunits, one about 30 kDa and the other about 53 kDa. The major band at 73 kDa was the mature protein. Multiple invertase subunits have been reported in tomato (Bucheli et al., 1994; Yelle et al., 1991), carrot (Unger, et al., 1992), potato (Bracho and Whitaker, 1989) and mung bean (Arai et al., 1992), and melon fruit (Iwatsubo et al., 1992) when β-methocapethanol was presented in the SDS loading buffer. In papaya, additonal β-methocapethanol did not increase the presence of lower molecular bands (Figure 6.14). The lower molecular band detected in the buffer-insoluble portion could be a different isoform or a newly synthesized protein. Another possibility was that the very high protease (papain) in papaya fruit flesh resulted in protein degradation. The mature protein molecular mass of papaya fruit invertase has been reported 275 kDa by gel filtration (Chan et al., 1976) and 52 kDa in non-denatured gel electrophoresis (Lopez et al., 1988). However, in our experiment, when the protein was loaded for electrophoresis without denaturing by boiling, only the low molecular weight protein was recognized by the carrot cell wall invertase antiserum (Figure 6.14). The non-denatured glycosylated peptide could have effectively shielded the protein from antibody binding (Weil and Rausch 1994).

When the extracellular invertase is active, sucrose is hydrolyzed into hexose monomers (McCollum et al., 1988). Thus, the transmembrane sucrose gradient between the phloem and cell wall is maintained or increased to drive pholem unloading of sucrose into the apoplast (cf. Sturm 1990). A high extracellular invertase activity increases sucrose transport to the sink organs and thus may increased or maintained sink strength (Morris, 1982; Ho, 1984). Invertase mRNA levels were higher in the late stage of fruit development than in younger fruit and stems, roots and petioles (Figure 6.12) suggesting that cell wall invertase may contribute to

carbohydrate partitioning between source and sink tissue during sink maturation and ripening. Western blots (Figure 6.11) and invertase activity assays in the presence of 1 M NaCl in the extraction buffer (Figure 6.10) further confirmed that cell wall invertase was a major form during the late stage of papaya fruit development. The increased invertase mRNA levels and protein levels coincident with increased invertase activities suggested that invertase in papaya fruit was probably regulated at the transcriptional posttranscriptional, as well as translational posttranslational levels. That is agreed with the model proposed by Eschrich (1980) for phloem unloading.

Sugar regulate the expression of invertase and SS genes in maize and other species. (Koch et al. 1996). Cell wall invertase activity in papaya seemed to also be regulated by the availability of assimilate supply. Previous experiments indicated that removal of fruit trigged an earlier increase in invertase activity. Defoliation, which reduced assimilate supply, delayed invertase enzyme activity relative to control during the late stage of fruit development. SS gene expression, in contrast, was highest in young fruit and decreased as the fruit developed. This decline in activity paralleled the decline in mRNA level. A post-translational mechanism may also exist to control SS activity at the late stage of fruit development. When SS mRNA declined to a very low level, enzyme activity remained fairly constant. This could be explained by slow turn over of the enzyme. The different expression patterns of invertase and SS in the sink tissues indicated that SS is a predominant enzyme in young fruit and petioles, while invertase is a more important enzyme in the young leaves, flowers and fruit with regard to sugar accumulation. The result implied that unloading pathway in the papaya fruit changed from symplastic in young fruit to apoplastic during sugar accumulation.

A




B

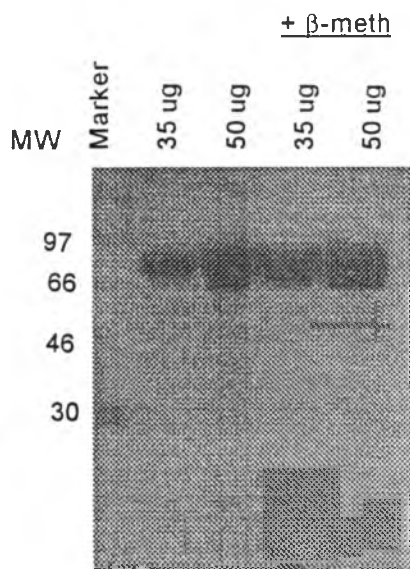



Figure 6.15. SDS-PAGE (A, right gel) and western blot (A, left and B) aanlysis of papaya invertase protein. A: Proteins were loaded without heat denaturing. 40μg/lane. B: Soluble proteins from full ripe papaya fruit were loaded after 3 min boiling with or without addition of β-methocaptoethanol.

# CHAPTER 7

## SUMMARY

Developing papaya fruit were strong sinks that imported carbohydrates from leaf (source) to fruit continually until harvest. The relationship between papaya fruit growth, respiration, sugar accumulation and the activities of SPS, SS, invertase enzyme were investigated. The growth of 'Sunset' papaya showed a double sigmoid growth curve in terms of the increase in fruit mass, length and diameter (Figure 4.2). The relationship between fruit fresh mass and fruit length and diameter was expressed as a linear regression after log transformation (Log fruit mass = 3.1253 X(log fruit length) - 0.9525, $r^2$ =0.97; Log fruit mass = 2.583 X (log fruit diameter) + 0.2513, $r^2$ =0.99, respectively, p = 0.0001). Fruit flesh dry mass percentage decreased from 14 to 56 DAA, then remained constant from 56 to 112 DAA, and then rapidly increased one month before harvest. Sugar accumulation in fruit flesh and dry matter growth rate increased after seed maturation. Fruit respiration rate decreased from 48 ml $CO_2$ $h^{-1}$ $Kg^{-1}$ in fruit 14 DAA to 12 ml $CO_2$ $h^{-1}$ $Kg^{-1}$ at 70 DAA, then remained constant until the fruit skin showed color break. Fruit sugar began to increase about one month before harvest, with a 40 to 50% of the total sugars as sucrose. SPS activity remained very low throughout fruit development and increased only slightly before harvest. SS activity was very high in 14 DAA fruit and decreased to less than 1/3 within 42 to 56 days, and remained constant during rest of fruit development. Acid invertase activity was very low in the young fruit and increased more than 10-fold 42 to 14 days before maturation. The development of flesh color in papaya fruit (Sunset) was correlated with sugar levels in the same tissue. The regression model between fruit flesh 'a' color and total soluble solids in full ripe fruit was CIE a = -0.2767 $(TSS)^2$ + 7.49144 (TSS) -32.708, (p=0.0001, $r^2$ =0.67, n=213).

SS activity was highly correlated with respiration ($r^2$ =0.9511, p=0.00003) and invertase was associated with sugar accumulation ($r^2$=0.738, P=0.05) during the last phase of fruit development. The relationship between enzyme activity and calculated fruit flesh carbon import

rate suggested that SS and acid invertase were the two major enzymes that determined papaya fruit sink strength in the early and late development phase, respectively.

Comparison of low sugar accumulation fruit type (UH801) with the commercial Solo types in sugar levels and enzyme activities demonstrated that the differences in invertase activities could account for the difference in final fruit sugar levels.

SPS, SS enzyme activities decreased and acid invertase activity significantly increased during postharvest ripening of papaya. The activity of these enzymes in ripe papaya tissue were not correlated with sugar composition and levels.

Source-sink balance was critical for fruit set, development and sugar accumulation The effect of defoliation and fruit thinning on fruit growth and sugar accumulation depended upon the time of defoliation and the number of leaves, or fruit removal, weather and cultivars. In papaya. 50% defoliation did not significantly alter new fruit set and ripe fruit TSS, 75% defoliation significantly reduced new flowers and fruit set, and decreased ripe fruit TSS. Removal of leaves in a spiral reduced the number of new flower and fruit set more than the defoliation from the bottom to top. Fruit thinning increased new fruit set and ripe fruit TSS level in 'Line-8', and 'Sunset' but not 'Kapoho'. Fruit thinning of 'Line-8' also increased young fruit TSS and sugar levels on the remaining fruit on plants compared to same aged fruit on the control and defoliated plants. There was no significant difference between defoliated and non-defoliated treatment in fruit production, average fruit mass, and percentage of fruit flesh and seed, seed mass ratio and seed dry mass during the test period.

Continual defoliation resulted in lower new fruit set, smaller fruit size, and lower TSS during the 168 days experimental period. In contrast, there were 52% and 100% more new fruit on the fruit removal treatment plants than on control plants within the first 56 days and 168 days, respectively. Larger fruit size, faster fruit development, lower respiration and higher sugar contents were observed in the immature fruit (140, 154, and 175 DAA) picked from the fruit removal treatment. Fruit removal plus defoliation gave the same number and mass of new fruit as the control and slightly lower TSS in mature fruit than in control.

Correlation analysis between fruit sugar and the enzyme activities during fruit removal and continual defoliation treatments further demonstrated that invertase was possibly responsible for sugar accumulation during the final stage of fruit development (r=0.833, p=0.001, invertase enzyme activity one stage earlier than sugar). Invertase activity varied with different extraction buffers, and it depended upon pH and extraction method. Total invertase activity increased continually from pre-maturation to the full ripe fruit stage. The total invertase activity was 4.7 fold higher than the buffer soluble invertase activity in the fruit one month before harvest and 18 fold higher in color break fruit, respectively. While total invertase activity was only 6% higher than soluble activity in full ripe fruit. The solubility of invertase dramatically changed after fruit was harvested during ripening. The soluble invertase increased 26-fold while total invertase activity increased only 1.5-fold during the 7 to 10 days postharvest. Carrot cell wall invertase antibody recognized three proteins of papaya invertase with one major of 73 kDa and two minor proteins at 53 and 30 kDa. Western blot analysis confirmed the increased invertase activity in vitro was partially the result of increase in invertase protein. No molecular mass difference was observed in the fractions of buffer soluble and pellet and suggested that the change of solubility was probably due to cell wall degradation during fruit ripening rather than change the location of invertase.

A cDNA library from immature green papaya fruit was constructed and a putative complete invertase cDNA was isolated and characterized. The complete deduced amino acid sequence of papaya invertase had an open reading frame that encoded a polypeptide chain of 582 residues and calculated molecular weight of 65, 684 Da. The protein was highly homologous to known plant cell wall invertase and 67% identical at the amino acid level with carrot cell wall invertase. The calculated iso-electric point was 6.94.

A SS gene fragment was isolated from papaya fruit two weeks before maturation and was highly homologous to a SS gene in A. *glutinosa* (X92378, 81% identical) and of many other species. The cloned fragment was used as a radioactive probe for Northen analysis.

Invertase mRNA levels were very low in 14 DAA fruit and higher in fruit two weeks before harvest than six to four weeks before harvest and in full ripe fruit. The highest invertase mRNA levels were observed in 30% yellow fruit. The mRNA levels in the late stage of fruit development were coincident with invertase protein levels and *in vitro* enzyme activity. SS gene expression, was highest in young fruit and decreased during fruit development and also paralleled SS activity *in vitro*. The different expression patterns of invertase and SS in all tested sink tissues confirmed that SS is a predominant enzyme in young fruit, and petioles while invertase is a more important enzyme in fruit sugar accumulation and the young leaf and, flower. Southern blot analysis indicated that both invertase and SS genes were coded by a low or single copy number genes.

The results of above suggested that cell wall invertase be a key enzyme involved in sugar unloading during late fruit development stage. A model for the accumulation of sugars in papaya fruit is given in figure 7.1.

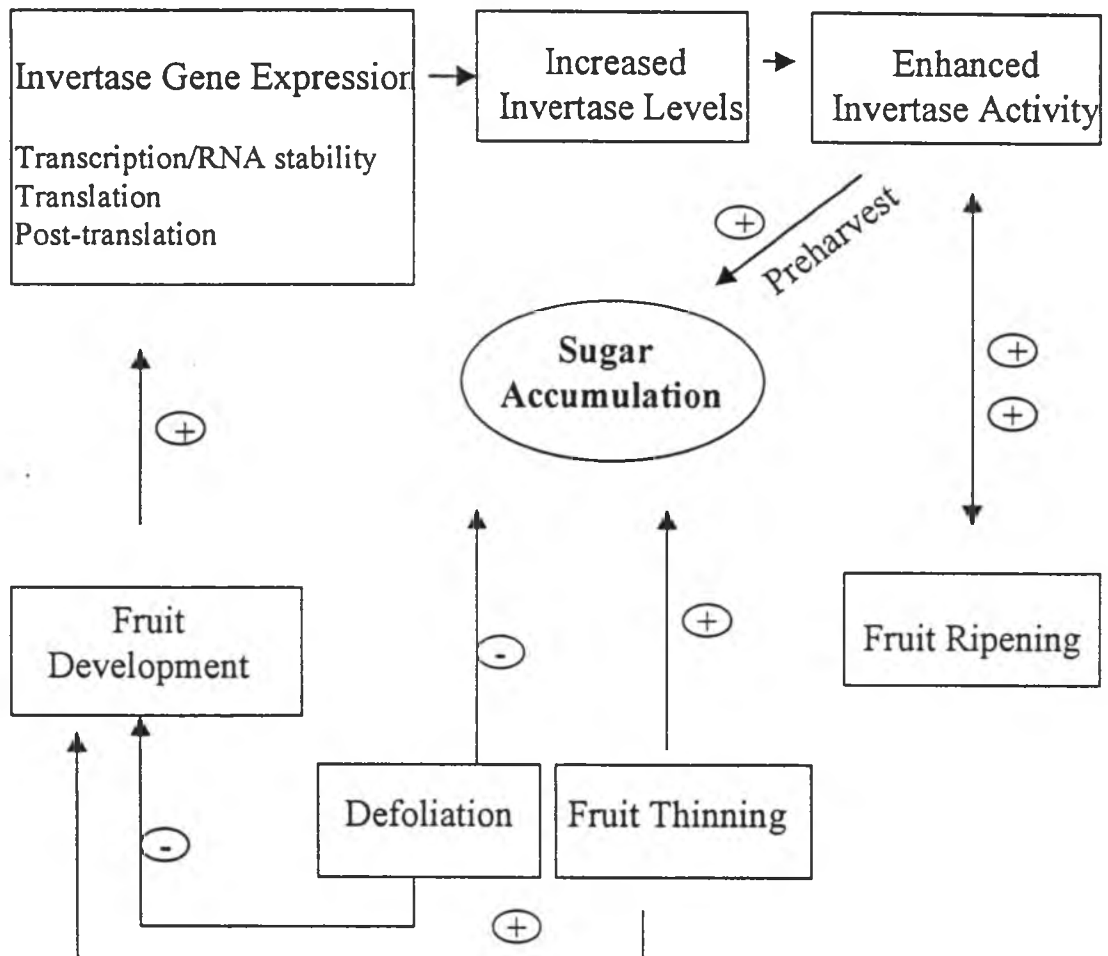



Figure 7.1. Model for the accumulation of sugars in papaya fruit. '+', increase, '-', decrease.

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