ALMA MATER STUDIORUM
UNIVERSITÀ DEGLI STUDI DI BOLOGNA

FACOLTÀ DI AGRARIA

Dipartimento di Colture Arboree

Dottorato di Ricerca in Colture Arboree ed Agrosistemi Forestali Ornamentali e Paesaggistici – AGR/03

XX Ciclo

Genotypic variation in *Actinidia deliciosa* fruit size and carbohydrate content

Dissertazione presentata dalla Dott.ssa SIMONA NARDOZZA

Tutore:
Chiar.mo Prof.
GUGLIELMO COSTA

Co-Tutore:
Dr. MICHAEL J. CLEARWATER

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To Massimo

To Mum and Dad

Abstract

In a global and increasingly competitive fresh produce market, more attention is being given to fruit quality traits and consumer satisfaction. Kiwifruit occupies a niche position in the worldwide market, when compared to apples, oranges or bananas. It is a fruit with extraordinarily good nutritional traits, and its benefits to human health have been widely described.

Until recently, international trade in kiwifruit was restricted to a single cultivar, but different types of kiwifruit are now becoming available in the market. Effective programmes of kiwifruit improvement start by considering the requirements of consumers, and recent surveys indicate that sweeter fruit with better flavour are generally preferred. There is a strong correlation between at-harvest dry matter and starch content, and soluble solid concentration and flavour when fruit are eating ripe. This suggests that carbon accumulation strongly influences the development of kiwifruit taste.

The overall aim of the present study was to determine what factors affect carbon accumulation during *Actinidia deliciosa* berry development. One way of doing this is by comparing kiwifruit genotypes that differ greatly in their ability to accumulate dry matter in their fruit. Starch is the major component of dry matter content. It was hypothesized that genotypes were different in sink strength. Sink strength, by definition, is the effect of sink size and sink activity.

Chapter 1 reviews fruit growth, kiwifruit growth and development and carbon metabolism.

Chapter 2 describes the materials and methods used.

Chapter 3, 4, 5 and 6 describes different types of experimental work.

Chapter 7 contains the final discussions and the conclusions

Three *Actinidia deliciosa* breeding populations were analysed in detail to confirm that observed differences in dry matter content were genetically determined. Fruit of the different genotypes differed in dry matter content mainly because of differences in starch concentrations and dry weight accumulation rates, irrespective of fruit size. More detailed experiments were therefore carried out on genotypes which varied most in fruit starch concentrations to determine why sink strengths were so different.

The kiwifruit berry comprises three tissues which differ in dry matter content. It was initially hypothesised that observed differences in starch content could be due to a larger proportion of one or other of these tissues, for example, of the central core which is highest in dry matter content. The study results showed that this was not the case.

Sink size, intended as cell number or cell size, was then investigated. The outer pericarp makes up about 60% of berry weight in ‘Hayward’ kiwifruit. The outer pericarp contains two types of parenchyma cells: large cells with low starch concentration, and small cells with high starch concentration. Large cell, small cell and total cell densities in the outer pericarp were shown to be not correlated with either dry matter content or fruit size but

further investigation of volume proportion among cell types seemed justified. It was then shown that genotypes with fruit having higher dry matter contents also had a higher proportion of small cells. However, the higher proportion of small cell volume could only explain half of the observed differences in starch content. So, sink activity, intended as sucrose to starch metabolism, was investigated.

In transiently starch storing sinks, such as tomato fruit and potato tubers, a pivotal role in carbon metabolism has been attributed to sucrose cleaving enzymes (mainly sucrose synthase and cell wall invertase) and to ADP-glucose pyrophosphorylase (the committed step in starch synthesis). Studies on tomato and potato genotypes differing in starch content or in final fruit soluble solid concentrations have demonstrated a strong link with either sucrose synthase or ADP-glucose pyrophosphorylase, at both enzyme activity and gene expression levels, depending on the case. Little is known about sucrose cleaving enzyme and ADP-glucose pyrophosphorylase isoforms. The HortResearch *Actinidia* EST database was then screened to identify sequences putatively encoding for sucrose synthase, invertase and ADP-glucose pyrophosphorylase isoforms and specific primers were designed. Sucrose synthase, invertase and ADP-glucose pyrophosphorylase isoform transcript levels were anlayzed throughout fruit development of a selection of four genotypes (two high dry matter and two low dry matter). High dry matter genotypes showed higher amounts of sucrose synthase transcripts (SUS1, SUS2 or both) and higher ADP-glucose pyrophosphorylase (AGPL4, large subunit 4) gene expression, mainly early in fruit development. SUS1-like gene expression has been linked with starch biosynthesis in several crop (tomato, potato and maize). An enhancement of its transcript level early in fruit development of high dry matter genotypes means that more activated glucose (UDP-glucose) is available for starch synthesis. This can be then correlated to the higher starch observed since soon after the onset of net starch accumulation. The higher expression level of AGPL4 observed in high dry matter genotypes suggests an involvement of this subunit in drive carbon flux into starch. Changes in both enzymes (SUSY and AGPse) are then responsible of higher starch concentrations. Low dry matter genotypes showed generally higher vacuolar invertase gene expression (and also enzyme activity), early in fruit development. This alternative cleavage strategy can possibly contribute to energy loss, in that invertases' products are not adenylated, and further reactions and transport are needed to convert carbon into starch. Although these elements match well with observed differences in starch contents, other factors could be involved in carbon metabolism control. From the microarray experiment, in fact, several kinases and transcription factors have been found to be differentially expressed.

Sink strength is known to be modified by application of regulators. In 'Hayward' kiwifruit, the synthetic cytokinin CPPU (N-(2-Chloro-4-Pyridyl)-N-Phenylurea) promotes a dramatic increase in fruit size, whereas dry matter content decreases. The behaviour of CPPU-treated 'Hayward' kiwifruit was similar to that of fruit from low dry matter genotypes: dry matter and starch concentrations were lower. However, the CPPU effect was strongly source limited, whereas in genotype variation it was not. Moreover, CPPU-treated fruit gene expression (at sucrose cleavage and AGPase levels) was similar to that in high dry matter genotypes. It was therefore concluded that CPPU promotes both sink size and sink activity,

but at different “speeds” and this ends in the observed decrease in dry matter content and starch concentration. The lower “speed” in sink activity is probably due to a differential partitioning of activated glucose between starch storage and cell wall synthesis to sustain cell expansion.

Starch is the main carbohydrate accumulated in growing *Actinidia deliciosa* fruit. Results obtained in the present study suggest that sucrose synthase and AGPase enzymes contribute to sucrose to starch conversion, and differences in their gene expression levels, mainly early in fruit development, strongly affect the rate at which starch is therefore accumulated. This results are interesting in that starch and *Actinidia deliciosa* fruit quality are tightly connected.

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How can I define the last three years of my life? There are not proper words, I think, to describe all the deep change that doing a PhD bring to your life, but one thing is definitely true: it has been an exiting challenge with myself. I have learn a lot about my work, about my life, about different way of living a life. But I am sure I could not get into my final goal without other people help. I think this is my chance to say thank you to everyone who has positively stepped into my life in the last three years.

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Contents

Keywords

Actinidia deliciosa, genotype, kiwifruit, starch, sucrose synthase, invertase, ADP-glucose pyrophosphorylase, phloem unloading, dry matter content, dry weight, large parenchyma cells, small parenchyma cells, sink strength, sink size, sink activity, breeding, carbohydrate metabolism, sucrose cleavage, CPPU, fruit development, fruit growth, outer pericarp, gene expression, enzyme activity.

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List of Abbreviations and symbols

#: number
ABA: abscissic acid
AGPase: ADP-glucose pyrophosphorylase
aka: also known as
BF: Breeding Family
Blk: Orchard Block
bp: base pair
Cat. #: Catalogue number
cDNA: complementary DNA
CPPU: (N-(2-Chloro-4-Pyridyl)-N-Phenylurea)
CTAB: Cetyltrimethylammonium Bromide
DA: *Actinidia deliciosa* as for HortResearch classification
DA: *Actinidia deliciosa* for HortReaserch internal codification
DAA: days after anthesis
dATP: Deoxyadenosine triphosphate
dCTP: Deoxycytidine triphosphate
dGTP: Deoxyadenosine triphosphate
DM: dry matter
DNA: Deoxyribonucleic acid
dNTPs: Deoxynucleotides mix, dATP, dGTP, dCTP, dTTP
dTTP: Deoxythymidine triphosphate
DW: dry weight
EDTA: Ethylenedinitrilotetraacetic acid
Eq.: equation
F: Forward primers
FB: full bloom
Fig.: Figure
FW: fresh weight
Glc: glucose
HR: HortResearch
IAA: Isoamyl Alcohol
ID: identity
IPTG: Isopropyl β-D-1-thiogalactopyranoside
LB: Lysogeny broth, Luria-Bertani broth or Luria broth
NaOH: sodium hydroxide
NTC: non-template control, negative control
NZ: New Zealand
PCR: Polymerase Chain Reaction
PES: Polyethersulfone
PVP: Polyvinylpyrrolidone
qPCR: Quantitative Real Time PCR
R: Reverse, for primers
RNA: Ribonucleic acid
RO water: reverse osmosis water
rpm: rotation per minute
RT: Reverse Transcriptase
SDS: sodium dodecyl sulphate
SSC: soluble solid concentration

SUSY: sucrose synthase
Tab.: Table
Taq: Termophylus acquaticus polymerase
TBE: Tris base, borate, EDTA buffer
TP: Te Puke, Research Centre
Tris: tris-hydroxy-methyl-amino-methane
WAFB: weeks after full bloom
X-gal: 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside
Yr: Year

1 GENERAL INTRODUCTION

Yield, fruit size and external appearance have traditionally been factors strongly affecting growers' returns for most of the fruit crops. "However, as consumers become more discerning and the international market place increasingly competitive, more emphasis is being placed on fruit flavour and internal quality" (Richardson *et al.*, 1997). Consumers' initial choice could be driven by appearance, whereas repurchase depends on other factors (McMath *et al.*, 1992), such as sensory quality traits. Most market research indicates that sensory characteristics (texture, taste, odour, and flavour) are the primary reason with which consumers purchase a particular type of fruit (Harker, 2002)

One of the most important plant breeding goal is to satisfy consumer's needs, identifying clear targets for expected or desired sensory characteristics. Improvement of fruit quality and size are a breeding goal for many horticultural crops, and the enhancement of the efficiency with which successful new products are released is a key point in plant breeding (Harker, 2002). The relationship between soluble sugar concentration (often highly correlated with fruit sensory quality), and fruit size/yield is often negative for a number of crops, such as tomato, peach, strawberry and peppers (Ben-Chaim and Paran, 2000; 2004; Dirlewanger *et al.*, 1999; Fulton *et al.*, 1997; Monma and Takada, 1991). This negative correlation is the result of a co-localization of QTL for fruit size and sugars, as reported in tomato and peach (Dirlewanger *et al.*, 1999; Saliba-Colombani *et al.*, 2001). However, few studies on tomato introgression lines showed no association of sugar content with fruit size (Causse *et al.*, 2004; Fridman *et al.*, 2002; Fridman *et al.*, 2000). A similar result was shown in *Actinidia chinensis*, where the occurrence of large fruit-high sugars concentration families was observed, suggesting that the negative correlation between size and sugar content might be overcome (Cheng *et al.*, 2004).

One of the fundamental ideas of biology – and certainly of applied genetics – concerns the relation between phenotype and genotype. The idea and the terminology go back to Johannsen (1909), who, in the early years of this century, showed that selection for small and large seed in an inbred line of bean (*Phaseolus vulgaris*) was ineffective. There was variation in seed size but it was not heritable. By contrast there were consistent differences between different lines which could regularly be recognized despite variability within lines and between samples. So some variability is genetic, some environmental. The genotype (or genetic constitution) determines a certain potential for development, environment determines the developmental track adopted and the phenotype is the outcome.

1.1 KIWIFRUIT

1.1.1 KIWIFRUIT: ORIGIN AND BOTANICAL OVERVIEW

Kiwifruit is China native dioecious, perennial, climbing, woody plant. Kiwifruit comprises more than 60 species belonging to the genus *Actinidia*, with a wide variability in fruit shape, size, colour and composition (Ferguson, 1990a). 'Hayward' is the green-fleshed cultivar of choice throughout the world outside China and belongs to the species *A. deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson (Huang and Ferguson, 2003). 'Hayward' was selected in New Zealand in 1920s by Hayward Wright (Ferguson and Bollard, 1990). Today 'Hayward' cultivar is grown commercially in many countries (Fig. 1-1), especially Italy, New Zealand and Chile (Cheng *et al.*, 2004), representing the 97.5% of kiwifruit plantings (Huang and Ferguson, 2003).

The scientific classification of the Taxon *Actinidia deliciosa* (A. Chev.) C. F. Liang & A. R. Ferguson var. *deliciosa* is reported in Tab. 1-1, whereas its distinguishing features are resumed in Tab. 1-1 (Ferguson, 1990b).

Tab. 1-1: Actinidia deliciosa scientific classification (Ferguson, 1990b).

Taxonomic Rank	Name
Kingdom	*Plantae*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Order	*Ericales*
Family	*Actinidiaceae* Hutchinson
Genera	*Actinidia*
Section:	*Stellatae* Li
Series	*Perfectae* C.F. Liang
Specie	*Actinidia deliciosa* (A. Chev.) C. F. Liang & A. R. Ferguson
Variety	*deliciosa*

1.1.2 KIWIFRUIT TRADE

In the last decade (1996-2005) the world's imported kiwifruit volume was estimated by FAO (2008) in US$ 1,038,309,000 per year. First 12 importer countries managed the 70% the total volume (51% EU's countries, 13% Asian countries- mainly Japan- and 6% North American countries).

Tab. 1-2: Distinguishing features of *Actinidia deliciosa* (A. Chev.) C. F. Liang & A. R. Ferguson, as reported by Ferguson (1990a).

Distinguishing features	Description
Buds	Bud base is large and protruding. The bud is almost completely submerged in the bark, with only a small aperture. The hairs are long, greyish-white, and downy.
Stems	Young growing tips are often covered with brilliant crimson hairs. Further back the hairs are long, stiff, and yellow-brown, not readily shed, but if shed, leaving distinct stubs. The branches are dark brown, thick, and prone to twisting.
Leaves	Generally obovate, attenuate to cordate, with mainly acuminate to cuspidate apices; larger and thinner than leaves of A. chinensis. The leaf margins are undulating, with frequent long serrations. The lower surface of the leaf is densely covered with long, greyish-brown, stellate hairs which are not readily shed.
Flowers	Many flowering shoots are 15-20 cm long and covered with long, stiff, yellow-brown hairs which are persistent. Pistillate flowers are usually larger than staminate flowers and their mean diameter is 5.5 cm. Mean stamens number is about 130. Pollen grains are oblate to spherical (22.5 x 25 μm); their surface is, indistinctly, finely reticulate.
Fruit	Most fruit are elongated – ovoid or cylindrical, 5-6 cm long. The long, hard, yellow-brown, stiff, bristle-like hairs are not readily shed, but if lost, numerous brown spots are revealed on the fruit surface. Fruit pulp is dark green of jade green.
Chromosome number	2n = 174 (Yan *et al.*, 1997) x = 29, hexaploid genome

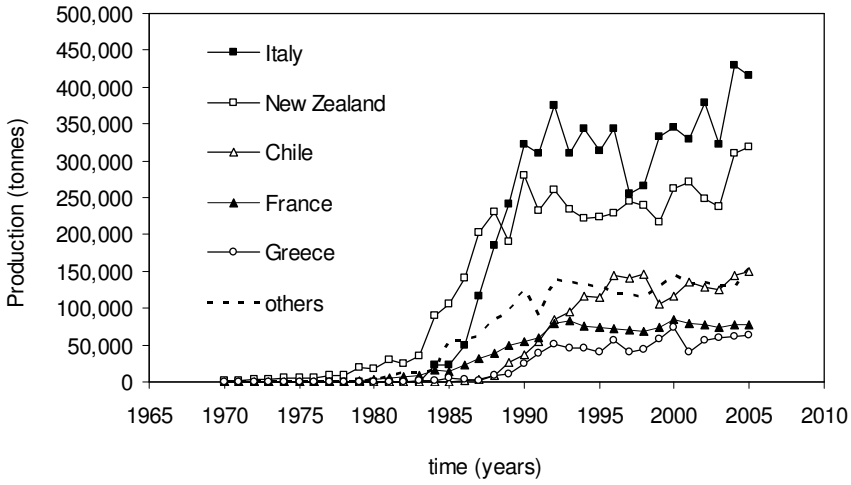


Fig. 1-1: Kiwifruit World's production. In the picture above first 5 producer countries in 2005 are reported. Production volume of first 5 countries was 87% of the World's production in 2005. Elaboration of FAO data (2008).

1.1.3 KIWIFRUIT: BERRY STRUCTURE

1.1.3.1 MORPHOLOGY

The fruit is a berry which develops from a superior multicarpellate ovary (Hopping, 1976b) borne on a 3- to 4-cm-long pedicel (Habart, 1974). The fruit is generally ovoid, subglobose, and length and width vary depending on the cultivar (Habart, 1974). The epidermis is composted of a multiseriate suberized periderm, brown in colour because of tannin deposits. The surface of the epidermis is covered with stiff uniseriate and multiseriate hairs. Sepals and stigma styles are persistent (Hopping, 1976b).

1.1.3.2 HISTOLOGY

The histology of the flower and the fruit has been widely described by Habart (1974) and Schmid (1978). The fruit compromises an outer pericarp of thin-walled parenchymatous cells, an inner pericarp of elongated septum cells which delimit the carpel locules, and a central core of small parenchyma cells. The vascular system originates from the vascular cylinder that enters the fruit from the pedicel. In the receptacle, each vascular bundle divides into three separate bundles. The central one diverges outwards to become the median dorsal carpellary bundle, whereas the other two fuse below the base of the locule to form the ventro-median carpllary bundle which extend the full length of the locule adjacent to the central core (Habart, 1974; Hopping, 1976b; Schmid, 1978).

The pericarp connsists of an outer layer of thin-walled parenchymatous cells which extend from the epidermis to the outer bundles (outer pericarp), and an inner layer of elongated septum cells which extended from the outer bundles to the core and surrounded the locules (inner pericarp). The outer pericarp could be further divided into an outer hypodermal layer, 10-15 cells wide, that extended inwards from the epidermis, and an inner layer of elliptical cells that extended from the hypodermal layer to the outer bundles.

During pericarp expansion, cells of the hypodermal layer elongate in the tangential plane whereas those of the inner layer increases several fold in diameter and become essentially spherical. Hopping (1976b) observed that some cells in both layers did not enlarge appreciably and, at maturity, filled the spaces between cells that had increased in size. A further study shown that the outer pericarp is composed of small, isodiametric parenchyma cells (cross-sectional diameter about 0.1-0.2 mm) containing abundant starch, and large ovoid parenchyma cells (cross-sectional diameter about 0.5-0.8 mm) containing very few starch grains. The final starch content of a cell might be equivalent (Hallett *et al.*, 1992). Enlargement of septum cells, which enclose the locules and collectively make up the inner pericarp, is almost entirely in the radial direction although some tangential enlargement occur in the zone immediately adjacent to the outer pericarp. The cells of both pericarp layers contain chloroplasts that persisted until maturity. During maturation chlorophyll is lost from the inner pericarp but not from the outer pericarp (Hopping, 1976b).

1.1.3.2.1 Berry Tissue Proportions

As described in §1.1.3.2 kiwifruit berry consists of three different tissues. Each of them contributes to the final fruit fresh weight as reported by MacRae *et al.* (1989) and shown in Tab. 1-3. The DM concentration increase centripetally from the outer pericarp to the core Tab. 1-3.

Tab. 1-3: Tissue contribution to final kiwifruit berry fresh weight and relative DM content. DM content significantly increase centripetally (MacRae et al., 1989)

Tissue	Fresh weight (%)	DM (%)
Core	7%	24-25%
Inner Pericarp	36%	17-18%
Outer Pericarp	57%	15-16%

The starch accumulation pattern in the entire fruit reflects changes in outer pericarp, whereas the starch concentration follow a distribution similar to the DM concentration one. Fructose and glucose concentrations increase at harvest time in all tissues, whereas sucrose general level does not change in pericarp tissues, but in the core. Acid content is lower in the core. Quinic acid concentration increases from the core to the outer pericarp, whereas citric acid is higher in the inner pericarp (MacRae *et al.*, 1989). All these proportion needs to be considered at the time of sampling, if a representative sample of the fruit wants to be collected.

1.2 FRUIT DEVELOPMENT

The term fruit development is used to refer to the series of processes from the initiation of growth to death (Watada *et al.*, 1984). The chronological development of the fruit from flowering to maturity and senescence involves a sequence of physical and biochemical changes, both macro- and micro-levels. The molecular, cellular and physiological mechanisms involved in fruit development (Bollard, 1970; Coombe, 1976; Gillaspy *et al.*, 1993), the regulation of assimilation supply to fruit (Ho, 1992; Marshall and Grace, 1992), and the role of endogenous auxins (Miller, 1990) in developing fruit are well documented. The entire period during which these developmental changes occur can be broadly classified into four stages: growth, maturation, ripening, and senescence (Fig. 1-2).

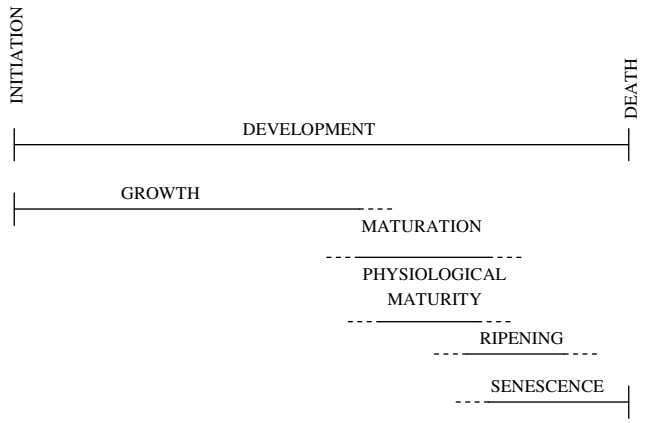


Fig. 1-2: Plant and fruit developmental stages (Watada *et al.*, 1984).

1.2.1 GROWTH

All living organism are capable of 'growth' in the sense of change in size, change in form and change in number, given suitable conditions. These three processes together form an important part of the phenomena of life itself (Hunt, 1982). Hunt (1979) define 'growth' as 'all the irreversible changes with time, mainly in size (however measured), often in form, and occasionally in number that occur in living organisms'.

As part of a living organism, fruit are able to grow. The growth starts at the onset of fruit development, and consists in increase in size, change in shape, and mobilization reserves from other parts of the plant. Fruit growth is stimulated by pollination and commonly involves the enlargement of the ovary, the enlargement of the receptacle, or both. The ovary grows rapidly soon after the pollination, and this is the beginning of fruit development. Fruit growth is accompanied by significant changes in cellular structures, which results in observed changes in fruit size and shape. The fruit growth pattern is often characterized by an initial period of rapid cell division and development of cell walls, followed by a long period of slow cell expansion (Opara, 2000). The duration of cell division and its contribute to whole fruit growth vary considerably among species (Ho, 1992).

1.2.1.1 GROWTH ANALYSIS

Growth analysis is a helpful instruments to data interpretation, considering that when fruit growth is analyzed plotting primary data (fresh weight, fruit length, etc…) against time very little of the first phase of development is revealed (Hunt, 1990). Primary data analysis is therefore necessary to provide a detailed insight on fruit growth dynamics (Opara, 2000). The classical approach is the easiest and feasible to apply. Absolute growth rate and relative growth rate are then described.

Absolute growth rate (AGR) is a simple derived index of fruit growth, representing the rate of a change in size per unit time. In whole fruit studies primary data generally considered are fruit diameters, fruit fresh weight or fruit dry weight. The generic formula used to calculate AGR is:

$$AGR = \frac{dX}{dt} \qquad \textbf{Eq. 1-1}$$

where X is the primary datum and t is the time (Opara, 2000).

Unless a functional study is performed, generally a mean AGR (MAGR) is calculated over the interval of time t_n and t_{n-1}:

$$MAGR = \frac{(X_n - X_{n-1})}{t_n - t_{n-1}} \qquad \textbf{Eq. 1-2}$$

where X_n is the primary datum at t_n time and X_n is the primary datum at t_{n-1} time (Opara, 2000).

In Fig. 1-3 AGR of 'Hayward' and 'Hort16A' kiwifruit is reported from Minchin *et al.* (2003). The functional study of fruit growth allowed to get the AGR as first derivative, resulting in a smooth curve. This requires a relevant number of sampling dates.

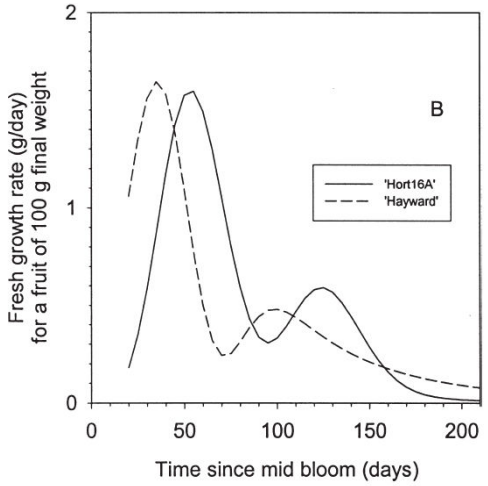


Fig. 1-3: AGR in 'Hayward' and 'Hort16A' kiwifruit calculated as the first derivative of the fresh weight growth curve (Minchin *et al.*, 2003).

AGR is an useful index to compare bodies of like data, such as comparison of fruit growth under different management conditions, whereas to compare the overall of unlike systems relative growth rate (RGR) is required (Opara, 2000). Therefore, AGRs do not account for the initial difference in size, which is a limiting factor of potential growth (Opara, 2000).

RGR (Eq. 1-3) expresses growth as the rate of increment in size per unit of initial size per unit of time (Opara, 2000). This index is suitable for quantitative analysis of fruit growth. It takes account of original size at the onset of growth measurement, and allow more equal comparisons than AGR (Hunt, 1990).

$$RGR = \frac{1}{X} \cdot \frac{dX}{dt} = \frac{d(\log_e X)}{dt} \qquad \textbf{Eq. 1-3}$$

As for AGR, unless a functional study is performed, a mean relative growth rate (MRGR) is used, and the logarithmic transformation of primary data (X) allow an homogenization of the variability (Eq. 1-4) (Opara, 2000).

$$MRGR = \frac{\log_e X_2 - \log_e X_1}{t_2 - t_1} \qquad \textbf{Eq. 1-4}$$

AGR is usually measured as size per time [size • time^{-1}], whereas RGR is usually measured as size per size per time [size • size^{-1} • time^{-1}] (Hunt, 1979).

An example of plots of AGR and RGR against time are reported in Fig. 1-4. They refers to dry weight accumulation, with a sigmoid pattern. AGR is initially increasing to a maximum that decrase, whereas RGR starts from a high level and progressively decrease (Opara, 2000).

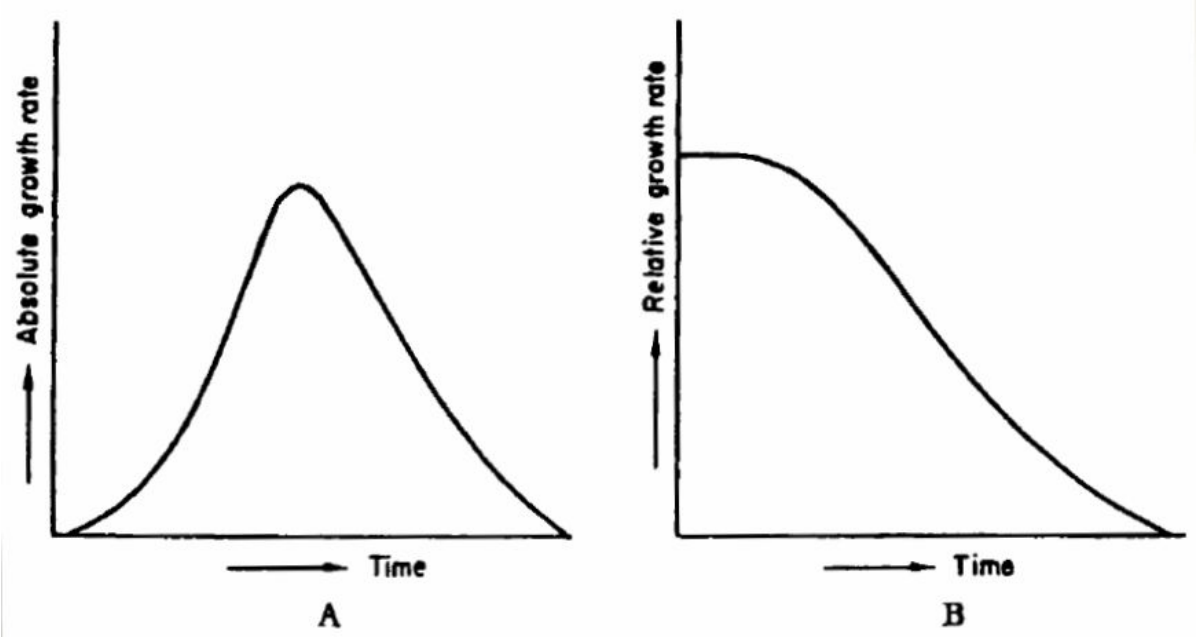


Fig. 1-4: Changes in (A) AGR and (B) RGR of dry weight (Opara, 2000).

1.2.1.2 FRUIT GROWTH IN KIWIFRUIT

There is a linear increase in total dry weight content of kiwifruit from fruit set to harvest (Clark and Smith, 1988; Hopping, 1976b; Richardson *et al.*, 1997). In contrast, the increase in fruit fresh weight follows a two or three phase curve. There has been some debate whether kiwifruit berries grown with a single sigmoid growth pattern (Walton and De Jong, 1990), a double sigmoid growth pattern (Hopping, 1976b) or triple sigmoid growth pattern (Pratt and Reid, 1974). These opinion are based on measurements of length and diameter of 'Monty' kiwifruit, fresh weight of 'Hayward' kiwifruit, and measurements of length and diameter of 'Bruno' kiwifruit respectively.

Hopping (1976b) divided the double sigmoid growth curve in three stages:

- Stage I (0-58 days after flowering): it is a period of rapid growth and weight gain due initially to cell division in all three different tissue, followed by cell enlargement;
- Stage II (58-76 days after flowering): it is a period of reduced growth and weight gain due to a slowing of cell enlargement in both the inner pericarp and central core;
- Stage III (76-160 days after flowering): it is a second period of growth and weight gain due to cell enlargement of the inner pericarp and central core. Fruit growth can still be detected at harvest as both weight and volume increment.

Walton and De Jong (1990) sustain that the growth of kiwifruit berries may be best described by a single sigmoid growth curve and any variations from this are due to sampling error and/or cultural conditions.

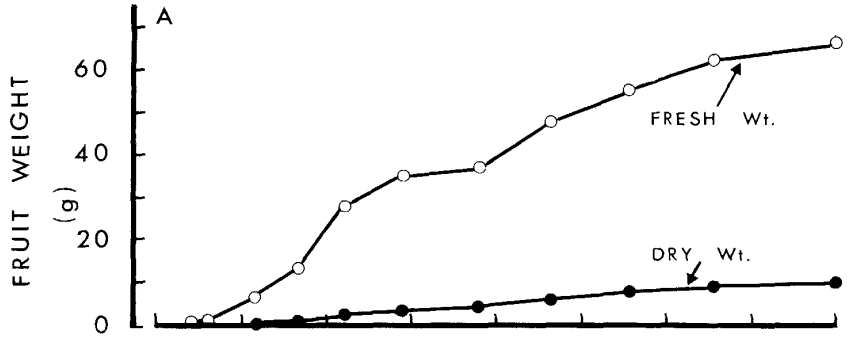


Fig. 1-5: Double sigmoid growth curve of developing kiwifruit observed by Hopping (1976b).

More recent study allow to divide the fruit growth according to the start of net starch accumulation (Fig. 1-6). According to Hopping (1976b), fruit growth is rapid in the first 50 days after anthesis, because of the cell division phase. Then a longer period (50–120 days) follows when starch accumulates in fruit and growth slows. In the final maturation period of 30–60 days fruit grow and accumulate starch very slowly or not at all, as seeds mature and the fruit starts to ripen (Richardson *et al.*, 2004). The relative timing of each phase appears to be strongly affected by growing environments (Richardson *et al.*, 1997; Tombesi *et al.*, 1994; Walton and De Jong, 1990). By commercial harvest, when fruit fresh weights typically change little (Currie *et al.*, 1999), both the total dry weight, and the DM concentration of the fruit, may still be increasing appreciably (Snelgar *et al.*, 2005).

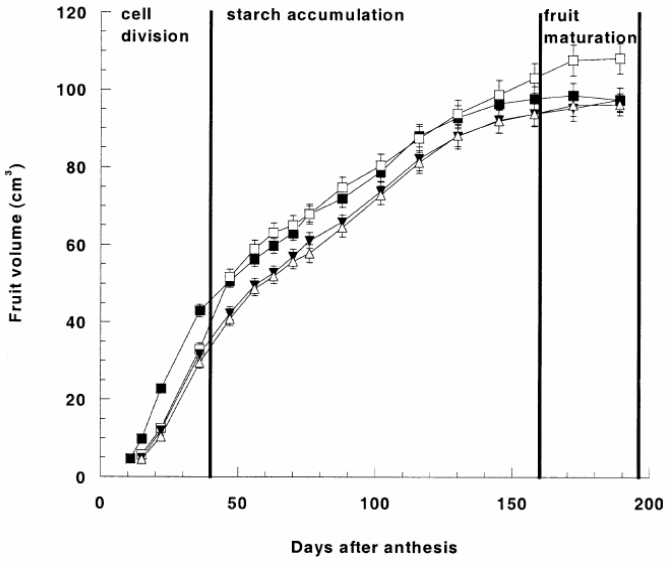


Fig. 1-6: Fruit growth division in three phases as reported in Richardson et al. (2004).

1.2.2 MATURATION

The maturation concept has got different meaning according to the context. In physiology, maturation is the process associated with completing natural growth and development and the attainment of full size. At physiological maturity, a fruit will continue ontogeny even if detached (Watada *et al.*, 1984).

In horticulture, maturity is the stage of development when the plant or part of the plant possesses the prerequisites for utilization by consumers (Watada *et al.*, 1984). According to the intended use, fruit may be horticulturally mature in the early stage, mid-stage, or late stage of development.

In postharvest technology, maturation is commonly defined as "that stage at which a commodity has reached a sufficient stage of development that after harvesting and postharvest handling, its quality will be at least the minimum acceptable to the ultimate consumer" (Reid, 1992).

1.2.3 RIPENING

The term ripening refers to the processes that qualitatively transform the mature fruit as it reaches the end of its growth period (Leopold, 1964). As reviewed by Opara (2000), the changes in ripening fruit are well documented in literature and generally include tissue softening, with the associated change in coloration and flavour, hydrolytic changes, which usually result in the rise of soluble sugar concentration, increased permeance of the cuticle to gases, respiratory climacteric and flavour production. Ripening is a precisely regulated developmental program (Brady, 1987). Based on the occurrence of a respiratory climacteric, the ripening biochemical pathways can be classified as climacteric or non-climacteric. Ripening of fleshy fruit terminates with senescence and decay of the tissue.

1.2.4 SENESCENCE

Senescence is used collectively to refer to the degradative changes that naturally lead to death whole plants or organs. The period of senescence is characterized by depressed growth rate, and termination of growth sets the stage for senescence. During this stage the deterioration of fruit structural integrity occur. These changes in fruit manifests as loss of firmness, colour degradation and increased skin membrane permeability (Leopold, 1964). During fruit growth and development, senescence marks the period during which fruit has lost the power of growth (Opara, 2000).

1.3 FRUIT QUALITY

Size, water, and the content of carbon compounds are the main criteria for assessing the quality for fresh fruits. Although abundant knowledge is available about the processes involved in growth and primary metabolism, the genetic and environmental improvement of fruit quality remains a complex task due to the antagonism between quality traits, for instance, size and composition. For cultivated tomato, for example, fruit concentration in carbon compounds can be enhanced by cultivation management, but this improvement is often paralleled with an undesirable reduction in yield, mainly due to the decrease of mean fruit size and the increasing incidence of growth disorders (Ho, 2003).

Fruit quality traits are quantitative, complex, and controlled by environment and genes. The variation of these traits with environment and genotype has been traditionally studied

following a quantitative genetics approach such as correlation and marker–trait association analysis (Liu *et al.*, 2007).

Fruit quality traits are strongly affected by fruit composition in many fruit crop, as well as in kikifruit.

1.3.1 KIWIFRUIT COMPOSITION: DRY MATTER AND QUALITY

Data from Richardson *et al.* (Richardson *et al.*, 1997) suggest that in kiwifruit a grower's ability to alter the soluble solids concentration at eating ripeness is limited and primary factors affecting fruit "quality" are climatic and genetic. In fact, the comparison between studies on kiwifruit berry composition during 'Hayward' fruit development indicate quite major differences according to the country of origin (MacRae *et al.*, 1989; Okuse and Ryugo, 1981; Walton and De Jong, 1990)

Taste is an important aspect of kiwifruit quality and is largely determined by the concentration and balance of sugars and acids. This affects perceptions of overall flavour intensity as well as sweetness and sourness (Cheng *et al.*, 2004). Sensory analyses (Stec *et al.*, 1989) have identified the soluble solids concentration at eating ripeness as one important determinant of consumer preference.

Dry matter (total solids) embraces both the soluble (largely sugars) and insoluble carbohydrate (mainly the structural carbohydrates and starch) pools in fruit (Beever and Hopkirk, 1990; Hopkirk, 1991; Scott *et al.*, 1986). Dry matter can therefore be taken as an indicator of the total fruit carbohydrate, of which at-harvest 40-70% may be starch. During fruit ripening, the starch is almost completely converted to soluble sugars. As a large proportion of the dry matter at-harvest is starch plus soluble sugars, this dry matter value can be related to the soluble sugars that will be present in the ripe fruit (Burdon *et al.*, 2004).

Further, Crisosto (1992) suggested that DM measurements made at any time after harvest provide a reliable predictor of ripe fruit soluble solids content and hence fruit quality. Relationships have also been shown between consumer acceptability and both the DM (Scott *et al.*, 1986) and the ripe SSC (MacRae *et al.*, 1989; Mitchell *et al.*, 1992) of the fruit. Dry matter in kiwifruit has been proposed as being both a maturity indicator for timing harvest and also as a predictor of the sensory quality of the fruit once ripe.

At harvest, much of the carbohydrate in kiwifruit is starch, which on ripening is hydrolysed to sugars (Given, 1993).

DMs extended from 14–19.5% FW, representing the typical range for 'Hayward' kiwifruit (Beever and Hopkirk, 1990).At harvest typical starch contents are about 7% FW (Richardson *et al.*, 1997; Walton and De Jong, 1990).

The high heritability of SSC, DM, vitamin C and TA, suggests that these characters will be amenable to change through selection in this population (Cheng *et al.*, 2004). QTL for fruit weight, SSC, DM, sugars and acids content were often co-localised in tomato (Saliba-Colombani *et al.*, 2001) and in peach (Dirlewanger *et al.*, 1999) but there was also a region on chromosome 9 where a QTL for SSC and DM occurred without an association with fruit

weight in tomato. Data from Cheng *et al.* (2004) suggest that a similar situation might occur in kiwifruit.

The DM concentration dips 50 days after fruit set followed by a rapid, curvilinear increase until about 140 days after fruit set (Richardson *et al.*, 1997) as shown in Fig. 1-7.

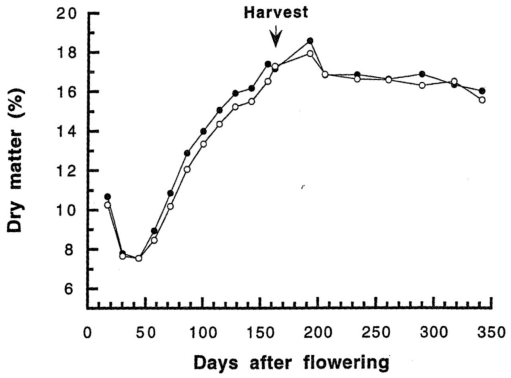


Fig. 1-7: Dry matter accumulation curve in kiwifruit (Richardson et al., 1997).

Scott *et al.* (1986) carried out an investigation into maturation of fruit growing in Australia. Small differences in SSC at harvest resulted in quite different SSC at eating ripeness, depending on fruit-growing district. They could not find no consistent correlation between flavour rating of eating-ripe fruit and starch, individual sugars or titratable acidity at harvest. The fruit dry weight provided a reasonable indication of good flavour. Their recommendation was that fruit attain 15% dry weight before harvest. They also found the soluble solids content to be useful as an indicator of eating quality when the fruit has been ripened, but not at the time of harvest. Percentage DM could be used at any stage and was thus a more useful predictor of harvest maturity.

1.3.1.1 VARIATION IN FRUIT DRY MATTER AND FRUIT QUALITY

Variation in DM concentration occurs between fruit from different vine in the same orchard, between different orchards. between different seasons, between individuals from breeding populations.

The relationship between climate and DM concentration of kiwifruit has not yet been determined, although it is usually assumed that climatic variation is a key factor influencing seasonal variation in DM concentration. In New Zealand the average DM concentration of kiwifruit from a large number of orchards in 2001 season reached only 14.3% as compared to the average of 17.3% for the same orchards in 1998 (Woodward, 2001).

Mowat and Amos (2002) reported that within one season, the DM concentration of individual fruit from a single orchard ranged from 11% to 21%, and in New Zealand most fruit are in the 14-17% range (Burdon *et al.*, 2004).

In the breeding population observed by Cheng and co-workers (2004) DM concentration ranged from 10.6% to 22.3%.

The linear increase in dry weight implies that the rate of carbohydrate partitioning to fruit is determined in the first month after flowering and remains the same despite variations in the climate that may effect fruit fresh weight (Richardson *et al.*, 1997). Stategies for rates of carbohydrate allocation to kiwifruit are established early in the season (Richardson *et al.*, 1997). Rates of dry matter accumulation in fruit, while strongly sensitive to crop load, remained virtually unchanged throughout the season.

1.3.1.2 FACTORS AFFECTING DRY MATTER CONCENTRATION

Dry matter concentration can vary to a greater or lesser extent depending on the season, timing of harvest, the orchard location and canopy management (Burdon *et al.*, 2004).

Spring high temperatures result in early accumulation of DM in the fruit that doesn't affect the final DM concentration at harvest. High summer temperature cause reduction of DM concentration mainly due to excessive vegetative vigour (Richardson *et al.*, 2004; Snelgar *et al.*, 2005).

1.3.2 KIWIFRUIT COMPOSITION: NON-STRUCTURAL CARBOHYDRATES AND ORGANIC ACIDS

Sugars and acids are two of many factor that contribute to fruit flavour and quality (Walton and De Jong, 1990).

1.3.2.1 CARBOHYDRATES IN KIWIFRUIT

Carbohydrates are divided into structural carbohydrates and non-structural carbohydrates. In kiwifruit berry, main structural carbohydrates are those involved in cell wall composition, such as cellulose, pectins, galctose and uronic acids (Gallego and Zarra, 1997).

Non-structural carbohydrates are mainly soluble sugars and starch. Seasonal trends in fruit carbohydrate levels of *Actinidia deliciosa* have been described in several studies (Klages *et al.*, 1998; Miller *et al.*, 1998; Okuse and Ryugo, 1981; Richardson *et al.*, 1997; Walton and De Jong, 1990).

Knowledge of the level and nature of non-structural carbohydrate composition in various plant organ can provide an important indicator of the balance between the supply and demand for photosynthate at different stages of development or under stress conditions, and of the availability of carbohydrates reserves. The level and nature of soluble carbohydrates can also influence the osmotic potential and water relations of cells and tissues (Boldingh *et al.*, 2000).

1.3.2.1.1 Soluble Carbohydrates

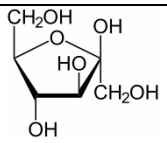
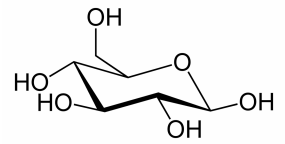
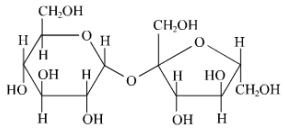
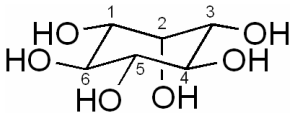
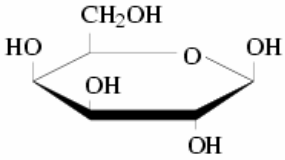
Major sugars found in kiwifruit berry are glucose, fructose and sucrose, whereas myo-inositol (a sugar-alcohol) and galactose are minor sugars.

Studies from fruit set onwards show an early peak in glucose concentration at 30-40 days after anthesis that coincides with a peak in fruit water content and the period of intense cell

diviosion in the central core and pericarp (Hopping, 1976b; Smith *et al.*, 1995b). The sharp increase in water content of the fruit during weeks 3-8, was associated with rapid fruit enlargement and changes in fruit shape. Smith and co-workers (1995b) found a relatively close relationship between the concentration of glucose measured by Walton and De Jong (1990) and the water content determined in their study consistent with fruit enlargement being osmotically driven. The role of glucose is associate with cell growth: cell become large, highly vacuolated and are filled primarily with simple metabolites (sugars and organic acids).

Fructose and sucrose concentrations are at lower levels than glucose throughout fruit development. Generally their concentrations increase when starch net loss begins and fruit start to ripe (Klages *et al.*, 1998). Sucrose is the major sugar in kiwifruit pedicel phloem exudates, contributing up to 95% of all sugars (Klages *et al.*, 1998). It was therefore assumed that carbon is mainly translocated as sucrose from source to sink tissues.

Tab. 1-4: Sugars in kiwifruit berry: majors and minors are here reported .

Compound	Characteristics	Formula
fructose	Monosaccharide, reducing sugar	
glucose	Monosaccharide, reducing sugar	
sucrose	Disaccharide, non reducing sugar	
myo-Inositol	Sugar-alcohol	
galactose	Monosaccharide, reducing sugar	

myo-Inositol is a 6-carbon cyclic sugar-alcohol that shows a peak in early fruit development in *Actinidia deliciosa* (Klages *et al.*, 1998; Miller *et al.*, 1998; Walton and De Jong, 1990). It could fulfil an osmoprotective function at that time as well as acting as substrate for cell wall precursors (Bohnert *et al.*, 1995; Loewus *et al.*, 1990). In *Actinidia deliciosa* a large proportion of the *myo*-inositol is likely to be synthesised in the fruit. The synthesis must be developmentally regulated and therefore be responsive to physiological or environmental signals (Klages *et al.*, 1998). *myo*-Inositol is the major soluble sugar in *Actinidia arguta*

(60% of total soluble sugars), whereas it counts for about 10% in *Actinidia deliciosa* (Boldingh *et al.*, 2000).

Galactose is also present in developing kiwifruit berry, but the concentration is one order of magnitude lower than other sucrose. Planteose is a trisaccharide (sucrose and galactose) found in *Actinidia* leaves by HPLC quantification. When sugars are analyzed by chromatography, galactose is shown to be a common sugar in *Actinidia* species. However it is possible that the amount measured as galactose is instead planteose (Klages *et al.*, 2004).

Ripening fruit of *Actinidia deliciosa* accumulate hexoses rather than sucrose, which is similar to ripening domesticated tomato cultivars (*Lycopersicon esculentum*) (Miron and Schaffer, 1991; Yelle *et al.*, 1988).

1.3.2.1.2 Insoluble Carbohydrates

Insoluble carbohydrates are structural (cellulose) and non-structural (starch). Starch is the end-product of photosynthesis in source tissues and is stored as energy reserves in sink tissues.

Starch is a huge (from 0.1 to over 50 μm in diameter) complex quaternary structure made of two distinct polysaccharide fractions: amylopectin and amylose. Amylopectin is composed of intermediate size α-1,4 linked glucans that are clustered together and hooked to longer spacer glucans by α-1,6 linkages. Amylopectine is the most relevant fraction of starch in many species. Amylose is often referred to as a smaller, essentially linear molecule with very few α-1,6 branches (Ball and Morell, 2003).

Actinidia deliciosa has a transiently accumulating starch fruit. Starch in kiwifruit is accumulated centripetally from the sub-epidermal zone (Okuse and Ryugo, 1981) from 40 to 60 days after full bloom. The onset of net starch degradation coincides with the onset of net sugar accumulation (Boldingh *et al.*, 2000). From several studies a consistent pattern emerges whereby starch begins to accumulate in fruit after the initial phase of rapid fruit expansion and by harvest accounts for some 50% of the total fruit dry matter (MacRae *et al.*, 1989; Okuse and Ryugo, 1981; Richardson *et al.*, 1997; Walton and De Jong, 1990)

1.3.2.2 ORGANIC ACIDS IN KIWIFRUIT

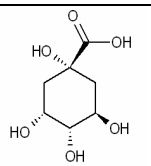
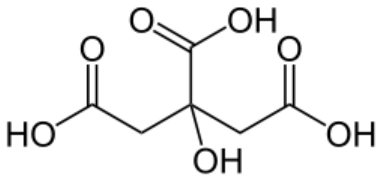
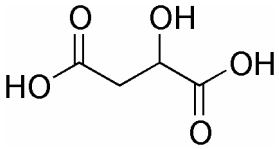
The characteristic acidity in 'Hayward' fruit is produced by fruit acid accumulation occurred during growth. The main organic acids detected and identified in the kiwifruit berry are quinic, citric and malic acids (Marsh *et al.*, 2004; Okuse and Ryugo, 1981; Walton and De Jong, 1990). At harvest, kiwifruit contain 0.9–2.5% total acidity, with 40–50% as citrate 40–50% as quinate, and 10% as malate. There are at least three distinct tissue zones within a kiwifruit and the balance of the different acids changes within these zones (MacRae *et al.*, 1989). The proportion of citrate is highest in the inner cortex, quinate is highest in the outer cortex, whereas the core has the lowest total acid content (about half the other zones), predominantly citrate.

Quinic acid is the main organic acid during early fruit development. It peaks between 20 and 50 days after flowering (Walton and De Jong, 1990) and it could be involved in cell enlargement as glucose is.

Citric acid is the second most prevalent acid reaching its greatest values at harvest.

Malic acid level is lower than quinate and citrate. It peaks after quinic acid, reaching its greatest values at harvest.

Tab. 1-5: Major organic acids in kiwifruit berry.

Compound	Formula
Quinic acid	
Citric acid	
Malic acid	

1.3.2.3 SUGARS, ACIDS AND FRUIT GROWTH IN KIWIFRUIT

In the first 30 days of fruit growth, *Actinidia deliciosa* shows a rapid increase in organic acids, primarily quinic acid, followed soon afterward, between 30 and 45 days after flowering, by a peak in the sugar content, mainly glucose (Boldingh *et al.*, 2000; Walton and De Jong, 1990). These rapid increase correlate with the period of rapid cell expansion described by Hopping (1976b) and therefore could be associated with the cell growth. Soluble sugar and organic acids role during early developmental stages is mainly associated with the onset of fruit enlargement (soon after cell division phase). In fact, cell vacuolation primarly consist in a accumulation of simple metabolites, as soluble sugars and organic acids are (Boldingh *et al.*, 2000).

As fruit grow, the concentration of sugars in the fruit declines, as consequence of energy and carbon skeleton demands, necessary for the synthesis of starch and bio-energetically expensive lipids. Between 90 and 120 days after flowering, the sugar concentration declines to a minimum and thereafter increases steadily to their final values. The rise of simple sugars is a consequence of the starch hydrolysis at about 150 days after flowering (Walton and De Jong, 1990).

The sugar to acid ratios for fruit at harvest are possibly affected by climate, being lower at warmer sites, probably due to the greater demand for substrate to support increased respiration rates at higher temperatures (Penning De Vries, 1975).

1.4 SINK STRENGTH

Sucrose transport from source leaves into sink organs is controlled by 'sink strength', the ability of a sink organ to attract sucrose (Ho, 1988). Many fruit, such as potato, tomato, kiwifruit, are starch storing sink. It might be therefore generalized that sink strength is the capacity of an organ to attract carbon. Sink strength is heavily affected by sink size and sink activity. Sink size is the results of cell number and cell size, whereas sink activity refers to sucrose, and generally to non-structural carbohydrate, metabolism, from phloem unloading to carbon accumulation/storage (Ho, 1992).

Metabolic pathways involved in starch biosynthesis are different between source and sink tissues. In addition to the structural difference in amylopectin and starch granules, the carbon of starch is derived from fructose-6-P in the Calvin– Benson cycle in photosynthetic tissues, while in sink tissues it is derived from sucrose, which is translocated from source tissues through the phloem. Therefore, sucrose must be metabolized to sugar-P or ADPglucose in the cytoplasm, and either or both of these compounds are translocated into the amyloplasts via the compound-specific hexose monophosphate translocator or the ADGglucose translocator, respectively (James *et al.*, 2003).

During the early phases of development, tomato fruit are strong carbohydrate sinks (Ho and Hewitt, 1986). Tomato fruit sink strength can be described as the product of sink size and sink activity (Warren-Wilson, 1972). Sink size is a physical restraint that includes cell number and cell size. Sink activity (RGR) is a physiological restraint that includes multiple factors and key enzymes involved in carbohydrate utilization and storage (Ho, 1984). Sucrose synthase appears to play a major role in tomato fruit sink establishment and maintenance by cleaving imported sucrose and providing UDP-glucose for biosynthetic reactions. The contribution of sucrose synthase is most important during the early stages of tomato fruit growth, because active starch accumulation occurs during this period. High sucrose synthase activity may lead to reduced concentrations of sucrose in pericarp cells and increased sucrose gradient from the source leaves to the fruit, resulting in greater sink strength (Walker *et al.*, 1978). Several authors (Claussen *et al.*, 1986; Sung *et al.*, 1989)have suggested that the activity of sucrose synthase could be used as a biochemical marker for sink strength.

Carbon autotrophy is the most prominent feature of higher plants and the non-reducing disaccharide sucrose plays a central role in plant metabolism. Carbohydrates are synthesized in source leaves and translocated to sink tissues in most species in the form of sucrose to sustain heterotrophic metabolism and growth, or to be stored as sucrose or starch. Growth and development of plants is accompanied by changes in source–sink relations (Roitsch and Gonzalez, 2004)

Sucrose, as opposed to hexoses, accumulation may contribute to an high soluble solids content of the fruit in several ways. If accumulating sucrose, a sink can accumulate twice as much soluble carbohydrate maintaining an equivalent osmotic potential. Cell turgor regulates sink activity and sucrose, relative to hexose, accumulation will result in lower turgor for equivalent levels of soluble carbohydrates and promote sink activity (Wyse *et al.*, 1986).

Fruit volume increase and accumulation of carbon compounds results from a number of processes such as sugar unloading and metabolism, water import, and cell wall expansion, which are intimately connected at the fruit level and regulated by several steps during fruit development (Liu *et al.*, 2007).

1.4.1 SINK SIZE: CELL NUMBER AND CELL SIZE

Sink size is considered as a physical constraint of an organ (Ho, 1992). Plant organs are a combination of different tissues, and tissue 'fundamental unit' is the cell. The combination of cell number and cell size determines the final size of an organ. Cell number of an organ is usually genetically determined, at least at order of magnitude level, whereas environmental conditions affect variations within a genotype (Ho, 1992). In fruit for a number of crop (Higashi *et al.*, 1999; Scorza *et al.*, 1991; Yamaguchi *et al.*, 2002; Yamaguchi *et al.*, 2004) cell number is strongly affected by cell division phase early in fruit development (Bohner and Bangerth, 1988). After this phase fruit growth is mainly driven by cell expansion (Gillaspy *et al.*, 1993).

Cell number affects also *Actinidia deliciosa* fruit size (Hopping, 1976b). As previously reported kiwifruit berry presents two different type of cells, strongly different in size and starch concentration. It is therefore possible that between-genotype variation in cell number and size is not enough to explain differences in sink strength, but the relative proportion of the two cell type populations should be considered.

1.4.2 SINK ACTIVITY: PHLOEM UNLOADING

Carbon autotrophy is the most prominent feature of higher plants and the non-reducing disaccharide sucrose plays a central role in plant metabolism. Carbohydrates are synthesized in source leaves and translocated to sink tissues in most species in the form of sucrose to sustain heterotrophic metabolism and growth, or to be stored as sucrose or starch. Growth and development of plants is accompanied by changes in source–sink relations (Roitsch and Gonzalez, 2004).

The partitioning of sugars in economically important sink organs such as fruits or seeds is governed by several complex physiological processes, including photosynthetic rate, phloem loading in the source leaf, long-distance translocation in the phloem, phloem unloading in sink organs, post-phloem transport and metabolism of imported sugars in sink cells (Oparka, 1990; Patrick, 1997). It is now well accepted that phloem unloading plays a key role in the partitioning of photoassimilate (Fisher and Oparka, 1996; Patrick, 1997). The process of phloem unloading has been studied extensively over the past 20 years remains poorly

understood. Elucidation of the cellular pathway of phloem unloading is central to this process, because to a large extent, the unloading path determines the key transport events responsible for assimilate movement from the sieve elements to the recipient sink cells (Fisher and Oparka, 1996; Patrick, 1997). A symplasmic phloem unloading pathway predominates in most sink tissues such as vegetative apices (Imlau *et al.*, 1999; Patrick, 1997), sink leaves (Haupt *et al.*, 2001; Imlau *et al.*, 1999; Roberts *et al.*, 1997) and potato tubers, which represent a typical terminal vegetative storage sink (Oparka and Santa Cruz, 2000; Viola *et al.*, 2001). Symplasmic unloading is also efficient in the maternal tissues of developing seeds, which represent a class of terminal reproductive storage sinks (Patrick, 1997; Patrick *et al.*, 1995). In some cases, symplasmic unloading also occurs in elongating zones of the stem and in the mature axial path where radial transport of assimilates from the phloem may follow sym- or apoplasmic routes (Patrick and Offler, 1996; Van Bel, 1996). The predominance of a symplasmic unloading pathway is associated with greater transport capacity and lower resistance (Patrick, 1997; Patrick and Offler, 1996). The unloading route may differ according not only to sink types, but also to sink development, sink function and even to growth conditions for a particular sink type, and alternative unloading pathways may exist in the sinks with symplasmically interconnecting phloem (Itaya *et al.*, 2002; Oparka and Turgeon, 1999; Patrick, 1997; Roberts *et al.*, 1997; Viola *et al.*, 2001).

1.4.2.1 FRUIT PHLOEM UNLOADING

The fruit models most investigated are tomato (Ho and Hewitt, 1986), grape (Coombe, 1992), citrus (Garcia-Luis *et al.*, 1991) and apples (Zhang *et al.*, 2004).

The early stages of tomato fruit development are characterized by symplasmic unloading routes to the storage parenchyma cells (Johnson *et al.*, 1988). This conclusion is based on the distribution of 6(5)carboxyfluorescein CF and [^{14}C]glucose (Ruan and Patrick, 1995), protection of phloem-imported sucrose from hydrolysis by an extracellular invertase (Dali *et al.*, 1992), and the absence of an energy-dependent retrieval system (Johnson *et al.*, 1988; Ruan and Patrick, 1995). The switch from starch to soluble sugar accumulation, during fruit development (Ho and Hewitt, 1986), is accompanied by changes in the post-sieve element pathway. The symplasmic route to the phloem parenchyma cells is maintained, but the symplasmic route to the storage parenchyma cells is structurally diminished (Johnson *et al.*, 1988). The development of an obligatory apoplasmic step accords with extracellular hydrolysis of phloem-imported sucrose (Dali *et al.*, 1992; Damon *et al.*, 1988), and the appearance of plasma membraneH_+-ATPase and hexose porter activities (Fieuw and Willenbrink, 1991; Ruan and Patrick, 1995). The putative hexose/proton symporter accounts for some 70 to 80% of the in vivo hexose accumulation flux by the fruit during the phase of cell expansion (Ruan *et al.*, 1997).

In contrast with tomato fruit, the grape berry and citrus fruit accumulate soluble solutes to high concentrations throughout their development (Coombe, 1992; Koch and Avigne, 1990).

In the case of the grape berry, symplasmic transport could be limited at the phloem/storage parenchyma interface where sufficient plasma membrane surface area is available to support

exchange to the fruit apoplasm. Consistent with this conclusion are the high sugar concentrations in the berry apoplasm (Brown and Coombe, 1985; Lang and During, 1991) that are sensitive to changes in phloem import rates (Brown and Coombe, 1985). In citrus fruit, disruption of the symplasmic continuity within the stalks of juice vesicles and [^{14}C]assimilate tracing suggest an apoplasmic post-phloem transport (Koch *et al.*, 1985; Koch and Avigne, 1990).

Also in apples, that predominately accumulates soluble sugars, fruit was shown to follow an extensive apoplasmic phloem unloading pathway. The predominant plasma membrane-localization of both the 52-kD putative monosaccharide transporter and the 90-kDH^+-ATPase in the sieve elements provides support for apoplasmic phloem unloading pathway that is furthermore sustained by both the symplasmic isolation of the SE-CC complex and 6(5)carboxyfluorescein (CF) confinement to the phloem strands (Zhang *et al.*, 2004). The predominantly cell wall-localization of invertases (Zhang *et al.*, 2001) is also in agreement with an apoplasmic phloem unloading pathway in apple.

Kiwifruit berry is a starch storing sink which accumulates low levels of soluble sugars during fruit development (Boldingh *et al.*, 2000; Walton and De Jong, 1990). The phloem unloading pathway throughout kiwifruit berry development is still unknown, but from observation listed above, there is high probability to be symplamic, unless till the onset of net starch degradation.

1.4.2.2 SHIFTS BETWEEN APOPLASMIC AND SYMPLASMIC ROUTES IN SINK

Shifts between apo- and symplasmic routes have been detected during sink development of tubers (Viola *et al.*, 2001) and fruits (Patrick and Offler, 1996; Ruan and Patrick, 1995). In both cases, the switch coincides with commencement of a grand phase of photoassimilate import. Prior to initiation of tuber development, plasmodesmata are closed and unloading from sieve element-companion cells (SE-CC) complexes follows an apoplasmic route (Viola *et al.*, 2001). Coincident with tuber swelling, developing internal and external phloem strands of tubers commence to unload symplasmically (Viola *et al.*, 2001). In contrast, developing tomato fruit exhibit a reverse behaviour switching from symplasmic to an apoplasmic path of unloading (Patrick and Offler, 1996; Ruan and Patrick, 1995). A major difference between potato tuber and tomato fruit sinks is that the former accumulates starch and the latter osmotically active hexoses. Furthermore, the recent finding that cotton fibre plasmodesmata are reversibly gated across their development (Ruan *et al.*, 2001) could equally apply to other unloading pathways allowing for greater flexibility in regulating photoassimilate fluxes from the phloem.

In grapes early in development (stage I and II) a comparable level of soluble sugars comparable between phloem in the pedicel and berry was found, and could support the symplasmic phloem unloading pathway. At berry veraison, an high cell wall invertase activity, an high soluble sugar concentration, a decrease in plasmodesmata conductivity and the hydraulic isolation of the berry, sustain whereas an apoplasmic phloem unloading pathway (Zhang *et al.*, 2006).

1.4.3 SINK ACTIVITY: SUCROSE CLEAVING ENZYMES

In recent years it has become evident that sugars, notably sucrose and its cleavage products, are important metabolic signals that affect the expression of different classes of genes (Koch, 1996b; Rolland *et al.*, 2002) and are involved in the regulation of development (Wobus and Weber, 1999).

Sucrose is a key compound in plant metabolism and it also plays an important signalling role in metabolic and morphologic development by regulation of gene expression in plants (Gibson, 2005; Lunn and MacRae, 2003). Source sucrose metabolism is focused on the synthetic process, whereas sink sucrose metabolism often emphasize on catabolism (Quick and Schaffer, 1996).

The different organs of plants have diverse tasks and biochemical requirements. One of the crucial functions of source leaves is the synthesis of energy-rich molecules for the transport of carbon, whereas heterotrophic sink organs, such as developing fruits, seeds, roots and tubers are dependent on the import and utilization of these compounds. In most plant species, assimilated carbon is transported as sucrose, a disaccharide in which glucose and fructose are linked via an *O*-glycosidic bond. Cleavage of this bond initiates sucrose utilization and in plants this reaction is catalyzed by two enzymes with entirely different properties: invertase (EC 3.2.1.26) and sucrose synthase (EC 2.4.1.13) (Copeland, 1990). Invertase is a hydrolase, and cleaves sucrose into the two monosaccharides. By contrast, sucrose synthase is a glycosyl transferase, which, in the presence of UDP, converts sucrose into UDP-glucose and fructose

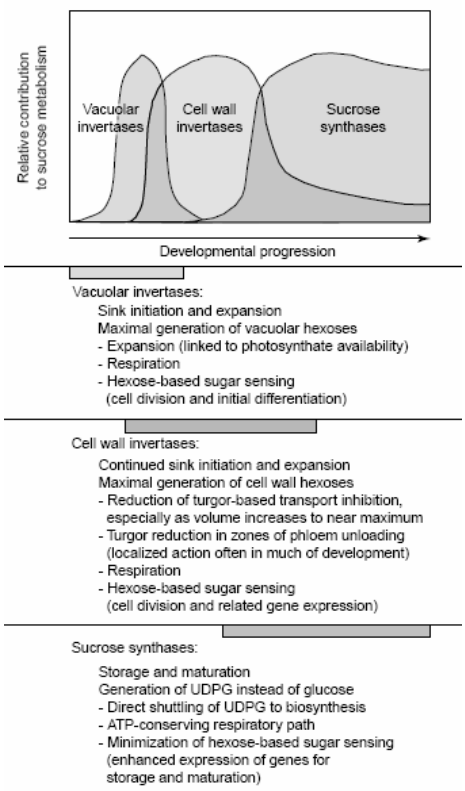


Fig. 1-8: A common developmental profile of the contributions of sucrose cleaving enzymes to sequential stages of sink initiation, expansion, and storage/maturation (Koch, 2004).

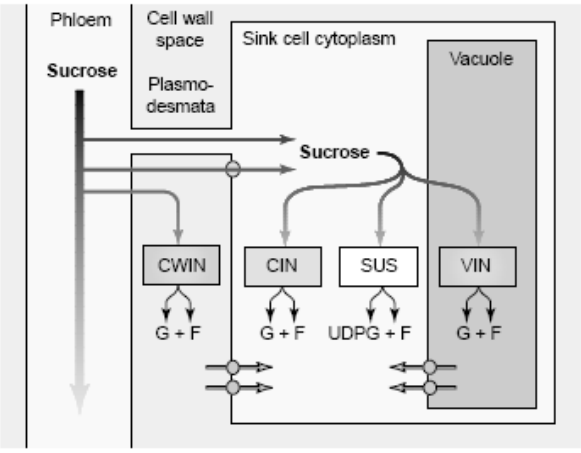


Fig. 1-9: The physical path of sucrose movement and site of its cleavage are central not only to mechanisms of import but also to the sugar signals generated. cell wall invertase (CWIN); cytoplasmic invertase (CIN); sucrose synthase (SUS); vacuolar invertase (VIN); fructose (F); glucose (G) (Roitsch and Gonzalez, 2004).

1.4.3.1 SUCROSE SYNTHASE

Sucrose synthase (UDP-glucose: D-fructose 2-glucosyl-transferase, EC 2.4.1.13) catalyses the reversible reactoin:

sucrose + UDP ⇔ UDP-glucose + fructose

It has a major role in energy metabolism and is involved in the movement of sucrose into diverse pathways important for metabolic structure and storage functions of the plant cell. Sucrose synthase (SUSY) activity correlates with sugar import (Sung *et al.*, 1989), cell wall synthesis (Chourey *et al.*, 1991) and sink strength (Sun *et al.*, 1992). Several pieces of evidence indicate that SUSY exists both free in the cytosol and in association with the plasmalemma (Amor *et al.*, 1995; Carlson and Chourey, 1996).

SUSY activity has been studied in various plants and has been shown to play a major role in energy metabolism, controlling the mobilization of sucrose into various pathways important for the metabolic, structural, and storage functions of the plant cell (Sturm and Tang, 1999). The role of SUSY in carbon import may involve a dual capacity to direct carbon toward either polysaccharide biosynthesis or an adenylate-conserving path of respiration (Koch, 2004). Main SUSY activity associated roles are listed below:

- Phloem Loading: SUSY activity associated with vascular tissues appears to play a key role in supplying energy in the companion cells for phloem loading by providing substrate for respiration (Fu and Park, 1995; Hänggi and Fleming, 2001).
- Sink Strength: SUSY cleavage activity correlates with the sink strength of storage organs, providing substrates for starch synthesis in potato tubers, maize kernels, or pea embryos (Déjardin *et al.*, 1997; Sun *et al.*, 1992; Zrenner *et al.*, 1995).
- Cell Wall Synthesis: SUSY is also proposed to supply UDP-glucose for cell wall biosynthesis in association with the cellulose synthase complex (Delmer and Amor, 1995; Haigler *et al.*, 2001; Ruan *et al.*, 2003).

Sucrose synthase is up-regulated under low-oxygen conditions (Koch *et al.*, 2000; Zeng *et al.*, 1998) and by carbon deprivation (Koch, 1996a). Sugar inducible up-regulation in potato requires SNF-related kinase (SnRK) (Halford *et al.*, 2003; McKibbin *et al.*, 2006).

Sucrose synthase is typically soluble in the cytoplasm, although it can move rapidly on and off membranes and cytoskeletal locations. Activities at plasma membranes and Golgi sites for cell wall biosynthesis, possible roles at the tonoplast level (storage/use of vacuolar sucrose) and activity at points on actin (to facilitate starch formation through plastid proximity) are some of the special function attributed to SUSY-bound forms as reviewed by Koch (2004).

Sucrose synthase activity is negatively affected by high levels of fructose (Morell and Copeland, 1985).

Here are reported some examples to show sucrose synthase importance in sink organs. The reduction of activity had as consequence a reduction of carbon availability synthesis of storage products and growth. The reductions in seed sucrose synthase activity and the reduced accumulation of starch was the effect of The *rugosus4* (*rug4*) mutation of pea (Craig *et al.*, 1999) and the *shrunken1* (*sh1*) and *sucrose synthase1* (*sus1*) mutations of maize (Chourey *et al.*, 1998). Those mutations eliminate isoforms of sucrose synthase that are highly expressed in the developing seed. The *rug4* mutation reduces sucrose synthase activity in the *Rhizobium* nodules of pea roots as well as in the seed, resulting in a loss of nitrogen fixing capacity and premature senescence of these organs (Craig *et al.*, 1999; Gordon *et al.*, 1999). Tubers of transgenic potato plants with reduced sucrose synthase activity also accumulate less starch than wild-type tubers (Zrenner *et al.*, 1995). In transgenic cotton plants with reduced sucrose synthase activity, fibre cell initiation and elongation and seed development are compromised (Ruan *et al.*, 2003).

1.4.3.1.1 Sucorse Synthases in Arabidopsis thaliana

In the model plant *Arabidopsis thaliana*, the complete sequencing of the genome reveals six putative members in the SUSY gene family (Barratt *et al.*, 2001). The complete *Arabidopsis* SUSY family comprises 6 isoforms: AtSUS1 (At5g20830), AtSUS2 (At5g49190), AtSUS3 (At4g02280), AtSUS4 (At3g43190), AtSUS5 (At5g37180) and AtSUS6 (At1g73370). All sucrose synthases are reported in Fig. 1-10. All isoform present two characteristic domains: the sucrose synthase domain and the glucosyl-transferase domain (Baud *et al.*, 2004). By sequence homology, sucrose synthases cluster in three groups: SUS1 group (AtSUS1, AtSUS4), SUSA (AtSUS2, AtSUS3) and a third group (AtSUS5, AtSUS6) (Baud *et al.*, 2004). The same behaviour has been shown by phylogenic analysis of sucrose synthase in pea (Barratt *et al.*, 2001), citrus (Komatsu *et al.*, 2002) and potato (Fu and Park, 1995).

AtSUS1 is expressed in the phloem of leaves and in roots (Martin *et al.*, 1993). AtSUS2 (At5g49190) was cloned and characterized by Chopra *et al.* (1992). The two genes were found to be strongly and differentially regulated in leaves exposed to environmental stresses (Déjardin *et al.*, 1999). To date, no data relative to AtSUS3 (At4g02280), AtSUS4 (At3g43190), AtSUS5 (At5g37180), or AtSUS6 (At1g73370) are available.

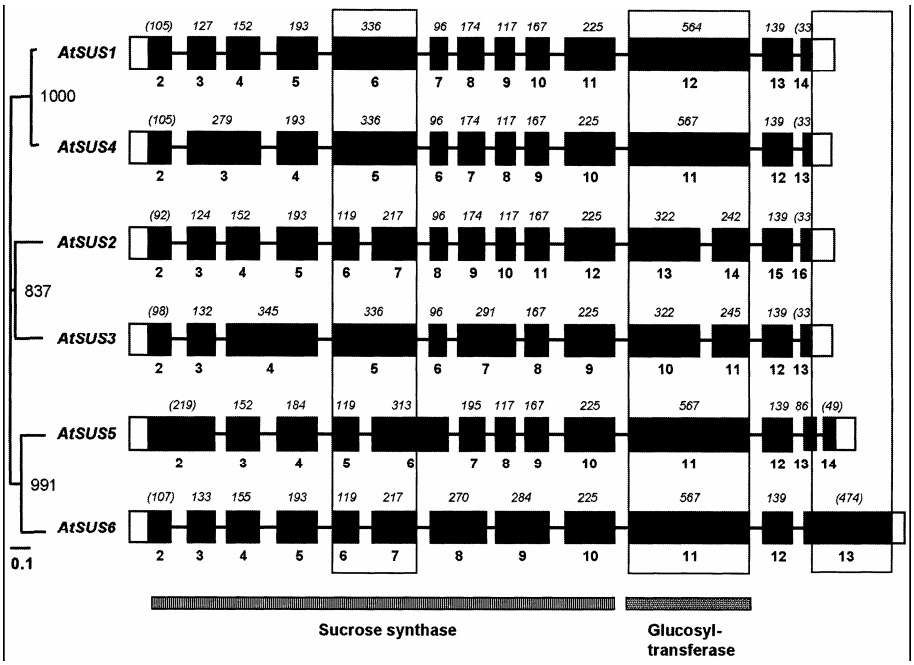


Fig. 1-10: Comparison of the six *Arabidopsis* SUSY genes as reported by Baud *et al.* (2004). On the left, a distance tree among the glucosyl-transferase (GT) domains from *A. thaliana* sucrose synthase proteins is shown. On the right, a comparison of the gene structures is presented. Numbered black boxes represent exons and the lines connecting them denote introns; the possible presence of a non-coding exon 1 was not considered. Sizes of exons are given above the structure, and the numbers in parentheses indicate the length of coding sequences.

Different sucrose synthase isoforms fulfil distinct metabolic functions being in many species spatially and developmentally differentially expressed (Barratt *et al.*, 2001; Fu and Park, 1995; Komatsu *et al.*, 2002; Martin *et al.*, 1993)

1.4.3.1.2 Sucrose Synthases in Kiwifruit

MacRae *et al.* (1992) have studied the carbohydrate metabolism during postharvest ripening in kiwifruit and sucrose synthase was one of the enzyme under investigation. During ripening, sucrose synthase activity was initially constant, increasing during the peak of climacteric.

The closest *Arabidopsis* sucrose synthases isoforms are AAC28175.1 on chromosome 4 for SUSA, and AAK59464.1 on chromosome 3 for SUS1. SUS1 is a sucrose synthase with greatest homology (83–88% amino acid identity) to sucrose synthases involved in sucrose unloading in storage organs. Richardson *et al.* (2004) showed SUS1 having the highest steady-state mRNA levels at 40 DAA, whereas its expression decreased as fruit developed.

SUSA is a sucrose synthase with the greatest homology to a sucrose synthase shown to highly expressed in mature mandarins (Komatsu *et al.*, 2002). In kiwifruit SUSA showed increased expression as fruit developed, with greatest expression in mature fruit 194 DAA (Richardson *et al.*, 2004).

1.4.3.2 INVERTASES

Invertases (EC 3.2.1.26, β-fructosidase, β-fructofuranosidase) catalyse the irreversible hydrolyses to glucose and fructose. The importance of invertases was only recognized when molecular studies were initiated in the early 1990s (Miller and Chourey, 1992; Sturm and Chrispeels, 1990; Vonschaewen *et al.*, 1990); thereafter, invertases attracted a lot of attention and to date there are >300 invertase sequences in the databases, representing >200 different isoenzymes from ~50 plant species. A still-growing number of studies have revealed that invertases play a crucial role in various aspects of plant growth and development (Roitsch and Gonzalez, 2004).

Based on their solubility, subcellular localization, pH-optima and isoelectric point, three different types of invertase isoenzymes can be distinguished: vacuolar (Inv-V), cell wall bound (Inv-CW) and neutral (Inv-N) invertases (Roitsch and Gonzalez, 2004) (Fig. 1-11, Tab. 1-6).

Tab. 1-6: Properties and functions of invertase isoenzymes. Modified from Roitsch and Gonzalez (2004). *, (Nonis *et al.*, 2007).

Invertase	pH-optimum	pI	Localization	Function
Vacuolar (Inv-V)	Acidic	Neutral	Vacuole	Control of sugar composition in fruit and storage organs Osmoregulation and cell enlargement Response to drought stress, hypoxia and gravitropism Response to wounding
Extracellular (Inv-CW)	Acidic	Basic	Apoplast	Regulation of sucrose partitioning Response to wounding and pathogen infection Regulation of seed and pollen development
Neutral (Inv-N)	Neutral or alkaline	Neutral	Cytoplasm	Regulation in plant development* Supportive role to other sugar metabolism enzymes*

As reported in §1.4.2, phloem unloading can be symplasmic or apoplasmic, and sucrose is then hydrolysed by different invertase isoenzymes, as reported in Fig. 1-11. In symplasmically isolated tissues, sucrose is unloaded from the sieve elements of the phloem into the apoplast by an assumed efflux sucrose transporter. Sucrose can be then cleaved by an extracellular invertase bound to the cell wall. The resulting hexoses will be transported into the sink cell by a hexose transporter. Sucrose can be also directly transported into the sink cell by a sucrose transporter. Sucrose unloaded in the sink cell, either through the apoplasm or via plasmodesmata, can be cleaved in the cytosol by neutral invertase or sucrose synthase, or in the vacuole by vacuolar invertase. The hexoses generated are not only substrates for heterotrophic growth but also function as regulators of gene expression. Coordinated regulation by metabolic stimuli has been shown for photosynthesis, sink metabolism and defence responses. Cell wall invertases play a special role because any stimulus up-regulating the activity will decrease the local sucrose concentration in the sink-tissue and thus increase the sink-strength, providing a feed-forward mechanism for

maintaining and amplifying diverse stimuli to enhance the flow of assimilates (Roitsch and Gonzalez, 2004).

The invertase activity is regulated at gene expression and enzyme activity levels. Invertase gene expression is under the control of a multigene family, and isoforms gene expression is organ and developmental stage specific (Lorenz *et al.*, 1995; Sturm *et al.*, 1995; Weber *et al.*, 1995a). Plant invertase gene expression or activity has been shown to be regulated by several factors, such as differential transcript formation (Cheng *et al.*, 1999), exon skipping (Bournay *et al.*, 1996), inhibition by proteinaceous inhibitors (Rausch and Greiner, 2004), as well as a novel mechanism that controls compartmentalization and breakdown (Rojo *et al.*, 2003).

Plant invertase multigene family comprises two different subfamilies: acid invertases (cell wall and vacuolar) and cytoplasmic invertases. Plant acid invertases are closely related to respiratory eukaryotes (bacteria and yeast) (Sturm and Chrispeels, 1990), wheres plant neutral/alkaline invertases showed affinity to cyanobacterial invertases (Vargas *et al.*, 2003).

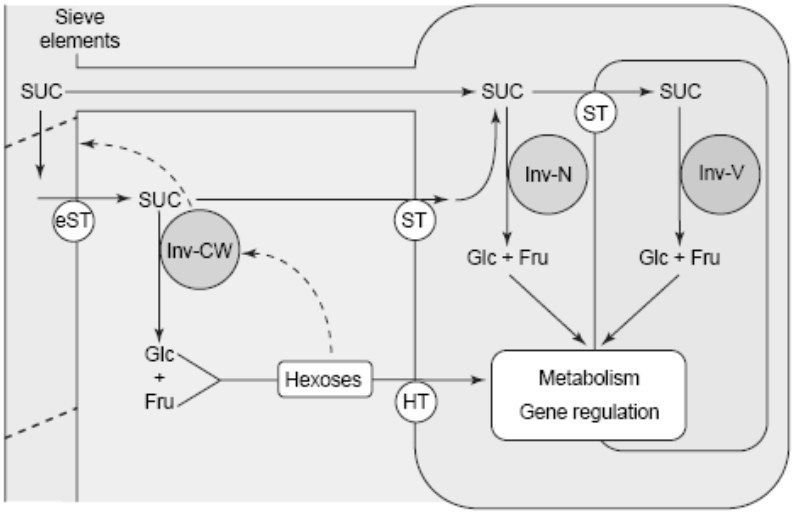


Fig. 1-11: Subcellular localization of invertase isoenzymes and phloem unloading pathways (Roitsch and Gonzalez, 2004). efflux sucrose transporter (eST); extracellular invertase (Inv-CW); hexose transporter (HT); sucrose transporter (ST); neutral invertase (Inv-N); vacuolar invertase (Inv-V); fructose (Fru); glucose (Glc); sucrose (SUC).

1.4.3.2.1 Invertses in Arabidopsis thaliana

In *Arabidopsis thaliana* two large gene families encode the acid invertases (cell wall and vacuolar) (Haouazine-Takvorian *et al.*, 1997; Sherson *et al.*, 2003) and the neutral/alkaline invertases of the cytosol (Vargas *et al.*, 2003).

Vacuolar invetases comprises two isoforms, Atβfruct3 and Atβfrut4 (Haouazine-Takvorian *et al.*, 1997), whereas four cell wall invertase isoforms have been identified: Atβfruct1 and Atβfrut6, Atβfruct2 and Atβfrut5 (Fridman and Zamir, 2003). Fridman and Zamir (2003) reported Atβfruct2 and Atβfrut5 to cluster with LIN5, the tomato invertase that plays a key role in the introgression line 9-2-5 promoting sugar accumulation. LIN5 is therefore sink

specific, whereas in Arabidposis, the homologous Atβfrut5 is the main expressed in the silique and together with Atβfrut6 is expressed in the pod (Fridman and Zamir, 2003).

1.4.3.2.2 Invertases in Kiwifruit

Little is known on invertase enzyme activity in kiwifruit. A study on carbohydrate metabolism during post-harvest ripening in kiwifruit shows soluble invertase activity to increase soon after harvest and then decrease to the initial level after 60 days post-harvest, for both acid and alkaline isoenzyme (MacRae *et al.*, 1992). At-harvest acid invertase activity was shown to be double than the alkaline one. No information are available on cell wall invertase. To date no studies have been published on invertase gene family in kiwifruit.

1.4.4 SINK ACTIVITY: STARCH SYNTHESIS

Starch is the major storage polysaccharide in plants and is accumulated as granules in many different organs such as leaves, roots, shoots, fruits, or grains, where it is used as a carbon and energy source (Sivak and Preiss, 1998).

The first step in starch synthesis is the formation of the adenosine 5'-diphosphate (ADP)-glucose from glucose 1-P and ATP, a reaction catalyzed by ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27). ADP-glucose is the soluble precursor of starch and the substrate of the following reaction. The glucosyl moiety of ADP-glucose is then transferred to an existing α-glucan chain through an α-1,4 glucosidic bond by glycogen/starch synthase (EC 2.4.1.21). Branching enzyme (EC 2.4.1.18) then catalyzes the formation of K-1,6 glucosidic branches in the growing glucan chain (Salamone *et al.*, 2000).

The regulatory and rate-limiting step of starch biosynthesis is the synthesis of the glucosyl donor, ADP-glucose, by ADP-glucose pyrophosphorylase.

Starch accumulation and degradation occurs on a diurnal basis in many green tissues and this regulation can be accounted for by photosynthetically mediated fluctuations in metabolites that regulate ADP-Glucose pyrophosphorylase (Preiss, 1984). In tomato fruit, as well as in kiwifruit, a major shift in starch metabolism occurs over a period of weeks and it seems possible that this regulation could occur through regulation of levels of enzymes contributing to either starch biosynthesis and degradation. Dinar and Stevens (1981) reported a positive correlation between starch levels and final soluble sugars levels in tomato fruit. Robinson *et al.* (1988) shown a positive correlation between high starch levels, high ADP-Glucose pyrophosphorylase and sucrose synthase activity, and high soluble sugar levels

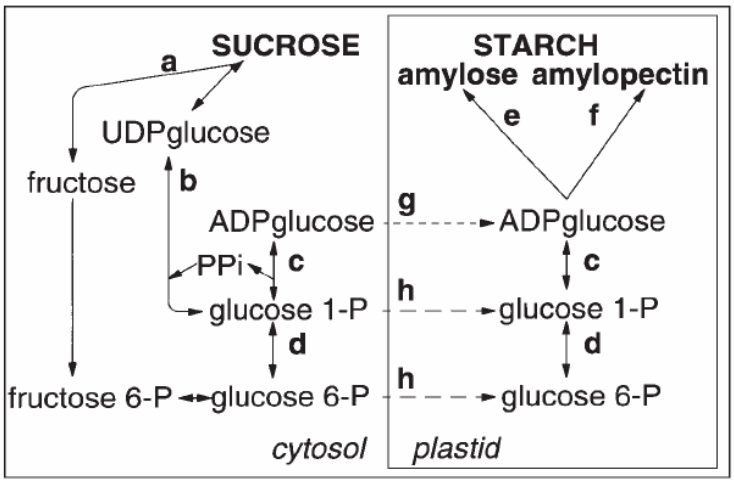


Fig. 1-12: The major metabolites and enzymes involved in the conversion of sucrose to starch in storage organs. Carbon is shown entering the plastid either as a hexose phosphate (Smith et al., 1995a) or as ADPglucose. Enzymes are: a, sucrose synthase; b, UDPglucose pyrophosphorylase; c, ADPglucose pyrophosphorylase; d, phosphoglucomutase; e, starch synthase (GBSSI); f, starch synthase and starch-branching enzyme; g, ADPglucose transporter; h, hexose phosphate transporter. PPi: inorganic pyrophosphate. From Smith et al. (1997).

1.4.4.1 ADP-GLUCOSE PYROPHOPHORYLASE

The native ADP-glucose pyrophosphorylase enzyme (ATP:α-glucose-1-P adenyltransferase, EC 2.7.7.27 – AGPase) is a heterotetramer ($\alpha_2\beta_2$) composed of two different types of subunits (Morell *et al.*, 1987; Sivak and Preiss, 1998). AGPase has a molecular mass of 200-240 kDa (Copeland and Preiss, 1981; Preiss *et al.*, 1987) and, in all plants studied to date, it consists of two small (50 kDa) and two large (51 kDa) subunits. The small subunit is considered the fully catalytic one and it is highly conserved between species. The large subunit has lost its catalytic function and it mainly plays an allosteric modulatory function regulating the activity of the enzyme (Ballicora *et al.*, 1995; Sivak and Preiss, 1998). Large subunit amino acid sequence is more divergent between different species. Although that, the large and small subunits are very similar to each other in the overall amino acid sequences, reflecting a common ancestry (Ballicora *et al.*, 2005): in fact, in bacteria, the enzyme is a homotetramer (Iglesias *et al.*, 1991).

Different studies on ADP-glucose pyrophosphorylase from maize and tomato have suggested that both subunits may have a regulatory role (Ballicora *et al.*, 1998; Cross *et al.*, 2004). The activity of most plant AGPase is finely regulated by an allosteric mechanism. The enzyme is activated by 3-phosphoglycerate (3-PGA) and inhibited by phosphate (Pi) (Chen and Janes, 1997; Kleczkowski *et al.*, 1993a; Kleczkowski *et al.*, 1993b; Sikka *et al.*, 2001), but the extent of regulation varies from organ to organ. Leaf, potato tuber and tomato fruit AGPases are dependent on 3-PGA for maximum catalytic activity and this 3PGA activation is reversed by Pi. Although the reaction is reversible in vivo, the cleavage of PPi drives the reaction forward.

ADP-Glucose pyrophosphorylase (AGPase) catalyzes a rate-limiting step in starch synthesis and the first committed step in the biosynthesis of both transient starch in

chloroplasts/chromoplasts, and storage starch in amyloplasts. In all plant tissues capable of starch biosynthesis, adenosine 5' diphosphate glucose (ADPGlc) pyrophosphorylase (AGPase, EC 2.7.7.27) is the enzyme responsible for the production of ADPGlc, the soluble precursor and substrate for starch synthases. Modification of the regulatory properties of this enzyme increases starch yields in potato (*Solanum tuberosum*) tubers, and maize (*Zea mays*), wheat (*Triticum aestivum*), and rice (*Oryza sativa*) seeds (Giroux *et al.*, 1996; Smidansky *et al.*, 2002; Smidansky *et al.*, 2003; Stark *et al.*, 1992).

In addition to the plastidial AGPase present in all starch-synthesizing tissues, biochemical evidence indicates the presence of at least two distinct AGPase enzymes in the endosperms of maize (Denyer *et al.*, 1996), barley (Thorbjørnsen *et al.*, 1996a), rice (Sikka *et al.*, 2001), and wheat (Tetlow *et al.*, 2003) which have been shown to correspond to plastidial and cytosolic isoforms of AGPase. In the developing endosperms of wheat, maize, barley, and rice the cytosolic isoform accounts for 65–95% of the total AGPase activity, implying that most of the storage starch biosynthesis in these tissues occurs through import of ADPGlc into amyloplasts. Plants possess multiple genes encoding either the AGPase Large or AGPase Small subunits, or both, and these are differentially expressed in different plant organs. This means that the AGPase subunit composition may vary in different parts of the same plant in tissues such as potato (La Cognata *et al.*, 1995), rice (Nakamura and Kawaguchi, 1992), and barley (Villand *et al.*, 1992a). The multiple genes encoding the AGPase Large subunits show strong specificity in their expression, for example, being restricted to leaf, or root and endosperm in both barley and wheat (Olive *et al.*, 1989; Villand *et al.*, 1992a; Villand *et al.*, 1992b) or induced under specific conditions, such as increased sucrose or glucose levels in potato (Duwenig *et al.*, 1997; Müller-Röber *et al.*, 1990). Multiple isoforms of the AGPase Small subunit in bean show organ-specific expression patterns; one form is expressed only in leaves, the other in both leaves and cotyledons (Weber *et al.*, 1995b). Different cDNAs encoding the AGPase Small subunit in maize also have distinct tissue expression patterns (Giroux and Hannah, 1994; Prioul *et al.*, 1994). The differential expression of subunits in different tissues may produce AGPases with varying degrees of sensitivity to allosteric effectors (see the section on allosteric regulation), which are suited to the particular metabolic demands of a given plant tissue/organ. In cereal endosperms, the subcellular localization of AGPase isoforms is thought to be regulated by differential splicing of AGPase genes. Studies with barley indicate that the plastidial and cytosolic AGP-S subunit mRNAs are produced from a single gene by the use of two alternate first exons (Thorbjørnsen *et al.*, 1996b).

Recently has been reported the role of a sugar non fermenting kinase (SnFR1) in the transcriptional regulation of both AGPase subunits in potato, as well a redox regulation that control the transition of the heterotetrameric enzyme from an inactivated form to an active one (Tiessen *et al.*, 2002; Tiessen *et al.*, 2003). This findings implement the already highly regulated process of AGPase activity. A schematic representation of all the mechanism controlling AGPase is reported in Fig. 1-13.

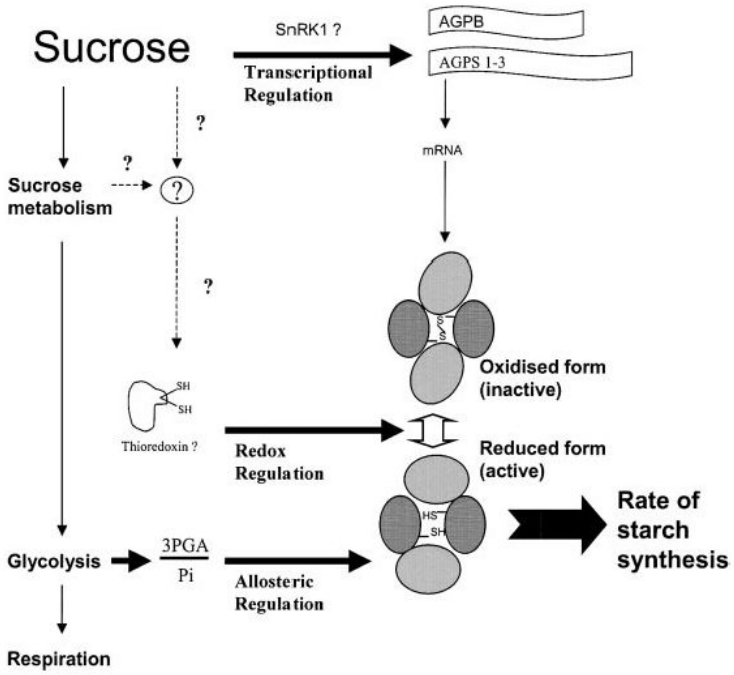


Fig. 1-13: Starch synthesis is regulated by ADP-glucose pyrophophorylase at different time scales and different levels of control in growing potato tubers. Diagram from Tiessen *et al.* (2002) modified by Geigenberger (2003).

1.4.4.1.1 AGPase Gene Family in Dicots

Generally, in dicots, a gene family of three or four members encodes for the large subunit of AGPase enzyme, whereas a single gene is responsible for the small catalytic subunit transcription. The large subunit genes have a tissue-specific localization and their expression is temporally differentiated, whereas the small subunit is generally ubiquitous, and it is not therefore temporally and spatially controlled (Crevillen *et al.*, 2005). *Arabidopsis*, one of the most studied dicot plant model, comprises four genes encoding for the large subunit (Apl1, Apl2, Apl3, Apl4) and a single gene for the small one (Aps1), as well as a small subunit-like gene (Aps2) which is not functional with any of the large subunit in *E. coli* (Crevillen *et al.*, 2003).

1.4.4.1.2 AGPase Gene Family in Tomato

Tomato is a model plant with similarity in fruit growth with kiwifruit. Starch accumulates in early stages of fruit development, contributing for about 20% of dry weight, in the period before the onset of maturation. Starch is completely degraded in ripe fruit, and the result of this is the enhancement of soluble sugar concentration, positively correlated with previously stored starch (Dinar and Stevens, 1981; Ho, 1996). Studies on sucrose to starch metabolism support a limiting role of AGPase in tomato fruit starch synthesis (Schaffer *et al.*, 2000; Yelle *et al.*, 1988).

The tomato genome, as well as the potato one, consists of three independently segregating genes encoding for the large subunit isoforms (L1, L2 and L3) and one for the small subunit (Chen *et al.*, 1998; Park and Chung, 1998).

L1 is the tomato large subunit gene mainly expressed in developing fruit, followed by L2, whereas L3 is source located. The main tomato fruit AGPase holoenzyme is the heterotetramer S1-L1 (Chen *et al.*, 1998; Li *et al.*, 2002; Park and Chung, 1998). The S1 subunit gene expression remains until 40-45 days after anthesis, whereas L1 and L2 expression was observed till 25 days after anthesis (Li *et al.*, 2002). In a recent study as been shown how L1 large subunit expression has a role in heterotetramer enzyme stability, and its temporally limited expression is a limiting factor for AGPase activity in tomato (Petreikov *et al.*, 2006). In fact, AGPase activity was observed to be strongly linked to the transient L1 gene expression.

1.4.4.1.3 AGPase in Kiwifruit

Very little is known about AGPase in kiwifruit up to date, even if kiwifruit is known to be a strong sink attracting up to 50% in dry weight of starch.

Antognozzi *et al.* (1996) reported in their study on CPPU (N-(2-Chloro-4-Pyridyl)-N-Phenylurea) effects on fruit carbohydrate metabolism the pattern of AGPase enzyme activity throughout fruit development (Fig. 1-14). AGPase enzyme activity was shown to be high at the onset of net starch accumulation, and then decrease gradually.

Fig. 1-14: AGPase enzyme activity in *Actinidia deliciosa* cv 'Hayward' throughout fruit development: data from untreated fruit (Antognozzi *et al.*, 1996).

1.4.5 CYTOKININ, A GROWTH REGULATOR INVOLVED IN SINK STRENGTH: SINK SIZE OR SINK ACTIVITY?

Hormone by definitions are "chemical messengers". They are substances produced in one part of the body and transported to a site of action elsewhere in the body, causing changes in physiological activity affecting growth and development (definition based on animals). Their action occurs at very small (10^{-6} – 10^{-8} M) quantities (Taiz and Zeiger, 2002).

Main classes of plant hormones or growth regulators are: auxins, cytokinins, gibberellins, abscissic acids and ethylene. Each hormones plays specific roles in promotion of growth and development, and they are usually named with the principal mechanism they control. Auxins and gibberellins are well known to be involved in cell extension and growth related processes, cytokinins promote cell division, ethylene is the ripening hormone and abscissic acid is involved in senescence processes. All plant hormones are interconnected into a network of mutual regulations, let them then preside an indefinite number of processes (Taiz and Zeiger, 2002).

Sink strength of different reproductive and vegetative organs has been also linked to the direct or indirect influence of citokinin levels (Gersani and Lips, 1980; Leonard *et al.*, 1983; Thomas, 1985). Tomato plants transformed to express *ipt* gene (key enzyme in cytokinin biosynthesis) in ovary tissues, showed increased cytokinin level throughout fruit development and higher at-harvest soluble solid concentration (Martineau *et al.*, 1995). has revealed that the cytokinin signal is transduced by two-component systems to the nucleus where target genes are activated. A recent study from Brenner *et al.* (2005) has shown some of the early and late responsive gene affected by cytokinin. Some sugar metabolic enzymes were also affected: SUS1 and monosaccharide transporters were up-regulated whereas a sucrose transporter and an hexokinase were down-regulated.

Natural cytokinins are N^6-substituted adenine derivatives, generally containing an isoprenoid derivative side chain. Cytokinins affect several aspects of plant development and physiology, such as seed germination, de-etiolation, chloroplast differentiation, apical dominance, plant pathogen interactions, flower and fruit development, and leaf senescence. Cytokinin are classified as natural and synthetic. Natural cytokinins are isoprenoid derivatives and they are the most abundant form (several plant species contain adenine derivatives with aromatic substituents). Synthetic cytokinins are diphenylurea derivatives that are structurally unrelated to the adeninetype cytokinins (Haberer and Kieber, 2002).

1.4.5.1 CYTOKININ-LIKE SUBSTANCES APPLICATION ON KIWIFRUIT

First studies about exogenous application of cytokinins to affect kiwifruit berry growth goes back to 70s, where it was shown that zeatin and benzyl adenine application did not affect significantly the fruit growth (Hopping, 1976a). Both cytokinins were natural.

Applications of the synthetic cytokinin CPPU (N-(2-Chloro-4-Pyridyl)-N-Phenylurea) showed interesting results in promoting fruit development in several studies (Antognozzi *et al.*, 1996; Biasi *et al.*, 1991; Biasi *et al.*, 1993; Blank *et al.*, 1992; Cruz-Castillo *et al.*, 2002; Iwahori *et al.*, 1988; Kim *et al.*, 2006; Lewis *et al.*, 1996; Patterson *et al.*, 1993; Woolley *et al.*, 1991). All the authors agree on the main growth regulators observed effects (fruit growth promotion, higher soluble sugar levels at harvest, negative side effects in early applications), whereas they did not about the mechanism of action. Some authors sustain that CPPU promotes cell division (Cruz-Castillo *et al.*, 2002; Iwahori *et al.*, 1988), others showed an effect on cell distension (Lewis *et al.*, 1996; Patterson *et al.*, 1993), or both were affected as per Woolley and co-workers (1991).

Dramatic fruit growth promoted by CPPU ends with an average 10% reduction in dry matter content (Antognozzi *et al.*, 1996; Patterson *et al.*, 1993).

After several years of study, although, the mechanism of action is not well understood yet. CPPU has little mobility: only few millimetre in depth from the application point, as shown in a ^{14}C-tracking study (Biasi *et al.*, 1993). It is therefore a possibility that signal transduction and transcription regulation mechanisms are involved.

1.5 THESIS GOALS AND OBJECTIVES

Fruit quality improvement is one of the actual breeding target for many fruit crop, as well as for kiwifruit. Fruit quality in kiwifruit is strongly related to dry matter and starch accumulated during fruit development. The overall aim of the present study was to determine what factors affect carbon accumulation during *Actinidia deliciosa* berry development. One way of doing this is by comparing *Actinidia deliciosa* genotypes that differ greatly in their ability to accumulate starch in their fruit.

Firstly fruit development, from anthesis to maturity, was studied for a range of genotypes selected from 15 *Actinidia deliciosa* breeding families to represent the extremes of DM content within the overall population, while also spanning a range of average fruit fresh weights at maturity. This was leading to understand how and when the contrasting genotypes differ in their patterns of fresh and dry weight growth, and the accumulation of carbohydrates, particularly starch. Fruit development was also examined over four seasons to determine how consistent the developmental patterns were. Secondly, being genotypes different in sink strength, both the components of sink strength were investigated. Are genotypes different because they are different in cell size or cell number? Are genotypes different because of a difference in carbohydrate metabolism that affect sink activity? Thirdly, a comparison of endogenous (genotypic) and exogenous (growth regulator application) modification of dry matter accumulation was carried out.

Thesis is structured into different chapters, and after a detailed report of all the generic method used for the individual experiments in chapter 2, in the following four chapter singular questions are answed as below reported:

Chapter 3 Are observed differences in dry matter among different genotypes related starch content and genetically determined?

Chapter 4 Are genotypes different because of strongly different anatomical structures?

Chapter 5 Is the starch metabolic pathway different between high and low dry matter genotypes?

Chapter 6 Is CPPU acting as low dry matter genotypes in limiting dry matter accumulation into kiwifruit berry?

In Chapter 7 a general discussion binding results of previous chapter is reported.

2 GENERAL MATERIAL AND METHODS

Material and Methods described in this chapter have been used for some of the experiments. Some techniques are here widely described in a step by step way, whereas in each following chapter a brief resume will be reported.

2.1 PLANT MATERIAL

2.1.1 FRUIT DEVELOPMENT STUDY

For the study of *Actindia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* fruit development, a total of 24 vines were selected in 2003 and 2004 from three factorial seedling populations. These were planted in 2 contiguous orchard blocks at Te Puke Research Centre (Bay of Plenty, NZ; 37° 49' S - 176° 19' E) in 1999 for breeding purposes. Vine selection was accurate. A total of 818 female seedling were screened and ranked according records of fruit dry matter content at harvest in previous years. An equal number of extreme high and low fruit dry matter plants characterized by a large or small final fruit size were selected. Vines were allocated in 4 classes with contrasting rates of fruit fresh weight and dry weight accumulation (Fig. 2-1).

		Fruit Size	
		Large	Small
Dry matter	High	CLASS High-Large	CLASS High-Small
	Low	CLASS Low-Large	CLASS Low-Small

Fig. 2-1: Fruit classes according to vines were grouped in.

All vines were growing on a T-bar trellis at 0.8 m spacing on the row and 5 m spacing between rows. The first selection step was carried out in 2003 when18 vines were selected. The following year only 4 seedlings from the original bunch were kept and 6 more extreme vines were added, for a total of 10. In 2006 only 9 of the 10 selected seedlings were assessed, due to the eradication of all the vine in one of the two blocks. In 2005 scions from the 10 selected vine were grafted on 4 'Bruno' rootstocks each genotype, following a complete randomized block design (Mead *et al.*, 1993) obtained using SAS® software. Grafted vines were growing in the same orchard block at Te Puke Research Centre spanning 4 rows on a T-bar trellis at 2 m spacing on the row and 5 m spacing between rows. Two polliniser varieties, King (early flowering) and M56 (mid-late flowering) were grafted both on each stump in a 1:5 male to female ratio each.

Blocks were managed according normal commercial practises, pruned using one year old cane replacement and a low intensity summer pruning. A basic ferlizer level was used.

In Appendix III Plant Pedigree Summaries of all the three breeding populations are reported. All the family used are genetically related to each other. ID codes used to name each individual ware created at HortResearch.

2.1.1.1 BREEDING POPULATIONS

Here are reported some informations on each breeding family (BF) object of the study.

2.1.1.1.1 Breeding Family C15

The breeding family C15 was obtained crossing the female parent 35-02-11f.92 and the male parent C-2-115.86. 310 vines, out of the 543 seedlings planted, were females.

2.1.1.1.2 Breeding Families A16 and B16

The breeding family A16 was obtained crossing the female parent 40-12-15d.92 (-2 generation male parent is C15's male parent) and the male parent 32-06-15a.91. 295 vines, out of the 300 seedlings planted, were females (of which 113 hermaphrodite).

2.1.1.1.3 Breeding Families A01...A03, A05...A07, A09...A14

The breeding families A01...A03, A05...A07, A09...A14 were obtained from an F2 Male Progeny Test crossing the female parent 40-12-15d.92 (-2 generation male parent is C15's male parent) and 12 of its male siblings (male parent) 32-06-15a.91 as shown in Tab. 2-1. The female parent was the same used for A16 crossing. 213 vines, out of the 451 seedlings planted, were females. Dots between family codes mean 'from-to'.

Tab. 2-1: Male Parents of F2 Male Progeny Test.

Family	Male Parent	Family	Male Parent
A01	40-08-14e.92	A09	47-01-07a.92
A02	40-09-14f.92	A10	47-01-07b.92
A03	40-12-15c.92	A11	47-03-07a.92
A05	40-13-15d.92	A12	47-03-07b.92
A06	46-02-04d.92	A14	47-04-09-f.92
A07	46-03-04c.92	A15	47-06-09f.92

2.1.1.2 SELECTED SEEDLINGS

In Tab. 2-2 seedling selected in 2003 and 2004 are reported. All seedlings were spanning the three breeding populations. 7 vines were from BF C15, 5 vines were from BF A16 and 11 vines were from the F2 Male Progeny Test. According to the criteria described in §2.2 and the classes showed in Fig. 2-1, 6 High-Large, 6 High-Small, 8 Low-Large and 4 Low-Small seedlings were selected.

Tab. 2-2: List of all vines selected and objects of the present study. Here are reported the progressive plant number (some gaps are due to a poor fruit load of the vine), the HR identification, the year of selection, the family of origin, Female and Male Parents, the DM and size classes of each seedling.

#	HR-ID	Year	Family	Female Parent	Male Parent	DM	Size
1	40-12-08f	2003	A14	40-12-15d-92	47-06-09f.92	Low	Large
3	40-11-09b	2003	A11	40-12-15d-92	47-03-07a.92	High	Large
4	40-11-09f	2003	A12	40-12-15d-92	47-03-07b.92	High	Small
5	40-10-05b	2003	C15	35-02-11f.92	C-2-115.86	High	Large
8	40-04-18b	2003	A16	40-12-15d.92	32-06-15a.91	High	Small
11	40-04-12b	2003	A06	40-12-15d-92	46-02-04d.92	Low	Small
12	40-04-04c	2003	C15	35-02-11f.92	C-2-115.86	Low	Large
13	46-01-11d	2003	A01	40-12-15d-92	40-08-14e.92	High	Large
14	46-01-14a	2003	A01	40-12-15d-92	40-08-14e.92	High	Small
15	46-03-14f	2003	A01	40-12-15d-92	40-08-14e.92	Low	Large
17	40-06-00b	2003	C15	35-02-11f.92	C-2-115.86	Low	Small
18	40-11-01d	2003	C15	35-02-11f.92	C-2-115.86	High	Large
19	40-05-18a	2003	A16	40-12-15d.92	32-06-15a.91	Low	Large
20	40-07-14d	2003	A16	40-12-15d.92	32-06-15a.91	Low	Large
21	40-03-17e	2003	B16	40-10-14e.92	32-06-15a.91	High	Large
22	40-03-08c	2003	A07	40-12-15d-92	46-03-04c.92	Low	Large
23	40-03-06a	2003	C15	35-02-11f.92	C-2-115.86	Low	Small
24	46-06-04b	2003	A01	40-12-15d-92	40-08-14e.92	High	Small
25	40-06-14a	2004	A16	40-12-15d.92	32-06-15a.91	Low	Large
26	40-09-15g	2004	A16	40-12-15d.92	32-06-15a.91	Low	Large
27	46-04-15g	2004	A01	40-12-15d-92	40-08-14e.92	Low	Small
28	40-13-03b	2004	C15	35-02-11f.92	C-2-115.86	High	Large
29	40-08-03e	2004	C15	35-02-11f.92	C-2-115.86	High	Small
30	40-11-09e	2004	A12	40-12-15d-92	47-03-07b.92	High	Small

2.1.2 GROWTH REGULATOR APPLICATION

The growth regulator experiment was carried out on green 'Hayward' kiwifruit [*Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa*] in a 1991 block orchard at Te Puke Research Centre (Bay of Plenty, NZ; 37^{o} 49' S - 176^{o} 19' E). Vines were managed for commercial production (except for pruining, thinning and girdling practices) on a pergola-trained system at 6 m spacing on the row and 5 m spacing between rows. All vines were growing on open-pollinated 'Kaimai' seedling rootstocks.

2.2 SELECTION CRITERIA FOR SIZE AND DRY MATTER

Size and dry matter are two continuous variable, so there is not a clear separation between large and small size, and high and low dry matter, as it can be easily found for qualitative characters such as colour.

It was the positioned a threshold for both variable, considering for example that one of the objective for dry matter was select really extreme genotypes. Dry matter threshold was between 17.5 and 18% of dry matter content at 154 DAA, referred to first years of population observations. Size was less important then dry matter for the experiment, and the goal of this selection was to have an equal distribution of fruit size between dry matter classes. A virtual threshold was then decided also for size, and it was 90 g of average fruit final fresh weight.

2.3 SAMPLE COLLECTION AND SAMPLING DATES FOR FRUIT DEVELOPMENT STUDY

Sampling dates were decided from the observation of general kiwifruit growth curves and dry matter accumulation from population studies. Particular attention was placed early in fruit development, when fruit grows fast. From 6 to 10 sampling dates were planned, according to the vine crop load. In 2006-07 season, when replicate vines were available, 10 time points were analyzed.

Samples were collected randomly from the vine at each time point.

Tab. 2-3: Seasons, number of genotypes and sampling dates for fruit development study. DAA: days after anthesis.

Season	Genotypes (No.)		Time (DAA)										Time points (No.)
	Hi-DM	9											
	Lo-DM	9											
2003-04	total	18	7	14	28	42	56	70	98	126	154		9
	Hi-DM	5											
	Lo-DM	5											
2004-05	total	10		14	28	42	56	70	98	126	154		8
	Hi-DM	5											
	Lo-DM	4											
2005-06	total	9		14		42	56	70		126	154		6
	Hi-DM	5											
	Lo-DM	5											
2006-07	total	10	7	14	28	42	56	70	98	126	154	182	10

2.4 PHYSICAL MEASUREMENTS

Physical measurements consist in average fresh weight determination and tissue proportion analysis, dry matter content and dry weight calculation.

2.4.1 FRESH WEIGHT DETERMINATION

At each sampling date 10 fruit [unless total plant fruit was lower than (10Xsampling date number)] were singularly weighed on a two decimal position electronic scale and the weight recorded.

2.4.2 TISSUE PROPORTION

At each sampling date fruit a picture of the whole fruit, a transversal and a longitudinal section were taken using a digital camera (Nikon, Coolpix 990). For early sampling dates (up to 2 WAFB) picture were taken using the camera on a stereo-microscope (Nikon, SMZ1500). As ruler was placed as scale in each picture.

2.4.2.1 IMAGE ANALYSIS

Images were analyzed using ImageJ (Rosband, 1997-2006). The plugins option 'Analyze: Area Calculation' was used.

2.4.3 DRY MATTER

Each equatorial slice was oven-dried to a constant weight at 60-65^{o}C, and the dry weight of the slice recorded and expressed as a percentage of the fresh weight (DM (%)). The dehydration was carried out by an Ezidri^{TM} Home Food Dehydrator model Ultra FD 1000 from Hidraflow Industries Limited (NZ). The weight was determined using a 3 decimals scale (Mattler Toledo, PB303-S/FACT). Slices were placed on 60 mm diameter Petri dishes (Sarstedt, Cat. #821194).

2.4.4 DRY WEIGHT CALCULATION

Dry weight was calculated using the formula reported in Eq. 2-1 dry weight. (DW) and fresh weight (FW) were expressed in g, whereas dry matter (DM) was reported as percentage.

$$DW = \frac{FW \times DM}{100} \qquad \textbf{Eq. 2-1}$$

2.5 ANATOMY

2.5.1 SPECIMEN PREPARATION FOR LIGHT MICROSCOPY

Specimen preparation was carried out as for Hallett and Sutherland (2005) and it consists of 6 phases: fixation, dehydration, resin embedding and capsule inclusion-polymerization, block trimming and sectioning, slide staining and mounting.

2.5.1.1 FIXATION

Before start to process sample, excess fixative was poured into labelled vials (specimen name, date and fixative type). Fine forceps/tweezers and scalpel with fresh blade were also prepared.

As soon as fruit were collected a central 1-5 mm transversal slice was cut from each berry. A sector comprehensive of all three tissues was then cut off as shown in Fig. 2-2 and quickly immersed in the Glutaraldehyde-Formaldehyde fixative (2.5%-2%).

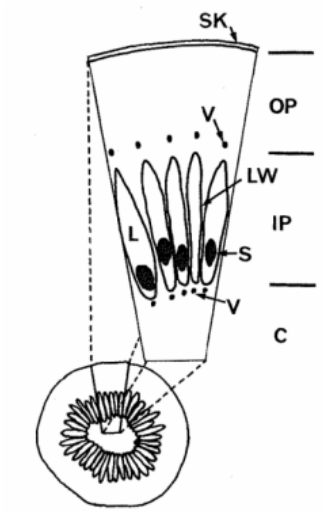


Fig. 2-2: Diagrammatic cross-section of a segment of kiwifruit. C = core, IP = inner pericarp, L = locule, LW = locule wall, OP = outer pericarp, S = skin layers (epicarp), V = location of major vascular trace (Hallett et al., 1992).

Fixative penetration (particularly for plant material) was aided by light evacuation (to pull out trapped air). Loosely capped vials were put in the vacuum desiccator and a light vacuum was pulled using the water aspirator. When bubbles were generated the vacuum was close off, but it was kept running (about 1 hour). When the vacuum was released it was checked that samples were not floating on the fixative surface. Cap was then securely fastened. Samples were than stored at 4ºC for 1-2 weeks.

2.5.1.2 DEHYDRATION

After fixation the samples were washed for 1 hour in 50 mM phosphate buffer (3 changes over 1 hour period using the rotator to agitate), rinsed quickly in distilled water, then replaced with 10% ethanol.

Dehydration was held with progressively more concentrated ethanol solutions to 100% 10-20 minutes per step using the rotator to agitate. Last step was a double change of 100% dried ethanol.

2.5.1.3 RESIN EMBEDDING

The last 100% ethanol was replaced with LR White Resin (London Resin, Reading, UK). Vials were agitated on the rotator overnight. The second day, two changes were carried out. Then sample resin was changed on each of the next two days.

2.5.1.4 CAPSULE INCLUSION AND POLYMERIZATION

A small label with specimen number and LR White Resin were placed into gelatine capsules, the specimen was quickly included and then the cap sealed. The resin was hardened by heat polymerization in embedding oven in fume hood at 60ºC for at least 8 hours. Then the blocks were let cool down and stored in a small labelled bag.

2.5.1.5 SLIDE PREPARATION: BLOCK TRIMMING AND SECTIONING

Block surface was trimmed first with a sharp blade to a trapezium shape surface. A more fine trimming was performed with a glass knife at the microtome (Leica, UltraCut UCT). 10 sections were then cut 1 µm thick, collected with a loop and placed on a drop of water on a glass slide. Slide was dried on a hot plate at 50ºC.

2.5.1.6 SLIDE PREPARATION: STAINING AND MOUNTING

Sections of resin-embedded material were stained in a 0.05% solution of toluidine blue in benzoate buffer (pH 4.4) for 5 minutes, 3 time water rinsed, air dried, and mounted in Shurmount (Triangle Biomedical Sciences, Durham, NC).

2.5.2 LIGHT MICROSCOPE OBSERVATIONS

Sections were viewed using an Olympus Vanox AHT3 microscope (Olympus Optical, Tokyo), coupled to a CoolSnap (Roper Scientific Ltd, Tucson, Arizona) digital camera, and the image was projected on a computer screen using RSImage software (Roper Scientific Ltd, Tucson, Arizona) and the magnification recorded. Observations were held from 40X to 400X magnifications. A reference scale picture was taken at each used magnification, for further measurements.

2.5.3 SPECIMEN PREPARATION FOR STEREO-MICROSCOPY

Samples were collected, fixed and dehydrated as for §§ 2.5.1.1 and 2.5.1.2. After dehydration, samples were stained and pictures were taken as following reported.

2.5.3.1 STAINING TECHNIQUE FOR CELL COUNTING

The cell walls at the surface of each slice were stained as reported in (Goffinet *et al.*, 1995) by immersing sectors for three minutes in 5% aqueous tannic acid, rinsing briefly in tap-

water, and immersing in 1% ferric ammonium sulphate (iron alum) for one minute, then rinsed again in water.

2.5.4 STEREO-MICROSCOPE OBSERVATIONS

After a second water rinse the sectors were pinned to the bottom of water-filled Petri dishes and examined at a 6X magnification with a stereo-microscope (Nikon, SMZ1500). Images were obtained using a Camera Control Unit (Nikon, SU-1) and analyzed by using ImageJ software (Rosband, 1997-2006). Before start each set of image captures, a picture of a scale was taken as zoom camera reference (Maiji Techno, 1 mm in 100 divisions).

2.6 BIOCHEMICAL TECHNIQUES

2.6.1 CARBOHYDRATE AND ACID ANALYSIS

Samples were ground in liquid nitrogen. A subsample was extracted twice using 80% ethanol at 60ºC for 1 h. Adonitol (Sigma, Cat. #A5502) and Tartrate (Sigma, Cat. #T0375) were added as internal standard for sugar and acid determination respectively. Suspension was then gravity filtered (modification of Cranswick and Zabkiewicz, 1979).

The insoluble residue was transferred from filter paper into Erlenmeyer flasks and analysed for starch as per Smith *et al.* (1992). The residue was autoclaved (1 h), and incubated at 55ºC with amyloglucosidase (Sigma, Cat. #A7255) in acetate buffer (0.25 M, pH 4.5) for a further hour. The glucose concentration in filtrates was measured colorimetrically at 510 nm (UV-Vis Spectrophotometer - Shimadzu, UV-160A) using a manual glucose oxidase method (Trinder, 1969) (Glucose oxidase- Sigma, Cat. #G6125; Peroxidase- Sigma, Cat. #P8250). Glucose concentrations were subsequently converted to starch equivalents and expressed as mg starch g^{-1} of fresh weight (FW).

Separation and quantification of individual sugars was carried out as per Klages *et al.* (1998) whereas separation and quantification of individual acids was carried out as per Cheng *et al.* (2004) as following reported.

The filtrate was evaporated to dryness in a rotary evaporator, re-dissolved in a minimum of water and passed through two Sephadex ion exchange columns (Sigma, Cat. #SPC25120 and #Q25120) (Redgwell, 1980) of 0.625 ml bed volume each. The eluate from the columns was immediately frozen and lyophilised and the sample was then redissolved in 10% isopropanol. An aliquot was transferred to an autosampler vial and dried under vacuum over P_2O_5. Sugars were derivatised with pyridine (Sigma, Cat. #P57506) and trimethylsilylimidazole (Aldrich, Cat. #153583) (1:1) (70ºC for 30 min). Acids were derivatised with 1:1 (v/v) pyridine and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA Thermo Scientific, Cat. #48913) before GLC analysis (60ºC for 15 min).

Sugars and acids were analysed by gas chromatography (Hewlit Packard, 6890 GC system) using split injection and a DB 1701 30 m column (Agilent J&W) with temperature

programming from 150ºC to 270ºC over 45 min for sugars and 130ºC to 270ºC over 35mins . Peaks of sugars (glucose, fructose, sucrose and *myo*-inositol) and acids (quinic, citric and malic) were confirmed by comparison with standard mixtures using GC/MS.

The individual sugar and acid contents were expressed as the milligrams per gram fresh fruit weight (mg g^{-1} FW).

2.6.2 ENZYME ACTIVITY

The methodology used for enzyme activity analysis was previously described for kiwifruit (MacRae *et al.*, 1992).

2.6.2.1 LIST OF SOLUTIONS AND REAGENTS

All the solutions used are described in Appendix IV. Buffer solutions were made fresh each day. Amounts are for 8 extractions per day.

- BSA - 2% w/v solution
- DTT -100 mM solution
- Sucrose – 0.5M solution
- Extraction Buffer (75mL)
- Desalting Buffer and Equilibrating Buffer (100 mL + 100mL)

2.6.2.2 ENZYME EXTRACTION

Weighed tissue (1.000 g of tissue, with 3 decimal positions recorded) was accurately ground with mortar and pestle in liquid N_2 to fine powder. Then PVPP and 3 mL of frozen extraction buffer were added and a further grounding step was performed. The mixture was let thaw in the mortar and then immediately transferred into two 2 mL clean Eppendorf tubes (Eppendorf). Phases were separated by centrifugation at 12,000 rpm at 4ºC for 20 minutes (Micro-centrifuge - Eppendorf, 5415R). Eight 'Sephadex G-25 Medium' PD-10 Desalting Columns (Amersham Bioscience) were equilibrated with Equilibrating Buffer at 4ºC, following manufacture's instructions.

2.5 mL of the supernatant were desalted through the column and the left over was measured. Enzymes were eluted with 3.5 mL of elution buffer. Eluted fraction was then aliquot into 1.5 mL eppendorf tubes and immediately frozen and stored in liquid N_2 before assaying.

After each use, columns were cleaned by pouring through 10-15 mL of distilled water and then stored with desalting buffer inside.

Cell Wall invertases are cell wall-bound enzymes. They were assessed using a suspension of the pellet. Pellet was washed twice in extraction buffer by vortexing. Pellet was collected by centrifugation (12,000 rpm at 4ºC for 5 minutes) and resuspend in 1 mL of Elution Buffer.

2.6.2.3 INVERTASE ASSAY

2.6.2.3.1 Stocks

Alkaline invertase Assay Buffer: 0.1 M HEPES pH 7.5

Acid invertase Assay Buffer: 0.5 M Sodium Acetate, pH 5

0.5 M Sucrose, make it fresh or store in freezer up to 15-20 days

5% Zinc sulphate

Saturated Barium hydroxide solution (80g/L)

2.6.2.3.2 Cytoplasmic Invertase (Soluble-Alkaline)

For blanks an extract aliquot of each sample (enough to give 2x120 µL blanks) was boiled for 15 minutes then clarified by centrifugation (5 minutes at max speed).

To four micro-tubes per sample (2 replicates, 2 blanks) were added:

- 40 µL assay buffer
- 40 µL sucrose
- 120 µL enzyme extract (fresh in 2, blanks in 2)

Replicate tubes were incubated 1 hour at 37 ºC (Thermoline Scientific, Dry Bath). Alkaline-invertase activity was stopped by heating the tubes at 95ºC for 4 minutes. Solutions were clarified by centrifugation (5 minutes at maximum speed).

2.6.2.3.3 Vacuolar Invertase (Soluble-Acid)

To four tubes per sample (2 replicates, 2 blanks) add:

- 40 µL assay buffer
- 40 µL sucrose
- 120 µL enzyme (fresh in 2, blank in 2)

To the blank tubes 200 µL of Zinc sulphate solution were added to precipitate enzymes. After 5 minutes on ice the reaction was neutralized by the addition of 80 µL of Barium hydroxide solution. Solutions were clarified by centrifugation (2 minutes at maximum speed).

Replicate tubes were incubate 3 hours at 37 ºC. The reaction was stopped as for blank tubes.

2.6.2.3.4 Cell Wall Acid Invertase (Insoluble-Acid)

To four tubes per sample (2 replicates, 2 blanks) add:

- 40 µL assay buffer

- 40 µL sucrose
- 120 µL cell wall suspension (fresh in 2, blank in 2)

To the blank tubes 200 µL of Zinc sulphate solution were added to allow enzyme precipitation. After 5 minutes on ice the reaction was neutralized by the addition of 80 µL of Barium hydroxide solution. Solutions were clarified by centrifugation (2 minutes at maximum speed).

Replicate tubes were incubated 3 hours at 37 ºC at 250 rpm. The reaction was stopped as for blank tubes.

2.6.2.4 GLUCOSE ASSAY USING HK/G6P-DH ENZYME.

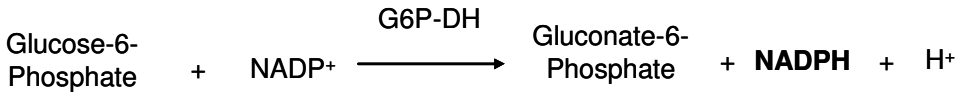
2.6.2.4.1 List of Reagents

- Enzyme for glucose determination: Hexokinase/Glucose-6-Phosphate Dehydrogenase: HK/G6P-DH(stored at 4ºC). The enzyme is added without any preparation. HK/G6P-DH ratio 2:1 in 3 mg/mL suspension in 3.2 M $(NH_4)_2SO_4$ solution. (Roche, Cat. # 10 737 275 001).
- Glucose Assay Buffer: 0.2 M Imidazole/3 mM $MgCl_2$, 5.4 mg/mL ATP, 3.2 mg/mL $NADP^+$. Make it fresh daily.
- Glucose Standard solution: 0.5 g/L, 2.78 nmol/µL (Roche, Cat. #0716260).

2.6.2.4.2 Glucose Determination

In Eq. 2-2 and in Eq. 2-3 the stechiometry of the reaction is reported. After the coupled activity of HK and G6P-DH for each mole of D-Glucose a mole of NADPH is generated. 1 g/L of Glucose gives 1 g/L of NADPH that gives 1 absorbance unit at a 340 nm wavelength.

Eq. 2-2



Eq. 2-3

2.6.2.4.3 Glucose Standard Curve

A Glucose Standard Curve was performed for each Glucose Assay's plate. Aliquots of 1X and 10X diluted Glucose Standard solution were loaded to span a 1.4-111.1 nmol of Glucose content, as reported in Tab. 2-4.

2.6.2.4.4 Spectrophotometer Enzyme Assay

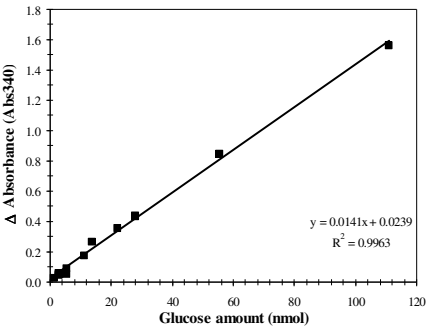
Glucose Assay was performed at 340 nm using a Spectramax micro-plates spectrophotometer (Molecular Devices, Spectramax®Plus384) and 96-200 µL well COSTAR UV-Plates (Corning, Cat. #3635) at 25 ºC.

For each plate loaded a diagram with sample locations was drawn. 2 Blank wells were designed for each plate. Replicates and sample's blanks were loaded twice. A standard curve was also drawn as stated in §2.6.2.4.3.

105 µL of sample, water or standard solution were placed in the well. Then 95 µl/well of Glucose Assay Buffer was added. A first reading was performed with Softmax Pro (Molecular Devices, v 3.1.2) and used as baseline (t_0, reading without enzyme). Then 2 µL of enzyme suspension were added and the plate was incubated at 25 ºC for 30 minutes. A second reading was then performed (t_{30}: reading after enzyme's reaction).

Tab. 2-4: Glucose Standard Curve example. On the last column on the right a standard curve plot is also reported

Dilution factor	Glc Std solution (µL)	Expected Abs	(X) Glucose amount (nmol)	(Y) Measured ΔAbs
10X	5	0.03	1.4	0.03
1X	1	0.05	2.8	0.06
10X	10	0.05	2.8	0.05
1X	2	0.10	5.6	0.09
10X	20	0.10	5.6	0.05
10X	40	0.20	11.1	0.17
1X	5	0.25	13.9	0.26
10X	80	0.40	22.2	0.36
1X	10	0.50	27.8	0.43
10X	100	0.50	27.8	0.44
1X	20	1.00	55.6	0.85
1X	40	2.00	111.1	1.56



2.6.2.4.5 Invertase Activity Calculation

Eq. 2-4 and Eq. 2-5 report formulas to calculate invertase enzyme activity. Information needed to solve the equations are: tissue fresh weight, total volume extracted after centrifugation, PD10 loaded volume, PD10 eluted volume, eluted volume used in enzyme's assay, enzyme's assay final volume, enzyme assay time, glucose standard curve slope, enzyme's assay volume loaded for glucose assay, t0 sample assay absorbance, t30 sample assay absorbance, t0 blank absorbance and t30 blank absorbance. Enzyme activity was expressed as µmol of glucose per g of FW per hour.

$$\frac{\left(\left(\text{Mean Abs well sample Assay t30} - \text{Mean Abs well sample Assay t0}\right) - \left(\text{Mean Abs well Blank killed t30} - \text{Mean Abs well Blank killed t0}\right)\right)}{\text{GLC STD CURVE SLOPE}} = \text{GLUCOSE/well (nmol)} \qquad \textbf{Eq. 2-4}$$

$$\text{GLUCOSE/well (nmol)} \times \frac{\text{Volume loaded in well (µl)}}{\text{Assay final volume (µl)}} \times \frac{\text{Eluted volume PD10 (µl)}}{\text{Eluted volume used for assay (µl)}} \times \frac{\text{Total volume extracted after centrifugation (ml)}}{\text{Volume loaded on PD10 (ml)}} \qquad \textbf{Eq. 2-5}$$

$$\frac{}{\text{Tissue FW (g)} \times \text{Enzyme assay time (h)} \times \text{1000 (nmol to µmol conv.)}} = \text{Invertase activity (µmol glucose} \cdot \text{gFW}^{-1} \cdot \text{h}^{-1}\text{)}$$

2.7 MOLECULAR BIOLOGY TECHNIQUES

2.7.1 RNA EXTRACTION FOR QPCR

The RNA extraction was performed following a method adapted from that described by Chang *et al.* (1993).

2.7.1.1 LIST OF LAB GLASSWARE NEEDED

All the glassware used was washed and baked in the oven at 250ºC for at least 5 hours.

- 2 bottles, 500 mL (just the bottle, without plastic cap and seal)
- 2 bottles, 50 mL (just the bottle, without plastic cap and seal)
- 1 beaker, 1 L
- 2 beakers, 100 mL
- 3 cylinders, 250 mL
- 10 glass pipettes, 25 mL

2.7.1.2 SOLUTIONS

- Pine Tree Method Extraction Buffer
- SSTE Buffer
- Absolute Cold Ethanol (Merck, Cat. #1.00983.2500)

- Chloroform:IAA - 24:1Solution
- 2-Mercaptoethanol (Sigma, Cat. #M3148)
- Lithium Chloride - 12 M Solution

2.7.1.3 METHODOLOGY

Before start the RNA extraction the water-bath (Julabo, SW21) was set at 65 °C, the fume hood was cleaned with absolute ethanol and the bench was covered with absorbent paper. Cleaned and dried pestles and mortars were rinsed with absolute ethanol and left under the fume hood till completely dried. 15 mL of Pine Tree Method Extraction Buffer was poured in sterile 50 mL Falcon™ Conical Tube (DB Bioscience, Cat. #352070) each sample and left in the water-bath to be warmed up to 65 °C. The tissue (~2 g) was ground accurately to a fine powder with a pestle and mortar in liquid nitrogen. 300 µL of 2-mercaptoethanol were added to the warm Pine Tree Method Extraction Buffer (under fume hood) and subsequently the ground tissue was gradually added by mixing thoroughly after each addition. The tube was returned to the water-bath briefly (max 1 minute) to allow the mixture to warm up again. The tube was then sat at room temperature whilst processing the rest of the samples.

When all samples were in buffer, each tube was vortexed vigorously for 30 seconds and then an equal volume of Chloroform:IAA Solution was added. The solution was mixed carefully by inverting the tube 60 times. Then all the tubes were centrifuged in a Bench-top Centrifuge (Heraeus, Multifuge 3S-R) at 8,500 rpm for 10 minutes at room temperature to separate the phases. The supernatant was carefully transfer into a new tube and an equal volume of Chloroform:IAA Solution was added. The solution was mixed carefully by inverting the tube 60 times. The previous step was reaped twice. After the last centrifugation the supernatant was transferred into a new tube, the volume was estimated and 1/5 of volume of 12 M Lithium Chloride Solution was added to a final concentration of 2 M to allow the precipitation of nucleic acids. The solution was gently mixed and left at 4°C overnight. Transfer the supernatant into a new falcon tube.

The following day tubes were centrifuged in a Bench-top Centrifuge at 8,500 rpm for 20 minutes at 4°C. In the mean time the SSTE Buffer was warmed up at 65°C.

The supernatant was then poured off and the obtained pellet dried by briefly inversion of tubes to drain excess solution. The pellet was re-suspended into750 µL of warm SSTE Buffer and the solution was transferred into a sterile 2 mL Eppendorf tube. Each tube was vortexed for 15 sec immediately before and after the addition of 750 µL of Chloroform:IAA Solution to allow the RNA-CTAB bound breakdown. Tubes were then centrifuged in a Micro-Centrifuge (Eppendorf, 5415R) at 14,000 rpm for 5 minutes at 4°C. The supernatant was then carefully transferred into a sterile 2 mL Eppendorf tube. 2 volumes of –20°C chilled absolute ethanol (RNase free) were added and the solution was gently mixed by inversion. Then tubes were placed in the –80°C freezer for 1 hour to let RNA to precipitate.

Tubes were micro-centrifuged at 14,000 rpm for 30 minutes at 4°C. The supernatant was discarded and tubes were inverted on a tissue to let them dry completely (5-10 minutes). According to size of the pellet obtained, RNase free water was added to obtain a similar RNA concentration for all samples. Usually 100-110 μL of water were added.

2.7.1.4 RNA QUALITY AND QUANTIFICATION FOR QPCR

2.5 μL of RNA solution was run on a 0.5X TBE (from 10X TBE - Invitrogen, Cat. #15581-028) 1% Agarose (Invitrogen, Cat. #15510027) 2 μL/100 mL EtBr (Sigma, Cat. #E1510) gel in a 0.5X TBE Buffer Solution using a gel electrophoresis apparatus (BioRad, SubCell® GT) to check for degradation.

RNA concentrations were obtained at a NANODROP® Spectrophotometer (NanoDrop Technologies, NanoDrop® ND-1000) using 2 μL each sample. A dilution series (usually 1X, 5X, 10X) was prepared for each sample. 3 readings for each sample and each dilution were recorded, including absorbance ratios (Abs260/280 and Abs260/230 close or higher than 2).

From 2 g of frozen tissue 100-120 μg (or more) of total RNA were generally expected.

2.7.2 DNASE TREATMENT

To avoid eventual DNA contamination all samples were DNase treated using the commercial kit RQ1 RNase-Free DNase (Promega, Cat. #M6101) and following the manufacturer's protocol.

2.7.3 FIRST-STRAND CDNA SYNTHESIS FOR QPCR

The First-Strand cDNA synthesis was carried out using the commercial kit SuperScript™ III Reverse Transcriptase (Invitrogen, Cat. #18080-44). The manufacturer's protocol was followed using 1μg of total RNA, oligo$(dT)_{20}$ Primers (Invitrogen, Cat. #18418-020), 10 mM dNTP Mix (Invitrogen, Cat. #18427-013) and RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen, Cat. #10777-019). All obtained cDNAs were Ribonuclease H (Invitrogen, Cat. #18021-014) treated, to remove the RNA complementary to the cDNA, following manufacturer's protocol.

cDNA solutions obtained were diluted 1:100, 100 μL aliquots were prepared from half of the solution and all of them were stored in -80°C freezer till ready to be use, avoiding multiple thawing out.

2.7.4 BIOINFORMATICS TOOLS

2.7.4.1 SEQUENCES SEARCH

Sequences encoding for putative target enzyme genes were identified in HortResearch *Actinidia* EST Database and translated amino acid sequences were blasted against

Arabidopsis sequences in the National Center for Biotechnology Information (NCBI)'s GenBank using the tblastn algorithm (Altschul *et al.*, 1990; Altschul *et al.*, 1997). Then, *Arabidposis* protein sequences were reversely blasted against HortResearch *Actinidia* EST Database to refine the selection. Then expectation values and identities were evaluated. From the bunch of ESTs selected the one from fruit library were preferred when available.

2.7.4.2 PRIMER DESIGN FOR QPCR

Genes encoding for target enzymes were identified by homology in the HortResearch *Actinidia* EST database, and, where putative gene family members existed, candidates were selected when they appeared in fruit library tissues. ESTs were aligned with respective Arabidopsis genomic DNA and Arbidopsis cDNA by AlignX software from the package Vector NTI Version 9.0.0 (Invitrogen, Cat. #12605050). *Arabidopsis* gene introns and exons were identified. Gene-specific primers corresponding to these genes were designed using Primer3 software (Rozen and Skaletsky, 2000) to a stringent set of criteria, enabling application of universal reaction conditions.

General criteria for primer design for real time PCR:

- 100-120 bp length
- melting temperature 59-61°C (optimum 60°C) and similar melting temperature of the forward and reverse primer
- primer pair must span at least an intron of not less than 300 bp (if possible)
- GC content must be at least 45-50%
- primers should be 20 base in length
- orientated to the most 3' end of the cDNA sequence (the degradation of the RNA starts from the 5' end of the sequence, and so it's worth to design the primer close to the poly-A tail. The probability to get the amplicon in most of the cDNA is much higher in this case.

2.7.4.3 PHYLOGENETIC TREES

Multiple alignment of deducted amino acid sequences was performed using AlignX (Invitrogen, Vector NTI package), a ClustalW program (ClustalX-algorithm based program). Output file was exported as *.msf, opened in GeneDoc and was exported in PHYLIP format as *.phy. Trees were constructed using PHYLIP package (Felsenstein, 2002). Sequenses were bootstrapped, and amino acid sequence distances calculated using the JTT matrix (Jones *et al.*, 1992). Distance tree was calculated using Neighbor-joining method (Saitou and Nei, 1987). The consensus tree was computed according to the majority-rule. Results were exported to TREEVIEW program (Page, 1996) for the visualization.

2.7.5 RT-PCR

To check for primers reaction specificity, RT-PCR reactions were carried out using Platinum® Taq DNA Polymerase (Invitrogen, Cat. #10966-034) and 10 mM dNTP Mix (Invitrogen, Cat. #18427-013), as reported in Tab. 2-5 with the thermal profile shown in Tab. 2-6.

Tab. 2-5: Reagent concentrations and amounts used for PCR reaction.

Reagent	[Start]	[Final]	Amount per reaction (µL)
Buffer 10X	10X	1X	2.00
Mg^{2+}	50 mM	1.5 mM	0.60
dNTP's	10 mM	0.2 mM	0.40
Primer F	10 µM	0.2 µM	0.40
Primer R	10 µM	0.2 µM	0.40
Taq	5 u/µl	0.04 u/µl	0.16
Purified Water			11.04
cDNA	100X	25X	5.00

Tab. 2-6: Thermal profile for PCR reaction.

Stage	Cycles	Step	Temperature	Time
1	1	Pre-incubation step	94 ºC	2 min
		Denaturing step	94 ºC	15 sec
2	30	Annealing step	60 ºC	30 sec
		Extension step	72 ºC	30 sec
3	1	Final Extension step	72 ºC	5 min

2.7.6 qPCR

qPCR DNA amplification and analysis was carried out using the AB 7500 Real Time PCR detection system (Applied Biosystems, AB 7500 Fast Real Time PCR). All reactions were performed using the AB SYBR® Green PCR Master Mix (Applied Biosystems, Cat. #4309155) according to the procedure described by the manufacturer, on 96-well plates (MicroAmpTM Fast Optical 96-well reaction plate – Applied Biosystems, Cat. #4346906; MicroAmpTM Optical Adhesive Film - Applied Biosystems, Cat. #4360954). Reactions were performed in triplicate using 7.5 µL 2X Master Mix, 0.2 µM each primer, 5 µL 100X diluted cDNA and nuclease-free water (BarnStead, NanoPure Diamond™) to a final volume of 15 µL. A negative water control was included in each run.

Fluorescence was measured at the end of each annealing step. Amplification was followed by a melting curve analysis with continual fluorescence data acquisition during the 60–95ºC melt. The raw data were analysed with the 7500 Fast System SDS software, version 3.1 (Applied Biosystems), and expression was normalized to *Actinidia deliciosa* Protein Phosphatase Phosphatase Regulatory Subunit A (PP2A HortResearch EST 312205) to

minimize variation in cDNA template levels. PP2A was selected for normalization due to its consistent transcript levels. For each gene, a standard curve was generated using a cDNA serial dilution, and the resultant PCR efficiency calculations (ranging between 1.87 and 2.02) were imported into relative expression data analysis. Relative expression was calculated as for Ren *et al.* (2007). Error bars shown in qPCR data are technical replicates, representing the means ± SE of three replicate qPCR reactions.

Tab. 2-7: AB 7500 Fast Real Time PCR thermal profile.

Stage	Cycles	Step	Temperature	Time
1	1	Pre-incubation step	95 ºC	10 min
2	40	Denaturing step	95 ºC	15 sec
		Annealing step	60 ºC	1 min

2.7.7 MICROARRAYS

The macroarray experiement was performed using the methodology described in Schaffer *et al.* (2007).

RNA extraction for the microarray experiment was held as for §2.7.1, but a more accurate quantification and quality determinations were performed as follow.

To enhance the quality of the extracted RNA a clean-up step was performed using RNeasy® midi kit (Qiagen, Cat. #75144) following manufacturer's instructions. RNA binds to the RNeasy silica-gel membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in water.

A preliminary quantification was performed as for §2.7.1.4.

2.7.7.1 RNA PRECIPITATION AND RE-SUSPENTION STEPS

The labelling reaction requires a minimum RNA concentration of 3 μg/μL. The column clean-up gives a lower concentration, so RNA precipitation and re-suspension was required. The precipitation was carried out adding 0.1 volumes of Sodium Acetate (3M pH 5.5) and 2 volumes of 100% Ethanol, and let samples stand in ice for 1 hour (RNase-free solutions, tubes and tips were recommended). Pellet was collected by a 30 minutes 13,200 rpm centrifugation at 4ºC. Pellet was then re-suspended according to the concentration obtained by the preliminary quantification at the NANODROP® spectrophotometer for an estimated final concentration of 6 μg/μL.

2.7.7.2 RNA QUALITY AND QUANTIFICATION BY AGILENT BIOANALYZER

The RNA electrophoresis was performed by a micro-fluidics technique using an Electrophoresis Bioanalyzer (Agilent Technologies, Agilent 2100). The methodology used is here reported.

2.7.7.2.1 Materials Needed:

- RNA 6000 Nano LabChip Kit (Agilent, Cat. #5065-4476):
  - RNA gel matrix
  - RNA dye concentrate
  - RNA chip
  - RNA 6000 Nano Markers
  - Spin filters
- RNA 6000 Ladder (Ambion, Cat. #7152)

2.7.7.2.2 Preparation:

Before start the kit was equilibrated at room temperature for 30 minutes and RNA ladder thaw on ice. Then the gel-dye mix was prepared by putting 400 μL of RNA gel matrix (red top) into a spin filter. The spin filter was centrifuged at 1500g at 20 ºC for 10 minutes. Filtered gel must be used within 4 weeks. 65 μL of filtered RNA gel matrix were mixed with 1 μL of RNA dye concentrate (blue top) in a RNase free 1.5 mL micro-centrifuge tube and the solution was vortexed well. Solution must be protected from light and stored at 4 ºC and used within a week.

An aliquot of RNA samples were diluted considering that the sensibility range of the Bioanalyzer is between 50 and 500 ng.

2.7.7.2.3 Procedure:

- Loading the gel-dye mix

A new RNA chip was put on the Chip Priming Station. 9.0 μL of gel-dye mix were pipetted into the well marked “G” in a black circle. The Chip Priming Station was then closed and the plunger was pressed until it was held by the clip. Exactly after 30 seconds the clip was gently released and the chip was checked for air bubbles presence. Finally 9 μL of gel-dye mix were pipetted in the wells Marked “G” in a purple square.

- Loading the RNA 6000 Nano Marker

First, 5 μL of RNA 6000 Nano Marker (green top) was pipetted into the well with the ladder picture next to it. Then 5 μl of RNA 600 Nano Marker were pipetted into all twelve sample wells

- Loading the Ladder

1μL of RNA 6000 Ladder was loaded into the well with the ladder picture next to it.

- Loading the Samples

1μL of sample was loaded in each of the twelve sample wells. 1μL of RNA 6000 Nano marker was loaded in each unused sample well. The chip was put in the adapter and vortexed for one minute at the set-point of the IKA vortexer. The chip was run in the Bioanalyzer within 5 minutes.

Data were acquired and analyzed by the 2100 Expert Software (Agilent Technologies, Version B.02.03).

2.7.7.3 cDNA Labeling and Hybridization

RNA was labeled with either Cy3 or Cy5 flourescent dye (GE Healthcare) using an amino-allyl dye coupling reaction. RT of the RNA consisted of taking 50 μg of RNA was added to 3 μL Oligo dT 23mer with a dAGC anchor (100 mM) in a total volume of 19.5 μL. This was heated to 70ºC for 10 min and cooled to 4 ºC on ice, 6 μL transcriptor buffer, 2 μL dithiothreitol (100 mM), and 2 μL dNTP mix (dA, G, CTP 7.5 mM, dTTP and amino allyl dUTP 3.75 mM), and 10 units of Transcriptor was added (total reaction volume 30 μL) and incubated for 42ºC for 30 min. The reaction was stopped by adding 1 μL 20 mM EDTA, the RNA degraded by adding 1 μL NaOH (500 mM), heating at 70ºC for 10 min, and cooling on ice, the reaction was neutralized by adding 1 μL HCl (500 mM). cDNA was precipitated with ethanol and reasuspended in 5 μL Na_2CO_3 (100 mM pH 9.0). Cy dye NHS esters (GE Healthcare) were resuspended in 22 μL dimethyl sulfoxide, and 5 μL was added to the resuspended cDNA, incubated for 2 h in the dark at room temperature, and cleaned using a PCR purification column (Qiagen) as described in the manufacturer's protocols, eluting from the column with 52 μL of water.

Kiwifruit microarrays containing 17,472 45-55 mer oligonucleotides, representing 17,212 non redundant *Actinidia* sequences with a constant melting temperature, designed to *Actinidia spp.* ESTs as reported in Crowhurst *et al.* (2008), were used to measure global gene expression patterns. Oligos were printed on epoxy slides (MWG) using a Biorobotics II robot. Each array was hybridized with sheared genomic DNA from *A. deliciosa* 'Hayward' and *A. eriantha* (genotype 11-6-15e) (Crowhurst *et al.*, 2008) in one channel to allow direct comparison between arrays. A total of 3.2 μg of sheared genomic DNA was labeled using a radprime labeling kit (Invitrogen) as described in the manufacturer's protocol, except 2 μL of 3mM dAGCTP and 1.5 mM dT and amino allyl dUTP was used instead of the kit supplied nucleotides. Amino allyl incorporated DNA was ethanol precipitated and Cy dyes (GE Healthcare) added as described in the cDNA labeling protocol. Labeled cDNA and gDNA were mixed, and put through a further PCR cleanup column, eluting in 52 μL water. A total of 33 μL 203 SSC, 8.8 μL 5% SDS, 13.5 μL Liquid Block (GE Healthcare), and 114.7 μL water were added. The mixed DNA was denatured at 95ºC for 10 min then kept at 60ºC for hybridization. Hybridizations were performed eight in a time in a Lucidea Hybridization (GE Healthcare). Microarrays were prehybridized at 45ºC with 220 μL hybridization mixture without any labeled nuceotides for 15 min, using the mix step. The

microarrays were then washed with wash 1 (23 SSC 0.3% SDS) and flushed with air. Hybridization mixtures containing the labeled cDNA and gDNAwere injected onto the slide and hybridized for a minimum of 16 h at 45ºC again using the mix step. The microarrays were washed with wash 1 for 1.2 min and cooled to 30ºC, the microarrays were washed again with wash 1 for 1.2 min, wash 1 for 2.4 min, wash 2 (0.53 SSC, 0.3% SDS) for 2.4 min (twice), and then once with wash 3 (0.53 SSC). The microarrays were then air dried and scanned using a Genepix 4000B scanner (Axon). Spots were aligned using Genepix 5 software (Axon).

2.7.7.4 MICROARRAY'S DATA PROCESSING

All analysis was done in the statistical software package "R" (R Development Core Team, 2008). Microarrays were analysed using the limma package in BioConductor (Smyth and Speed, 2003). Normalisation using an in-house protocol that had the following steps: All RNA and DNA channels were normalized with global mean normalization, combined into two files (containing all gDNA and all cDNA channels), and the distribution of intensities were normalized using quantile normalization (Bioconductor). A ratio (M) of the Cy3 and Cy5 values for each slide were calculated and the M values of the dye swaps were then smoothed with loess smoothing to remove dye bias. An absolute value for each spot was achieved by multiplying each ratio with the median gDNA value for that spot.

The normalised data was modelled in the limma package, firstly to high and low dry matter genotypes following this it was modelled to low and high dry matter genotypes harvested at the two time points selected for dry matter accumulation (56 and 98 DAA). Genes were selected using a non-adaptive FDR (False Discovery Rate) control (Benjamini and Hochberg, 1995) with an adjusted p value of 0.01 and a greater than 2 fold change of expression.

2.7.8 SEQUENCING

To confirm the correspondence between target amplified sequence and database amplicon, target sequence was cloned by inclusion in a plasmid vector, *E. coli* cells transformation and proliferation. Plasmid DNA was then purified and sequenced as reported in the following paragraphs.

2.7.8.1 RT-PCR REACTION

10 µL of cDNA were amplified in 2 replicates each primer's pair in a 50 µL PCR reaction. PCR reaction was performed using Platinum® Taq DNA Polymerase (Invitrogen, Cat. #10966-034) as shown in Tab. 2-8 using the thermal profile reported in Tab. 2-6.

2.7.8.2 PCR'S PRODUCT PURIFICATION

PCR's product purification was carried on using QIAquick® PCR Purification (Qiagen, Cat. #28104) following manufacturer's microcentrifuge protocol. DNA was eluted using 30 µL elution buffer.

Tab. 2-8: Reagents, concentration and amount used to amplify target cDNA sequences.

Reagent	[Start]	[Final]	Amount per reaction (µL)
Buffer 10X	10X	1X	5.00
Mg^{2+}	50 mM	1.5 mM	1.50
dNTP's	10 mM	0.2 mM	3.50
Primer F	10 µM	0.2 µM	3.50
Primer R	10 µM	0.2 µM	3.50
Taq	5 u/µl	0.04 u/µl	1.40
Purified Water			30.10
cDNA	100X	20X	10.00

2.7.8.3 LIGATION

Ligation of the purified PCR product was carried on by Vector: pGEM®-T Easy (Promega, Cat. # A1360) following the manufacturer's indications as reported in Tab. 2-9.

Tab. 2-9: Reagents and concentrations for pGEM®-T Easy ligation reaction.

Reagent	[Start]	[Final]	Amount per reaction (µL)
2X Ligase Buffer	2X	1X	5
pGEM®-T Easy Vector	50 ng/µl	5 ng/ µl	1
PCR cleaned product			3
T4 DNA Ligase	3 units/µl	0.3 units/µl	1

Reaction was performed at room temperature for at least 1 hour.

2.7.8.4 TRANSFORMATION

Transformation with the vector was performed on MAX Efficiency® DH5α™ Competent Cells (Invitrogen, 18258-012) as reported in Tab. 2-10.

Tab. 2-10: Reagents and amount used for transformation.

Reagent	Amount per reaction (µL)
Competent Cells DH5α®	20
Ligation product	4

Cells and ligation product were poured in a 2 mL Safe Lock Eppendorf tube and placed in an ice bath for 2 minutes. Then they were heat shocked at 42 ºC for 30 seconds and placed

again in an ice bath for 2 minutes. Cells were then incubated at 37 ºC, 200 rpm for 1 hour in 500 µL of liquid LB medium.

2.7.8.5 SOLID MEDIUM COLONY GROWTH

Solid LB medium X-gal – IPTG were used for colony growth and transformed colony discrimination. For each primer couple 2 replicate plates were designed. 200 µL of transformation product was pipetted in each plate and spread with an L spatula. Plates were incubated overnight at 37ºC. To better discriminate white and blue colonies, plates were sealed with Parafilm and kept in the fridge for 2 hours. Petri dishes 92 mm diameter (Sarstedt, Cat. #821472).

2.7.8.6 COLONY SELECTION AND COLONY PCR

From each plate 5 full-white colony were chosen. 20 µL colony PCR reaction was performed on each chose colony to assess the transformation result using gene-specific primer's pair. Reagents and concentration of PCR reaction used are reported in Tab. 2-11, whereas PCR thermal profile is shown in Tab. 2-12.

Tab. 2-11: Reagents, concentration and amount used for Colony PCR reaction.

Reagent	[Start]	[Final]	Amount per reaction (µL)
Buffer 10X	10X	1X	2.00
Mg2+	50 mM	1.5 mM	0.60
DNTPs	10 mM	0.2 mM	0.40
Primer F	10 µM	0.2 µM	0.40
Primer R	10 µM	0.2 µM	0.40
Taq	5 u/µL	0.04 u/µL	0.16
Water			16.04

Tab. 2-12: PCR thermal profile for colony PCR.

Stage	Cycles	Step	Temperature	Time
1	1	First Denaturing step	94 ºC	5 min
2	30	Denaturing step	94 ºC	30 sec
		Annealing step	60 ºC	30 sec
		Extension step	72 ºC	1 min
3	1	Final Extension step	72 ºC	10 min

PCR products were separated by electrophoresis on a 0.5X TBE, 2% Agarose 2 µL/100 mL EtBr gel in a 0.5X TBE Buffer Solution. Fragment size was compared with 1 Kb Plus DNA Ladder™ (Invitrogen, Cat. #10787-018).

2.7.8.7 OVERNIGHT LIQUID COLONY GROWTH

5 colonies for each primer's pair were grown overnight in 3 mL of liquid LB-Ampicillin medium (100 µg/mL, final Ampicillin concentration) in 13 mL tubes (Sarstedt, Cat. 62.515.006) at 37 ºC and 200 rpm for 12-16 hours.

2.7.8.8 PLASMID DNA PURIFICATION

2 mL of each colony were transferred into safe lock 2 mL Eppendorf tubes and centrifuged for 5 minutes at 13,000 rpm. The supernatant was discarded, 1 mL of each colony was added and centrifuged at 13,000 rpm for 3 minutes. The supernatant was discarded and the pellet was dried with a one minute spin at 13,000 rpm.

The plasmid was then extracted from the pelletted colonies using the QIAprep® Spin Miniprep Kit (Qiagen, cat. # 27104) and following the manufacturer's micro-centrifuge protocol.

Plasmid DNA was eluted in 50 µL Buffer EB (10 mM Tris·Cl, pH 8.5).

2.7.8.9 PLASMID DNA CONCENTRATION

1 µL of plasmid DNA was separated by electrophoresis on a 0.5X TBE 2% Agarose 2 µL/100 mL EtBr gel in a 0.5X TBE Buffer Solution.. Plasmid mass was estimated by Low DNA Mass Ladder (Invitrogen, Cat. #10068-013).

300 ng of plasmid DNA in 14 µL solution were placed in a 200 µL tube for each sequencing reaction.

2.7.8.10 SEQUENCING SERVICE

Sequencing reactions were performed by "The Allan Wilson Center for Molecular Ecology and Evolution", Massey University, Auckland, New Zealand (http://awcmee.massey.ac.nz/index.htm), following a standard method with 15 µL reaction final volume: 14 µL plasmid DNA solution (300 ng) and 1 µL of T7 primer (5' TAA TAC GAC TCA CTA TAG GG 3') or SP6primer (5' ATT TAG GTG ACA CTA TAG 3').

2.7.8.11 SEQUENCE ANALYSIS

Sequence analysis was held using the software ContigExpress from the package Vector NTI Version 9.0.0 (Invitrogen, Cat. #12605050).

2.8 CHEMICALS, LABORATORY EQUIPMENTS AND SOLUTIONS USED

Chemical reagents, laboratory equipment and solution recipes are listed in Appendix I, Appendix II and Appendix IV respectively.

2.9 STATISTICAL ANALYSIS

Time series measurement data were analyzed as reaped measures.

A mixed effects model was fitted using the Mixed Procedure in the SAS software package (SAS Institute Inc, 2000) for Windows to determine the effect treatments and time on selected variables. An auto regressive of order one (AR1) covariance structure was used to model the repeated measures on the individual units. Type 3 Tests of Fixed Effects were used to assess the significance of the main effects, then the least-squares means (LSMs) were calculated, and tests for a difference between treatments at each time point were carried out using the Slice option in the LSMEANS statement. These tests effect slice were used when a significant interaction between treatment and time was present.

Comparison between coupled data sets were held using t-test for unequal variances.

Differences were considered different when probability was less than 0.05. Probability levels were indicated using increasing number of star symbols (*).

3 FRUIT DEVELOPMENT AND CARBOHYDRATE ACCUMULATION

3.1 INTRODUCTION

An important goal for breeding of kiwifruit is to develop new cultivars with improved and more consistent flavour (Clearwater *et al.*, 2007). Taste is an important aspect of kiwifruit quality and is largely determined by the concentration and balance of sugars and acids. This affects perceptions of overall flavour intensity as well as sweetness and sourness. In fruit of 'Hayward', the main soluble sugars are glucose, fructose and sucrose, and the main organic acids are citric, quinic, malic and ascorbic (Okuse and Ryugo, 1981; Paterson *et al.*, 1991; Richardson *et al.*, 1997). Soluble solids content (SSC) of ripe fruit and fruit dry matter content (DM) at harvest are both correlated with increased fruit flavour acceptability (Jaeger *et al.*, 2003; MacRae *et al.*, 1989; Marsh *et al.*, 2004; Paterson *et al.*, 1991). Dry matter in kiwifruit has been proposed as being both a maturity indicator for timing harvest and also as a predictor of the sensory quality of the fruit once ripe (Scott *et al.*, 1986). It is reasonably constant during ripening with only small losses due to respiration (Mitchell *et al.*, 1992). It is useful because the fruit DM is dominated by the large carbohydrate component (around 75% of DM—Beever and Hopkirk, 1990) that is sugar and starch at harvest and which mostly becomes sugar when eating ripe. Hence the DM indicates either the potential or actual sugar level of the fruit.

The growth in fresh weight of a kiwifruit can generally be described as biphasic, with an initial rapid increase in fresh weight for 40-50 days after anthesis, followed by a period of slower growth until to commercial maturity between 150-200 days after anthesis (Clearwater *et al.*, 2007). The accumulation of fruit dry weight is described as linear (Richardson *et al.*, 1997) or it better fits an expo-linear curve, with a slow starting accumulation in the first few weeks (Hall *et al.*, 2006). DM concentration is high at anthesis, then it dips 40-50 days after fruit set followed by a rapid, curvilinear increase until about 140 days after fruit set (Richardson *et al.*, 1997). Towards harvest the increase in dry matter content may slow or stop (Clearwater *et al.*, 2007).

In the *Actinidia* breeding population observed by Cheng and co-workers (2004) DM content ranged from 10.6% to 22.3%. A previous study on genotype differing in DM content has shown that there were no consistent links between easily measured vegetative traits and gross measures of fruit number, weight or dry matter content (Clearwater *et al.*, 2007). It was concluded that a large proportion of the differences in fruit phenotype observed between contrasting *Actinidia* genotypes could be attributed to the effect of genetically determined processes that directly influence flower and fruit development, rather than to the indirect effect of vegetative traits associated with plant productivity. In tomato starch is the main carbohydrate stored in early developmental stages. Ho *et al*. (1996) suggested that the level of starch in the first stage determines the level of hexose accumulation. It has recently been

demonstrated that the peak of starch accumulation, observed at early developmental stages in the introgression line 9-2-5, characterized by an higher at harvest soluble solid concentration (SSC), was the effect of an enhanced capacity of sucrose uptake (Baxter *et al.*, 2005).

The goal of the present study was to describe fruit development from anthesis to maturity and understand how and when the contrasting genotypes differ in their patterns of fresh and dry weight growth, and the accumulation of carbohydrates, particularly starch. A range of genotypes was selected from 15 *Actinidia deliciosa* breeding families to represent the extremes of DM content within the overall population (the highest and lowest fruit DM contents at harvest), while also spanning a range of average fruit fresh weights at maturity. Fruit development was examined over four seasons to determine how consistent the developmental patterns were.

The hypothesis was that high and low DM genotypes differ in DM content (%) and dry weight (DW) accumulation rates throughout fruit development from anthesis to maturity. An alternative hypothesis was that high and low DM genotypes differ primarily in starch content, and that they only begin to differ in DM content and DW accumulation after the onset of net starch accumulation, which usually begins approximately 50-60 days after anthesis. It was expected that differences in fresh weight growth between genotypes would interact with dry weight and starch accumulation through dilution effects. We therefore tested a variety of indices for their ability to rank the genotypes based on the rate at which dry weight and starch were accumulated relative to fresh or dry weight.

3.2 MATERIALS AND METHODS

3.2.1 PLANT MATERIAL

For the study of *Actindia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* fruit development, a total of 24 vines were selected in 2003 and 2004 from three factorial seedling populations. A total of 15 breeding families were screened and selected vines were belonging to 9 different families. These were planted in 2 contiguous orchard blocks at Te Puke Research Centre (Bay of Plenty, NZ; 37^{o} 49’ S - 176^{o} 19’ E) in 1999 for breeding purposes. An equal number of extreme high and low fruit dry matter plants characterized by a large or small final fruit size were selected. All vines were growing on a T-bar trellis at 0.8 m spacing on the row and 5 m spacing between rows. The first selection step was carried out in 2003 when 18 vines were selected. The following year only 4 seedlings from the original bunch were kept and 6 more extreme vines were added, for a total of 10. In 2006 only 9 of the 10 selected seedlings were assessed, due to the eradication of all the vines in one of the two blocks. In 2005 scions from the 10 selected vine were grafted on 4 ‘Bruno’ rootstocks each genotype, following a complete randomized block design (Mead *et al.*, 1993) obtained using SAS® software (SAS Institute Inc, 2000). Grafted vines were growing in the same orchard block at Te Puke Research Centre spanning 4 rows on a T-bar trellis at 2 m spacing on the row and 5 m spacing between rows. Blocks were managed according normal

commercial practises, pruned using one year old cane replacement and a low intensity summer pruning. A basic fertilizer level was used.

3.2.2 PHYSICAL MEASUREMENTS

At anthesis time, total flower number was counted for selected vine, and number of open flowers recorded in a number of observations to pick the anthesis time (50% open flowers). A number of 5 to 20 fruit each time point from each genotype was randomly collected and fruit fresh weight recorded (Tab. 3-1). Dry matter content, as percentage of fresh weight, was determined for individual fruit as for Burdon *et al.* (2004). Fruit dry weight was then calculated multiplying fresh weight and dry matter (%).

Mean absolute growth rates (AGR) were calculated for dry weight as reviewed by Opara (2000).

3.2.3 NON-STRUCTURAL CARBOHYDRATE AND ORGANIC ACID ANALYSIS

In 2003-04 and 2004-05 seasons 5-6 representative genotypes, both high and low dry matter, were chosen, wedges of tissue (providing representative proportions of all tissue types, radially and longitudinally) were then cut and snap-frozen in liquid nitrogen for later analysis of starch (Smith *et al.*, 1992), soluble sugars (glucose, fructose, sucrose, myo-inositol and galactose) as per Klages *et al* (1998) and organic acids as per Cheng *et al.* (2004). Four biological replicates were analyzed (each genotype at each time point). The individual starch, sugar and acid contents were expressed as the milligrams per gram fresh fruit weight (mg g^{-1} FW).

Tab. 3-1: Seasons, number of genotypes and sampling dates for fruit development study. DAA: days after anthesis.

Season	Genotypes (No.)		Time (DAA)										Time points (No.)
	Hi-DM	9											
	Lo-DM	9											
2003-04	total	18	7	14	28	42	56	70	98	126	154		9
	Hi-DM	5											
	Lo-DM	5											
2004-05	total	10		14	28	42	56	70	98	126	154		8
	Hi-DM	5											
	Lo-DM	4											
2005-06	total	9		14		42	56	70		126	154		6
	Hi-DM	5											
	Lo-DM	5											
2006-07	total	10	7	14	28	42	56	70	98	126	154	182	10

3.2.4 INDEXES

Efficiency indexes were calculated as following explained. Starch specific conversion rate (SCR), fruit efficiency in starch accumulation, was calculated using Hunt's equation (1973) to estimate root efficiency. Starch accumulation rate (AGR) was divided by dry weight. Fruit dry weight pondered AGR was calculated dividing dry weight AGR by the respective interval average fresh weight, to smooth size dilution effect. Starch pondered AGR was calculated dividing starch accumulation rate (AGR) by the respective interval average fresh weight, to smooth size dilution effect.

3.2.5 STATISTICAL ANALYSIS

A mixed effects model was fitted using the Mixed Procedure in the SAS software package for Windows (SAS Institute Inc, 2000) to determine the effect of dry matter class and time on fruit dry weight, fruit dry matter concentration, starch concentration, sucrose concentration and dry weight absolute growth rate. An auto regressive of order one (AR1) covariance structure was used to model the repeated measures on the individual plants. Type 3 Tests of Fixed Effects were used to assess the significance of the main effects, then the least-squares means (LSMs) were calculated, and tests for a difference between treatments at each time point were carried out using the Slice option in the LSMEANS statement. These tests effect slice were used when a significant interaction between treatment and time was present.

3.3 RESULTS

3.3.1 FRUIT PHYSICAL MEASUREMENTS FOR DRY MATTER CLASSES

Genotype dry matter classes were different in dry matter content (%) (Fig. 3-1C, F, I, L) in all the observed seasons. Small differences in dry matter content were showed since early in development, but only from 70 DAA classes were consistently different in dry matter content over all the observed seasons. The accumulation pattern was similar between the two classes and after 70 DAA curves were parallel. At-harvest dry matter content was about 20-21% for high dry matter genotypes and 15.5% in low dry matter genotypes, varying little from season to season. In 2005-06 season genotypes were significantly different at 14 DAA. In 2006-07 season dry matter content was significantly different at all sampling dates, but 42 DAA.

Dry weight accumulation rate was higher for high dry matter genotypes, but differences were evident late in development (about 100 DAA). Early in development dry weight accumulation pattern were similar between the two classes. 2005-06 season dry weight accumulation was not consistently different because highly affected by low crop load (Fig. 3-1B, E, H, K).

Genotype dry matter classes were not different in fresh weight accumulation patterns because classes were equally comprising large and small size genotypes (Fig. 3-1A, D, G, J). Fresh weight growth followed a sigmoid pattern, characterized by an early fast period and a

slow constant growth till harvest. Fresh weight was about 90 g for all the seasons, but in 2005-06 season when it was affected by low crop load (Fig. 3-1G)

Anthesis dates were consistent throughout seasons (Tab. Appendix V- 1) and were not related with final dry matter concentration (data not shown).

Dry weight absolute growth rate was generally higher for high dry matter genotypes late during the development (70-126 DAA), as shown in Fig. 3-2.This enforce the evidence of an higher dry weight accumulation curve slope particularly observed in (Fig. 3-1E, K).Dry weight absolute growth rate showed an evident peak in both years between 70 and 98 DAA in high dry matter genotypes.

3.3.2 NON-STRUCTURAL CARBOHYDRATES AND ORGANIC ACIDS FOR DRY MATTER CLASSES

Genotype dry matter classes were strongly different in starch concentration from 98 DAA, and starch level for high dry matter genotypes was above the low dry matter genotype ones from early in development (Fig. 3-3A). At-harvest average starch concentration was 70% higher in high dry matter genotypes (84 mg g^{-1} FW in high dry matter genotypes and 49 mg g^{-1} FW in low dry matter genotypes).

At-harvest sucrose concentration was significantly different between the two dry matter classes (Fig. 3-3D). High dry matter genotypes showed a significantly higher sucrose concentration at 154 DAA ($p<.0001$, Tests of Effect Slices).

All other soluble sugars and organic acids did not shown any consistent difference between the two observed genotype classes (Fig. 3-3). Glucose peaked for both dry matter classes at 42 DAA (Fig. 3-3B).

Data showed in Fig. 3-3 referred to 2004-05 season. Pattern were similar in 2003-04 season.

Starch contribution to dry matter was different between dry matter genotype classes from 70 DAA. In Fig. 3-4 dry matter components for genotype dry matter classes in 2004-05 season are reported. Starch was 13-27% higher in high dry matter genotype class (Fig. 3-4A) from 70 DAA to 154 DAA.

Soluble sugar contribution to total dry matter was higher in low dry matter class at 98 and 126 DAA (Fig. 3-4B), whereas organic acid contribution was 25% higher in high dry matter genotypes at 56 DAA (Fig. 3-4A).

3.3.3 GENOTYPES' CONSISTENCY

Dry matter content was consistent between seasons whereas fruit size was affected by a higher variability, particularly in large size genotypes. Fluctuations in dry matter content where not consistently strong to rearrange genotype classification. In Fig. 3-5, dry matter concentration and fresh weight, of two season values at 154 DAA, were plotted. Circles and numbers were added to highlight genotype behaviours in two different seasons and check for consistency in the characteristics.

Small size fruit classes were more consistent in fruit size and dry matter content than large size fruit classes. The dry matter gap between high and low dry matter genotypes in this class was 7% (average), that means about 50% more dry matter in high dry matter genotypes. The gap between high and low dry matter genotypes in large size classes was 4% (average), that means 25% more dry matter in high dry matter genotypes. Vine 28, large size genotype, was influenced by dry matter dilution effect in 2004-05 season: 15% more fresh weight ended into a 10% less at-harvest dry matter content. A high crop load of 2006-07 season genotype 3 vines ended into a loss of 27% of at-harvest fresh weight.

Higher values in dry matter content were found in small sized genotypes.

3.3.4 GENOTYPES' COMPARISON

The comparison of the most extreme genotype (Fig. 3-6), for both dry matter content and fruit size, showed a trend for fresh weight, dry weight, dry matter and starch similar to those reported in Fig. 3-1 and Fig. 3-3A.

In both class size dry matter content was significantly different in high dry matter genotypes at all observed time points (Fig. 3-6E, F).

Starch concentration was consistently higher for both high dry matter genotypes from 98 DAA. At-harvest starch concentration of high dry matter genotypes was double than in low dry matter genotypes (Fig. 3-6G, H).

Dry weight accumulation rate was higher for high dry matter genotypes in both size classes from 98 DAA (Fig. 3-6C, D).

Fruit fresh weight growth was similar within size classes, and at-harvest mass of large size genotypes was double of the small size ones (Fig. 3-6A, B).

3.3.5 GENOTYPES' RANKING

Between 70 and 98 DAA it was possible to rank genotypes according to both dry weight and starch accumulation efficiency (Fig. 3-7).

All 3 indexes were from 2 to 3 time higher in high dry matter genotypes (Fig. 3-7). High dry matter genotypes presented a similar efficiency in all 3 indexes, whereas low dry matter genotype 17 was more efficient than genotype 25 (Fig. 3-7B, C).

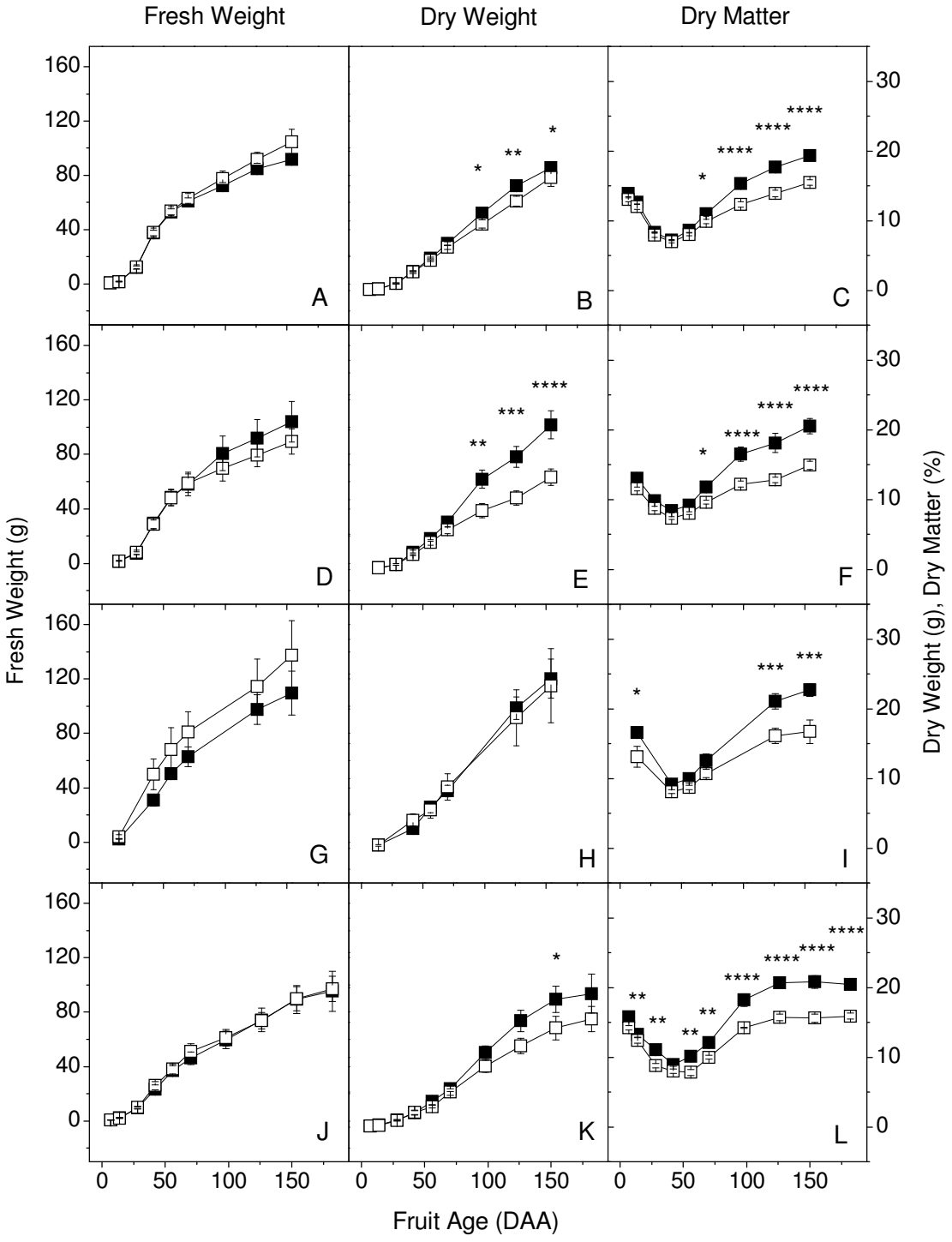


Fig. 3-1: Growth curves for *Actinidia deliciosa* genotypes in different seasons. Genotypes were subdivided according dry matter concentration into high and low dry matter classes. Fruit fresh weight (A), fruit dry weight (B) and dry matter concentration (C) in 2003-04 season. n= 9 vines per class. Fruit fresh weight (D), fruit dry weight (E) and dry matter concentration (F) in 2004-05 season. n= 5 vines per class. Fruit fresh weight (G), fruit dry weight (H) and dry matter concentration (I) in 2005-06 season. n= 5-4 vines per class. Fruit fresh weight (J), fruit dry weight (K) and dry matter concentration (L) in 2006-07 season. n= 5 vines per class. Values are average ±SE of the mean. High dry matter genotypes, (■); low dry matter genotypes, (□). Tests of Effect Slices: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; $p<0.0001$; blank, not significant.

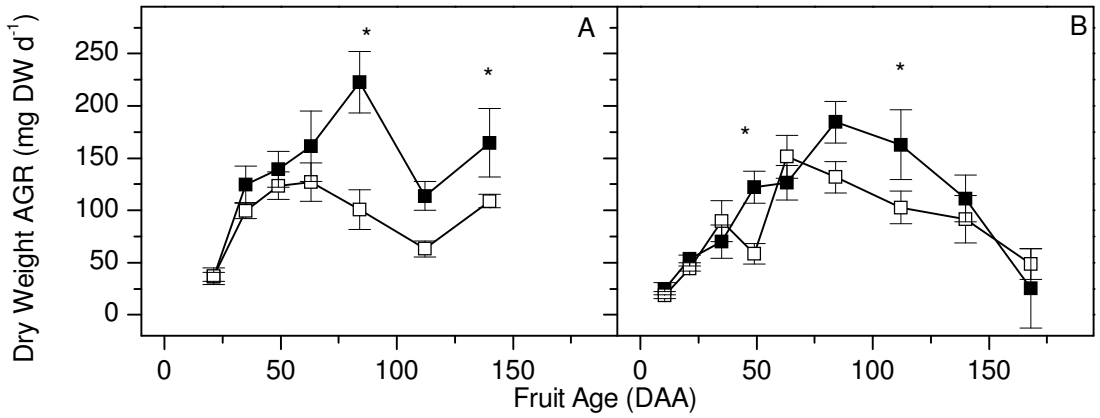


Fig. 3-2: Absolute Growth Rate (AGR) of fruit dry weight for *Actinidia deliciosa* genotypes in 2004-05 (A) and in 2006-07 (B) seasons. Genotypes were subdivided according dry matter concentration into high and low dry matter classes. n = 5 vines per class. Values are average ±SE of the mean. t-test results are also reported: *, p<0.05; **, p<0.01; non significant if blank. High dry matter genotypes (■), low dry matter genotypes (□).Tests of Effect Slices: *, p<0.05; **, p<0.01; ***, p<0.001; p<0.0001; blank, not significant.

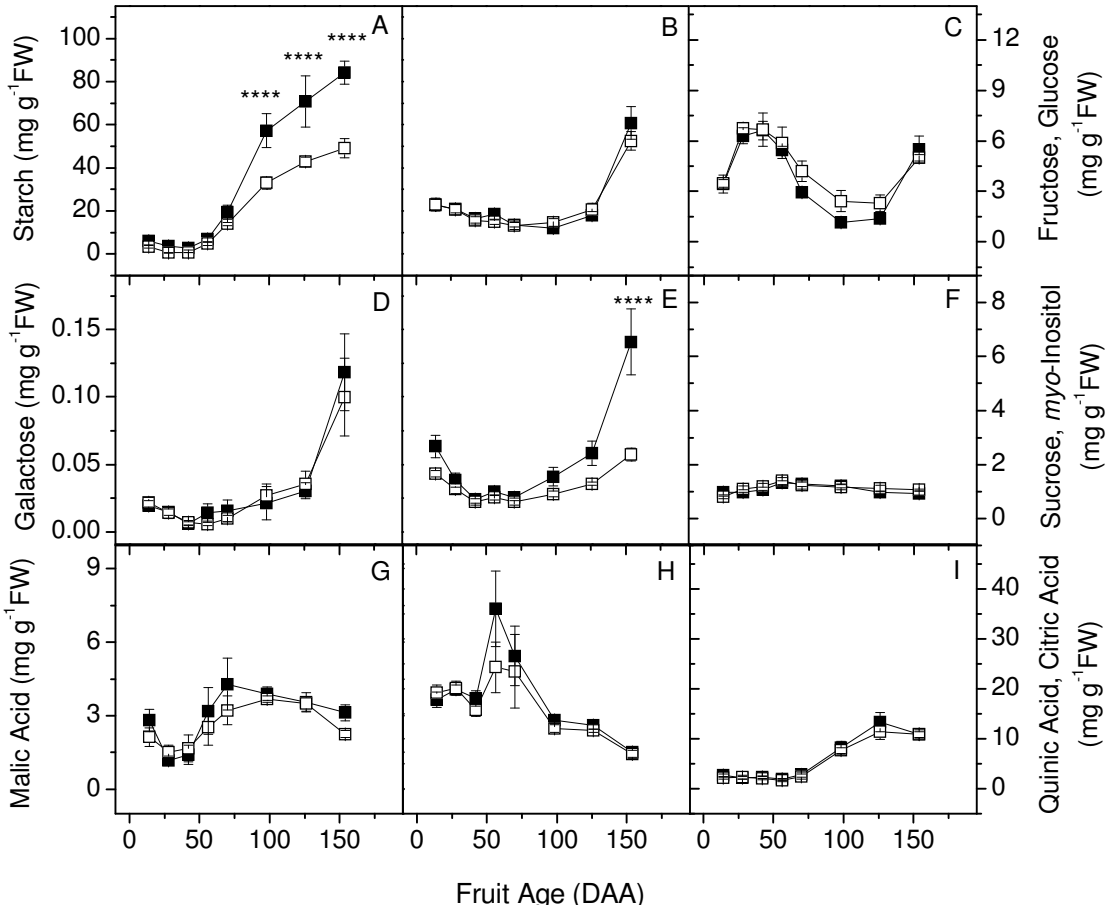


Fig. 3-3: Changes in carbohydrate and organic acid concentrations during fruit growth in 2004-05 season for *Actinidia deliciosa* genotypes. Genotypes were subdivided according dry matter concentration into high and low dry matter classes. Starch (A), fructose (B), glucose (C), galactose (D), sucrose (E) and myo-inositol (F), malic acid (G), quinic acid (H) and citric acid (I) concentrations are here reported. n = 5 vines per class. Values are average ±SE of the mean. Tests of Effect Slices: *, p<0.05; **, p<0.01; ***, p<0.001; p<0.0001; blank, not significant.

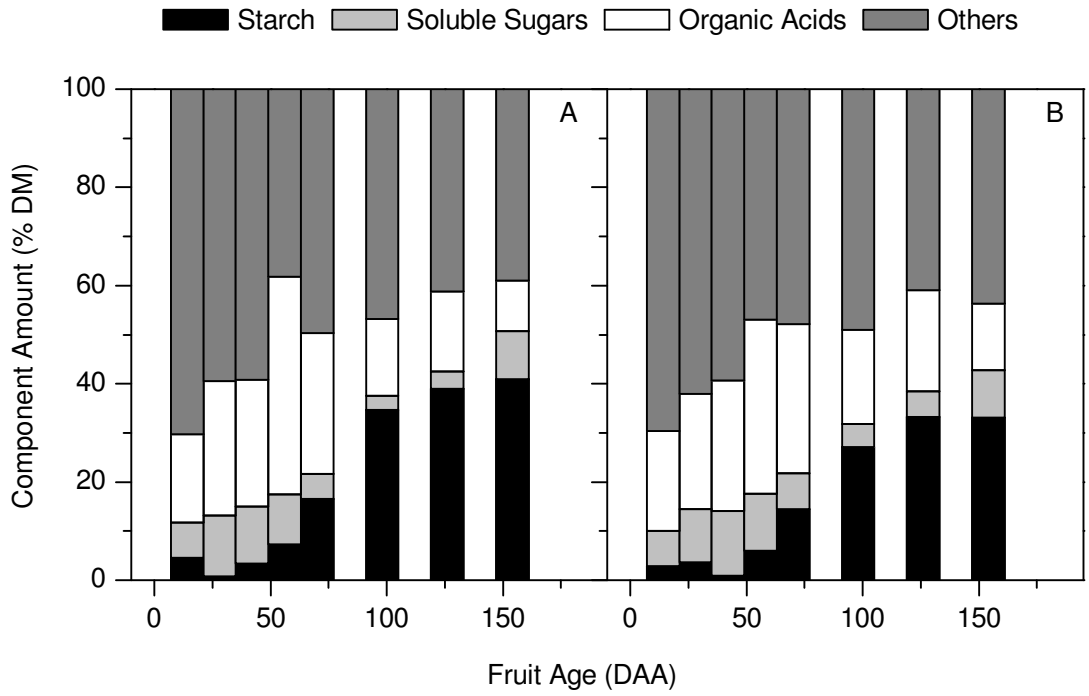


Fig. 3-4: Dry matter components during fruit growth in 2004-05 season for *Actinidia deliciosa* genotypes. Genotypes were subdivided according dry matter concentration into high and low dry matter classes. Comparison between high dry matter (A) and lo dry matter (B) genotypes for dry matter components: starch, soluble sugars, acids and other non-detected compounds in 2004-05 season. n = 5 vines per class.

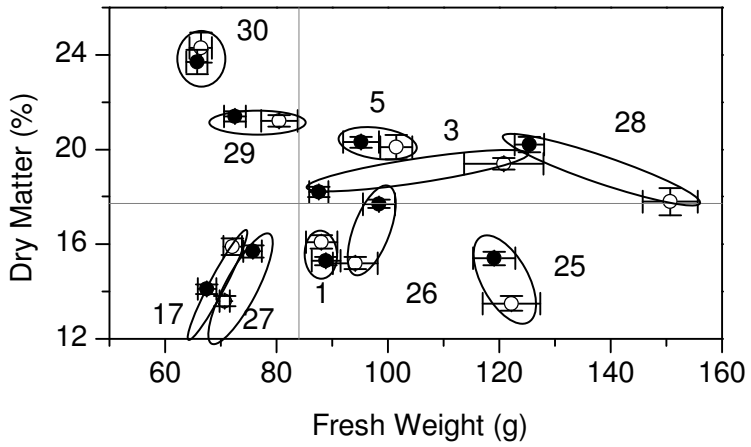


Fig. 3-5: Genotype subdivision in dry matter (DM) and fresh weight classes (values at 154 DAA). Circles and numbers indicate which genotype dots refer to (numbers are on the right side of the respective circle). Two seasons data are here reported: 2004-05 (○) and 2006-07 (●) seasons. Crossing lines subdivide genotypes in classes. From up left in clock wise order: Small fruit-high dry matter, Large fruit- high dry matter, Large fruit-low dry matter, Small fruit-low dry matter. n = 5-10 (2004-05 season) and n = 15-20 (2006-07 season). Values are averages ±SE.

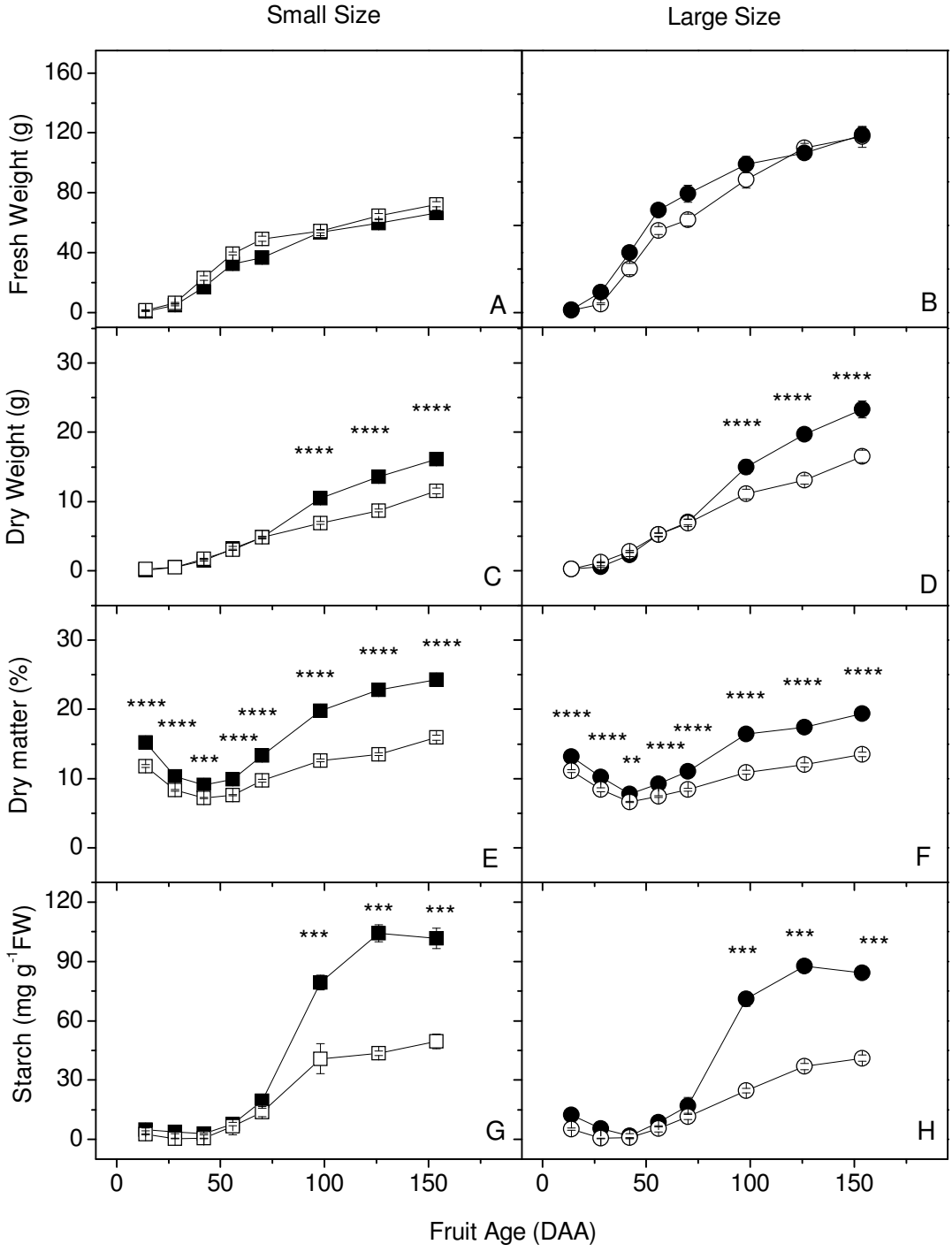


Fig. 3-6: Fresh weight (A-B), dry weight (C-D), dry matter (E-F) and starch concentration (G-H) of 4 *Actinidia deliciosa* genotypes in 2004-05 season. Data are averages ±SE of the mean. n = 10 for physical measurement; n = 4 for starch. Small fruit high dry matter genotype 30, (■); small fruit low dry matter genotype 17, (□); large fruit high dry matter genotype 3, (●); large fruit low dry matter genotype 25, (○).Tests of Effect Slices: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; $p<0.0001$; blank, not significant.

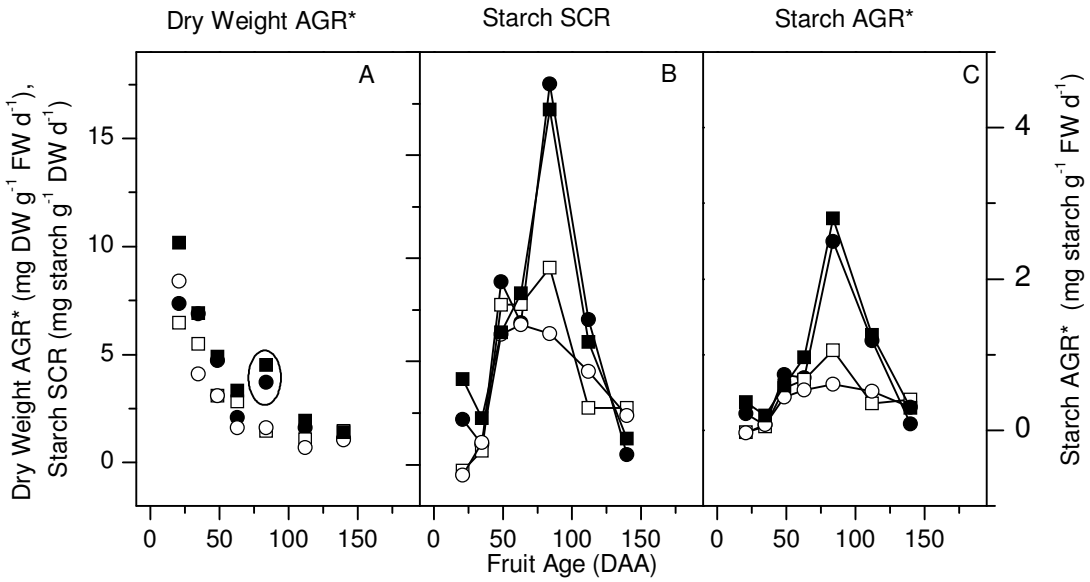


Fig. 3-7: Genotype ranking according to efficiency indexes for dry weight and starch accumulation in selected *Actinidia deliciosa* genotypes. Dry weight ponderate AGR (AGR*, A), starch specific conversion rate (SCR, B), starch ponderate AGR (AGR*, C). Small fruit high dry matter genotype 30, (■); small fruit low dry matter genotype 17, (□); large fruit high dry matter genotype 3, (●); large fruit low dry matter genotype 25, (○).

3.4 DISCUSSION

Genotypes were predicted to be either high or low in dry matter concentration, whether or not fruit were small or large, based on larger scale seedling experiments and three years of prior analysis early soon after establishment (Marsh, 1999). The actual experiments have demonstrated that the selected genotypes have remained true to prediction over a further three seasons, despite variations in growing conditions and crop loading. In addition the selected genotypes behaved as predicted when grafted onto genetically identical rootstocks and planted in a more normal orchard layout as replicates. Fresh weight was expected not to differ because there were equal numbers of small and large sized genotypes among the classes. Therefore there was no dilution effect in differential accumulation of dry matter. These data overwhelmingly indicate the key driver of DM accumulation is genetic and not environmental in these genotypes.

Some genotypes could accumulate higher dry matter because of increased sink strength (Causse *et al.*, 2004; Ho, 1992; Sun *et al.*, 1992). This could be because more sugar downloaded in the fruit through increased phloem unloading (Baxter *et al.*, 2005; Fridman *et al.*, 2004), or because there was greater allocation to new metabolites such as starch through enzymatic activity or allocation to cellular sub-compartments (Chopra *et al.*, 2005; Ho, 1996; McKibbin *et al.*, 2006; Tiessen *et al.*, 2002). Hydrolysis of sucrose, for example, has long been suggested as the rate-limiting step for carbon accumulation in tomato fruit (Walker *et al.*, 1978). High dry matter genotypes differed primarily in net accumulation of starch, which became significantly different from approximately 100 DAA onwards. Starch accumulated at higher rates in high dry matter genotypes. In some seasons (and mix of genotypes) starch concentrations were significantly higher earlier in development (data not

shown). In particular, when a subset of genotypes were replicated on the same rootstocks, the accumulation of higher dry matter in the fruit occurred either before or at anthesis. These results suggest that the fruit have a higher sink activity in high dry matter genotypes compared to low dry matter genotypes and that this overrides fruit growth and expansion.

As sink strength is the effect of sink size and sink activity (Ho, 1992), a difference in one of the two components might be expected to change the specific fruit sink strength.

A study on two genotypes with different invertase enzyme activity showed no differences in sucrose uptake between the two lines. It was then demonstrated that sucrose synthases were the key enzyme in sucrose unloading and starch content was then related to sucrose availability (N'Tchobo *et al.*, 1999). The introgression line IL9-2-5 in tomato presents higher dry matter level and higher at-harvest soluble sugars than the wild type. This increase has been linked to an higher sucrose unloading capacity early in development because of a different activity in a fruit specific cell wall invertase encoded by *LIN5* gene (Baxter *et al.*, 2005; Fridman *et al.*, 2004). IL9-2-5 genotype did not differ in other characteristic, such as photosynthetic efficiency (Baxter *et al.*, 2005). In potato tubers expressing an antisense *AGPB* (large subunit) construct, enzyme activity resulted to be decreased of a 50% compared to the wild type and ended into a lower starch content (Geigenberger *et al.*, 1999; Müller-Röber *et al.*, 1992). A tomato introgression line harbouring a wild specie allele for an AGPase large subunit was observed to have young fruit with increased AGPase activity and starch content, as well higher soluble solids in mature fruit (Schaffer *et al.*, 2000). A recent study showed that a temporally extended period of that particular AGPase large subunit was responsible of the observed increased starch accumulation (Petreikov *et al.*, 2006). Transgenic potato lines over-expressing a sucrose non-fermenting-1-related protein kinase-1 gene (SnRK1) showed decrease glucose levels and increased starch levels. Gene expression analysis showed increased sucrose synthase and AGPase gene expression in the transgenic lines, as well as the enzyme activity (McKibbin *et al.*, 2006). These are just some of the many studies about differences between genotypes in gaining and accumulate assimilates. It can be supposed than that observed differences in starch content can be affected by a change in sucrose unloading, or a change in the sucrose to starch conversion. Kiwifruit, as potato and tomato, accumulates starch. Being the starch the main change affecting dry matter content, it is obvious that further investigation are needed to elucidate which factors drive extra carbon into the berry. It might be a change at unloading level, or a more efficient sucrose synthase or a different behaviour in AGPase subunits (temporal, different isoforms action, etc…).

A sink with a higher number of cells and those cells are larger in size, is theoretically a stronger sink (Ho, 1992). A study in banana fruit bunch showed that differences in dry matter within the same bunch were related to the pulp cell number (Jullien *et al.*, 2001). Little is known about genetic determinants of sink size. A comparison between two melon genotypes differing in fruit size, ended that they differ mainly in cell number, and then maybe in cell size. The main determinant of fruit size was a genetic control of cell division (Higashi *et al.*, 1999). All studies up to date, however, did not matched both size and carbon

accumulation in a comparative study when differences in genotypes were found. This is a strong limit, because the size can mask important characteristics.

Generally, a negative correlation links sink carbon sequestration and sink size for several fruit crops (Ben-Chaim and Paran, 2000; 2004; Dirlewanger *et al.*, 1999; Fulton *et al.*, 1997; Monma and Takada, 1991). This is usually due to a co-localization of QTL for fruit size and sink activity (Dirlewanger *et al.*, 1999; Saliba-Colombani *et al.*, 2001). So, the selection heading to improve fruit size often has a reduction in fruit soluble solid concentration. Recent studies, however, indicate that this rule can be sometimes broken (Causse *et al.*, 2004; Cheng *et al.*, 2004; Fridman *et al.*, 2002; Fridman *et al.*, 2000), resulting in a genotypic selection of all possible combination of size and dry matter, as the present study shows.

Differences in dry matter content were consistent over four seasons, but primarily genotypes were different in starch content. Starch is the major non-structural and storage carbohydrate during *Actinidia deliciosa* berry development (Boldingh *et al.*, 2000; Richardson *et al.*, 1997). According to several studies a consistent pattern emerges whereby starch begins to accumulate in fruit after the initial phase of rapid fruit expansion and by harvest accounts for some 50% of the total fruit dry matter (MacRae *et al.*, 1989; Okuse and Ryugo, 1981; Richardson *et al.*, 1997; Walton and De Jong, 1990). In studied genotypes dry matter content started to be different between genotype classes after the onset of net starch accumulation, that was observed between 50-60 DAA. Starch was accumulated at higher rates in high dry matter genotypes. Starch was about 40% of total dry matter after the onset of net starch accumulation in high dry matter genotypes, whereas it was evidently lower in low dry matter genotypes (Fig. 3-4). While other non detected compounds (amino acids, cell wall polysaccharides, etc…) were similar after 70 DAA, the higher proportion of acids observed in low dry matter genotypes could be ascribed to a difference in primary metabolism between the two observed groups. Soluble sugars and acid concentration behaved as previously reported in *Actinidia deliciosa* (Boldingh *et al.*, 2000; Marsh *et al.*, 2004). Both dry matter classes showed the typical glucose peak at about 40 DAA, as well as a peak in quinic acid 15 days later (Fig. 3-3). The higher at-harvest sucrose content observed in high dry matter genotypes can be due to an earlier onset of fruit maturation in high dry matter genotypes.

Differences in dry matter content, dry weight and starch concentration observed did not affected fresh weight accumulation. Fruit growth followed a sigmoid pattern similar to those reported for 'Hayward' (Richardson *et al.*, 2004; Walton and De Jong, 1990).

Small size fruit were more consistent in both dry matter content and size, and extreme in values, whereas large size genotypes were more affected by fruit weight fluctuations. More extreme genotypes were observed in small sized fruit. Dry matter content was strongly consistent within single genotypes throughout different years (Fig. 3-5), while fruit fresh weight was more affected by crop load and environmental condition. These founding support the hypothesis that dry matter content is genetically determined. Observed genotypes were still falling in respective arbitrary classes. Small size fruit were more consistent in both dry

matter content and size, and extreme in values, whereas large size genotypes were more affected by fruit weight fluctuations. More extreme genotypes were observed in small sized fruit.

The comparison of single genotypes (Fig. 3-6) were consistent with 'population' behaviour, and enforced group's results. Starch was consistently different in both size classes. Dry matter was strongly different at each time point between high and low dry matter genotypes in both small and large sized fruit. Dry weight started to be different only later in development, after the onset of net starch accumulation, and only when significantly differences in starch concentration were evident. Within size classes, fruit fresh weight was not different. Small sized fruit genotype (30) was the one with the highest dry matter content (about 25 % at-harvest dry matter content), and the highest starch concentration (about 10% of fresh weight). Reported values for starch are about 7% of fresh weight (Richardson *et al.*, 1997; Walton and De Jong, 1990), so high dry matter genotypes had 20-30% more starch than 'Hayward'. High dry matter genotypes are therefore stronger starch accumulators.

Genotypes were selected according to at-harvest DM concentration and fruit size, then grouped in 4 classes: Small fruit-Hi-DM, Large fruit- Hi-DM, Large fruit-Lo-DM, Small fruit-Lo-DM. Fruit size and DM content are quantitative continuous variables and a class subdivision is much harder than for qualitative variables. Furthermore, it is evident that size can interact with dry matter and starch accumulation obscuring the real genotype efficiency, through dilution effects. This limitation requires an appropriate index, to rank genotypes, that weight starch accumulation (or dry weight accumulation) for fruit size. Three indexes were tested and all of them agree in pointing the 70-98 DAA stage as a critical step in fruit development when it was possible to predict the final dry matter content or starch concentration. At this stage, all tested indexes were about double in high dry matter genotypes than in low dry matter genotypes. Genotype 30 and 3, high in dry matter content, were the most efficient in starch accumulation, whereas genotype 17 and 25 were slower in carbohydrate accumulation. The dry weight index showed a result similar to the starch based one. Starch content determination is a time consuming analytical process and require laboratory instrumentation. The dry weight based index is a practical system that could be use to screen genotypes and select the more efficients. It could be verified applying it on a mapping population, organizing so a new phenotyping experiment. However, it has a list of disadvantages: it requires to have fruit, it referrers to a specific developing window, it needs lots of measures-replicates. So, further genomic investigations for high starch markers are needed to develop a quick and efficient method of breeding population screening.

3.5 CONCLUSIONS

In conclusion, genotypes dramatically differ in starch content primarily, then in dry matter content and dry weight. Differences were consistent in several years and genetically determined. Considering that dry matter is highly heritable (Cheng *et al.*, 2004), and that is strongly affected by starch concentration, it can be concluded that differences in carbohydrate metabolism can possibly contribute to the observed genotypic variations, as

observed in tomato studies (Baxter *et al.*, 2005; Fridman *et al.*, 2004). Further investigation are then needed to elucidate this hypothesis.

4 SINK STRENGTH: SINK SIZE

4.1 INTRODUCTION

The taste and quality of kiwifruit berry are strongly linked to the total soluble solids content of the mature fruit and the dry matter-starch accumulated during fruit development (Burdon *et al.*, 2004; Jaeger *et al.*, 2003). Various approaches have been taken in order to increase dry matter up to date in kiwifruit, mainly through agronomical practices, such as a combination of adjusted leaves to fruit ratios and cane or trunk girdling (Lai *et al.*, 1989; Smith *et al.*, 1992; Snelgar *et al.*, 1998). Breeding might be a more efficient approach to find improved new cultivar able to attract more carbon. In fact, the breeding history in *Actinidia* is only few generations old and potentiality for improvement given by germoplasm is high (Ferguson, 2007).

From a previous study on individuals from several breeding families, it was found a consistent difference in sink strength between genotype classes with contrasting dry matter content (chapter 3). In tomato, sink strength is defined as the product of sink size and sink activity (Warren-Wilson, 1972). Sink size is a physical restraint that includes cell number and cell size, whereas sink activity is a physiological restraint that includes multiple factors and key enzymes involved in carbohydrate utilization and storage (Ho, 1984).

Cell multiplication is a process in which division of a cell creates two or several cells, copy of the mother cell, and which undergo division. A proportion of cells in a population is not-dividing and is often reported that the proportion of cells dividing each following cycle progressively declines (Bertin *et al.*, 2003). Fruit growth starts after pollination, and initially growth is mostly driven by cell proliferation activity. At a certain point, varying from species to species, proliferation activity slows down progressively till mitosis cease and obtained cell population enters the cell enlargement stage (Bain and Robertson, 1951; Gillaspy *et al.*, 1993; Yamaguchi *et al.*, 2002). For several crop, final fruit size is largely determined by cellular production during ovary formation and during the few weeks after pollination (Bunger-Kibler and Bangerth, 1983; Hopping, 1976b). Duration and intensity of cytokinesis then can strongly affect fruit size (Bohner and Bangerth, 1988). Within- cultivar, fruit size can vary as a result in canopy source-sink relation, or even spatial location in the canopy (Snelgar *et al.*, 1998; Tombesi *et al.*, 1993; Whiting *et al.*, 2005). Between-cultivar variations in fruit size are usually refer to differences in cell number and cell size. In peach and melon differences in fruit size between cultivars have been shown to be influenced by mesocarp cell number (Higashi *et al.*, 1999; Scorza *et al.*, 1991). In peach and apricot, differences between genotypes were linked to both cell number and cell sice (Yamaguchi *et al.*, 2002; Yamaguchi *et al.*, 2004).

However, most of the studies were mainly addressed to give an explanation only to observed genotypic differences in size. None of them was trying to compare differences in sink

strength that were not counting of the size dilution effect. The present chapter is focused on how sink size (mainly at cellular level) can affect sink strength, and drive sink activity.

The anatomy of 'Hayward' kiwifruit berry has been deeply investigated. Kiwifruit berry is constituted by three different tissues: outer pericarp, inner pericarp (carpellar locules and seeds) and central core. Each tissue has a different impact on total fresh weight. Each tissue has different DM content and composition (MacRae *et al.*, 1989). 'Hayward' kiwifruit tissue mainly contributing to the final fresh weight is the outer pericarp. The outer pericarp is a parenchymatous tissue that according to Hopping (1976b) can be further subdivided into an outer hypodermal layer, 10-15 cells wide, that extended inwards from the epidermis and an inner layer of elliptical cells that extended from the hypodermal layer to the inner pericarp. During pericarp expansion, cells of the hypodermal layer elongate in the tangential plane whereas those of the inner layer increase several fold in diameter and became essentially spherical. However, some cells in both layers do not enlarge appreciably and, at maturity, fill the spaces between cells that have increased in size. The outer pericarp is composed of small, isodiametric parenchyma cells (cross-sectional diameter about 0.1-0.2 mm) containing abundant starch, and large ovoid parenchyma cells (cross-sectional diameter about 0.5-0.8 mm) containing very few starch grains. The final starch content of a cell might be equivalent (Hallett *et al.*, 1992).

The goal of this study was to understand if observed differences in either DM content and fruit size was an effect of different tissue proportion, of a different cell number, size or relative volume occupied by each cell class.

It was hypothesised that a larger proportion of central core in high DM genotypes, being the tissue with the higher DM concentration, could affect significantly the final DM content. Being starch concentration higher in small cells, it was hypothesised that an higher outer pericarp small cells density could explain an higher DM content. When many sections are observed the percentage of area occupied by a type of cell could be a good estimator of cell occupied volume. It was then supposed that high DM genotypes had a larger outer pericarp volume occupied by small cells more dense in starch. Within a DM class it was expected that differences in fruit size were mainly determined by a larger size of both small and large cells, and consequently a lower density.

It was then tried to verify if sink size, seen as a combination of cell number and cell size, was different between selected genotypes and if there was any significant change counting for measured starch or dry matter differences.

4.2 MATERIALS AND METHODS

4.2.1 PLANT MATERIAL

For the present study of *Actindia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* fruit anatomy, a total of 24 vines were selected in 2003-04 and 2004-05 seasons from three factorial seedling populations. These were planted in 2 contiguous orchard blocks at Te Puke Research Centre (Bay of Plenty, NZ; 37° 49' S - 176° 19' E) in 1999 for breeding

purposes. An equal number of extreme high and low fruit dry matter plants characterized by a large or small final fruit size were selected. All vines were growing on a T-bar trellis at 0.8 m spacing on the row and 5 m spacing between rows. Each genotype fruit development and dry matter content was known for a number of sampling dates from anthesis to harvest.

4.2.2 TISSUE PROPORTIONS

For tissue proportion measurements, at each sampling date, a picture of either a transversal and longitudinal section was taken with a digital camera (Nikon, CoolPix 900). A reference scale was included in each shot. Images were analyzed by using ImageJ software (Rosband, 1997-2006) and outer pericarp, inner pericarp and central core areas were represented as a percentage.

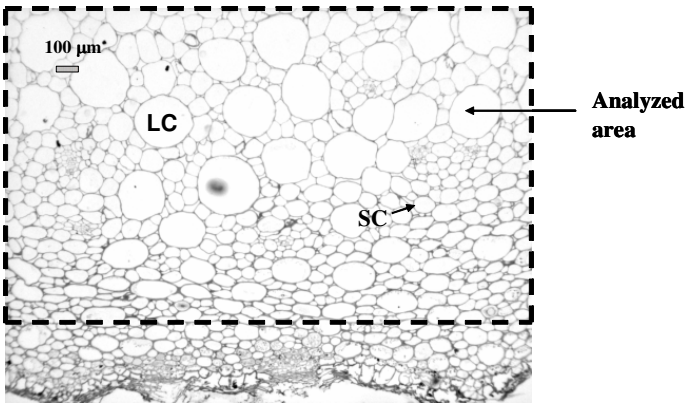


Fig. 4-1: Berry outer pericarp analyzed area for cell counting and volume estimation measurements. Large cells (LC) and small cells (SC) are also represented. First few cell layers close to the skin were not considered.

4.2.3 CELL COUNTING AND CELL VOLUME ESTIMATION

A median-transversal slice, 5 mm thick, was cut from each fruit, and a sector was then fixed in 2.5%-2% Gluteraldehyde-Formaldehyde in 25 mM Phosphate buffer (6.8 pH). Samples were left under vacuum for 1 h, then washed three times in buffer, dehydrated in an ethanol series and stored under 100% ethanol.

For cell counting and cell volume proportion Sectors were then stained as for Goffinet *et al.* (1995). Samples were examined at a 6X magnification with a stereo-microscope (Nikon, SMZ1500). Images were obtained using a Camera Control Unit (Nikon, SU-1) and analyzed by using ImageJ software (Rosband, 1997-2006). Before start each set of image captures, a picture of a scale was taken as zoom camera reference. Two different pictures were taken from each sector, trying to include also the skin. Cell were counted from about 6 layers out of the hypodermal tissue, cut cells were considered for 2 borders only, and either large and small cells were counted. The analyzed area (Fig. 4-1) was then measured. For cell volume proportion a grid of 13 rows and 18 column, for a total of 234 crossing points, was laid on each picture. Crossing points were classified in 4 classes (Fig. 4-2, only 2 classes reported): large cells, small cells, intercellular spaces and vasculature. A percentage of each was then calculated.

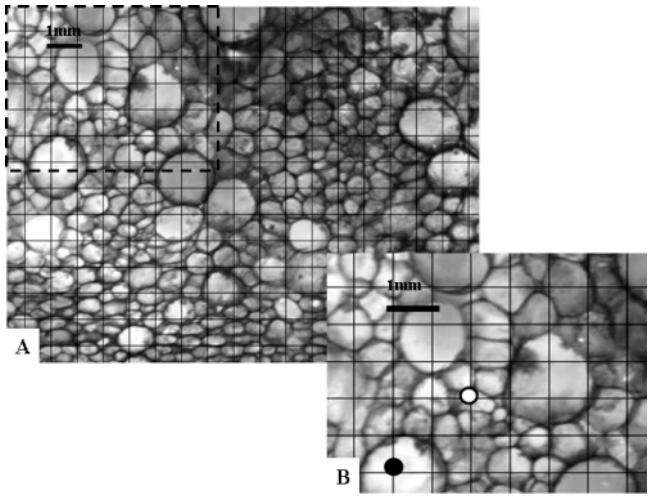


Fig. 4-2: Cell volume estimation grid technique (A). Each edge was named differently according to the type of tissue component underneath. An example is reported in picture B: large cells were pointed with closed dot (●) whereas small cells were named as opened dot (○). Image J program has a function able to keep track of assigned names and count then totals.

4.2.4 CELL SIZE MEASUREMENTS

For cell measurements samples were embedded in LR White Resin (London Resin, Reading, UK). Structural observation was carried out as for Hallett and Sutherland (2005)on 1 μm sections of resin-embedded material stained in a 0.05% solution of toluidine blue in benzoate buffer (pH 4.4), dried, and mounted in Shurmount (Triangle Biomedical Sciences, Durham, NC). Sections were viewed at 40X and 100X magnification using an Olympus Vanox AHT3 microscope (Olympus Optical, Tokyo) and resulting images were analyzed by using ImageJ software (Rosband, 1997-2006). From each section 40X, ten 100X pictures were saved and from each about 50 cells measured (maximum diameter and area). Data were then converted to μm using a reference scale.

4.2.5 STATISTICAL ANALYSIS

Statistical analysis was performed using the t-test two samples assuming unequal variances.

4.3 RESULTS

4.3.1 TISSUE PROPORTIONS

The fruit tissue proportion observations, carried out in 3 following seasons, showed no differences in the relative percentage occupied by each of the 3 berry tissues in transversal sections between high and low dry matter genotypes (Fig. 4-3A, B, C).

The outer pericarp was the most represented tissue occupying about 50% of the slice surface. Then the inner pericarp counted for about 40%, whereas the core represented the about 5-10%.

In 2005-06 season also the longitudinal profile was observed and tissue proportion measured (Fig. 4-3D). There were still no evident differences counting for the measured higher level of dry matter or starch.

Longitudinal slice tissue proportions resulted to be different compared to the transversal one. The core proportion was about 20%, whereas pericarp tissues occupied both 30-40% of the slice surface.

4.3.2 CELL DENSITY

There is not a clear correlation between cell density (total, small and large) and either final fruit size or final dry matter content (Fig. 4-4). All four genotype classes are equally distributed among increasing cell density (total, small and large).

Total cell density varied from about 60 to 100 cells per mm^2, small cell density ranged between 50 to 80 cells per mm^2 and large cells from 7 to 20 cells per mm^2.

4.3.3 ESTIMATED CELL VOLUME

Small to large cell volume ratio resulted to be significantly different between high and low dry matter genotypes (Tab. 4-1). Outer pericarp volume in high dry matter genotypes was mostly occupied by large cells in absolute terms (54.2%) and it resulted to be significantly different when compared to low dry matter genotypes (43.2%). High dry matter genotype showed an average 38.6% of the estimated outer pericarp volume occupied by large cells, whereas it was about 50% in low dry matter genotypes. Volumes occupied by either intercellular spaces and vascular tissues were marginal and not significantly different among the two observed groups.

4.3.4 LARGE TO SMALL FRUIT COMPARISON

A more fine investigation at the light microscope was held to understand how large and small fruit with equal dry matter content could be different in outer pericarp cells profile.

The two observed genotypes were significantly different in total cell number, being the small genotype the one with the higher total cell density (Fig. 4-5A). Also the small cell density was significantly different and higher in small size fruit, whereas the number of large cells was unchanged (Fig. 4-5B, C). Total cells density was then 30% higher in small size fruit.

When cell area was measured, it was clear that the difference in cell number was the effect of the larger size of large parenchyma cells in large size fruit, rather than to a variation in small cells, which area was unchanged (Fig. 4-6). Large cells were one order of magnitude bigger than small cells.

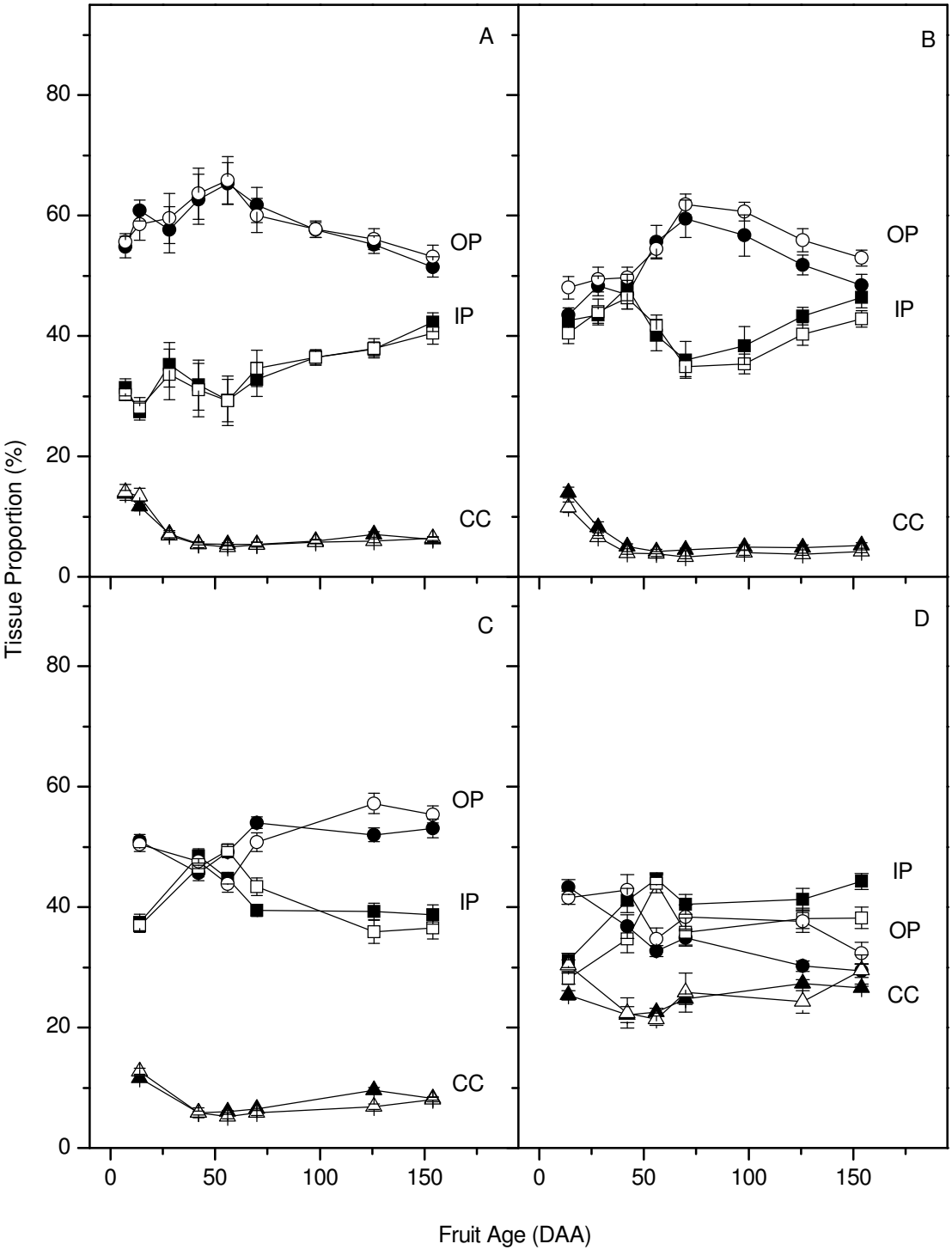


Fig. 4-3: Transversal (2003-04 season - A, 2004-05 season - B, 2005-06 season - C) and longitudinal (2005-06 season - D) slice tissue proportions. OP, outer pericarp (●, ○); IP, inner pericarp (■, □); CC, central core (▲, △). Closed symbols represent high dry matter class, opened symbols represent low dry matter class. n = 9 vines each dry matter class in 2003-04 season; n = 5 vines each dry matter class in 2004-05 season; n = 3-2 vines each dry matter class in 2005-06 season. Values are average ±SE of the mean.

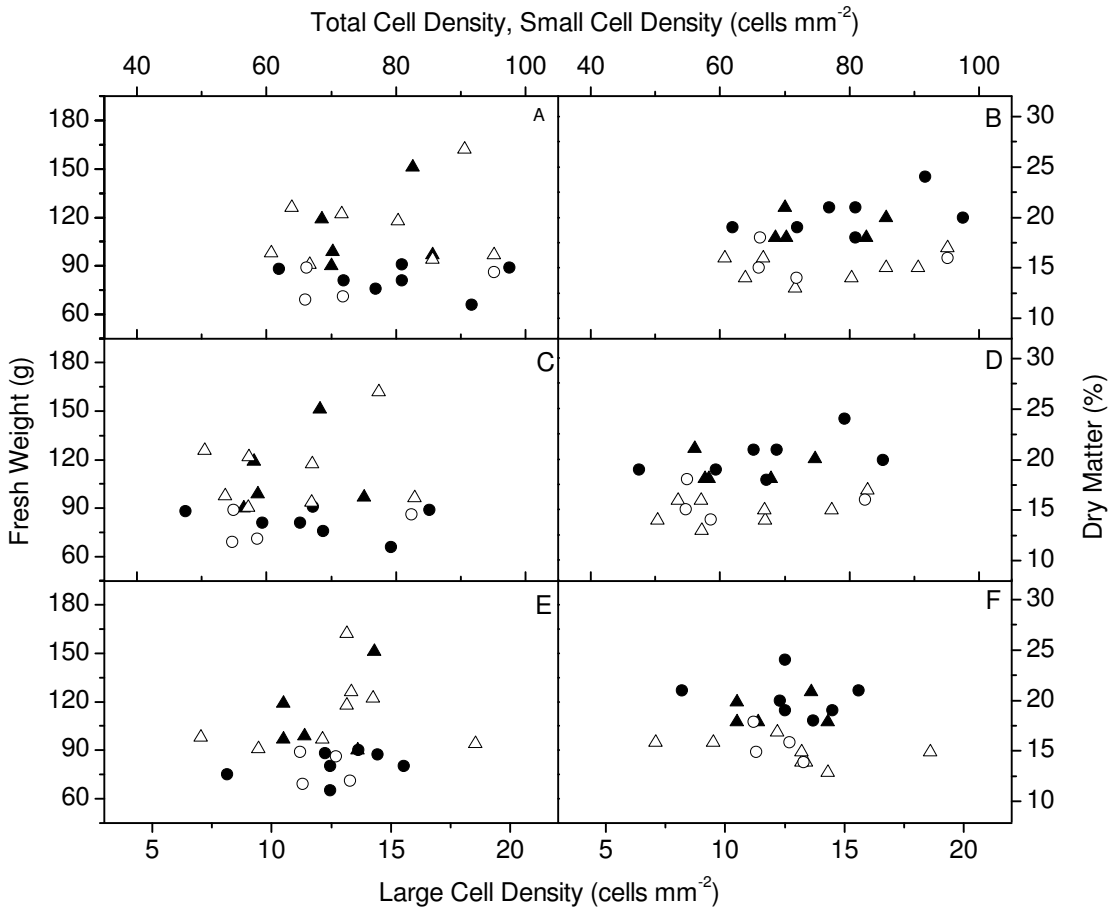


Fig. 4-4: Genotypes distribution according to fruit size or DM (%) and outer pericarp cell density (total A-B, small C-D and large E-F). n = 9 + 3 high DM and 9 + 3 low DM genotypes. 154 days old fruit sections were used. Large size genotypes (▲, △); small size genotypes (●, ○). Closed symbols represent high dry matter class and opened symbols represent low dry matter class.

Tab. 4-1: Estimated volumes for outer pericarp section components: large cells (LC), small cells (SM), intercellular spaces (IS), vascular tissues (VT). n = 5 vines each dry matter class; n = 6-4 vines each size class. Values are averages ±SE of the mean. t-test significance: ns, not significant; *, p<0.05; **, p<0.01.

	Estimated Volumes			
	LC (%)	**SC** (%)	**IS** (%)	**VT** (%)
High DM	38.6 ± 2.1	54.2 ± 3.1	4.2 ± 1.8	3.1 ± 0.6
Low DM	50.0 ± 2.4	43.2 ± 3.1	3.3 ± 1.2	3.5 ± 0.7
t-test	**	*	ns	ns
Large size fruit	44.9 ± 2.1	47.7 ± 3.4	4.3 ± 2.0	3.1 ± 0.7
Small size fruit	43.7 ± 3.1	49.7 ± 3.2	3.2 ± 1.2	3.4 ± 0.8
t-test	ns	ns	ns	ns

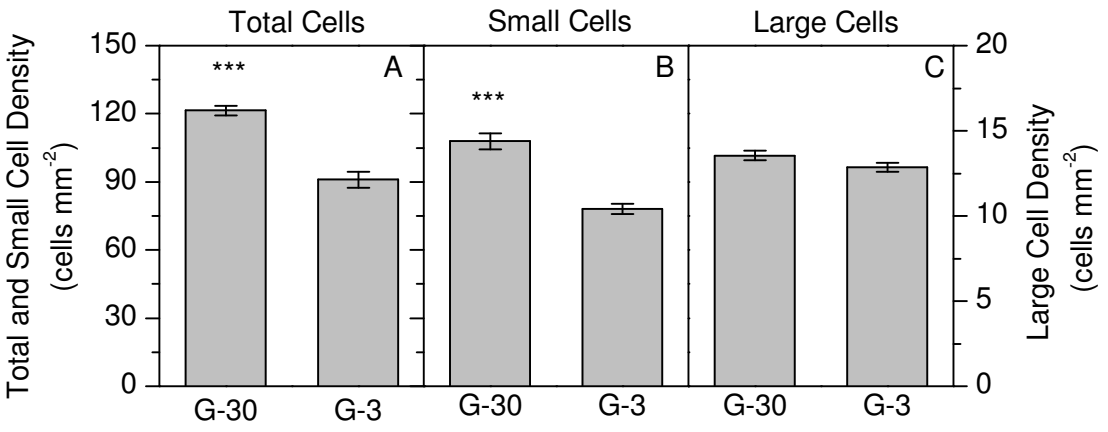


Fig. 4-5: Comparison of total, small and large cells density in outer pericarp between two high DM genotypes different in fruit average size. n = 10 outer pericarp sections (5 fruit and 2 outer pericarp areas) at 126 DAA in 2005-06 season. Values are average ±SE of the mean. t-test: ***, p<0.01.

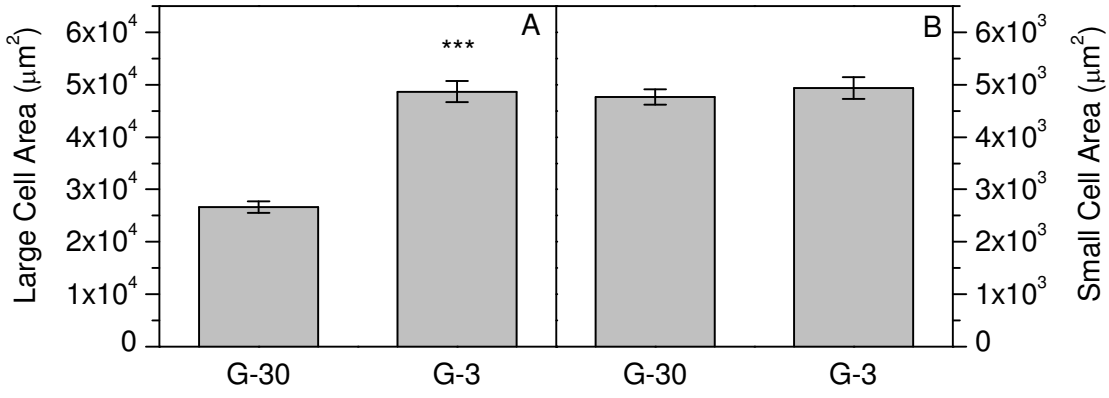


Fig. 4-6: Comparison of average large (A) and small cells (B) area in outer pericarp between two high DM genotypes different in fruit average size. n = 10 outer pericarp sections (5 fruit and 2 outer pericarp blocks) at 126 DAA in 2005-06 season. Values are averages ±SE of the mean. t-test: ***, p<0.01; blank, not significant.

4.4 DISCUSSION

Genotype dry matter classes observed to be different mainly in starch content in chapter 3, were hypothesized to differ also at anatomical level. And they differ in sink size, but as a combination of both cell number and cell size. It was not possible therefore discriminate genotypes only on a cell number base, but an estimate volume measurement was necessary. Genotypes were different according to a multiple organization of large and small cells, in number and size.

In kiwifruit berry the three tissues differ in dry matter content (MacRae *et al.*, 1989), but not evidence of a significant higher proportion of a tissue on another was observed between the observed classes. This result was consistent among several years of observations. Cell number is often related to cell division efficiency for several crop, and potential final fruit size is then affected by length of cytokinesis (Bertin *et al.*, 2003). Then the cell density was considered, but from a scatted plot of small, large and total cell density no clear genotype

clusters were identified. From a visual comparison of outer pericarp sections differences were evident between high and low dry matter genotypes, but they were a combination of both cell number and size. In fact, when an estimation of the volume occupied by each cell type, it was possible to see that in high dry matter genotypes small cell total volume was higher than in low dry matter genotypes (and *vice versa* was for large cell volume).

Considering that as previously observed in chapter 3, high dry matter genotypes have about 50% more starch than low dry matter genotypes. So, is the observed difference in estimated volume occupied by large and small cell enough to explain starch difference? A quick verification calculation can be easily made. Large and small cells in 'Hayward' have the same starch amount, what is different is of course the concentration. Small cell is spherical, whereas large cells are elongated (Hallett *et al.*, 1992). Data on large cell length are not available, but it can be assumed to be 4 time the radius and equal between the two genotype classes (to simplify the calculation). From the data previously observed, small cell and large cells area differ by an order of magnitude. If we make an approximate calculation of a per cell volume, large cells are two order of magnitude bigger than small cells. If the starch content in large and small cell is the same, the total content of starch for same volume is therefore two order of magnitude higher in small cells. The calculation of the amount of starch is done multiplying the estimated volume occupied by each type of cell and the starch for volume unit (100 time higher for small cells). The significant difference found in large and small cell volumes occupied in high and low dry matter counted only for half of the observed increase of starch in high dry matter genotypes (+25%). Then, the starch concentration in small cell between high and low dry matter genotypes might be different. For example, a 20% more starch in high dry matter genotypes small cells combined with the different volume can count for the observed 50% more starch. Further studies are then needed to verify this hypothesis and furthermore, it is useful to focus more on sink activity, being any higher capacity of a cell to accumulate starch a consequence of enzymatic differences (in unloading, regulation, activity, etc…).

If the previous arguing are true, it is expected that from the comparison of two genotypes with equal dry matter content, observed differences in cell number are referred then to a differences in cell size. Small sized genotypes showed an higher density of small cells than the large one, but they did not significantly differ in size. Large cell density was unchanged, but large cells were double sized in large fruit. A whole fruit basis can drive hypothesis in the wrong direction, considering that often sink strength is a matter of concentration. So, it might be that the total cell number in large fruit is higher than in small fruit, but this is not enough to explain the double size and moreover to explain an equal capacity to attract carbon among the two different sizes.

Previous study on cell size or cell number were referred to 'Hayward' kiwifruit and mainly addressed to explain hormonal role in fruit enlargement (Antognozzi *et al.*, 1997; Patterson *et al.*, 1993). A study similar to the present one is not present in kiwifruit literature, whereas anatomical characterization of different genotypes was performed on peach and melon (Higashi *et al.*, 1999; Scorza *et al.*, 1991). These studies, however, were more focused to

explain differences in fruit size rather than in carbon accumulation. In fact a small fruit size has usually been related to a low number of cell (Cowan *et al.*, 1997; Higashi *et al.*, 1999; Jullien *et al.*, 2001).

This study is emphasising more previous suggestion (chapter 3) that genotypic differences drive carbon allocation within kiwifruit berry. Also cell number, cell type distribution and cell size seem to be a peculiarity of each genotype, and the right combination of each variable ends into a different impact on the carbon uptake potentiality.

4.5 CONCLUSIONS

Anatomy investigations suggest that there were significant differences in the anatomy of the outer pericarp between genotypes, but there is no an unique combination of anatomical traits associated with either fruit size or starch content. So, anatomical differences by themselves are not sufficient to explain the observed differences in starch (or DM) accumulation. Considering that the concept of sink strength is referred to both sink size (here reported) and sink activity, further investigation on unloading and starch metabolic enzyme are then required.

5 SINK STRENGTH: FACTORS AFFECTING SINK ACTIVITY IN KIWIFRUIT BERRY

5.1 INTRODUCTION

Starch is one of the plant products most important to man (Burrell, 2003), and the biochemical pathways for starch synthesis in leaves and sink organs, such as cereal caryopsis, tomato fruit and tubers, have therefore been extensively studied (Geigenberger, 2003; N'Tchobo *et al.*, 1999; Tetlow *et al.*, 2004; Yang *et al.*, 2004; Zeeman *et al.*, 2007). For several model plants, therefore, all of the key enzymes have recently been described, and their corresponding genes cloned. Increasing interest has been placed to clarify mechanisms involved in the regulation of the carbon flux from sucrose to starch, with a priority goal in the improvement of quantity and quality of accumulated starch by staple crop such as wheat, rice, maize and potato.

Starch plays a key role in the growth and development of several crop fruit such as tomato where the transiently accumulation starch reserve affects the final soluble sugar content of the ripe organ (Dinar and Stevens, 1981; Ho, 1996). In tomato fruit, the temporal starch accumulation correlates with changes in activity of sucrose-to-starch metabolism involved enzymes (Robinson *et al.*, 1988; Schaffer and Petreikov, 1997; Wang *et al.*, 1993). In several crops, pivotal steps limiting starch synthesis are sucrose cleavage enzymes and ADP-glucose pyrophosphorylase (Fridman *et al.*, 2004; Petreikov *et al.*, 2006; Schaffer *et al.*, 2000; Zrenner *et al.*, 1995).

Sucrose is the most frequent sugar transported in higher plant phloem from source to sink organs (Ziegler, 1975). Once sucrose gets into the fruit, phloem unloading is required to guarantee a continuum carbon flux. The strategy used in phloem unloading is highly sink- and species-specific, and whether it is symplasmic or apoplasmic is determined by the preferential sucrose cleavage pathway involved. Both sucrose synthase and invertase enzymes attend sucrose cleavage, but frequently their role is temporally and spatially regulated (Koch, 2004).

Sucrose synthase (Susy, EC 2.4.1.13) catalyze the reversible sucrose cleavage into UDPGlucose and fructose, furnishing then activated glucose for both starch and cellulose synthesis (Delmer and Amor, 1995; Quick and Schaffer, 1996). Susy exists free in the cytosol or associated with cellulose synthase complex on the plasmalemma (Barratt *et al.*, 2001). In sink organ it is often associated with vascular tissue (Nolte and Koch, 1993). Fructose is the main inhibitor of the enzyme activity (Morell and Copeland, 1985). Sucrose synthase is a small multigene family in many species (from 2 to 6 isoforms), comprising three groups: SUS1, SUSA and third group, comprising homologues to *Arabidopsis* SUS5 and SUS6 (Barratt *et al.*, 2001; Baud *et al.*, 2004; Komatsu *et al.*, 2002; Kortstee *et al.*, 2007; Zrenner *et al.*, 1995). Transcription of sucrose synthase is tissue specific (Bieniawska *et al.*, 2007; Wang *et al.*, 1994).

Invertases (EC 3.2.1.26) catalyze the irreversible sucrose cleavage into glucose and fructose. Invertases are subdivided into three isoenzyme classes according to solubility, subcellular localization, pH-optima and isoelectric point (Godt and Roitsch, 1997). Two large multigene families, instead, encode acid invertase: acid invertase, both cell wall bound and vacuolar (Haouazine-Takvorian *et al.*, 1997; Sherson *et al.*, 2003), and the neutral alkaline invertase, located in the cytosol (Vargas *et al.*, 2003). Cell wall bound invertases are mainly involved in apoplasmic phloem unloading, vacuolar invertases play a role in osmoregulation, whereas cytoplasmic invertases sustain sink development (Fridman and Zamir, 2003).

In higher plants, ADPGlucose pyrophosphorylase (AGPase, EC 2.7.7.27) is a highly regulated enzyme that catalyze the committed step of starch synthesis by the reversible reaction (Glucose1P + ATP ↔ ADPGlucose + PPi) that provides ADPGlucose, the substrate for starch synthase (Preiss and Sivak, 1996). Plant AGPase is a heterotetrameric enzyme comprising two large and two small subunits. Plants generally have three or four gene isoforms encoding the large regulatory subunit, whereas only one gene controls the transcription of the catalytic small subunit (Crevillen *et al.*, 2005; Kim *et al.*, 2007; Kortstee *et al.*, 2007). In many plant tissues AGPases are confined to the plastid (ap Rees, 1995), whereas it has been shown that some *Graminaceae* do not behave so. In fact, AGPase isoforms were localized in the cytosol of developing endosperm of maize, wheat and barley (Denyer *et al.*, 1996; Thorbjørnsen *et al.*, 1996a; Vardy *et al.*, 2002).

Sink strength is the capacity of an organ to attract carbon (Ho, 1988). Sink strength is heavily affected by sink size and sink activity, and sink activity is therefore strongly linked to carbon metabolism. A large number of studies have demonstrated whether each of the previous enzyme was significant to drive carbon into a fruit, or generally into a sink, in genotypes with divergent capacity to attract and store carbohydrates, mainly in tomato, potato and maize (Baxter *et al.*, 2005; Giroux and Hannah, 1994; Müller-Röber *et al.*, 1992). In potato it has been demonstrated that sucrose synthase plays a crucial role in determining sink strength, and in transformed potato tubers starch synthesis and dry matter accumulation are depressed (Zrenner *et al.*, 1995). In tomato, studies on introgression lines showed that sucrose synthase is a key enzyme for starch accumulation in developing tomato (N'Tchobo *et al.*, 1999) and how wild specie alleles can affect sugar metabolism introducing a more efficient cell wall invertase (Fridman *et al.*, 2004). Temporally extended AGPase large subunit gene expression is responsible in a tomato introgression line of the observed starch increment (Petreikov *et al.*, 2006).

It is generally accepted that sink strength is highly related to source efficiency, but with some exception. For example, a study on the tomato introgression line IL9-2-5 showed that increase soluble sugars were not related to improved photosynthetic capacity of those plant and then it was a genetic determined effect (Baxter *et al.*, 2005). Also for a *A. chinensis* breeding population available in New Zealand has already been determined that there are no gross correlations between vegetative, floral or phenological traits and starch accumulation (Clearwater *et al.*, 2007). This suggests that source derived effects are not always

contributing significantly to the differences observed, and that partitioning can be strongly controlled by genes expressed in the developing fruit.

Sensory studies on kiwifruit found that fruit containing more starch at harvest are sweeter and more desirable when ready to eat (Jaeger *et al.*, 2003). During fruit development carbon is transported from the leaves by the phloem as sucrose. In fact, sucrose is the major sugar found by Klages and co-workers (1998) in the phloem exudates, contributing up to 95% of all sugar pools. The strategy used by kiwifruit berry to unload sucrose has not been clarified yet. It is therefore known that late in development fruit flesh cells are densely populated by plasmodesmata (Sutherland *et al.*, 1999), and then the probability to have a simplasmic phloem unloading mechanism is high. Little is also known about sucrose cleavage enzymes during fruit development, whereas their activity have been studied at post-harvest ripening stage (MacRae *et al.*, 1992). Recently, two sucrose synthase isoforms have been identified in kiwifruit, a SUS1 and a SUSA. SUS1 is more expressed early in fruit development whereas SUSA increases close to harvest time (Richardson *et al.*, 2004). Kiwifruit accumulates transiently starch throughout fruit development (up to 50% of dry weigth), from about 50 DAA to maturation, when starch breakdown starts, soluble sugar concentration increase and fruit are picked (Beever and Hopkirk, 1990; Richardson *et al.*, 1997). AGPase have been little studied for kiwifruit, even if starch is the main dry matter component of kiwifruit. AGPase activity has been shown to increase up to about 100 DAA and to be then lower at harvest (Antognozzi *et al.*, 1996), but no further information are known about genes encoding for unit isoforms. This lack of knowledge hampers the ability to breed new high starch kiwifruit cultivars, develop management techniques to increase fruit starch content, and predict how the environment influences fruit starch content.

Tomato, one of the model plant which breeding is worldwide active, has been highly improved and cultivated varieties today are far away from the wild ones. Recently, breeding strategies to improve yield or fruit composition make use of natural variation, introducing wild characteristics into cultivated tomato (Eshed and Zamir, 1995). Increased starch accumulation in young tomato fruit was obtained with the *Solanum habrochaites*-derived allele for AGPase through an interspecific cross (Schaffer *et al.*, 2000). The higher yield and soluble sugar content of an introgression line from *Solanum pennelii* reflect the potential of the wild cell wall invertase *LIN5* to enhance growth and sink strength (Fridman *et al.*, 2004). Although *Actinidia* breeding history is quite recent, and the domesticated varieties are only few generation far form the wild (Ferguson, 2007), the high variability of the available germoplasm and the interest in developing new succefull varieties introducing attracting traits, are a prospect to get similar results to those found in tomato.

Final kiwifruit berry size and starch content are important traits that influence total yield and consumer preference for a particular cultivar. As previous observed, no an unique combination of anatomical traits is able to explain genotypic differences in kiwifruit berry sink strength. The goal of this research was to understand the developmental mechanisms contributing to genotypic variation in starch content, and to identify factors controlling carbon partitioning in the developing fruit.

Three critical points have been observed in several fruit crop and by several authors for sucrose to starch conversion: sucrose unloading, sucrose cleavage, starch synthesis. The first hypothesis was that changes in sink strength were primarily determined by a difference in sucrose synthase gene expression between high and low dry matter genotypes. Then, being unknown the phloem unloading pathway operating in kiwifruit berry, also other sucrose cleaving enzyme were investigated in both gene expression and activity. Differences in dry matter were evident since early in fruit development, so it was then hypothesized that this is a critical phase and possible changes could be find in this window of time. An alternative hypothesis was that the starch synthesis pathway was affected by changes in the committed step that drives carbon into starch through AGPase, whether those changes were temporally or quantitatively affecting gene expression. Literature about gene involved in carbohydrate metabolism in kiwifruit is poor, with some exception for sucrose synthase (Richardson *et al.*, 2004). So a screen of HortResearch *Actinidia* EST libraries was first of all necessary to find and classify fruit expressed target genes (sucrose synthase, invertases and AGPases). Furthermore, for a broader view of which genes affects carbon metabolism in kiwifruit, a fruit development microarray experiment was designed. It was than hypothesized that other genes not directly involved in carbon metabolism could play a regulatory role over the sucrose to starch pathway.

5.2 MATERIALS AND METHODS

5.2.1 PLANT MATERIAL

For the present study, further selection of *Actindia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* genotypes used in chapter 3 was performed, and 4 genotypes more extreme for dry matter-starch content and fruit size were selected (in both direction).

Genotype 3 (high dry matter) and genotype 25 (low dry matter) were chosen as large fruit genotypes, whereas genotype 30 (high dry matter) and genotype 17 (low dry matter) were selected as small fruit genotypes (Tab. 5-1). These were planted in 2 contiguous orchard blocks at Te Puke Research Centre (Bay of Plenty, NZ; 37^{o} 49’ S - 176^{o} 19’ E) in 1999 for breeding purposes. All vines were growing on a T-bar trellis at 0.8 m spacing on the row and 5 m spacing between rows. These are referred to 2004-05 season samples. 2006-07 season samples for the selected genotypes were from grafted vines. In 2005 scions from the 10 selected vine were grafted on 4 ‘Bruno’ rootstocks each genotype, following a complete randomized block design (Mead *et al.*, 1993) obtained using SAS® software (SAS Institute Inc, 2000). Grafted vines were growing in the same orchard block at Te Puke Research Centre spanning 4 rows on a T-bar trellis at 2 m spacing on the row and 5 m spacing between rows.

Blocks were managed according normal commercial practises, pruned using one year old cane replacement and a low intensity summer pruning. A basic ferlizer level was used.

Tab. 5-1: List of genotypes used in the present study, reporting respecting features and sampling dates of two different years of observations.

Season	Genotypes			Time (DAA)								
	Size	DM	Code									
2004-05	Large fruit	High DM	30			28		56		98		154
		Low DM	17									
	Small fruit	High DM	3									
		Low DM	25									
2006-07	Large fruit	High DM	30	7	14	28	42	56	70	98	126	154
		Low DM	17									
	Small fruit	High DM	3									
		Low DM	25									

Actinidia deliciosa 'Hayward' and *Actinidia eriantha* were also used as control material and were the same used in Cruwhurst *et al.* (2008) for the vitamin C study (sampling dates: ovary, 7, 14, 28, 42, 84 and 189 DAA) . Vines were also from Te Puke Research Centre located in block 4 and block 11 respectively.

5.2.2 SAMPLING

At each time point, wedges of fruit tissue (providing representative proportions of all tissue types, radially and longitudinally) were cut and frozen in liquid nitrogen for both carbohydrates and organic acids analysis, and gene expression.

5.2.3 NON-STRUCTURAL CARBOHYDRATE AND ORGANIC ACID ANALYSIS

Starch was determined as reported in Smith *et al.* (1992). Soluble sugars (glucose, fructose, sucrose, myo-inositol and galactose) were analyzed as per Klages *et al* (1998) and organic acids as per Cheng *et al.* (2004). Four biological replicates were analyzed (each treatment at each time point). The individual starch, sugar and acid contents were expressed as the milligrams per gram fresh fruit weight (mg g^{-1} FW).

5.2.4 qPCR GENE EXPRESSION STUDY: RNA EXTRACTION, cDNA SYNTHESIS AND REAL TIME PCR AMPLIFICATION

RNA was isolated from 2 g of fruit tissues as for Chang *et al.* (1993). Following DNase treatment, first-strand cDNA synthesis was carried out using oligo$(dT)_{20}$ according to the manufacturer's instructions (SuperScript^{TM} III Reverse Transcriptase; Invitrogen). Genes encoding sucrose cleaving enzymes and ADP-glucose pyrophosphorylase enzymes and were identified by homology in the HortResearch *Actinidia* EST database, and, where putative gene family members existed, candidates were selected when they appeared in fruit library tissues. Gene-specific primers corresponding to these genes were designed using Primer3 software (Rozen and Skaletsky, 2000) to a stringent set of criteria, enabling application of

universal reaction conditions. To check reaction specificity, RT-PCR reactions were carried out according to the manufacturer's instructions (Platinum Taq, Invitrogen). Primer used for sucrose cleaving enzyme genes and AGPase subunit genes were tested and primers sequences are reported in Tab. Appendix VI- 2.

cDNA from *A. deliciosa* 'Hayward' and *A. erientha* fruit developmental series were provided by Dr Bulley at HortResearch (Crowhurst *et al.*, 2008).

qPCR cDNA amplification and analysis was carried out using the AB 7500 Fast Real Time PCR detection system (Applied Biosystems). All reactions were performed using the AB SYBR® Green PCR Master Mix (Applied Biosystems) according to the procedure described by the manufacturer, on 96-well plates (Applied Biosystems). Reactions were performed in triplicate using 7.5 μL 2X Master Mix, 0.2 μM each primer, 5 μL 100X diluted cDNA and nuclease-free water to a final volume of 15 μL. A negative water control was included in each run. Fluorescence was measured at the end of each annealing step. Amplification was followed by a melting curve analysis with continual fluorescence data acquisition during the 60–95ºC melt. The raw data were analysed with the 7500 Fast System SDS software, version 3.1 (Applied Biosystems), and expression was normalized to *Actinidia deliciosa* Protein Phosphatase Regulatory Subunit 2A (PP2A HortResearch EST 312205) to minimize variation in cDNA template levels. PP2A was selected for normalization due to its consistent transcript levels. For each gene, a standard curve was generated using a cDNA serial dilution, and the resultant PCR efficiency calculations (ranging between 1.87 and 2.02) were imported into relative expression data analysis. Relative expression was calculated as for Ren *et al.* (2007). Error bars shown in qPCR data are technical replicates, representing the means ± SE of three replicate qPCR reactions.

5.2.5 CONSTRUCTION OF PHYLOGENETIC TREES

Multiple alignment of deducted amino acid sequences was performed using AlignX (Invitrogen, Vector NTI package), a ClustalW program (ClustalX-algorithm based program). Output file was exported as *.msf, opened in GeneDoc and was exported in PHYLIP format as *.phy. Trees were constructed using PHYLIP package (Felsenstein, 2002). Sequenses were bootstrapped, and amino acid sequence distances calculated using the JTT matrix (Jones *et al.*, 1992). Distance tree was calculated using Neighbor-joining method (Saitou and Nei, 1987). The consensus tree was computed according to the majority-rule. Results were exported to TREEVIEW program (Page, 1996) for the visualization.

5.2.6 INVERTASE ACTIVITY

Invertase enzyme activity was assessed on 2004-05 season samples, for four genotypes (2 high and 2 low dry matter, either large or small size fruit), at four different time point throughout fruit development (28, 56, 98 and 154 DAA).

Enzyme extraction and cytoplasmic, vacuolar and cell wall invertase activity assays were performed as previously reported for kiwifruit (MacRae *et al.*, 1992), with some

modification. A complete protease inhibitor cocktail was used (Roche) instead of each single protease inhibitor. Cell wall invertase activity was assessed on aliquot of a washed centrifuged pellet suspension. Glucose enzymatically determined, and activity was expressed as μmol (or nmol) of glucose per g of fresh weight per hour. Each sample was repeated in 4-6 biological replicates, whereas each replicate was the average of 2 independent *in vitro* reactions per 2 replicate glucose determinations.

5.2.7 MICROARRAYS: RNA PREPARATION, PROBE LABELLING, HYBRIDIZATION AND ANALYSIS

To establish difference of gene expression between high and low dry matter accumulating kiwifruit berries, two high and two low dry matter genotypes were chosen, and both of them were paired in fruit size. 16 microarrays were hybridised, each sample was repeated twice with a dye swap experimental design as reported in Fig. Appendix VII- 1. Considering chapter 3 results, it was established fundamental changes in fruit physiology might happen between 56 and 98 DAA, so these two sampling dates were also compared. Genotype 1 and 25 (low dry matter), and genotype 29 and 3 (high dry matter) were selected. Genotype 1 and 29 replaced genotype 30 and 17 small size fruit genotypes, because they were closer in dry matter content to the respective large size fruit genotypes (3 and 25), and they were used in the experiment as biological replicates.

Total RNA was extracted as for Chang *et al.* (1993) and purified throughout midi RNAeasy cleanup kit (Qiagen). Integrity of RNAs was checked using an Agilent 2100 Bioanalyzer (Agilent Technology) and the results are reported in Fig. Appendix VII- 2.

RNA labeling, cDNA synthesis and hybridization were held as reported in Schaffer *et al.* (2007) on *Acinidia* microarrays containing 17,472 45-55 mer oligonucleotides, representing 17,212 non redundant *Actinidia* sequences. Genomic DNA from *A. deliciosa* 'Hayward' and *A. eriantha* (genotype 11-6-15e) was labelled and each microarray hybridized as described previously (Schaffer *et al.*, 2007).

For each array, the number of spots selected for analysis including control spots were as reported in Tab. Appendix VII- 1.

Data were normalized using quantile normalization in the Bioconductor package Limma (Smyth and Speed, 2003).

The normalised data was modelled in the limma package, firstly to high and low dry matter genotypes following this it was modelled to low and high dry matter genotypes harvested at the two time points selected for dry matter accumulation (56 and 98 DAA). Genes were selected using a non-adaptive FDR (False Discovery Rate) control (Benjamini and Hochberg, 1995) with an adjusted p value of 0.01 and a greater than 2 fold change of expression.

High and low dry matter selected genes selected using the above cut off values were then ranked according to the time fold changes and the adjusted p value. High dry matter genotypes were used as reference, so gene more expressed in high dry matter genotypes

were classified as up-regulated, whereas genes more expressed in low dry matter genotypes were named down-regulated. For each EST-Oligo the putative gene name and the closest Arabidopsis gene were reported, and a classification according to biological and molecular functions created.

5.3 RESULTS

5.3.1 NON-STRUCTURAL CARBOHYDRATES AND ORGANIC ACIDS

Non-structural carbohydrates profile 4 selected genotypes behaved as previously reported in chapter 3 among dry matter classes. The most evident difference was starch content. At-harvest starch content was double in high dry matter genotypes for both fruit size classes (Fig. 5-1A and Fig. 5-2A). The sucrose peak was observed to be higher in high dry matter genotypes (Fig. 5-1E and Fig. 5-2E).

Organic acid concentration evolution throughout fruit development was similar to those observed for dry matter classes (Fig. 5-1G, H, I and Fig. 5-2 G, H, I).

5.3.2 PHYLOGENETIC RELATIONSHIP AMONG THE GENES FOR SUCROSE CLEAVAGE AND AGPASE SUBUNITS IN *ACTINIDA* AND OTHER SPECIES

BLAST searches for sucrose synthase genes using HortReaserch *Actinidia* Sequence Database revealed 90 ESTs with homology to *Arabidopsis* SUS4 (At3g43190), 12 ESTs with homology to Arabidopsis SUS1 (At5g20830), 136 ESTs with homology to *Arabidopsis* SUS3 (At4g02280) and 5 ESTs with homology to *Arabidopsis* SUS6 (At1g73370) of >138,000 ESTs (expected value <1E-40). These came from a range of libraries comprising different tissues: fruit, leaves, petal, meristems, and buds. Selected ESTs are reported in Tab. Appendix VI- 1 with relative identity (*Arabidopsis* tblastn *Actinidia*).

The dendrogram reported in Fig. 5-3 shows that sucrose synthase gene family comprises 3 main groups: AtSUS1-AtSUS4 group, AtSUS3-AtSUS2 group, and AtSUS5-AtSUS6 group. Predicted amino acid sequences from selected ESTs fell in the appropriate group. *Actinidia* SUS1 (AdSUS1) and *Actinidia* SUS2 (AdSUS2) are still in the same branch of *Arabidopsis* genes, but they are closer to tomato, potato and carrot genes.

BLAST searches for acid invertase genes using HortReaserch *Actinidia* Sequence Database revealed 26 ESTs with homology to *Arabidopsis* At-FRUCT3 (At1g62660), 13 ESTs with homology to Arabidopsis At-FRUCT4 (At1g12240) and 9 ESTs with homology to Arabidopsis At-FRUCT1 (At3g13790) of >138,000 ESTs (expected value <1E-40). These came from a range of libraries comprising different tissues: fruit, leaves, petal, meristems, and buds. Selected ESTs are reported in Tab. Appendix VI- 1, with relative identity (*Arabidopsis* tblastn *Actinidia*).

The dendrogram reported in Fig. 5-4 shows that acid invertase gene subfamily comprises 2 main distinct groups: soluble acid invertases and insoluble acid invertases. Predicted amino

acid sequences from selected ESTs fell in the respective group. Insoluble inverteses can be additionally subdivide into alpha and beta subgroup. Kiwifruit insoluble invertase belongs to the beta-group. No ESTs were detected for alpha-group insoluble invertases. AdVINV4 (*Actinidia* vacuolar invertase 4) is close to tomato and potato, whereas AdVINV3 (*Actinidia* vacuolar invertase 3) is similar to the grape one.

BLAST searches for cytoplasmic invertase genes using HortReaserch *Actinidia* Sequence Database revealed 7 ESTs with homology to *Arabidopsis* At-A/N-INVD (At1g22650), 9 ESTs with homology to *Arabidopsis* At-A/N-INVE (At5g22510), 18 ESTs with homology to *Arabidopsis* At-A/N-INVI (At4g09510), and 2 ESTs with homology to *Arabidopsis* At-A/N-INVI (At4g34860)of >138,000 ESTs (expected value <1E-40). These came from a range of libraries comprising different tissues: fruit, leaves, petal, meristems, and buds. Selected ESTs are reported in Tab. Appendix VI- 1, with relative identity (*Arabidopsis* tblastn *Actinidia*).

The dendrogram reported in Fig. 5-5 shows that cyplasmic invertase gene subfamily comprises 2 main distinct groups: alpha-group and beta-group. Predicted amino acid sequences from selected ESTs fell in the respective group. All the kiwifruit predicted amino acid sequences fell close to the respective *Arabidopsis* one, but the isoform D. Isoform D is between two rice genes.

BLAST searches for AGPase genes using HortReaserch *Actinidia* Sequence Database revealed 2 ESTs with homology to *Arabidopsis* At-AGPL1 (At5g19220), 18 ESTs with homology to *Arabidopsis* At-AGPL2 (At1g27680), 2 ESTs with homology to Arabidopsis At-AGPL4 (At2g21590), and 1 ESTs with homology to *Arabidopsis* At-AGPS1 (At5g48300)of >138,000 ESTs (expected value <1E-40). These came from a range of libraries comprising different tissues: fruit, leaves, petal, meristems, and buds. Selected ESTs are reported in Tab. Appendix VI- 1, with relative identity (*Arabidopsis* tblastn *Actinidia*).

The dendrogram reported in Fig. 5-6 shows that AGPase gene encoding for the 2 different subunits clusters into 3 groups: small subunit group, large subunit 1 group and large subunit 2-3-4 group. Mays genes clearly diverges from dicots genes, in all clusters, whereas *Actinidia* genes fall between *Arabidopsis* and potato ones.

5.3.3 SUCROSE CLEAVAGE ENZYMES GENE EXPRESSION

Gene expression of sucrose cleaving enzymes was firstly tested on *Actinidia deliciosa* 'Hayward' fruit at different developmental stages (Fig. 5-7). SUS1 was mainly expressed early in fruit development. A peak in expression was observed at about 50 DAA. SUS2 was expressed early in development but at a low level (about 1 order of magnitude less). SUS2's EST comes from an *Actinidia eriantha* library, so the primer was tested on a similar *Actinidia eriantha* fruit development series (and also SUS1). Results are clearly showing that SUS2 was expressed in *A. eriantha* at a similar level of SUS1 in *A. deliciosa*, and that SUS2 was the main isoform early in development. SUS1 was also expressed in *A. eriantha* and both isoforms showed a peak at about 30 DAA. SUSA was expressed at low level early in development and showed a peak at harvest time.

Beta-group cytoplasmic invertases showed an higher expression level compared with the alpha-group one (invertase E). Expression levels were quite stable all over fruit development, but AdInvK was high soon after anthesis.

Soluble invertase isoforms are differentially expressed. The overall expression level is low compared to sucrose synthases. Transcripts were more abundant for AdVINV3 compared to the isoform 4.

Transcript levels for some of the genes previously described were assessed on high and low dry matter genotypes fruit developmental series. SUS1 and SUS2 were chosen because of and early expression, InvD was chosen out of the cytoplasmic genes (considering that all of them had similar expression levels) and also both vacuolar invertases. In Fig. 5-9 a summary of the 5 genes profile in high and low dry matter genotypes, over 2 different seasons and for both small and large size genotypes, is reported.

AdSUS1 was more expressed in high dry matter genotypes early in development (28 and 56 DAA): from 2 to 3 time according to the genotype (Fig. 5-9A, B, C, D). Data are also consistent from season to season. AdSUS2 transcript levels were higher in high dry matter large size genotype 3 (more than 3 times) (Fig. 5-9E, F, G, H).

Cytoplasmic invertase transcripts did not show any consistent difference among different genotypes (Fig. 5-9I, J,. K, L).

Vacuolar invertase expression levels were less constant and not completely consistent throughout the seasons. Large and small size genotypes behaved differently. High dry matter large size genotype showed a general higher level for both isoforms compared to low dry matter large size genotype whereas small size genotypes behaved *vice-versa*.

5.3.4 AGPase Subunits Gene Expression

Gene expression of AGPase enzyme subunits was firstly tested on *Actinidia deliciosa* 'Hayward' fruit at different developmental stages (Fig. 5-8). Both AdAGPL4 and AdAGPS1 transcripts were increasing up to 80 DAA. Small subunit transcripts showed a lower expression level.

Transcript levels for the same gene and for an additional large subunit (AdAGPL2) were assessed for Actinidia deliciosa high and low dry matter genotypes and expression levels are reported in Fig. 5-10. AdAGPL4 was more expressed in high dry matter genotypes, and transcript levels were up to 3 times higher at about 100 DAA. AdAGPL2 transcript levels were one order of magnitude inferior than large subunit 4, and resulted to be differentially expressed late in development high dry matter small size genotype (expression level lower than all other genotypes). Transcripts for the gene encoding for the small subunit were more stable and resulted to be more expressed in high dry matter small size genotype (up to 2.5 time in 2004-05 season), but differences were less evident in the other season.

5.3.5 Early development Sucrose Cleavage Enzymes and AGPase Subunits Gene Expression

AdSUS1 expression levels were higher in high dry matter genotypes since early in development (Fig. 5-11). AdSUS2 transcript resulted to be dramatically higher in high dry matter genotype 3 up to 56 DAA, where a clear peak was showed.

AdAGPL4 was generally higher in high dry matter genotypes also early in fruit development, whereas the other isoform did not show evident differences. The gene encoding for the AGPase small subunit was generally higher early in fruit development in high dry matter small fruit genotype.

5.3.6 INVERTASES ENZYME ACTIVITY

Invertase enzyme activity results are shown in Fig. 5-12. Soluble invertase activity, both cytoplasmic and vacuolar isoforms, were more active that the insoluble one (one order of magnitude). Both soluble invertases had a significant higher activity in low dry matter genotypes early in fruit development (28 DAA). Activity was generally increasing in both cytoplasmic and vacuolar invertases, to reach a stable level at about 100 DAA. Cell wall invertase activity was generally higher early in fruit development, decreasing then to close to zero levels. High dry matter small size fruit genotype showed a peak of cell wall invertase activity at 56 DAA, and was significantly different from the low dry matter one.

5.3.7 MICROARRAY EXPERIMENT

A verification of the microarray technique is shown in Fig. Appendix VII- 3. The scattered diagrams of the signal strength of each spot (indicating each gene) in the "dye-swap" experiment" for each sample is shown. Spots were linearly distributed. It was then concluded that the microarrys technique could be used for gene expression analysis.

The genes for which the signal intensity changed by more than 2-fold compared with high dry matter genotypes ($p<0.01$) were considered to be differentially expressed. Only few genes were found to be changed.

From the comparison of high and low dry matter genotypes (at both 56 DAA and 98 DAA), a total of 52 genes exhibited a differential expression. 17 genes resulted to be up-regulated and 35 down-regulated in high dry matter. First 30 changed genes were then selected: 17 resulted to be up-regulated and 13 down-regulated in high dry matter genotypes. The list of up-regulated and down-regulated is reported in Tab. 5-2 and Tab. 5-3 respectively.

The most interesting genes found to be up-regulated are two transcription factors, which gene expression changed by 3- and 2.5-folds (TCP family transcription factor and CCR4-NOT transcription factor). The carbohydrate metabolic enzyme mannose-6-P isomerase (MPI) was 3.6 time more expressed. Other changes were found in calcium binding and electron transport oligos. Nine of the up-regulated genes resulted to be unknown.

The most interesting gene found to be down-regulated by about 3-folds in high dry matter genotypes is an annexin, responsible of calcium binding and response to osmotic stress and ABA. Then a protein-zinc ion binding gene involved in multicellular organism development

was found to be 3-folds less expressed in high dry matter genotypes. A transcription factor was 2.4-folds down-regulated. Several oligos refer to unknown genes.

The comparison of high and low dry matter genotypes at 56 DAA showed 168 genes differentially expressed, equally distributed into up- and down-regulated. When high and low dry matter genotypes were compared at 98 DAA, less variability was shown, and only 24 genes resulted to be changed more than 2-folds (15 up- and 9 down-regulated in high dry matter genotypes) with an higher probability ($p<0.05$). So, only 56 DAA changes were deeply investigated.

A full list of all the genes changed at 56 DAA is reported in Tab. Appendix VII- 2 (up-regulated in high dry matter genotypes) and Tab. Appendix VII- 3 (down-regulated in high dry matter genotypes). Genes were classified according to the general function class into gene expressed in cell compartments and components (chloroplast, cell wall, etc…), stress related genes, kinases, gene involved in primary metabolism, transcription factors, translational or post-transcriptional regulators, transporters, binding proteins, secondary metabolism, energy transport and DNA silencing genes. Most interesting up-regulated genes were transcription, translation and post-transcriptional factors and those involved in energy transport and encoding for protein with kinase activity. Most interesting down-regulated genes in high dry matter genotypes were again transcription, translation and post-transcriptional factors and those involved in cell wall biosynthesis. Several kinase activity protein genes were also down-regulated.

First 30 changed genes were then selected: 9 resulted to be up-regulated and 21 down-regulated in high dry matter genotypes. The list of up-regulated and down-regulated is reported in Tab. 5-4 and Tab. 5-5 respectively. The most interesting up-regulated gene is an unknown gene, that changed by 8-folds. Then the TCP transcription factor was 4.3-folds up-regulated. The MPI gene was found to be 5-folds changed. A gibberellin stimulus response gene, structural constituent of the cell wall was found to be 4-folds less expressed in high dry matter genotypes. Clones for a cellulose synthase subunit was 3.7-folds down-regulated. Other gene changed comprised transcription factors, calcium binding proteins and a kinase. A relevant number of oligos referred to unknown genes.

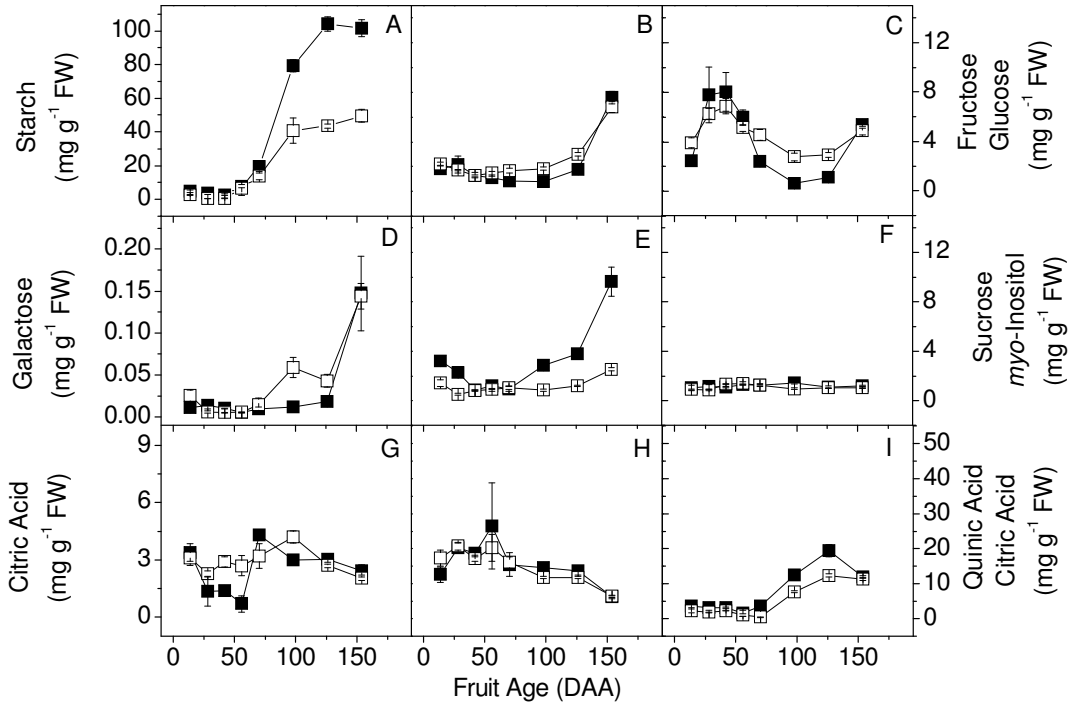


Fig. 5-1: Changes in carbohydrate and organic acid concentrations during fruit growth of two small sized fruit *Actinidia deliciosa* genotypes in 2004-05 season. Starch (A), glucose (B), fructose (C), sucrose (D), myo-inositol (E) and galactose (F), malic acid (G), quinic acid (H) and citric acid (I) concentrations are here reported. n = 4 biological replicates each time point. Values are average ±SE of the mean. Small fruit high dry matter genotype 30, (■); small fruit low dry matter genotype 17, (□).

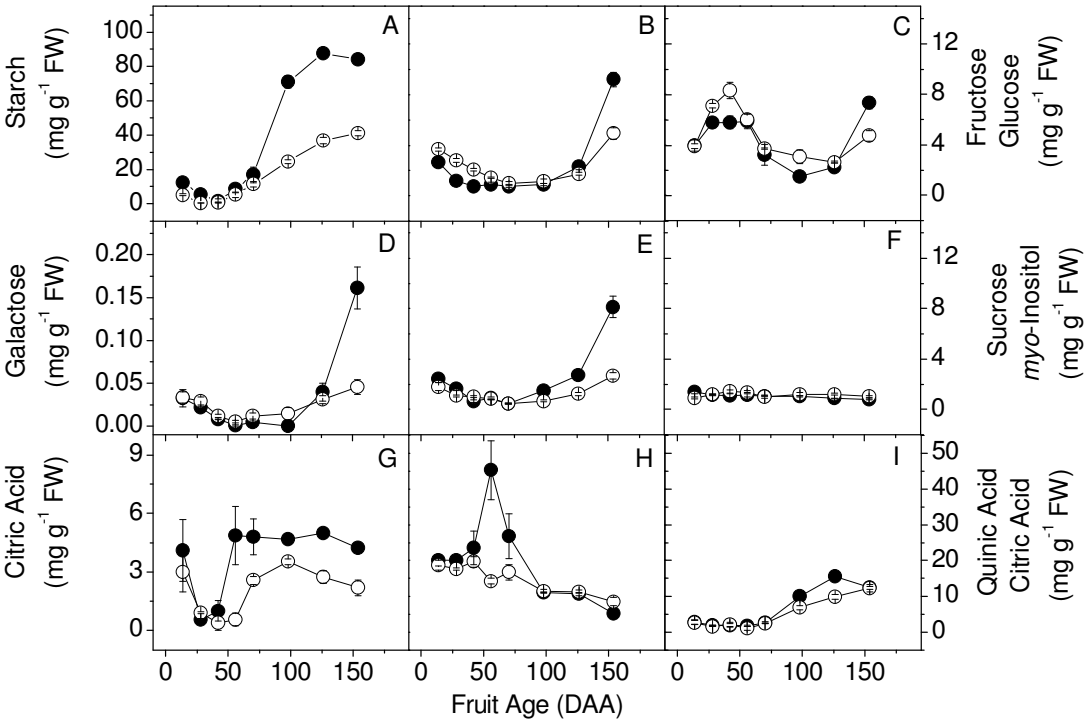


Fig. 5-2 Changes in carbohydrate and organic acid concentrations during fruit growth of two large sized fruit *Actinidia deliciosa* genotypes in 2004-05 season. Starch (A), glucose (B), fructose (C), sucrose (D), myo-inositol (E) and galactose (F), malic acid (G), quinic acid (H) and citric acid (I) concentrations are here reported. n = 4 biological replicates each time point. Values are average ±SE of the mean. Large fruit high dry matter genotype 3, (●); large fruit low dry matter genotype 25, (○).

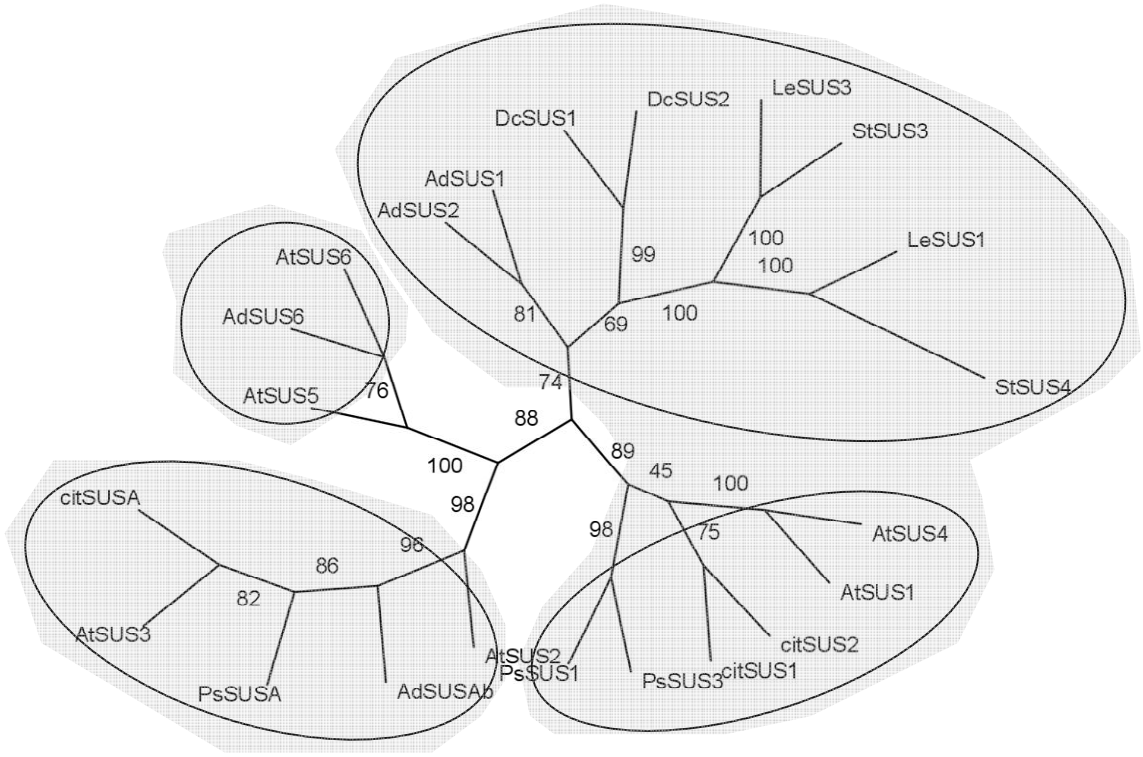


Fig. 5-3: Unrooted phylogenetic tree of plant SUSY. The tree was constructed with *Actinidia spp.* translated EST sequences for SUSYs (AdSUS1, AdSUS2, AdSUSA, AdSUS6) and other SUSY sequences from NCBI dababase using AlignX for the alignment and PHYLIP program (Felsenstein, 2002) to create the tree. NCBI database accession numbers of the displayed SUSYs are as follows: *Arabidopsis thaliana* (At SUS1 – X70990; At SUS2 – Q00917; At SUS3 – AL161494; At SUS4 – AL353871; At SUS5 – BAB11375; At SUS6 – AAG30975), *Solanum tuberosum* (St SUS4 – U24087; St SUS3 – U24088), *Pisum sativum* (Ps SUS1 – AJ012080; Ps SUS3 – AJ311496; Ps SUSA – AJ001071), *Lycopersicon esculentum* (Le SUS1 – L19762; Le SUS3 – AJ011319), *Daucus carota* (Dc SUS1 – X75332; Dc SUS2 – Y16091), *Citrus unshiu* (citSUS1 – AB022092; citSUS2 – AB021745; citSUSA – AB022091) (At, *Arabidopsis thaliana*; Ad, *Actinidia spp.*; Ps, *Pisum sativum*; St, *Solanum tuberosum*; Le, *Lycopersicon esculentum*; cit, *Citrus unshiu*; Dc, *Daucus carota*). Literature information's from Barratt *et al.* (2001) Komatsu *et al.* (2002) and Richardson *et al.* (2004).

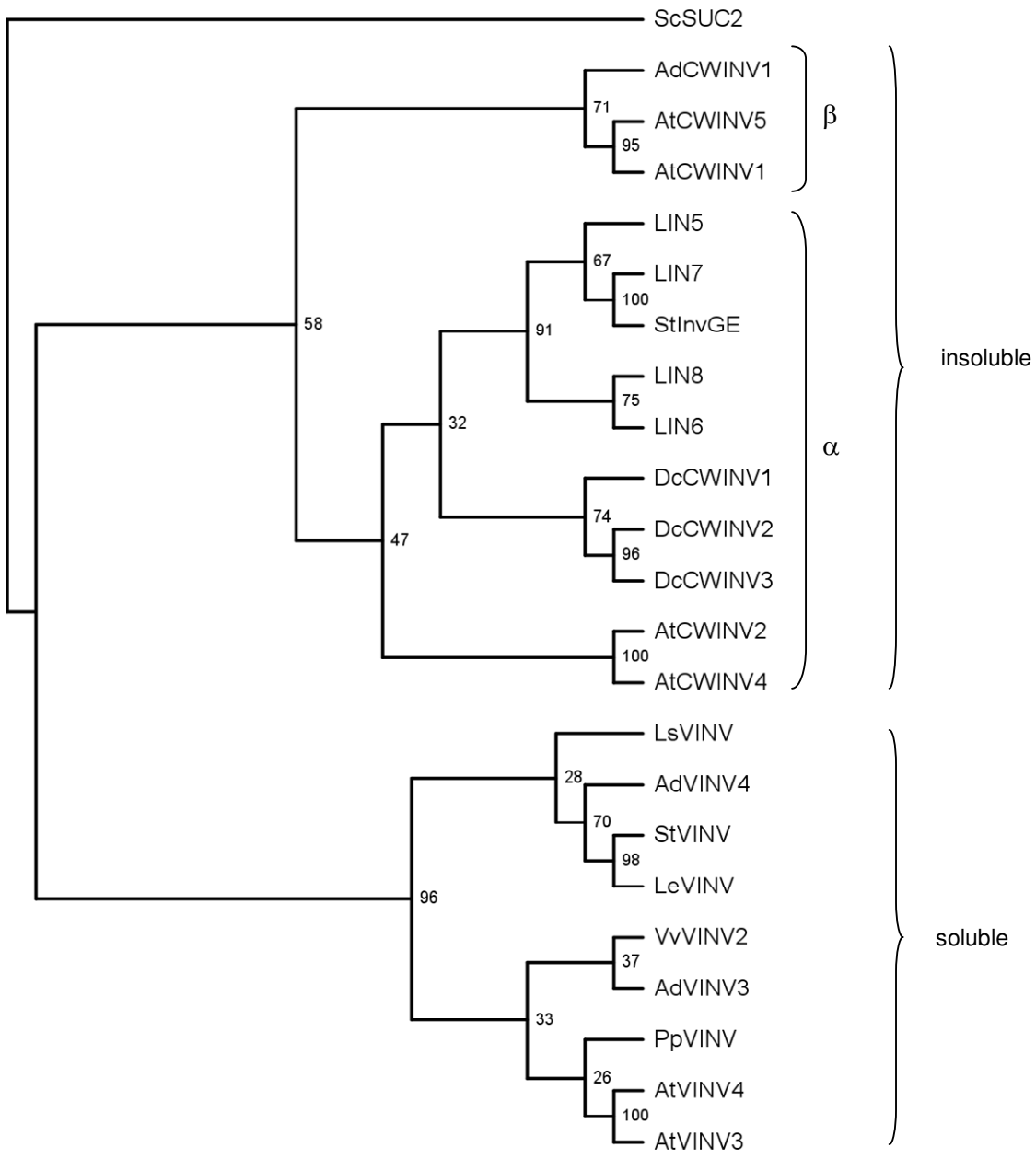


Fig. 5-4: Phylogenetic tree of plant acid invertases showing the two groups of alfa and beta cell wall invertase proteins identified by Ji *et al.* (2005). The yest cell wall invertse SUC2 of *Saccharomyces cerevisiae* (NP_012104) was used as outliner in agreement with Ji *et al.* (2005). The tree was constructed with *Actinidia spp.* translated EST sequences for acid invertases (AdCWINV1, AdVINV3, AdVINV4) and other acid invertase sequences from NCBI dababase using AlignX for the alignment and PHYLIP program (Felsenstein, 2002) to create the tree. NCBI database accession numbers of the displayed acid invertases are as follows: *Arabidopsis thaliana* (AtCWINV1 – CAA52619; AtCWINV2 – ABE66012; AtCWINV4 – BAB83031; AtCWINV5 – AB01929; AtVAC4– AAN13204; AtVAC3 – AAL32559), *Solanum tuberosum* (StInvGE – AJ133765; StVINV – CAA49831),), *Lycopersicon esculentum* (LIN5 – AJ272304; LIN7 – AF506006; LIN6 – AF506005; LIN8 – AF506007; LeVINV – P2900), *Daucus carota* (DcCWINV1 – P26792; DcCWINV2 – Q39692; DcCCWINV3 – Q39693), *Lagenaria siceraria* (LsVINV – AF519809), *Pyrus pyrifolia* (PpVINV – BAF35859), *Vitis vinifera* (VvVINV2 – AAB47172). (Sc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana*; Ad, *Actinidia spp.*; St, *Solanum tuberosum*; Le, *Lycopersicon esculentum*; Dc: *Daucus carota*; Ls, *Lagenaria siceraria*; Pp, *Pyrus pyrifolia*; Vv, *Vitis vinifera*). Literature informations from Fridman and Zamir (2003), Ji *et al*. (2005), Li *et al*. (2004), Maddison *et al.* (1999), Tymowska-Lalanne and Kreis (1998).

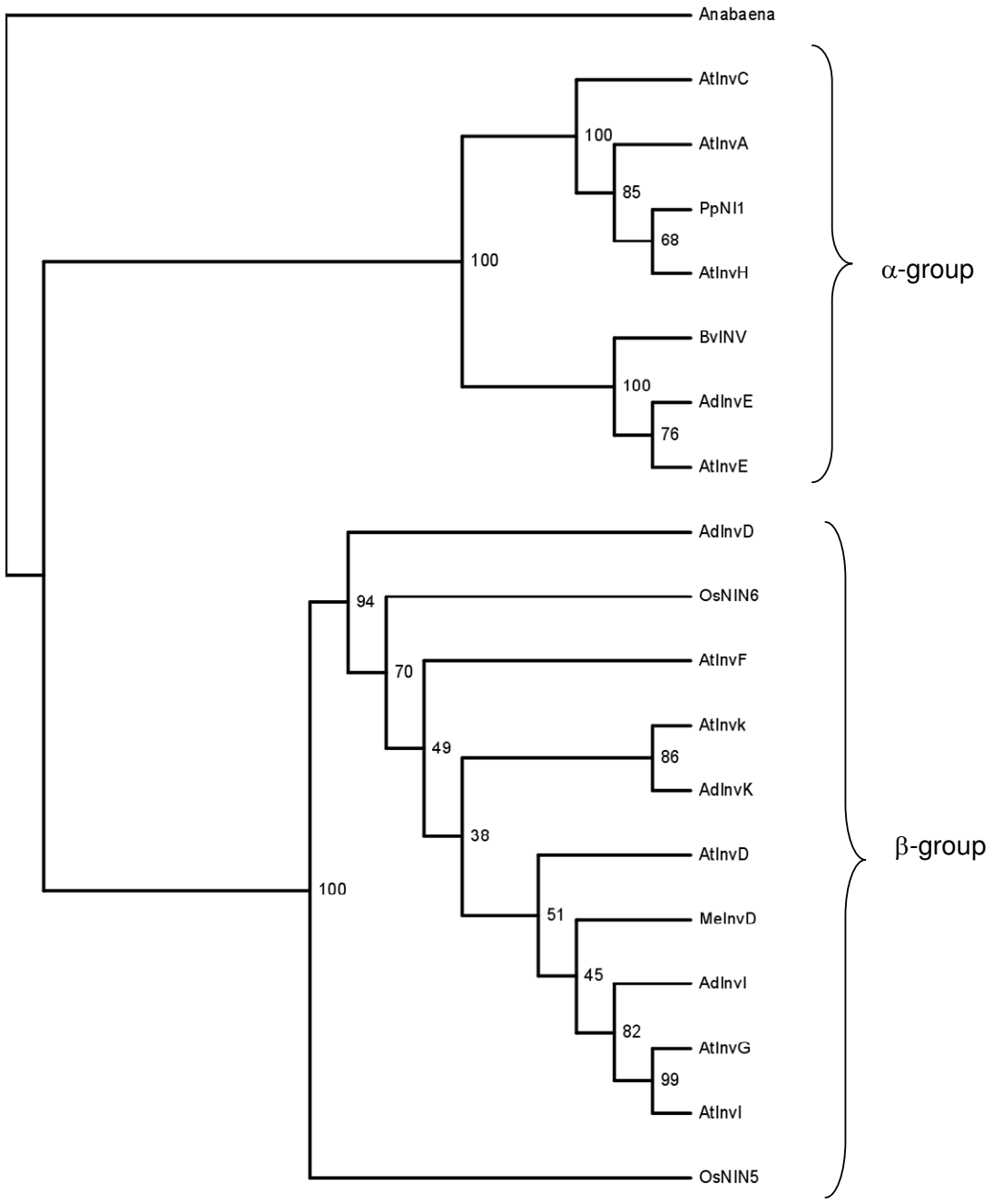


Fig. 5-5: Phylogenetic tree of plant cytoplasmic invertases showing the two groups of alfa and beta invertase proteins identified by Ji *et al.* (2005). The alkaline invertase of the cyanobacterium *Anabaena sp.* (AJ491788) was used as outliner in agreement with Ji *et al.* (2005) and Nonis *et al.* (2007). The tree was constructed with *Actinidia spp.* translated EST sequences for cytoplasmic invertases (AdInvD, AdInvE, AdInvI, AdInvK) and other cytoplasmic invertase sequences from NCBI dababase using AlignX for the alignment and PHYLIP program (Felsenstein, 2002) to create the tree. NCBI database accession numbers of the displayed cytoplasmic invertases are as follows: *Arabidopsis thaliana* (AtInvA – AY120777; AtInvC - AC020580; AtInvD – AY088388; AtInvE – BAB09123; AtInvF– AC069273; AtInvG – AY065247; AtInvH – AAF26084; AtInvI – NM_117019; AtInvK – CAB80203), *Manihot esculenta* (MeInvD – ABA08442), *Prunus persica* (PpNI1 – AM409095), *Oryza sativa* (OsNIN5 – AP005311; OsNIN6 – AC120539), *Beta vulgaris* (BvINV – CAD19320). (At, *Arabidopsis thaliana*; Ad, *Actinidia spp.*; Me, *Manihot esculenta*; Bv, *Beta vulgaris*; Pp: *Prunus persica*; Os, *Oryza sativa*). Literature information's from Vargas *et al.* (2003), Ji *et al.* (2005) and Nonis *et al.* (2007).

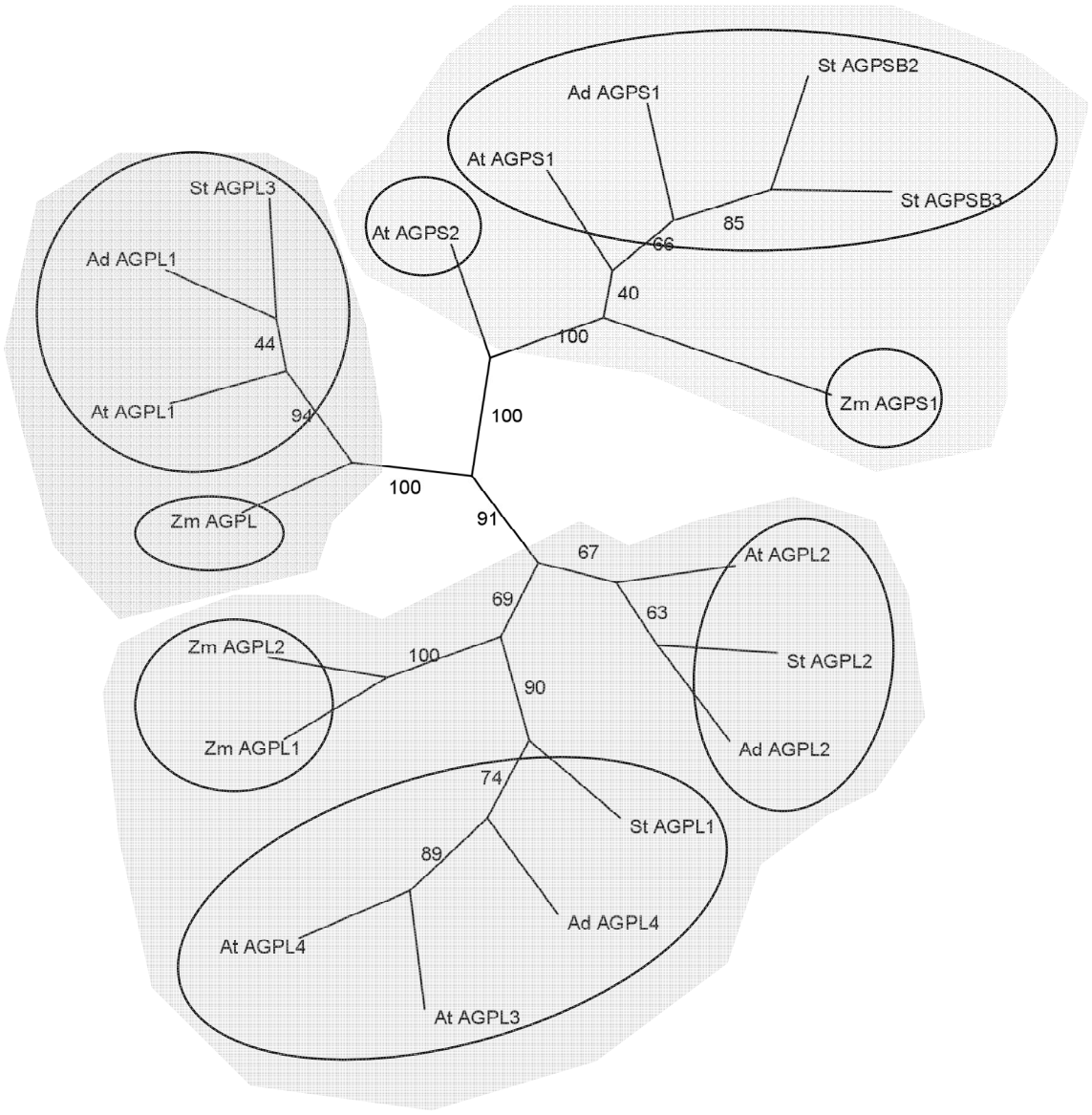


Fig. 5-6: Unrooted phylogenetic tree of plant ADP-glucose pyrophosphorylases. The tree was constructed with *Actinidia deliciosa* and *Actinidia chinensis* translated EST sequences for AGPases (Ad AGPL1, Ad AGPL2, Ad AGPL4, Ad AGPS1) and other AGPase sequences from NCBI dababase using AlignX for the alignment and PHYLIP program (Felsenstein, 2002) to create the tree. NCBI database accession numbers of the displayed AGPases are as follows: *Arabidopsis thaliana* (At AGPL1 – P55229; At AGPL2 – NP174089; At AGPL3 – NP195632; At AGPL4 – NP001031391; At AGPS1 – NP199641; At AGPS2 – NP172052), *Solanum tuberosum* (St AGPL1 –; St AGPL2 – P55242; St AGPL3 – NP55243; St AGPSB2 – ABB99399; St AGPSB3 – AAO23573), *Zea mays* (Zm AGPL1 – P55241; Zm AGPL2 – P55234; Zm AGPLP – ABD66656; Zm AGPS1 – P55240) (At, *Arabidopsis thaliana*; Ad, *Actinidia spp*.; Zm, *Zea mays*; St, *Solanum tuberosum*). Literature information's from Nakata *et al.* (1991) and Ohdan *et al.* (2005).

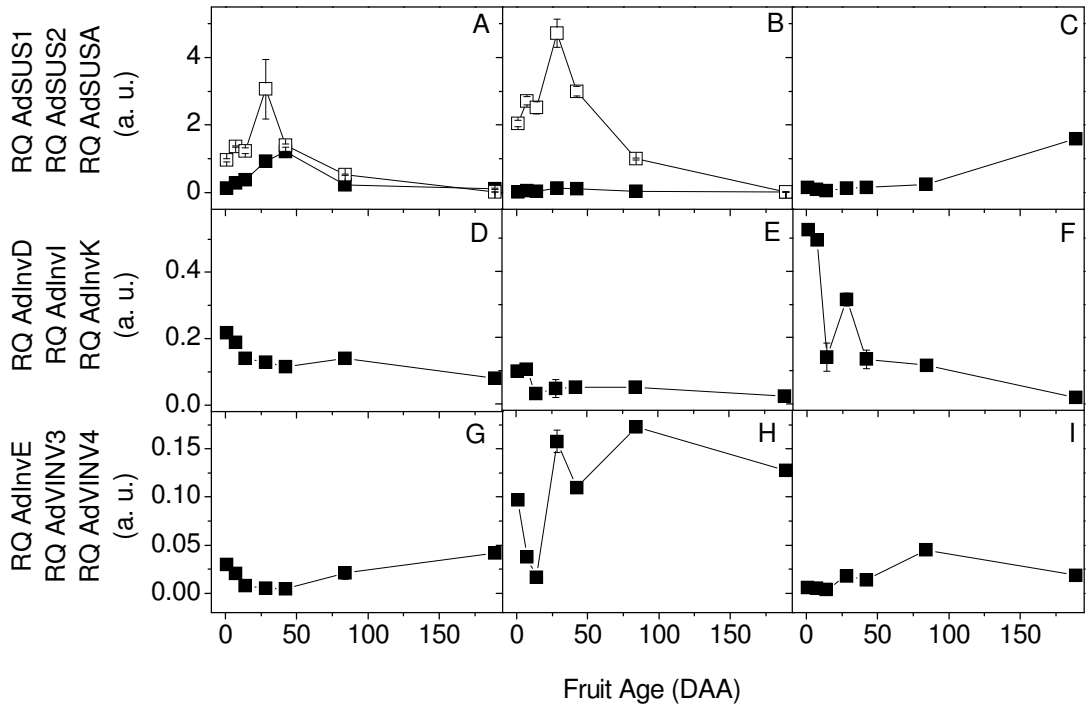


Fig. 5-7: Relative expression levels of sucrose cleaving enzyme transcripts in 'Hayward' *Actinidia deliciosa* (■) during fruit development. Expression levels for Sucrose synthase 1 (AdSUS1 - A), sucrose synthase 2 (AdSUS2 - B), sucrose synthase A (AdSUSA - C), cytoplasmic invertase D (AdInvD - D), cytoplasmic invertase I (AdInvI - E), cytoplasmic invertse K (AdInvK - F), cytoplasmic invertase E (AdInvE - G), vacuolar invertase 3 (AdVINV3 - H) and vacuolar invertase 4 (AdVINV4 - I) were analyzed. A different expression level was observed for *Actinidia eriantha* (□) for AdSUS1 and AdSUS2. Values are averages of 3 replicates ± SE of the mean.

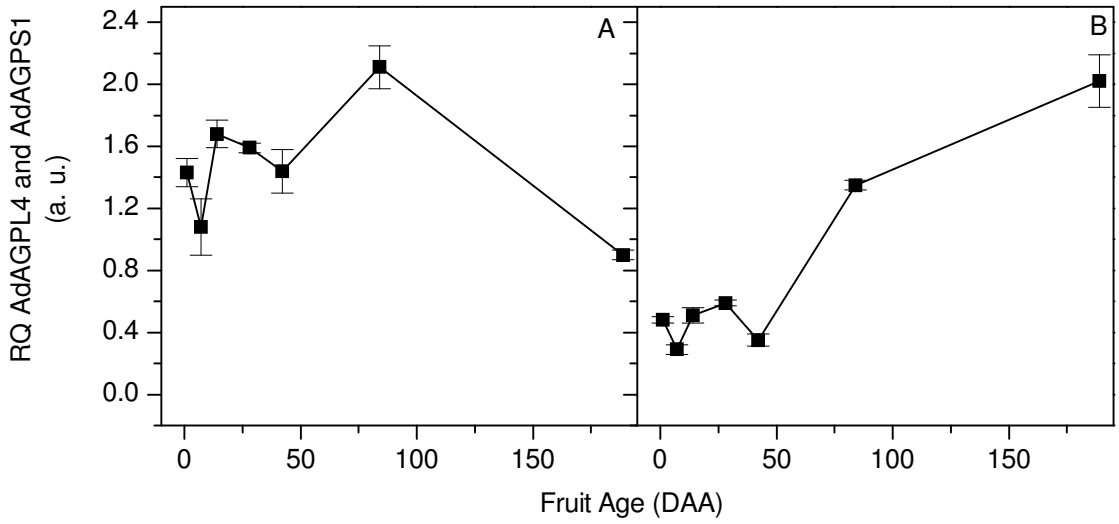


Fig. 5-8: Relative expression levels of ADP-glucose pyrophosphorylase (AGPase) enzyme transcripts in 'Hayward' *Actinidia deliciosa* during fruit development. Expression levels for AGPase large unit 4 (AdAGPL4 - A) and AGPase small unit 1 (AdAGPS1 - B) were analyzed. Values are averages of 3 replicates ± SE of the mean.

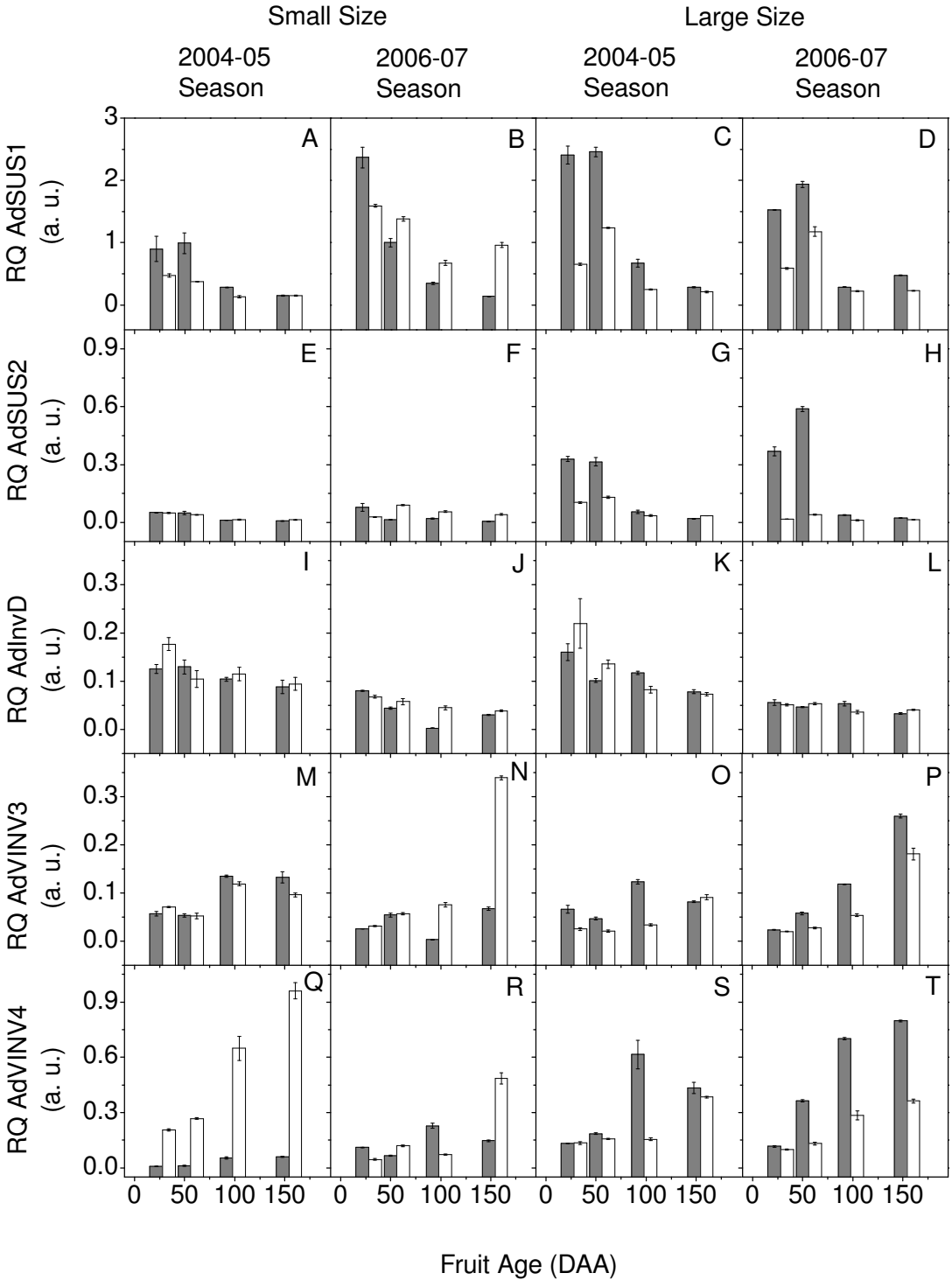


Fig. 5-9: Relative expression levels of sucrose cleaving enzyme transcripts in 4 *Actinidia deliciosa* genotypes during fruit development. Expression levels for Sucrose synthase 1 (AdSUS1 – A, B, C, D), sucrose synthase 2 (AdSUS2 – E, F, G, H), cytoplasmic invertase D (AdInvD – I, J, K, L), vacuolar invertase 3 (AdVINV3 – M,N, O, P) and vacuolar invertase 4 (AdVINV4 - Q, R, S, T) were analyzed. 2004-05 and 2006-07 season's data are here reported at 4 time points: 28, 56, 98 and 154 DAA. Small fruit: high dry matter genotype 30 and low dry matter genotype 17. Large fruit: high dry matter genotype 3 and low dry matter genotype 25. High dry matter genotypes (grey bars), low dry matter genotypes (white bars). Values are averages of 3 replicates ± SE of the mean.

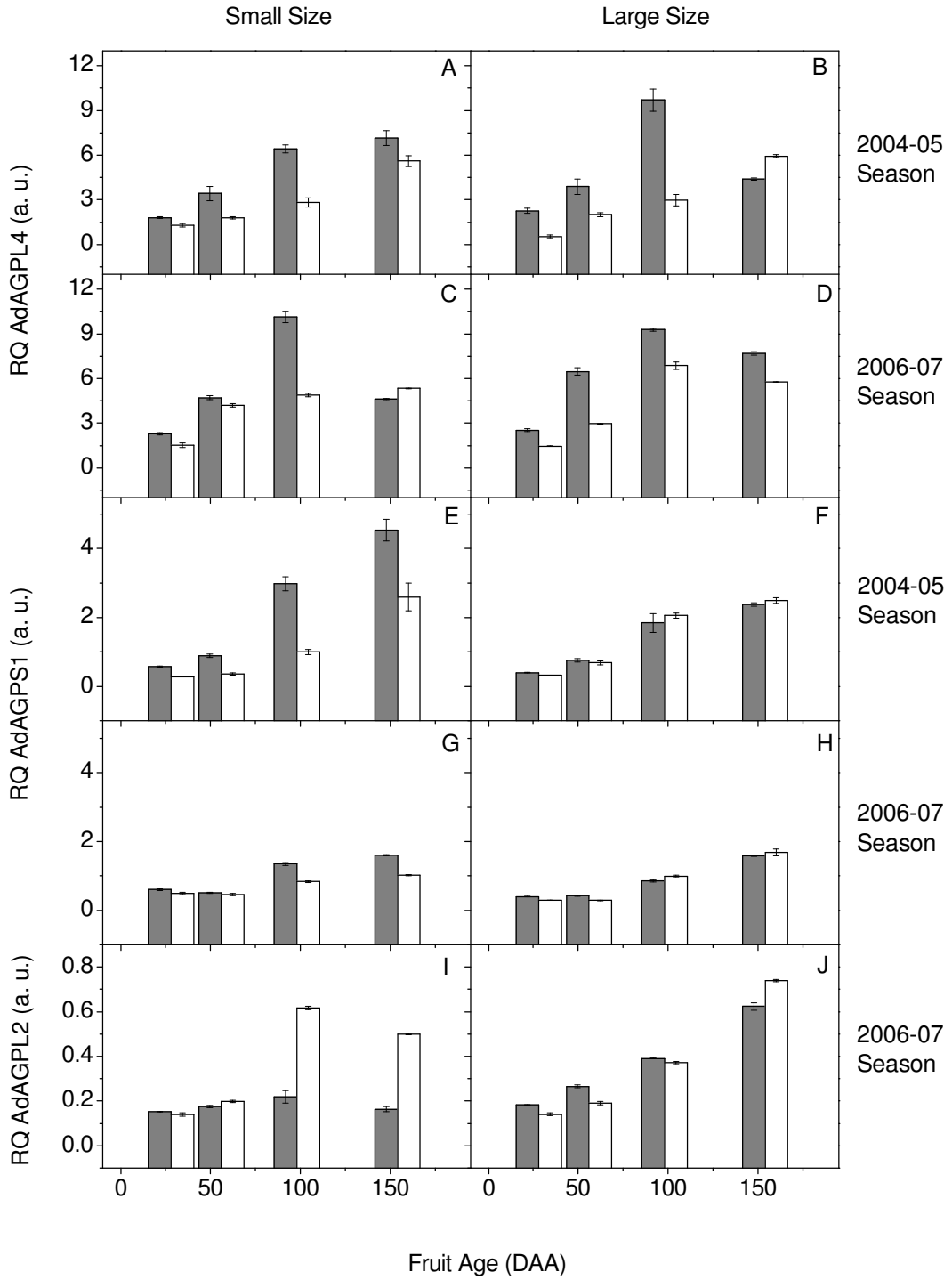


Fig. 5-10: Relative expression levels of AGPase enzyme transcripts in 4 *Actinidia deliciosa* genotypes during fruit development. Expression levels for AGPL4 (Ad AGPL4 – A, B, C, D), AGPS1 (AdAGPS1 – E, F, G, H) and AGPL2 (AdAGPL2 – I, J) were analyzed. 2004-05 and 2006-07 season's data are here reported at 4 time points: 28, 56, 98 and 154 DAA (AdAGPL2 data for 2006-07 season only). Small fruit: high dry matter genotype 30 and low dry matter genotype 17. Large fruit: high dry matter genotype 3 and low dry matter genotype 25. High dry matter genotypes (grey bars), low dry matter genotypes (white bars). Values are averages of 3 replicates ± SE of the mean.

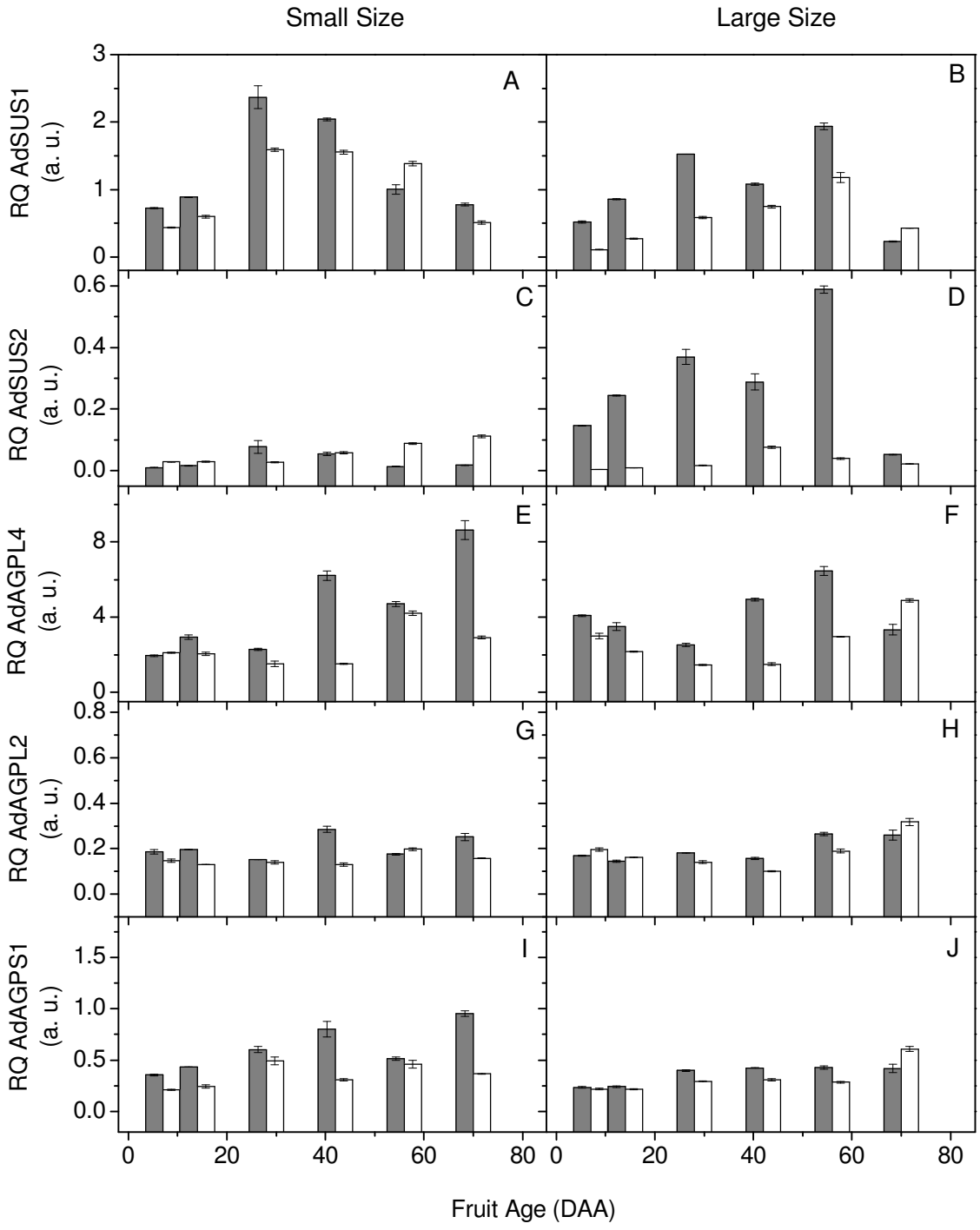


Fig. 5-11: Relative expression levels of 2 SUSY and AGPase enzyme transcripts in 4 *Actinidia deliciosa* genotypes during early stages in fruit development. Expression levels for Sucrose synthase 1 (AdSUS1 – A, B), sucrose synthase 2 (AdSUS2 – C, D), AGPL4 (Ad AGPL4 – E, F), AGPL2 (AdAGPL2 – G, H) and AGPS1 (AdAGPS1 – I, J) were analyzed. 2006-07 season's data are here reported at 6 time points: 7, 14, 28, 42, 56 and 70 DAA. Small fruit: high dry matter genotype 30 and low dry matter genotype 17. Large fruit: high dry matter genotype 3 and low dry matter genotype 25. High dry matter genotypes (grey bars), low dry matter genotypes (white bars). Values are averages of 3 replicates ± SE of the mean.

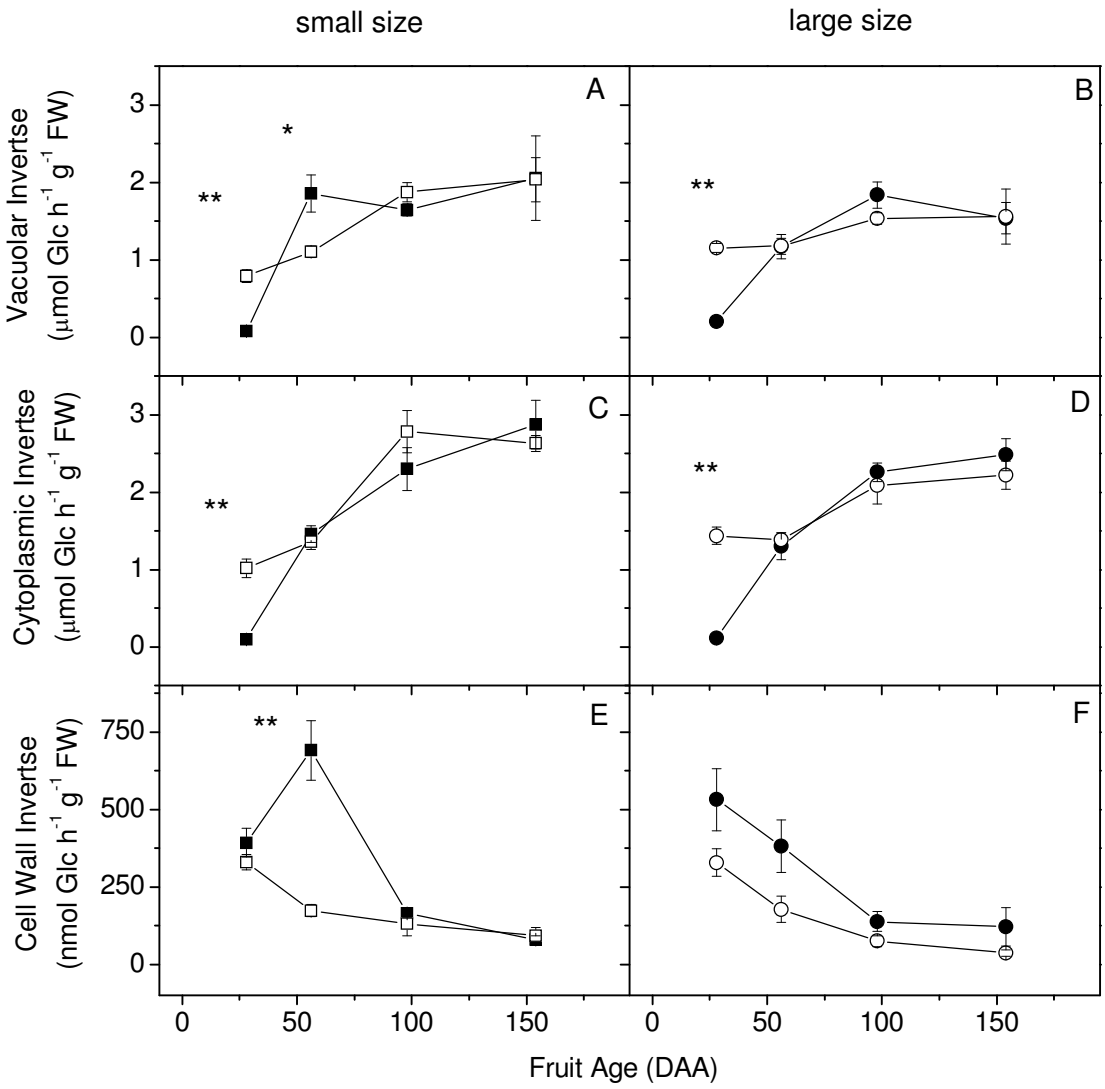


Fig. 5-12: Vacuolar invertase activity at 4 time points of fruit growth in 2004-05 season Small (A) and large (B) sized genotypes sub-group were tested Cytoplasmic invertase activity at 4 time points of fruit growth in 2004-05 season. Small (C) and large (D) sized genotypes sub-group were tested. Both group had an Hi- and a Lo-DM genotype. n = 4 (or 6 when significance occurred). t-test: *, p<0.05; **, p<0.01; ***, p,0.001; blank, not significant. Cell wall invertase activity at 4 time points of fruit growth in 2004-05 season. Small (E) and large (F) sizedgenotypes sub-group were tested. Both group had an Hi- and a Lo-DM genotype. n = 4 (or 6 when significance occurred). t-test: *, p<0.05; **, p<0.01; ***, p,0.001; blank, not significant.

Tab. 5-2: List of up-regulated genes in high dry matter genotypes at both 56 and 98 DAA (17 out of first 30 genes changed). FC, folds change; nd, not determined.

Oligo-EST (*Arbidopsis* gene)	FC	Description	Biological function	Molecular function
240637 (nd)	5.3	not determined by homology	unknown	unknown
194921 (At5g46850)	3.6	Cluster: Arabidopsis thaliana genomic DNA, chromosome 5, P1 clone:MSD23	unknown	unknown
310530 (At3g02570)	3.6	Mannose-6-phosphate isomerase	carbohydrate metabolic process, embryonic development ending in seed dormancy	mannose-6-phosphate isomerase activity
299414 (nd)	3.0	not determined by homology	unknown	unknown
100090 (At3g27010)	3.0	TCP family transcription factor	anatomical structure morphogenesis	transcription factor activity
306180 (nd)	2.9	not determined by homology	unknown	unknown
131107 (nd)	2.8	not determined by homology	unknown	unknown
34834 (nd)	2.7	not determined by homology	unknown	unknown
269905 (At1g53140)	2.7	unknown	unknown	unknown
230444 (At3g08850)	2.7	ABC transporter protein; PDR ABC transporter;	cell growth, embryonic development ending in seed dormancy	nucleotide binding, protein binding
322451 (At1g57770)	2.6	Cluster: COG1233: Phytoene dehydrogenase and related proteins	carotenoid biosynthetic process, electron transport	oxidoreductase activity
236259 (At4g32060)	2.5	Cluster: Hypothetical protein F10N7.140	unknown	calcium ion binding
122523 (nd)	2.5	not determined by homology	unknown	unknown
324355 (At5g05690)	2.5	cytochrome P450; cytochrome P450 family protein	electron transport	heme binding iron ion binding monooxygenase activity
305839 (At5g18230)	2.5	CCR4-NOT transcription complex subunit 3 (CCR4 associated factor 3);	regulation of transcription	transcription regulator activity
179237 (At3g05390)	2.4	expressed protein;	release of virus from host	unknown
311579 (At5g49210)	2.4	expressed	unknown	unknown

Tab. 5-3: List of down-regulated genes in high dry matter genotypes at both 56 and 98 DAA (13 out of first 30 genes changed). FC, folds change; nd, not determined.

Oligo-EST (*Arbidopsis* gene)	FC	Description	Biological function	Molecular function
235922 (nd)	-5.3	not determined by homology	unknown	unknown
311482 (nd)	-3.7	not determined by homology	unknown	unknown
233449 (nd)	-3.2	not determined by homology	unknown	unknown
313668 (At2g38750)	-3.1	annexin; Annexin A11	response to osmotic stress, response to abscisic acid stimulus	calcium ion binding, calcium-dependent phospholipid binding
312391 (At3g58040)	-3.1	Cluster: SINA2p	multicellular organismal development, ubiquitin-dependent protein catabolic process	protein binding, zinc ion binding
125043 (AtCg00340)	-2.8	Photosystem I P700 chlorophyll a apoprotein A2 (PsaB) (PSI-B)	photosynthesis, light harvesting in photosystem I, photosynthesis, light harvesting in photosystem II	chlorophyll binding
313588 (nd)	-2.8	not determined by homology	unknown	unknown
198058 (At4g01520)	-2.7	unknown	unknown	unknown
312547 (nd)	-2.7	not determined by homology	unknown	unknown
312739 (nd)	-2.6	not determined by homology	unknown	unknown
310182 (At5g08640)	-2.6	Leucoanthocyanidin dioxygenase (LDOX)	flavonoid biosynthetic process	flavonol synthase activity
249897 (At2g47800)	-2.4	MRP ABC transporter	response to water deprivation, multidrug transport, response to nematode, stomatal movement	folic acid transporter activity, ATPase activity, coupled to transmembrane movement of substances
234876 (At3g47640)	-2.4	Cluster: Hypothetical protein	regulation of transcription	DNA binding transcription factor activity

Tab. 5-4: List of up-regulated genes in high dry matter genotypes at 56 DAA (9 out of first 30 genes changed). FC, folds change; nd, not determined.

Oligo-EST (*Arbidopsis* gene)	FC	Description	Biological function	Molecular function
240637 (nd)	8.2	not determined by homology	unknown	unknown
310530 (At3g02570)	5.0	Mannose-6-phosphate isomerase	carbohydrate metabolic process, embryonic development ending in seed dormancy	mannose-6-phosphate isomerase activity
299414 (nd)	4.7	not determined by homology	unknown	unknown
305117 (At4g23930)	4.6	not determined by homology	unknown	unknown
100090 (At3g27010)	4.3	TCP family transcription factor	anatomical structure morphogenesis	transcription factor activity
131107 (nd)	3.8	not determined by homology	unknown	unknown
194921 (At5g46850)	3.4	Cluster: Arabidopsis thaliana genomic DNA, chromosome 5, P1 clone:MSD23	unknown	unknown
125041 (nd)	3.4	not determined by homology	unknown	unknown
322451 (At1g57770)	3.1	Cluster: COG1233: Phytoene dehydrogenase and related proteins	electron transport	oxidoreductase activity

Tab. 5-5: List of down-regulated genes in high dry matter genotypes at 56 DAA (21 out of first 30 genes changed). FC, folds change; nd, not determined.

Oligo-EST (*Arbidopsis* gene)	FC	Description	Biological function	Molecular function
235922 (nd)	-6.1	not determined by homology	unknown	unknown
312805 (At4g22880)	-5.7	oxidoreductase, 2OG-Fe(II) oxygenase family protein; Flavonol synthase/flavanone 3-hydroxylase (FLS)	response to wounding, vacuole organization and biogenesis, anthocyanin biosynthetic process, response to jasmonic acid stimulus, proanthocyanidin biosynthetic process	leucocyanidin oxygenase activity
237954 (At5g45960)	-5.1	GDSL-motif lipase/hydrolase family protein	lipid metabolism	hydrolase activity, acting on ester bonds
311482 (nd)	-4.6	not determined by homology	unknown	unknown
298926 (At1g76160)	-4.5	multi-copper oxidase type I family protein	unknown	copper ion binding oxidoreductase activity
312391 (At3g58040)	-4.0	Cluster: SINA2p	development ubiquitin-dependent protein catabolism	unknown
240153 (At5g14920)	-4.0	Cluster: Gibberellin-regulated protein 1 precursor	response to gibberellin stimulus	structural constituent of cell wall
313588 (nd)	-3.8	not determined by homology	unknown	unknown
321725 (At5g05170)	-3.7	cellulose synthase, catalytic subunit	cellulose biosynthesis	cellulose synthase UDP-forming activity
286459 (At2g04240)	-3.6	zinc finger (C3HC4-type RING finger) family protein;C3H TF Family	response to osmotic stress, response to salt stress	transcription factor protein binding zinc ion binding
310182 (At5g08640)	-3.5	Leucoanthocyanidin dioxygenase (LDOX)	flavonoid biosynthetic process	flavonol synthase activity
125043 (AtCg00340)	-3.5	Photosystem I P700 chlorophyll a apoprotein A2 (PsaB) (PSI-B)	photosynthesis, light harvesting in photosystem I, photosynthesis, light harvesting in photosystem II	chlorophyll binding chloroplast thylakoid membrane, plastoglobule
312739 (nd)	-3.5	not determined by homology	unknown	unknown
115884 (At1g31850)	-3.3	dehydration-responsive protein	unknown	unknown
234876 (At3g47640)	-3.3	Cluster: Hypothetical protein; basic Helix-Loop-Helix (bHLH) TF	regulation of transcription	transcription factor activity DNA binding
313668 (At2g38750)	-3.3	annexin; Annexin A11	response to osmotic stress, response to abscisic acid stimulus	calcium ion binding, calcium-dependent phospholipid binding
312939 (At4g20260)	-3.3	DREPP plasma membrane polypeptide family protein	response to cold	unknown
258325 (At5g21090)	-3.2	Cluster: protein kinase	signal transduction	protein binding
260703 (At1g16470)	-3.2	Proteasome subunit alpha type 2	ubiquitin-dependent protein catabolism	threonine endopeptidase activity
319604 (At4g27435)	-3.2	expressed protein;	unknown	unknown
195236 (nd)	-3.1	not determined by homology	unknown	unknown

5.4 DISCUSSION

Transient starch accumulation is a feature of many developing sinks, such as growing fruit and potato tubers. Kiwifruit berry behaves as those sink, and accumulates starch during fruit development. Starch is then remobilized in ripening fruit to constitute the pool of soluble sugars characterizing the full ripe fruit (MacRae *et al.*, 1992; Richardson *et al.*, 1997). There is therefore a strong correlation between stored starch and potential fruit final soluble solid concentration.

Genotypes characterized by strong differences in at-harvest starch content were different since early in fruit development as demonstrated in chapter 3. It was therefore hypothesized that observed dissimilar starch concentration could be determined by differential carbohydrate metabolism. The actual experiments have demonstrated that selected genotypes, whether or not fruit were small or large, have differential gene expression patterns for sucrose synthase and AGPase large subunit genes mainly early in fruit development. Also other genes not directly involved in carbohydrate metabolism, such as transcription factors and kinases, were shown to be differentially expressed mainly earlier in fruit development. An interesting role is played by vacuolar invertase, which both gene expression and enzyme activities were shown to be consistently higher in low dry matter genotypes (although not always significant).

5.4.1 SUCROSE CLEAVAGE: SUCROSE SYNTHASE PLAYS A KEY ROLE ON STARCH ACCUMULATION EARLY IN FRUIT DEVELOPMENT

Sucrose cleavage is tightly linked to the phloem unloading pathway used. Phloem unloading pathway during kiwifruit berry development has not been determined yet, although some of the literature information combined with present experiments' results lead to the conclusion that, at least early in fruit development, kiwifruit is a symplasmic unloader. Sucrose synthase transcrips were high earlier in the beginning compared to all other identified sucrose cleavage enzymes. There was also no evidence of ESTs encoding for *LIN5*-like cell wall invertases, that in tomato are linked with apoplasmic unloading. Furthermore, kiwifruit flesh cell walls are densely populated by plasmodesmata late in fruit development (Sutherland *et al.*, 1999). To assess if whether or not this was true also early in fruit development, an attempt to easily identify plasmodesmata was done by callose localization. Callose is a poly-sugar molecule in the form of β-1,3-glucan, reversibly and transiently deposited in cell walls as result of stresses or during many developmental changes (Kauss, 1996). Callose deposition occurs in the cell wall surrounding the plasmodesmata at both ends of the channel, compressing the plasma membrane inward, thus creating a narrowed neck region (Radford *et al.*, 1998). A preliminary study on early developmental stage fruit, using an immuno-labelling technique for callose (Ruan *et al.*, 2004), showed an abundance of plasmodesmata connections all around the vascular tissues and the parenchyma (data not shown). The observations were held at a light microscope so it was hard to discriminate and count plasmodesmata frequencies in the phloem unloading region. Further investigation are needed to confirm the data, but the high sucrose synthase gene expression, the no evidence

of ESTs encoding for fruit expressed cell wall invertases and the high plasmodesmata frequency sustain the hypothesis of a symplasmic phloem unloading pathway, at least early in fruit development. Kiwifruit is a starch storing sink, and it is a feature of that type of sink a symplasmic phloem unloading. Shift from apoplasmic to symplasmic phloem unloading has been observed in tomato fruit at the stage in which a net starch degradation start and soluble sugars are then accumulated (Lalonde *et al.*, 2003; Patrick and Offler, 1996; Ruan and Patrick, 1995).

Sucrose synthase is therefore the main sucrose cleavage enzyme in kiwifruit cytoplasm. To the two already described sucrose synthase genes (Richardson *et al.*, 2004), a further one has been identified and called SUS2. From the phylogenetic analysis SUS2 clusters with SUS1-like isoforms. The phylogenetic analysis shows also a redundant behaviour in *Actinidia* gene, being evolutionary close to the *Solanaceae* homologous(Langenkämper *et al.*, 2002). A SUS6-like ESTs was also found in the HortReaserch database, and it clusters with the third sucrose synthase group. The phylogenetic tree obtained is also in agreement with the three isoenzyme classes precedently defined by Baud *et al.* (2004).

From the tblastn sequence analysis, SUS1 is homologous to *Arabidopsis* SUS4, whereas SUS2 is homologous of *Arabidopsis* SUS1.

The expression level of sucrose synthase genes on 'Hayward' fruit developmental series are in line with Richardson *et al.* (2004) previous observations. It was here emphasised that SUS1 is strongly expressed early in fruit development. SUS2 behaved similarly to SUS1, but at lower levels. However SUS1 and SUS2 similar levels where found in *Actinidia eriantha*. In several crops expression of SUS1-like genes is sink-specific and its role is related with starch biosynthesis and phloem sucrose unloading (D'Aoust *et al.*, 1999; Zrenner *et al.*, 1995). Results here reported shown that SUS1 expression pattern in genotypes is overall similar to those previously observed in kiwifruit. SUS1 higher expression levels in high dry matter genotypes fit well with the increased starch concentration. In fact higher SUS1 could be responsible of a higher UDP-glucose availability and promote an enhanced carbon flow to starch synthesis. ADP-glucose pyrophosphorylase activity is moreover regulated by substrate concentration, and UDP-glucose is the direct precursor of ADP-glucose. Another factor that sustains sucrose synthase as the main sucrose cleavage enzyme early in fruit development is the low fructose concentration in kiwifruit (Fig. 5-1B and Fig. 5-2B). Fructose, in fact, inhibits sucrose synthase activity (Morell and Copeland, 1985). Kiwifruit is a metabolically highly active organ (at least early in development, when a dramatic increase of size occur in a short lag of time). In many metabolically highly active or bulky organs oxygen tensions are naturally low, and ATP synthesis may be thereafter limited (Guglielmetti *et al.*, 1995; Rolletschek *et al.*, 2002). Sucrose synthase requires less ATP than the conversion of sucrose to hexose phosphate via invertase does, and in low oxygen conditions its expression is promoted (Koch, 2004).

Interesting is the role of SUS2. Its expression was significantly high in one of the high dry matter genotypes. It is highly expressed in *Actinidia eriantha* (diploid specie) and then it might be a rare allele of SUS1 in Actinidia deliciosa (hexaploid specie). Further

investigation are then needed to clarify its role and involvement in sucrose cleavage and starch accumulation.

Other sucrose cleavage enzyme showed low expression levels when compared to sucrose synthase, and vacuolar invertases were in average more expressed in low dry matter genotypes (this was confirmed for vacuolar invertase 4 in microarray experiment, but it was not in the second year of experiment). Observed higher level of both vacuolar and cytoplasmic invertase activities in low dry matter genotypes might be seen as competitors for the same substrate of sucrose synthase and affect the carbon flux into starch. Vacuolar invertases are known to play an osmoregulatory role at cellular level (Roitsch and Gonzalez, 2004). Carbon accumulation into the vacuole can promote cell expansion, but this was not the case being high and low dry matter similar in fruit. Then, in low dry matter genotypes an alternative route of carbon starting from a vacuolar sucrose cleavage can be hypothesised.

5.4.2 DOES APL4 PLAY A DETERMINANT ROLE IN STARCH SYNTHESIS IN HIGH DRY MATTER GENOTYPES?

In dicots a small gene family of three or four members encodes for the AGPase large subunit, whilst usually a single gene encodes for the small one (Crevillen *et al.*, 2005). The results previously reported shown that *Actinidia* contains three isoforms for the large subunit (AGPL1, AGPL2 and AGPL4) and one isoform for the small subunit (AGPS1). AGPL1 ESTs were only from vegetative tissues library. AGPase subunit gene expression is tissue specific (Crevillen *et al.*, 2005) and the AGPL1-like tomato isoform (L3, as confirmed by the phylogenetic analysis) was found to be primarily expressed in leaves (Li *et al.*, 2002; Park and Chung, 1998). For those reasons, AGPL1 expression profile was not studied, and more attention was then concentrated on the other two large and the small subunit isoforms. As previously observed in this study for sucrose synthase and in Langenkämper *et al.* (2002) for sucrose phosphate synthases, also *Actinidia* AGPase subunit isorforms behave clustering close to *Solanaceae* in the phylogenetic analysis. As per tomato L1 isoform was the most strongly expressed in fruit tissues, followed by L2 (Li *et al.*, 2002; Park and Chung, 1998), a similar situation was found in *Actinidia* fruit, where AGPL4 isoform was the prevalent one, and AGPL2 was 5 to 10 time lower in transcript levels.

AGPL4 expression levels were higher in high dry matter genotypes, mainly early in development. At 40 DAA a dramatic increase (3-4 times) was observed in both high dry matter genotypes. The higher expression of AGPase subunit gene or a temporally extended expression, particularly the one encoding for the large subunit, lead to an increase in enzyme activity and starch accumulation (McKibbin *et al.*, 2006; Petreikov *et al.*, 2006). AGPase are highly regulated enzymes, and so the higher expression observed might be just one of the factors affecting the final higher starch result. In potato was observed that higher sucrose synthase and AGPase expression and activity, resulting in high starch potato, were a consequence of the regulatory role of a sucrose non-fermenting-kinase 1 protein (SnFR1) (Petreikov *et al.*, 2006). This might be a suggestion for a future investigation, being the

kiwifruit high dry matter a similar situation. Sucrose synthase and AGPase are hightly expressed.

5.4.3 OTHER CARBON-RELATED PATHWAYS

Starch synthesis is only one of the several pathway of carbon metabolism occurring in sink organ. A complicate network of different pathways is interconnected: products of one pathway might be the substrate for another, or two different pathways may compete for the same substrate. Some of the main pathways co-existing in a developing sink, as kiwifruit berry is, having carbon as key element are: starch synthesis, other non-structural carbohydrate metabolism, respiration, structural carbohydrate synthesis, vitamin C synthesis (Gallego and Zarra, 1997; Laing *et al.*, 2004; Richardson *et al.*, 2004; Sutherland *et al.*, 1999).

Vitamin C content has been shown to behave similarly to dry matter or starch content at altered conditions. So when vines were heated, both dry matter and ascorbate decreased (Richardson *et al.*, 2004). There are still hypothesis about vitamin C accumulation in kiwifruit berry, whether or not is the results of *in situ* synthesis (Richardson *et al.*, 2004). From the microarray experiment two enzyme of the L-galactose pathway (Smirnoff *et al.*, 2001; Wheeler *et al.*, 1998) resulted to be differentially expressed: mannose-P-6-isomerase (MPI) was 5 time more expressed in high dry matter genotypes whereas phospho-manno-mutase (PMM) was 2.5 time down-regulated. Little is known in kiwifruit about enzyme activity and gene expression in vitamin C pathway. PMM has been recently shown to be directly involved in vitamin C synthesis (Qian *et al.*, 2007), and if the lack of this enzyme in tobacco plants showed decreases in ascorbic acid, whereas the over-expression did not reflected the expected result of increase in ascorbate concentration, and it was concluded that PMM is not a rate-limiting step in ascorbate biosynthesis. It was then hypothesised a role in cell wall biosynthesis by Qian *et al.* (2007), similar to the one played by GDP-mannose pyrophosphorylase in potato and *Arabidopsis* (Conklin *et al.*, 1999; Keller *et al.*, 1999; Lukowitz *et al.*, 2001). Kiwifruit berry cell wall is poor of mannose, whereas main components are cellulose, galactose and uronic acid (Gallego and Zarra, 1997). It is therefore possible argue that most of the mannose is formed is directed into vitamin C metabolism, if this is located in the fruit. A change in the conversion ratio of D-glucose into mannose could drive carbon to one metabolic way rather than another.

myo-Inositol, a sugar-alcohol counting for about 20% of soluble sugar in *Actinidia deliciosa* fruit (Boldingh *et al.*, 2000; Klages *et al.*, 1998; Walton and De Jong, 1990), has been shown to have a central role in several biochemical pathways (Bohnert *et al.*, 1995; Loewus and Murthy, 2000). Interesting pathways for kiwifruit berry growth are: ascorbate biosynthesis and biosynthesis of cell wall precursors. Also inositol is a D-glucose derivate, and *myo*-inositol 1 phosphate synthase (MI-1-P synthase) is the committed step in its biosynthesis (Loewus and Murthy, 2000). In low dry matter genotypes MI-1-P synthase is more expressed compared to high dry matter genotypes. *myo*-Inositol was not different in concentration between high and low dry matter genotype, though. Extra-synthesized *myo*-

inositol could be directed into cell wall synthesis in low dry matter genotypes, being ascorbic acid and dry matter positively correlated, and it is reasonable to propose a different carbon partitioning strategy.

A promotion of cell wall metabolism is enforced by the higher expression level of cellulose synthase in low dry matter genotypes. UDP-glucose, the product of sucrose synthase activity, is the substrate for both starch and cellulose synthesis (Amor *et al.*, 1995; N'Tchobo *et al.*, 1999). An higher cellulose synthase expression level could be translated into an higher enzyme activity and enhance the competitiveness of cellulose synthesis on starch synthesis.

Polygalacturonase (PG) and Xyloglucan Endotransglycosylase (XET) are two enzymes usually associated with the maturation syndrome for many fruit, as well for kiwifruit (Percy *et al.*, 1996; Wang *et al.*, 2000), being involved in the cell wall loosening. PG are responsible of cell wall pectines solubilization, whereas XET act on the cleavage of the hemicellulosic component. However, early studies on XET investigate its role in cell wall loosening in high growing tissues (Fry *et al.*, 1992). The observed differences in expression of the two genes in high and low dry matter could be reflected into a differential cell wall composition.

More investigation are then needed on carbohydrate partitioning within a fruit, being a lot of the involved factors cell wall-related. Further gene expression studies and enzyme activity are then needed to confirm microarray results. Cell wall composition of high and low dry matter genotypes could be also investigated.

5.4.4 SUCROSE TO STARCH CONVERSION REGULATION FACTORS

Many of the following consideration refers to the microarray results reported in Tab. Appendix VII- 2 and Tab. Appendix VII- 3.

Potato is a model plant for the study of sucrose to starch metabolism in growing sink. Several studies showed how transcriptional changes of singular enzymes involved in the pathway not always had significant effect on starch accumulation in growing tuber (Müller-Röber *et al.*, 1992; Zrenner *et al.*, 1995). The sucrose to starch metabolic process is highly regulated, at both sucrose synthase and AGPase levels (Geigenberger, 2003). One factor that positively regulates levels of both SUSY and AGPase subunits transcripts is sucrose (Müller-Röber *et al.*, 1990; Salanoubat and Belliard, 1989). In response to sucrose supply there is a co-ordinated up regulation of both SUSY and AGPase in potato tuber (Geigenberger, 2003). The signalling mediated processes involved in this regulation have not been clarified yet. Both SUSY and AGPase expression are regulated by a sucrose non-fermenting-1-related protein kinase-1 gene, SnRK1 (McKibbin *et al.*, 2006). However, in potato, sucrose mediated transcriptional regulation allows only gradual changes in enzyme activity, and changes in transcriptional regulation partially counts in the alteration of the sucrose to starch pathway (Geigenberger, 2003). This can be true also for *Actinidia deliciosa* fruit. Microarrays results showed a number of transcription factors and protein kinases to be differentially expressed early in fruit development among high and low dry matter genotypes. Although further investigation at qPCR are needed to confirm the array data,

speculation about an hypothetical function can be still postulated. Protein kinases are known to play a role in protein phosphorylation: by changing the activity of the target protein they are involved in protein regulation (Hardie, 1995). It is possible that one of the reported protein kinase is involved into sucrose to starch metabolism, or plays a role in a network of signal transductions.

Recently it has been clarified that potato AGPase is regulated by a further mechanism, which involves post-translational redox-modifications (Tiessen *et al.*, 2002). AGPase are then in an active form when reduced by the opening of a disulphide bridge between small subunits (Fu *et al.*, 1998). The signalling components involved in the redox modulation of AGPase are still unknown. It has been proposed that thioredoxins (Ballicora *et al.*, 2000)or putative sugar sensors (Smeekens, 2000) might be involved. From the microarray experiment a thioredoxin (protein disulfide isomerase) was down-regulated (-2.5 FC) in high dry matter genotypes. A differential post-translational regulation of AGPase between high and low dry matter genotypes could end in the observed starch differences if this particular thioredoxin acts mainly in the oxidative direction in low dry matter genotypes. An higher proportion of AGPase inactivated form can strongly affect protein activity. Further studies are then required to confirm gene expression first, and to verify the *in vivo* role of the thioredoxin on kiwifruit AGPase.

Calcium is a second universal messenger, and its role is the mediation of stimulus-response coupling in the regulation of several cellular function (Trewavas and Malho, 1998). Several calcium sensing or binding proteins have been identified in plants, such as calmodulin, one of the several 'EF-hand' containing proteins involved in calcium binding (Snedden and Fromm, 2001). It has been hypothesized that calmodulin genes are plant specific and it is possible that target protein are extremely diversified (Yang and Poovaiah, 2003). Several calcium binding protein and calmodulin have been shown to be differentially expressd between high and low dry matter genotypes. It is therefore possible that a differential calcium content in high and low dry matter genotype fruit can affect the expression of its regulatory proteins.

Development of plant organs is determined by differential expression of genes, that are therefore controlled at different levels. One of the major mechanism controlling gene expression is transcriptional regulation (Lee and Young, 2000). A lot of transcription have been associated with the regulation of metabolic pathways, and probably the most studied is the secondary metabolism (Broun *et al.*, 2006). Several transcription factor, as well as post-transcriptional and post-traslational regulators, have been shown to be up- or down-regulated in high dry matter genotype suggesting that a better understanding in which is their contribution to the observed starch differences. Zinc-finger proteins, which belong to a transcription factor family, were differentially expressed early in development between a wild type rice genotype and an endosperm-less rice genotype (Li *et al.*, 2005), but a specific role was not attributed.

5.5 CONCLUSIONS

Genotypes were different in SUS1 and AGPL4, mainly early in fruit development, suggesting a role in driving sucrose into starch. It is therefore not excluded that other carbon-related metabolic pathways are more efficient in low dry matter genotypes, with a consequent differential carbon partitioning. Transcription factors, kinases, and other regulatory mechanism needs further study to elucidate their role in the overall carbon metabolism.

A pleiotropic mechanism is not therefore excluded, and high or low starch accumulation can be one of the effects of a mutation occurred in a wild type gene, able then to affect multiple phenotypic traits.

6 EFFECTS OF CPPU APPLICATION AND SOURCE TO SINK BALANCE ON CARBOHYDRATE ACCUMULATION BY KIWIFRUIT BERRIES

6.1 INTRODUCTION

CPPU is a synthetic cytokinin able to influence fruit set and development of several fruit crops and vegetable crops, such as apples (Barngerth and Schröder, 1994), grapes (Zabadal and Bukovac, 2006), watermelon (Hayata *et al.*, 1995), *Lagenaria leucantha* (Li *et al.*, 2004) and also kiwifruit (Antognozzi *et al.*, 1996; Kim *et al.*, 2006; Lewis *et al.*, 1996; Patterson *et al.*, 1993).

Although several studies have been carried out in the past no clear explanation of the mechanism of action is available. It is known that CPPU application dramatically increases kiwifruit berry size but in some situations this effect is accompanied with a decrease in final dry matter content (%DM). Kiwifruit berry maturation is known to be advanced by CPPU treatments (higher at-harvest soluble solid concentration and lower flesh firmness), but differences are not maintained during storage (Antognozzi *et al.*, 1996; Patterson *et al.*, 1993).

Antognozzi *et al.* (1996) found CPPU treated fruit to be lower in dry matter (%), higher in starch concentration and in ADP-glucose pyrophosphorylase activity. Sucrose is transported via phloem into kiwifruit berry (Klages *et al.*, 1998). Patterson *et al.* (1993) found that increased size was related to outer pericarp larger small cells rather than to an higher cell number. This can be driven by an osmotic regulation. Antognozzi *et al.* (1996) found an higher hexose concentrations soon after the CPPU treatment.

There are only two known sucrose cleavage pathways, catalyzed either in a reversible manner by sucrose synthase enzyme or in an irreversible way, catalyzed by invertases (Koch, 2004). Sucrose cleavage can be located in the apoplast (cell wall), cytoplasm or vacuole.

The major role commonly attributed to sucrose synthase enzyme in cytoplasm of sink organ is the conversion of sucrose into UDP-glucose, which is then transformed stepwise to glucose-1-phosphate, glucose-6-phosphate and ADP-glucose necessary for starch biosynthesis (ap Rees, 1995; Kossmann and Lloyd, 2000; Müller-Röber *et al.*, 1992; Tauberger *et al.*, 2000; Tiessen *et al.*, 2002). The evidence of an involvement of sucrose synthase in starch biosynthesis has been demonstrated in carrot and potato (Tang and Sturm, 1999; Zrenner *et al.*, 1995). The abundance of sucrose synthese in the Golgi apparatus and plasma membranes has evoked the idea that it is involved also in directing the carbon flow to cell wall synthesis (Amor *et al.*, 1995; Nakai *et al.*, 1999). In kiwifruit two different sucrose synthase isoforms have been identified: SUSA and SUS1 (Richardson *et al.*, 2004). SUS1 is mainly expressed early in fruit development whereas SUSA increases at the onset of fruit

maturation. In *Arabidopsis*, both sucrose synthases from SUS1 group (At5g20830 and At3g43190) have been shown to be up-regulated 120 minutes after cytokinin treatment (Brenner *et al.*, 2005).

Invertases are subdivided according localization and optimum pH into tree isoenzymes families: acid cell wall invertase, acid vacuolar invertase and neutral-alkaline Cytoplasmic invertase. Vacuolar invertases are involved in cell enlargement via vacuole osmotic regulation. Cell wall invertases are linked with apoplasmic phloem unloading. Cytoplasmic invertases support sucrose cleavage in the cytoplasm but are minimally active in most systems (Koch, 2004; Roitsch and Gonzalez, 2004; Winter and Huber, 2000).

Cytoplasmic sucrose is often transported into the vacuole for cleavage (Koch, 2004). Vacuolar invertases determine the level of sucrose stored in the vacuole and remobilization of sucrose for metabolic processes. A well-established function is the regulation of the sugar balance in fruit tissues and mature tubers (Ohyama *et al.*, 1995; Scholes *et al.*, 1996).

In muskmelons (Hayata *et al.*, 2001) has been shown that vacuolar invertase are one of the key enzymes responsible for regulating fruit growth in CPPU treated fruit. It was proposed that CPPU promotes fruit growth via activation of this enzyme. In *Lagenaria leucantha* (Li *et al.*, 2004) CPPU increases vacuolar invertases activity and gene expression early in fruit development. An involvement during peach fruit growth of a neutral invertase has recently been described (Nonis *et al.*, 2007). Abundance of plasmodesmata in kiwifruit cell wall (Sutherland *et al.*, 1999) and no evidence of cell wall invertase sequences (unpublished, and perhaps we have not finished looking yet), are supporting the symplasmic phloem unloading pathway.

ADP-glucose pyrophosphorylase catalyze a rate-limiting step in starch synthesis. In several crop has been proved the fundamental importance of ADP-glucose pyrophosphorylase in starch synthesis, by an increased starch yield: potato (Stark *et al.*, 1992), maize and wheat (Giroux *et al.*, 1996; Smidansky *et al.*, 2002). Plant ADP-glucose pyrophosphorylase are heterotetramer enzymes of two small and two large subunits. Plant possess multiple genes encoding either the large or small subunits, or both, and these are differentially expressed in plant organs (Preiss and Sivak, 1996).In tomato, three large subunit genes and one small subunit gene have been described (Chen and Janes, 1997; Chen *et al.*, 1998).

Kiwifruit vines, in conditions of high assimilate availability, are able to perform a redistribution of carbon, even in fruit bearded by leafless canes (Tombesi *et al.*, 1993). Between two or three leaves per fruit kiwifruit vine in able to satisfy a normal carbohydrate demand (Lai *et al.*, 1989), and on a whole plant basis fruit size increases up to a 5:1 leaf to fruit ratio (Snelgar *et al.*, 1986). In kiwifruit, as for many other fruit crop, there is a negative correlation between fruit size and crop load. Being the fruit a strong competitor for carbon, a decrease in 'Hayward' fruit size can be attributed to a limited photosynthate supply (Woolley *et al.*, 1991).

The aim of this study was to find the rate-limiting factor for dry matter accumulation after CPPU application, and verify if the mechanism is comparable to one of those observed in genotypic variations.

CPPU was hypothesised to enhance fruit carbohydrate demand. Leaves to fruit ratio was adjusted to two different crop loads, a standard and a low one, to test the effect of CPPU treatment when plenty of carbon is available. Canes were then girdled to avoid the redistribution of surplus carbon. It was firstly verified, as starting point, that CPPU treated fruit were larger in size and lower in dry matter content. It was hypothesised then that CPPU effect is source limited and an higher source availability can smooth the reduction in dry matter in CPPU treated fruit. It was hypothesised that CPPU treated dry matter was lower because of a reduction in starch concentration, as observed in low dry matter genotypes. It was finally supposed that changes in starch metabolism in CPPU treated fruit can be modulated by either sucrose cleaving enzyme or AGPase subunit changes in gene transcription.

6.2 MATERIALS AND METHODS

6.2.1 PLANT MATERIAL

The experiment was carried out on 15 green 'Hayward' kiwifruit vines [*Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa*] in a 1991 block orchard at Te Puke Research Centre (Bay of Plenty, NZ; 37^{o} 49' S - 176^{o} 19' E). Vines were managed for commercial production (except for pruining, thinning and girdling practices) on a pergola-trained system at 6 m spacing on the row and 5 m spacing between rows. All vines were growing on open-pollinated 'Kaimai' seedling rootstocks. The experiment was carried out in 2005-06 season. Anthesis was recorded the 23rd November (50% of open flowers).

6.2.2 TREATMENTS

A total of eight uniform canes were selected from each vine. On a half of the selected canes, CPPU was applied to fruitlet 28 days after anthesis as a 10 ppm dip, on girdled canes with either 3 leaves per fruit (standard fruit load) or 6 leaves per fruit (low fruit load). Untreated canes were used as control for both crop loads.

6.2.3 SAMPLING

Samples were collected 35, 49, 70, 126, 154 days after anthesis (DAA). A number of 6 fruit each time point was picked from 5 different canes per treatment and fruit fresh weight recorded. Dry matter content, as percentage of fresh weight, was determined for individual fruit as for Burdon *et al.* (2004). Fruit dry weight was then calculated multiplying fresh weight and dry matter (%).

At each time point, wedges of fruit tissue (providing representative proportions of all tissue types, radially and longitudinally) were cut and frozen in liquid nitrogen for both carbohydrates and organic acids analysis, and gene expression analysis.

6.2.4 NON-STRUCTURAL CARBOHYDRATE AND ORGANIC ACID ANALYSIS

Starch was determined as reported in Smith *et al.* (1992). Soluble sugars (glucose, fructose, sucrose, myo-inositol and galactose) were analyzed as per Klages *et al* (1998) and organic acids as per Cheng *et al.* (2004). Four biological replicates were analyzed (each treatment at each time point). The individual starch, sugar and acid contents were expressed as the milligrams per gram fresh fruit weight (mg g^{-1} FW).

6.2.5 QPCR GENE EXPRESSION STUDY: RNA EXTRACTION, CDNA SYNTHESIS AND REAL TIME PCR AMPLIFICATION

RNA was isolated from 2 g of fruit tissues as for (Chang *et al.*, 1993). Following DNase treatment, first-strand cDNA synthesis was carried out using oligo$(dT)_{20}$ according to the manufacturer's instructions (SuperScript™ III Reverse Transcriptase; Invitrogen). Genes encoding sucrose cleaving enzymes and ADP-glucose pyrophosphorylase enzymes and were identified by homology in the HortResearch *Actinidia* EST database, and, where putative gene family members existed, candidates were selected when they appeared in fruit library tissues. Gene-specific primers corresponding to these genes were designed using Primer3 software (Rozen and Skaletsky, 2000) to a stringent set of criteria, enabling application of universal reaction conditions. To check reaction specificity, RT-PCR reactions were carried out according to the manufacturer's instructions (Platinum Taq, Invitrogen). SUS1, V-INV3, V-INV4, A/N-INVD, AGPL4, AGPL2 and AGPS1 genes were tested and primers sequences are reported in Tab. Appendix VI- 2.

qPCR DNA amplification and analysis was carried out using the AB 7500 Fast Real Time PCR detection system (Applied Biosystems). All reactions were performed using the AB SYBR® Green PCR Master Mix (Applied Biosystems) according to the procedure described by the manufacturer, on 96-well plates (Applied Biosystems). Reactions were performed in triplicate using 7.5 µL 2X Master Mix, 0.2 µM each primer, 5 µL 100X diluted cDNA and nuclease-free water to a final volume of 15 µL. A negative water control was included in each run. Fluorescence was measured at the end of each annealing step. Amplification was followed by a melting curve analysis with continual fluorescence data acquisition during the 60–95ºC melt. The raw data were analysed with the 7500 Fast System SDS software, version 3.1 (Applied Biosystems), and expression was normalized to *Actinidia deliciosa* Protein Phosphatase Regulatory Subunit 2A (PP2A HortResearch EST 312205) to minimize variation in cDNA template levels. PP2A was selected for normalization due to its consistent transcript levels. For each gene, a standard curve was generated using a cDNA serial dilution, and the resultant PCR efficiency calculations (ranging between 1.87 and 2.02) were imported into relative expression data analysis. Relative expression was calculated as for Ren *et al.* (2007). Error bars shown in qPCR data are technical replicates, representing the means ± SE of three replicate qPCR reactions.

6.2.6 STATISTICAL ANALYSIS

A mixed effects model was fitted using the Mixed Procedure in the SAS software package (SAS Institute Inc, 2000) for Windows to determine the effect of CPPU treatment, crop load and time on fruit fresh weight, fruit dry weight, fruit dry matter concentration, starch concentration, glucose, fructose and sucrose concentrations and total starch content. An auto regressive of order one (AR1) covariance structure was used to model the repeated measures on the individual plants. Type 3 Tests of Fixed Effects were used to assess the significance of the main effects, then the least-squares means (LSMs) were calculated, and tests for a difference between treatments at each time point were carried out using the Slice option in the LSMEANS statement. These tests effect slice were used when a significant interaction between treatment and time was present.

6.3 RESULTS

6.3.1 CPPU INCREASES FRUIT SIZE

CPPU dramatically affected kiwifruit berry size, at both standard and low crop load. The final fruit fresh weight was 25% more at standard crop load in CPPU treated fruit, whereas it was about 50% higher when source were more abundant (Fig. 6-1A, B).

The treatment effect was evident in fruit fresh weight from about 20 days after the treatment in both crop loads.

Also fruit dry weight was affected by CPPU treatment, being higher in CPPU treated fruit, at both crop loads. Dry weight differences started to be evident at about 40 days after the treatment. Differences in dry weight accumulation resulted to be less dramatic than the fresh weight ones (Fig. 6-1C, D).

6.3.2 CPPU TREATMENT REDUCES DRY MATTER CONTENT

Dry matter content (%) was lowered by CPPU treatment of about 15% in the at-harvest content (Fig. 6-1E, F). Differences in dry matter content started to be evident at 40 days after the treatment (about 70 DAA).

6.3.3 OBSERVED DIFFERENCES IN DRY MATTER REFLECT STARCH ACCUMULATION AT STANDARD CROP LOAD

The analysis of non-structural carbohydrates and organic acids showed in both crop load a similar behaviour, being starch, fructose and glucose the most treatment-affected compounds (Fig. 6-2A, B, C and Fig. 6-3A, B, C).

Starch concentration was significantly lower in CPPU-treated fruit at standard crop load whereas it was unchanged in low crop load, but at-harvest sampling date. Both glucose and fructose levels were higher in CPPU-treated fruit.

From an analysis of the contribution of each analyzed compound class (being other non detected compound quote) on dry matter composition is evident that CPPU treated fruit at standard crop load accumulated a 25% less starch counting for dry matter content (Fig. 6-4).

This observation is referred to 126 DAA (being after this time point starch component decreasing).

Total starch content, on a per fruit basis, was increased by CPPU treatment, maily in low crop load canes (Fig. 6-5A, B).

6.3.4 HEXOSE TO SUCROSE RATIO IS AFFECTED BY CPPU AT BOTH CROP LOADS

Hexose to sucrose ratio was generally higher in CPPU-treated fruit throughout fruit development at both crop loads (Fig. 6-6).

In standard crop load trial differences were evident since early stages after the treatment whereas in low crop load trial, differences between CPPU-treated and control were shown after 70 DAA.

6.3.5 BOTH SUCROSE SYNTHASE AND VACUOLAR INVERTASE TRANSCRIPTS ARE CPPU AFFECTED

SUS1 transcript were generally higher in CPPU treated fruit at both crop loads, mainly in after-treatment stages (Fig. 6-7A, B). Also V-Inv3 and V-Inv4 transcript were higher in CPPU treated fruit early in fruit development (Fig. 6-7C, D, E, F).

Cytoplasmic invertase expression (A/N-InvD) was unaffected by CPPU-treatment whereas a low crop load enhanced early in development transcript levels (Fig. 6-7G, H).

6.3.6 CPPU EFFECT ON AGPASE SUBUNITS

AGPase Large subunit 4 (AGPL4) transcripts were higher in CPPU-treated fruit at 7 and 21 days after the treatment (35 and 42 DAA respectively) as shown in Fig. 6-8A-B. The other two subunits investigated did not show any appreciable change in expression levels between CPPU-treated and control fruit (Fig. 6-8C, D, E, F).

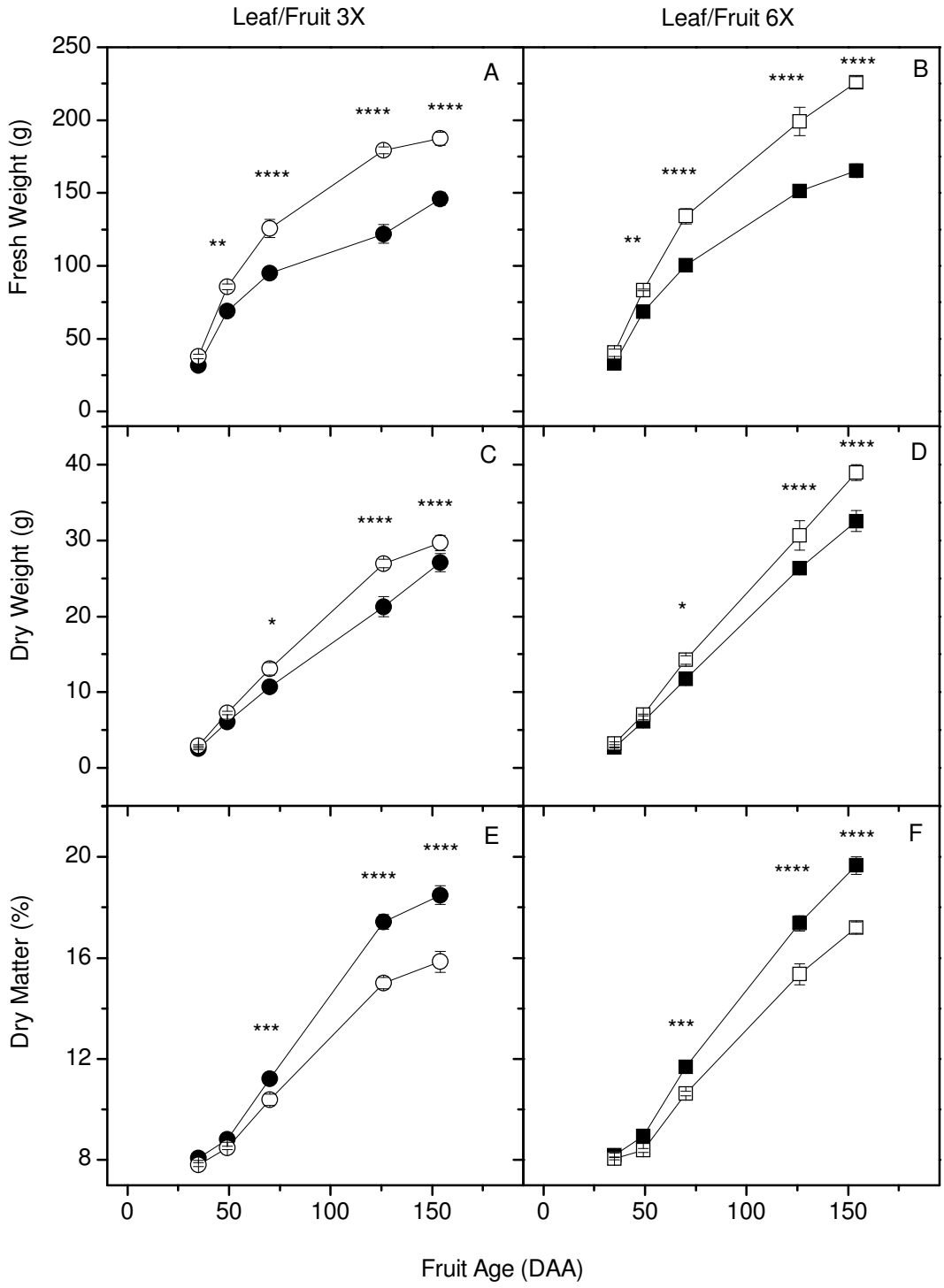


Fig. 6-1: Effects of CPPU on the growth of 'Hayward' kiwifruit at two different source to sink ratios. Fruit fresh weight (A, B), fruit dry weight (C, D) and dry matter (E, F) curves are here reported. n = 5 canes each time point. 3X leaf/fruit ratio (●, ○), 6X leaf/fruit ratio (■, □). Closed symbols are untreated fruit and opened symbols are CPPU treated fruit. Values are averages ±SE of the mean. Tests of Effect Slices: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; $p<0.0001$; blank, not significant.

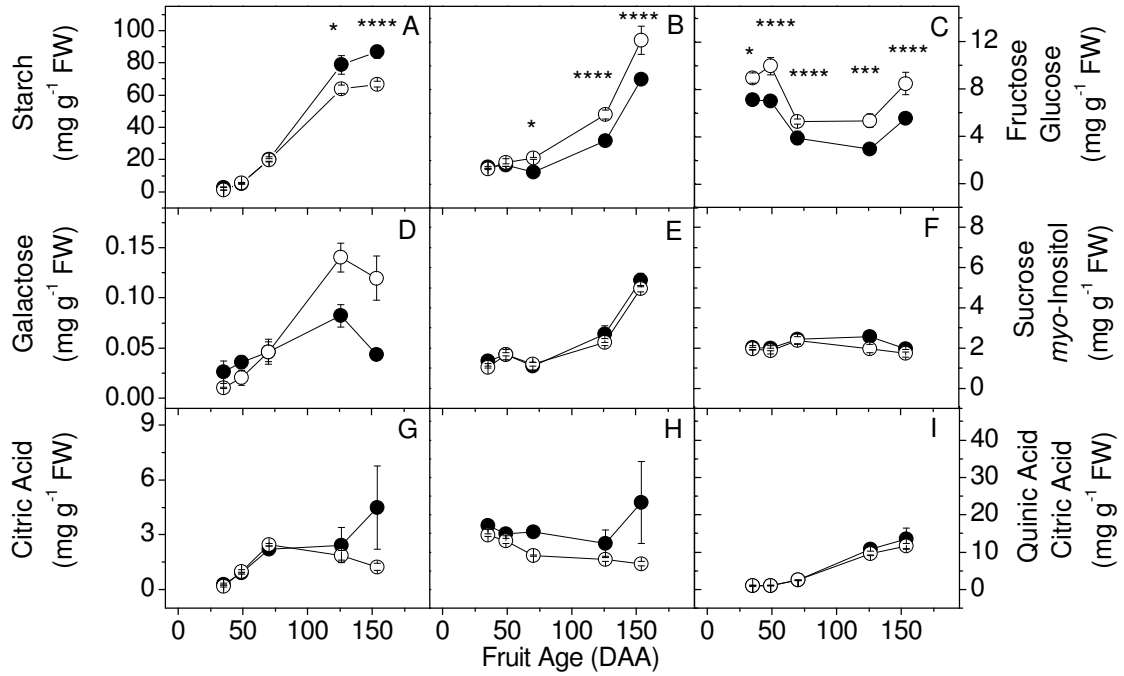


Fig. 6-2: Changes in carbohydrate and organic acid concentrations during fruit growth in of 'Hayward' kiwifruit at 3X leaf/fruit ratio. Untreated (●); CPPU treated (○). Starch (A), fructose (B), glucose (C), galactose (D), sucrose (E) and myo-inositol (F), malic acid (G), quinic acid (H) and citric acid (I) concentrations are here reported. n = 4 biological replicates each time point. Values are average ±SE of the mean. Tests of Effect Slices: *, p<0.05; **, p<0.01; ***, p<0.001; p<0.0001; blank, not significant.

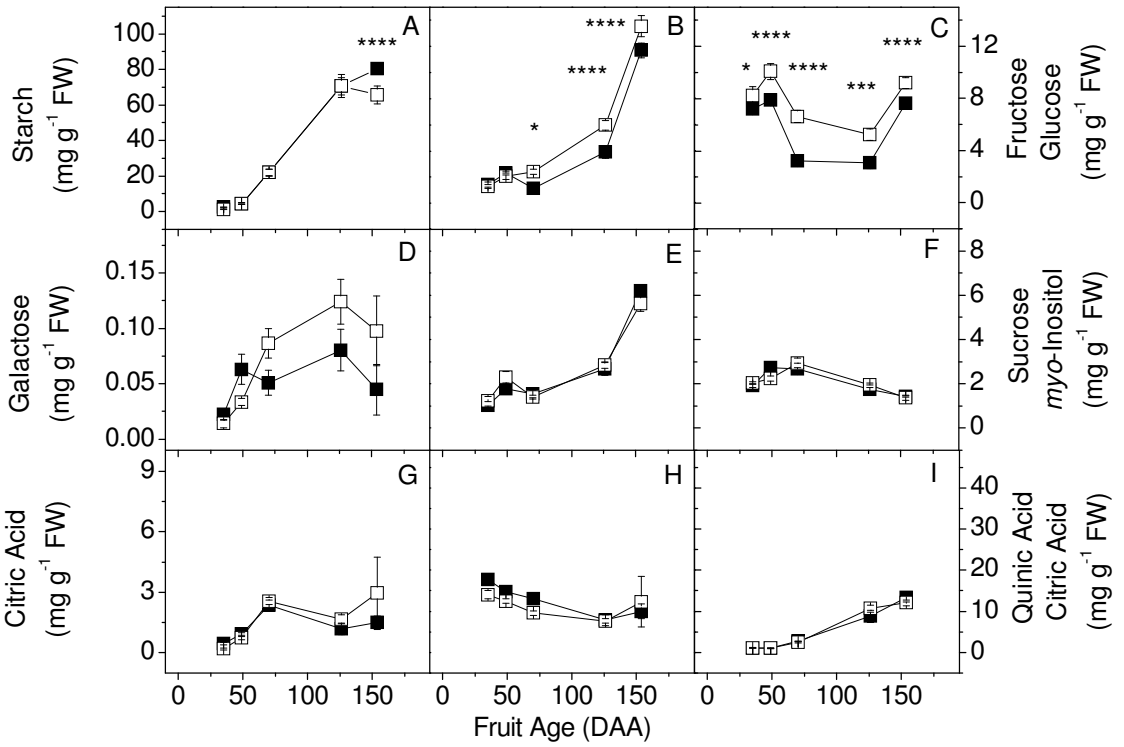


Fig. 6-3: Changes in carbohydrate and organic acid concentrations during fruit growth in of 'Hayward' kiwifruit at 6X leaf/fruit ratio. Untreated (■); CPPU treated (□). Starch (A), fructose (B), glucose (C), galactose (D), sucrose (E) and myo-inositol (F), malic acid (G), quinic acid (H) and citric acid (I) concentrations are here reported. n = 4 biological replicates each time point. Values are average ±SE of the mean. Tests of Effect Slices: *, p<0.05; **, p<0.01; ***, p<0.001; p<0.0001; blank, not significant.

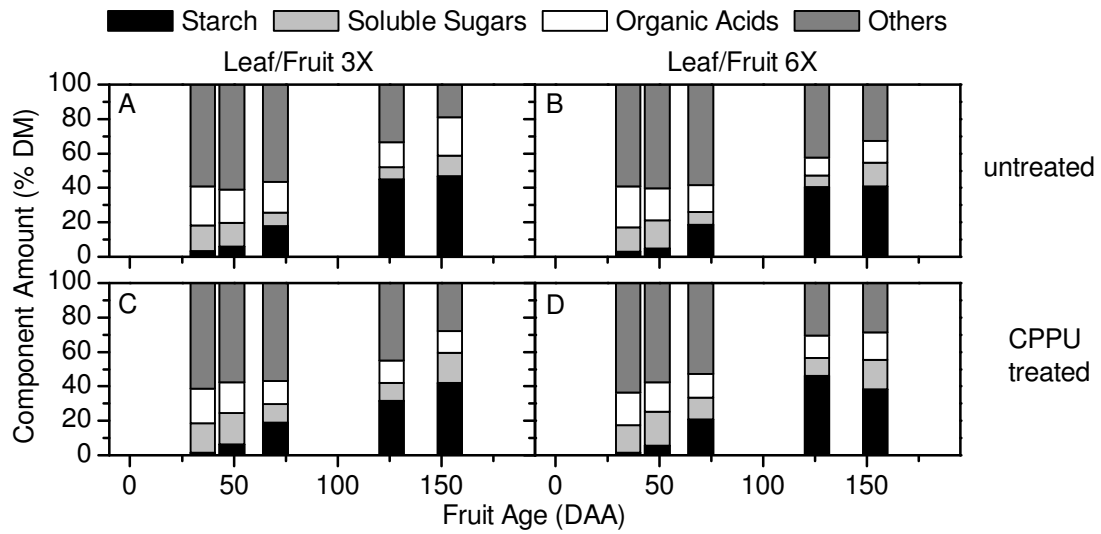


Fig. 6-4: Dry matter components during fruit growth in CPPU treated and untreated berries in both leaf/fruit 3X (●, ○) and leaf/fruit 6X (■, □). Dry matter components (starch, soluble sugars, acids and other non-detected compounds) are reported as a percentage of total dry matter.

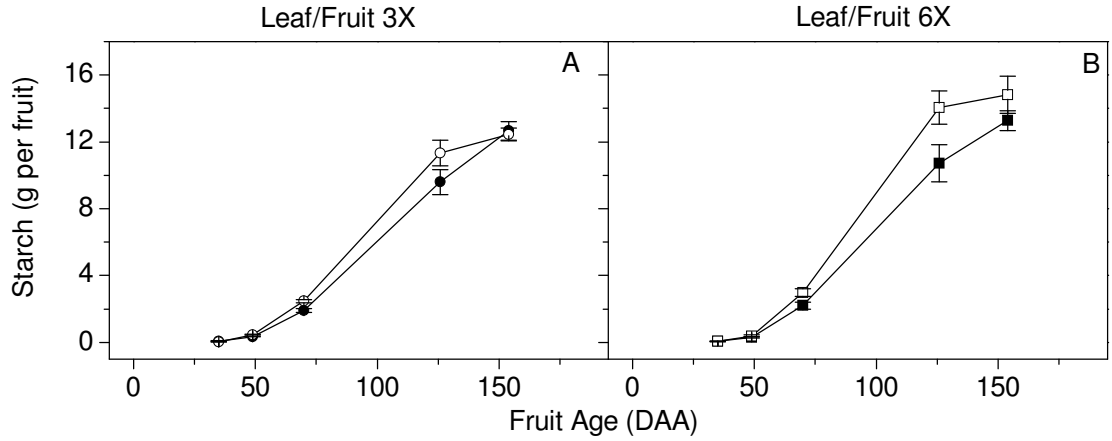


Fig. 6-5: Total starch per fruit in CPPU treated (opened symbols) and untreated (closed symbols) berries in both leaf/fruit 3X (A: ●, ○) and leaf/fruit 6X (B: ■, □). Values are averages ±SE of the mean.

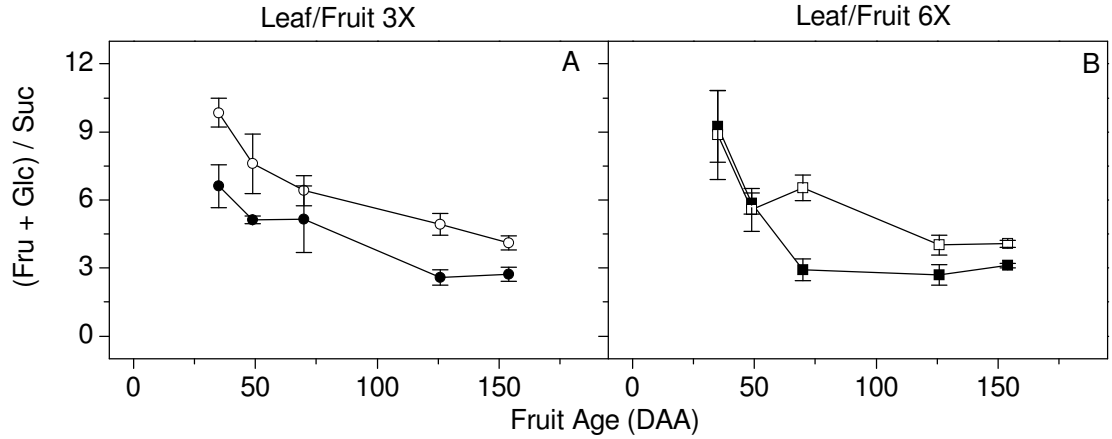


Fig. 6-6: Fructose plus glucose to sucrose ratio in CPPU treated (opened symbols) and untreated (closed symbols) berries in both leaf/fruit 3X (A: ●, ○) and leaf/fruit 6X (B: ■, □). Values are averages ±SE of the mean.

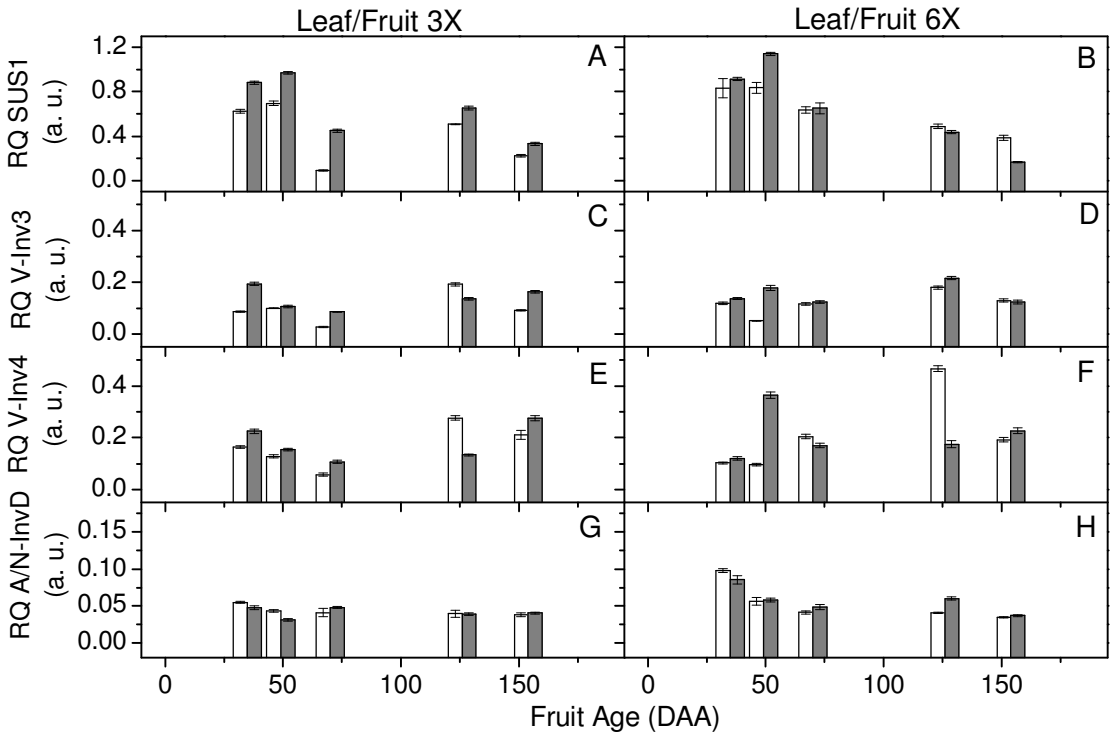


Fig. 6-7: Sucrose cleaving enzyme transcript expression patterns throughout fruit development of CPPU treated and untreated fruit, for both crop loads (control, white bars; CPPU, grey bars). Relative quantification (RQ) for susy (SUS1, A-B), vacuolar invertases (V-Inv3, C-D; V-Inv4, E- F) and cytoplasmic invertase (A/N-InvD, G-H) are here reported. Values are averages of 3 replicates ±SE of the mean. a.u. = arbitrary units.

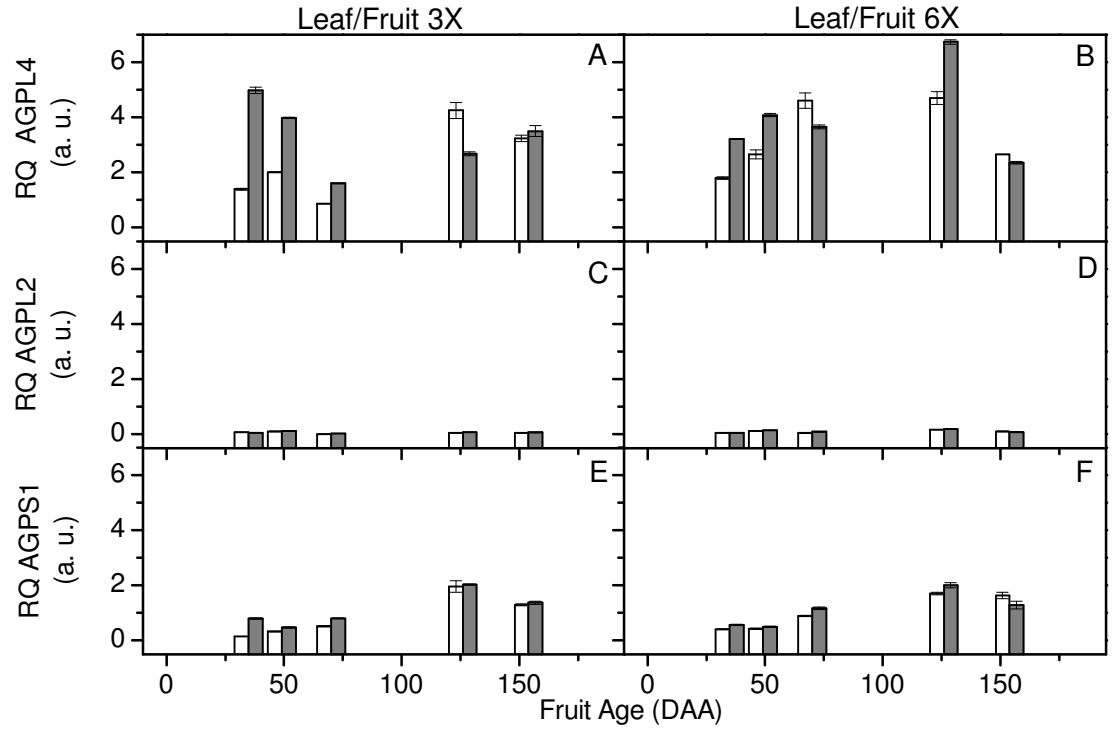


Fig. 6-8: AGPase enzyme transcript expression patterns throughout fruit development of CPPU treated and untreated fruit, for both crop loads (control, white bars; CPPU, grey bars). Relative quantification (RQ) for AGPase large subunit (AGPL4, A- B; AGPL2, C- D) and AGPase small subunit (AGPS1, E- F) are here reported. Values are averages of 3 replicates ±SE of the mean. a.u. = arbitrary units.

6.4 DISCUSSION

CPPU dramatically affected fruit size increasing berry fresh weight up to 50% more, whereas dry matter was reduced of about 15%. The change in dry matter at standard crop load was mainly the contribution of a change in starch concentration, whereas it was not observed when more sources were available. Per fruit starch content was enhanced by CPPU treatment, so were glucose, fructose and hexose to sucrose ratio. CPPU treated fruit shown higher transcript levels for SUS1, V-Inv3, V-Inv4 and APGL4 in the first few weeks after the treatment.

6.4.1 CPPU INCREASE FRUIT SIZE

The most obvious and widely reported CPPU effect is the dramatic fruit size increase, for kiwifruit as well for other fruit crop (Antognozzi *et al.*, 1996; Antognozzi *et al.*, 1997; Biasi *et al.*, 1991; Blank *et al.*, 1992; Hayata *et al.*, 2001; Hayata *et al.*, 1995; Hopping, 1976a; Kim *et al.*, 2006; Lewis *et al.*, 1996; Patterson *et al.*, 1993; Woolley *et al.*, 1991; Zabadal and Bukovac, 2006). The observed 25 and 50% increments at standard and low crop loads are in agreement with previous observation, but the girdling effect enhanced the average fruit size of all the compared treatments, being the control at standard crop load about 150 g. The combination of both CPPU and crop load ended into an about 70% higher fresh weight.

6.4.2 CPPU EFFECT IS SOURCE LIMITED

CPPU "side" and unwanted effect is a reduction in dry matter content (%), consistently observed in CPPU experiments (Antognozzi *et al.*, 1996; Patterson *et al.*, 1993). The control of source availability by girdling avoided photosynthate redistribution and showed an higher DM content (%) loss, up to 15-16%.

6.4.3 DRY MATTER REDUCTION CAN NOT COMPLETELY EXPLAINED BY A LOWER STARCH CONCENTRATION

Starch is one of the major component of dry matter in kiwifruit berry (Boldingh *et al.*, 2000; Richardson *et al.*, 1997), counting for about 50% of the total dry matter. It was previously showed that CPPU increases starch concentration in kiwifruit berry (Antognozzi *et al.*, 1996), although the dry matter content is reduced.

From the results here reported, there was no evidence of an increased starch concentration, whereas the loss of dry matter was followed by a decrease in starch concentration in standard crop load canes. In low crop load canes, as consequence of an higher carbon availability, starch did not differ between treated and control fruit. So a negative correlation between CPPU application and either dry matter or starch concentration was found.

Since all cytokinin responses are associated with growth or activation of biological processes, its involvement in carbohydrate supply has been suggested (Kuiper, 1993; Roitsch *et al.*, 2003). CPPU is a cytokinin-like compound so a similar effect can be expected. The

higher total starch content per fruit found on CPPU treated fruit in both crop loads, may suggest an higher absolute fruit capacity to attract carbohydrate.

6.4.4 SINK STRENGTH IS ENHANCED BY CPPU EARLY IN FRUIT DEVELOPMENT, BUT THEN FRUIT GROWS FASTER THAN ACCUMULATES CARBON

The higher hexose to sucrose ratio in CPPU treated fruit early in development suggests an involvement of invertases in sucrose cleavage. Transcripts level for both vacuolar invertase isoforms were found to be higher in CPPU treated fruit. CPPU effect on size was observed to be the effect of small cell enlargement in outer pericarp (Patterson *et al.*, 1993). Vacuolar invertases were shown to be affected by endogenous application of zeatin in maize (Ying *et al.*, 1999). So a CPPU effect on vacuolar invertases might affect fruit size by increasing cell vacuolation and the hexose to sucrose ratio. In fact, hexoses count for osmoregulation double than sucrose. Invertase reaction products are glucose and fructose.

SUS1 transcripts levels were higher in CPPU treated fruit. This founding contrasts with previous literature reports. In maize (Ying *et al.*, 1999) and *Chenopodium rubrum* (Ehness and Roitsch, 1997) the level of sucrose synthase transcript were not affected by cytokinin treatments, whereas MPIMP microarray experiments show a response of *Arabidopsis* seedling to kinetin application (MPIMP, 2008). Also Brenner and co-workers (2005) found sucrose synthases to be up-regulated by cytokinin applications. Sucrose synthase is a key enzyme not only for sucrose cleavage addressing activated glucose to starch synthesis, but it is also a source of carbon for cell wall carbohydrate synthesis (Amor *et al.*, 1995; Nakai *et al.*, 1999). Starch concentration was not increased in CPPU treated fruit, as expected to be as a consequence of an higher SUS1 expression. Dramatic fruit enlargement might require an higher synthesis of cell walls polysaccharides, so UDP-glucose could be more directed to cellulose synthesis rather than to starch accumulation.

The higher vacuolar invertase transcripts might affect the normal sucrose cleavage, and both sucrose synthase and vacuolar invertase compete for the same substrate. Glucose from invertase reaction is not activated, and a further reaction is required to drive it into starch synthesis.

AGPase large unit 4 transcripts were higher in CPPU treated fruit early in development. This is in agreement with the reported higher AGPase activity in CPPU treated fruit in Antognozzi *et al.* (1996). It has recently been suggested a role of a kinase (SnRK1) in the regulation of the signalling pathway of AGPase in *Arabidopsis* and an involvement of cytokinin in their transcriptional regulation (Chikano *et al.*, 2001; Tiessen *et al.*, 2003).

CPPU treated fruit showed a low dry matter content, but the biochemical and molecular determinants act differently from as previously observed in low dry matter genotypes.

Sink strength is affected by both sink size (cell number or size) and sink activity (carbon accumulation) (Ho, 1992). All the evidences here reported sustains a higher sink strength in CPPU treated fruit, in both sink size and sink activity. The key point is that the fruit growth

is faster than the carbohydrate accumulation, and furthermore a quote of carbohydrates might be used as energy source for higher respiration rates and structural modification that need to be supported.

6.5 CONCLUSIONS

CPPU increases sink strength, but as results here showed, sink grows faster than accumulate carbohydrates, and a quote of carbon is spent for fruit growth rather stored.

7 FINAL DISCUSSION

Kiwifruit quality and taste are strongly affected by at-harvest dry matter content or maximum starch concentration nearby the harvest time, or more generally by the capacity of the fruit to store carbon . Carbon metabolism in model plants has been deeply studied, so much is known to date about how tomato and potato deal with starch accumulation (Baxter *et al.*, 2005; Geigenberger, 2003; Kortstee *et al.*, 2007; Petreikov *et al.*, 2006). From literature investigations, however, it is easy to understand that carbon metabolism in storage organs is a complicate network of interaction finely regulated at each level (transcripton, translation, post-translation).

Kiwifruit carbon metabolism during fruit growth has been little investigated (Boldingh *et al.*, 2000; Richardson *et al.*, 1997; Walton and De Jong, 1990). Studies were mainly addressed to compositional characterization, and only few information are then available on the involved enzymes (Antognozzi *et al.*, 1996; Richardson *et al.*, 2004). Comparative studies on genotypes strongly different for the target characteristic have been shown to be highly efficient (Fridman *et al.*, 2004; Zrenner *et al.*, 1995). The approach is therefore addressed to the explanation of a clear and defined phenomenon. The present study was then approached in a similar way. Its point of strength is that it was possible find a break in the negative size-quality correlation (Dirlewanger *et al.*, 1999; Kortstee *et al.*, 2007) and have real carbon differences.

High and low dry matter genotypes were different since early in fruit development, and they differ in dry matter because starch was accumulated at different rates. Genotypes were consistently different in dry matter among several seasons, and differences were confirmed when genotypes were grafted on the same rootstock. All these elements bring to the conclusion that dry matter differences were genetic. Moreover they were not affected by dilution effect, being size-effect “neutralized” by equal distribution of size among the two different classes. The ability of a sink to attract carbon is strongly affected by two sink characteristics: one is its size, and the other is its activity (Ho, 1992).

Cell number (being cell type differentiation not a common trait to other fruit) is usually genetically determined (Olmstead *et al.*, 2007; Yamaguchi *et al.*, 2002). Studies, however, generally aim to explain differences in fruit size between genotypes or in the fruit ability to store carbon within a genotype (Jullien *et al.*, 2001), but do not combine the effect of both.

From the anatomical investigation, high and low dry matter genotypes were different in outer pericarp volume proportion occupied by small cells and large cells. Small cells were therefore occupying a larger proportion of volume in the high dry matter genotype outer pericarp. However cell size and cell number were found to be highly genotype-specific, for both the cell types. Then, the right combination of size and number of each cell type ends into a starch concentration difference. From the calculation of which is the incidence of those observed differences in terms of starch, it was shown that it counted only for half of the more starch found in high dry matter genotypes. This is true, unless high and low dry

matter genotypes differ in small cell starch concentrations. In fact, from a rough calculation, 10-20% more starch in small cell fits with the observed differences in at-harvest concentrations. These structural differences suggested sink activity could be unlike. Sink activity is the ability of an organ to accumulate carbon (Ho, 1992). In starch accumulating organ, as kiwifruit is, a lot of attention has therefore been addressed to the sucrose-to-starch metabolic pathway. Sucrose phloem unloading, sucrose cleavage and starch synthesis are the steps through which carbon goes once it leaves the sieve element. Starch-synthesising tissues and organs simultaneously expand, respire and store carbon (Quick and Schaffer, 1996). Organ-tissue expansion is dispatched in cell expansion that requires cell wall polymers' synthesis: carbon is therefore included in those polymers. Respiration is required to produce energy to support metabolism, and organic carbon is therefore turned into water, carbon dioxide and energy. When carbon is stored, simple sugars are reversibly converted to insoluble polymers (in starch storing organ); such a process can be seen as a delayed carbon utilization.

Sucrose is the main carbohydrate translocated in kiwifruit from source leaves to sink organs (Klages *et al.*, 1998). Sucrose phloem unloading strategy used by kiwifruit is still unknown, however, from literature findings and from the actual work, some consideration can be done. Plasmodesmata densely populate kiwifruit cells at either early stages (data not shown) or late in fruit development (Sutherland *et al.*, 1999). From the HortResearch *Actinidia* EST database there is no evidence of putative sequences for gene encoding sink expressed cell wall invertases. Sucrose synthase gene (SUS1), the key enzyme isoform driving the symplasmic unload, is highly expressed in kiwifruit early in fruit development as observed by Richardson *et al.* (2004) in 'Hayward' kiwifruit and confirmed in the actual work for both 'Hayward' and specific *A. deliciosa* genotypes. At the state of the art, sucrose unloading in kiwifruit berry is more likely to be simplasmic at least early in fruit development. Furthermore, sucrose synthase enzyme gene expression is promoted by anoxic conditions (Zeng *et al.*, 1998). From kiwifruit to the apoplasmic-unloader apple (late in fruit development) comparison in intercellular space volume (2% to 20% respectively), it can be assumed that kiwifruit berry oxygen levels might be quite low. In low oxygen conditions, respiration rate is low, and therefore energy must be saved. Sucrose cleavage operated by sucrose synthase is more energy conservative than the invertase one (Koch, 2004).

In several starch storing sink the key enzyme involved in sink strength determination, sucrose-unloading and sucrose-cleavage is sucrose synthase (SUSY) (Geigenberger and Stitt, 1993; Quick and Schaffer, 1996). Sucrose synthase activated product, UDP-glucose, plays a central role for the previously reported sink functions. UDP-glucose can be addressed into starch biosynthetic pathway, by conversion into glucose-phosphate (Wang *et al.*, 1993); UDP-glucose is the substrate for cellulose synthase, the main component of cell wall in several species (Amor *et al.*, 1995; Delmer and Amor, 1995); UDP-glucose can be also routed toward the glycolysis and respiration pathways (Quick and Schaffer, 1996).

Kiwifruit adopts the not-osmotically active carbon storage strategy, accumulating starch for most of its fruit development (Okuse and Ryugo, 1981). The results showed the SUS1 gene

to be more expressed early in fruit development in high dry matter genotypes rather than in low dry matter genotypes. The microarray results suggested that cellulose synthase was more expressed in low dry matter genotypes. It can be therefore speculated that a reduction of starch accumulation can be the effect of both a reduction in sucrose synthase gene expression and a differential carbon flow between high and low dry matter genotypes.

The second committed step of the sucrose-to-starch pathway is the ADP-glucose synthesis, operated by AGPase (Stark *et al.*, 1992). AGPase is an highly regulated enzyme throughout its 'biosynthetic pathway', from transcription to post-translational allosteric-modulation and redox-modification, as reviewed recently by Geigenberger (2003). High and low dry matter genotypes resulted to be different since early in fruit development in AGPL4 (large subunit 4) transcripts, being the gene more expressed in high dry matter genotypes. From the microarray experiment thioredoxin transcripts were differentially expressed, suggesting a possible role in post-transcriptional regulation of the enzyme similar to the potato one (Ballicora *et al.*, 2000).

Vacuolar invertases are known cleave the sucrose inside the vacuole (Husain *et al.*, 2001). Also starch storing sink, early in fruit development, the expansion stage prior the onset of net starch accumulation is driven by vacuolar invertases, in both tomato (Yelle *et al.*, 1988) and potato (Ross *et al.*, 1994). Large parenchyma cells are largely vacuolated compared to the small ones (IC Hallett, unpublished data), and results obtained in the present study showed that low dry matter genotypes have more outer pericarp volume occupied by those cells. It is therefore possible that the generally higher vacuolar invertase gene expression and activity observed early in development drives this low dry matter genotype feature. Vacuolar invertases might be then more competitive in low dry matter genotypes *versus* sucrose synthase for the same substrate (sucrose). Sucrose is transferred into the vacuole by tonoplast sucrose transporters. In a recent review on sucrose transporters, Sauer (Sauer, 2007) argued on the role of group 4-type sucrose transporters, about their tonoplast localization, as reported in a study on barley leaves (Endler *et al.*, 2006), and the sieve element (SE) vacuolar localization in potato tuber (Weise *et al.*, 2000), being this odd having SE no vacuoles at all. A differential role of a tonoplast sucrose transporter (group 4-type) can be hypothesized as well among the two dry matter classes.

CPPU has been considered as a final exogenous 'tester' of the genotypes' observation. CPPU is well known for its effects on increase fruit size and decrease dry matter content (Antognozzi *et al.*, 1996; Paterson *et al.*, 1991). It was then hypothesized that in CPPU-treated fruit the mechanism that limits carbon accumulation was similar to that observed in low dry matter genotypes, but it was not. Chapter 6 physical measurement and carbohydrate content results behaved similarly to those reported in literature and they were also comparable to low dry matter genotypes. From the gene expression profile, however, CPPU-treated fruit were more similar to high dry matter genotypes. These results lead to the conclusion that CPPU increase sink strength, but physical growth and carbon metabolism are unbalanced to the physical growth. When fruit is dramatically promoted, more energy for

metabolic functions and more carbon for the cell wall build up are thereafter needed. There might be a factor that drives UDP-glucose more in cellulose biosynthesis than in starch.

Kiwifruit sucrose-to-starch metabolic pivotal role might be attributed to SUSY and to the fate of its activity product UDP-glucose. The interaction of other factors in the complicate network might be able to change the equilibrium and end in a different capacity to store carbon as starch.

It is not however excluded that observed differences in starch might occur as a consequence of a pleiotropic affect of one changed gene, which affect more than one trait and therefore several genes (Falconer and Mackay, 1996).

7.1 FUTURE PERSPECTIVES

From the present study is then more clear that starch accumulation and dry matter content in kiwifruit are under polygenic control (Cheng *et al.*, 2004), as many quantitative traits are.

From the analysis of the results, however, more investigations are required to a better understating of the overall carbon metabolic network.

One of the first point that need a clear answer is which phloem unloading pathway is followed by kiwifruit berry during the overall fruit development. Another unanswered question is about cell wall invertases. Even if some activity was shown, no evidence of cell wall invertases gene sink expressed was found. A study should be then conduct trying to find the DNA region coding for the catalytic domain of *LIN5*-type cell wall invertase, usually conserved among the species.

In the present study, most of the enzyme were investigated at gene transcription levels. More enzyme activity, as well as metabolites, experiment should be carried out in the future. Other enzyme and regulation factors might be critical for kiwifruit sucrose-to-starch pathway. What is the oxygen content in growing kiwifruit and how does it affect SUSY gene expression? Is the thioredoxin gene involved in post-translational regulation of redox properties of AGPase enzyme? What is the role of cell wall carbohydrate synthesis in carbon metabolism?

As target genes are well identified, for a better analysis of their specific role in the overall starch pathway, antisense transgenic plants might be generated, either with model plants or kiwifruit.

The identification of those target genes could help to find markers, useful to see how they segregate against quantitative trait loci (QTL) for starch and size. It might be possible therefore to break the negative starch-size correlation.

7.2 CONCLUSIONS

Sucrose synthase and AGPase roles, at least at gene expression level, are important to drive kiwifruit starch accumulation. Results obtained in the present study suggest a strong contribute of both enzyme in the sucrose to starch conversion process. Differences in their

isoform gene expression pattern early in fruit development affect the rate at which starch is thereafter accumulated. Starch content and kiwifruit taste are tightly connected, and the key to improve kiwifruit organoleptic traits might be found in one of these two enzyme regulatory pathways, or in the enzyme themselves.

Appendix I: List of Chemicals and Lab Consumables

- **0.2 mL PCR micro-tubes** (Axygen, Cat. #PCR-02-L-C)
- **1 Kb Plus DNA Ladder™** (Invitrogen, Cat. #10787-018).
- **1.5 mL micro-tubes** (Axygen, Cat. #MCT-150-C)
- **10 mM dNTP Mix** (Invitrogen, Cat. #18427-013)
- **10 µL Filtered Tips** (Axygen, Cat. #TF-10-L-R-S)
- **1000 µL Filtered Tips** (Axygen, Cat. #TF-1000-L-R-S)
- **10X TBE Solution** (Invitrogen, Cat. #15581-028)
- **13 mL tubes** (Sarstedt, Cat. 62.515.006)
- **2 mL Safe Lock Eppendorf tubes** (Eppendorf, Cat. #0030 120.094).
- **20 µL Filtered Tips** (Axygen, Cat. #TF-20-L-R-S)
- **200 µL Filtered Tips** (Axygen, Cat. #TF-200-L-R-S)
- **2-Mercaptoethanol** (Sigma, Cat. #M3148)
- **AB SYBR® Green PCR Master Mix** (Applied Biosystems, Cat. #4309155)
- **Absolute Cold Ethanol** (Merck, Cat. #1.00983.2500)
- **Absolute Cold Ethanol** (Merck, Cat. #1.00983.2500)
- **Acetic Acid** (Merck, Cat. #1.00063.2500)
- **Adonitol** (Sigma, Cat. #A5502)
- **Ampicillin sodium salt** (Calbiochem, Cat. #171254)
- **Amyloglucosidase** (Sigma, Cat. #A7255)
- **ATP** (Sigma, Cat. #A-2383)
- **Barium hydroxide** (AnalR, Cat. #10048)
- **Bibasic sodium phosphate** (Merck AnalR, Cat. #10249.4C)
- **Broth Base** (Invitrogen, Cat. #12780-052)
- **BSA** (Sigma, Cat. #A3156)
- **Chloridric Acid** (Merck, Cat. #1.00312.2500)
- **Chloroform** (Merck, Cat. #1.02445.2500)
- **COSTAR UV-Plates** (Corning, Cat. #3635)
- **CTAB** (Merck, Cat. #1.02342.1000)

- **Cy dye NHS esters** (GE Healthcare)
- **Cy3 or Cy5 flourescent dye** (GE Healthcare)
- **DB 1701 30 m column** (Agilent J&W)
- **DTT** (Gibco BRL, Cat. #15508-013)
- **EDTA** (Sigma, Cat. #E5134)
- **EGTA** (Sigma, Cat. #E0396)
- **Ethanol, absolute** (Merck, Cat. #1.00983.2500)
- **Ethidium bromide, EtBr** (Sigma, Cat. #E1510)
- **Falcon™ 13 mL Conical Tube** (DB Bioscience, Cat. #352096)
- **Falcon™ 50 mL Conical Tube** (DB Bioscience, Cat. #352070)
- **Ferric Ammonium Sulphate** (BDH GPR, Cat. #27164.4M)
- **Glucose oxidase** (Sigma, Cat. #G6125)
- **Glucose Standard solution** (Roche, Cat. #0716260).
- **Glutaraldehyde** (Merck, Cat. # 1.04239.0250)
- **Glycerol** (Scharlaur, Cat. #GL0026)
- **HEPES** (Sigma, Cat. #H3375)
- **Hexokinase/Glucose-6-Phosphate Dehydrogenase, HK/G6P-DH** (Roche, Cat. #10 737 275 001)
- **Imidazole** (Sigma, Cat. #I0125)
- **IPTG** (Sigma, Cat. #I6758)
- **Isoamylacohol** (Merck, Cat. #1.00979.1000)
- **Lithium Chloride** (Sigma, Cat. #L9650)
- **Low DNA Mass Ladder** (Invitrogen, Cat. #10068-013).
- **Magnesium Chloride, MgCl2 • 6H20** (Sigma, Cat. #M0250)
- **MAX Efficiency® DH5□TM Competent Cells** (Invitrogen, 18258-012)
- **MicroAmpTM Fast Optical 96-well reaction plate** (Applied Biosystems, Cat. #4346906)
- **MicroAmpTM Optical Adhesive Film** (Applied Biosystems, Cat. #4360954)
- **Microarray epoxy slides** (MWG)
- **Minisart 20 μm filter** (Sartorius, Cat. #16534K)
- **Monobasic sodium phosphate** (Merck AnalaR, Cat. #10245.4R)

- **MOPS** (Sigma, Cat. #M1254)
- **$NADP^+$** (Sigma, Cat. #N-0505)
- **Nalgene Steriltop filter** (Millipore, Cat. #SCG PT02RE)
- **N-methyl-N-(trimethylsilyl) trifluoroacetamide** (MSTFA Thermo Scientific, Cat. #48913)
- **Oligo$(dT)_{20}$ Primers** (Invitrogen, Cat. #18418-020)
- **Paraformaldehyd**e (Merck, Cat. #1.04005.1000)
- **Peroxidase** (Sigma, Cat. #P8250)
- **Petri dishes 60 mm diameter** (Sarstedt, Cat. #821194)
- **Petri dishes 92 mm diameter** (Sarstedt, Cat. #821472)
- **Platinum® Taq DNA Polymerase** (Invitrogen, Cat. #10966-034)
- **PVP-40 K30** (Sigma, PVP40)
- **PVPP** (Sigma, Cat. #P6755)
- **Pyridine** (Sigma, Cat. #P57506)
- **QIAprep® Spin Miniprep Kit** (Qiagen, cat. # 27104)
- **Radprime labeling kit** (Invitrogen)
- **Ribonuclease H** (Invitrogen, Cat. #18021-014)
- **RNA 6000 Ladder** (Ambion, Cat. #7152)
- **RNA 6000 Nano LabChip Kit** (Agilent, Cat. #5065-4476):
- RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen, Cat. #10777-019).
- **RNeasy® midi kit** (Qiagen, Cat. #75144)
- **RQ1 RNase-Free DNase** (Promega, Cat. #M6101)
- **SDS** (Calbiochem, Cat. #42823)
- **Sephadex G-25 Medium' PD-10 Desalting Columns** (Amersham Bioscience, Cat. #17-0851-01)
- **Sephadex ion exchange column** (Sigma, Cat. #Q25120)
- **Sephadex ion exchange column** (Sigma,, Cat. #SPC25120)
- **Sodium Acetate** (Sigma, Cat. #S2889)
- **Sodium Chloride** (Merck, Cat. #1.06404.0500)
- **Sodium hydroxide, NaOH** (Merck, Cat. 1.06498.1000)
- **Spermidine** (Sigma, Cat. #S2501)

- **Sucrose** (Applichem, Cat. #A2211.0500)
- **SuperScriptTM III Reverse Transcriptase** (Invitrogen, Cat. #18080-44).
- **Tartrate** (Sigma, Cat. #T0375)
- **Trimethylsilylimidazole** (Aldrich, Cat. #153583)
- **Tris** (Merck, Cat. #1.08382.0500)
- **Triton® X-100** (BDH, Cat. #306324N)
- **Vector pGEM®-T Easy** (Promega, Cat. #A1360)
- **X-Gal** (Invitrogen, Cat. #15520-034)
- **Zinc Sulphate** (BDH, Cat. #306214Y)

Appendix II: List of Equipments and Software

- **7500 Fast System SDS software**: Applied Biosystems, version 3.1
- **Autoclave sterilizer** (Hirayama, Hi-Clave HV-85L)
- **Bench-top Centrifuge:** Heraeus, Multifuge 3S-R
- **Bioanalyzer**: Agilent 2100 Bioanalyzer from Agilent Technologies
- **Camera Control Unit**: Nikon, SU-1
- **Cycler:** Eppendorf, Mastercycler®
- **Dehydrator**: Ezidri™ Home Food Dehydrator model Ultra FD 1000 from Hidraflow Industries Limited (NZ)
- **Digital 3 decimal balance**: Mattler Toledo, PB303-S/FACT
- **Digital Camera** (Nikon, Coolpix 990)
- **Dry-bath**: Thermoline Scientific, Dry Bath
- **Gas chromatograph**: Hewlit Packard ,6890 GC system
- **Gel electrophoresis apparatus**: BioRad, SubCell® GT
- **Genepix 4 software** (Axon).
- **Genepix 4000B scanner** (Axon).
- **Laminar Flow Hood** (Total Air Care Ltd., Aircare900)
- **Lucidea Hybridization machine** (GE Healthcare)
- **Micro-Centrifuge:** Eppendorf, 5415R
- **Nuclease-free water machine** (BarnStead, NanoPure Diamond™)
- **Oligo's printer** (Biorobotics II robot)
- **Real-Time PCR**: 7500 Fast Real-Time PCR System from Applied Biosystems
- **Sequence Detection Software**: 7500 Fast System SDS software, version 3.1, Copyright© 2001-2004 Applied Biosystems
- **Softmax Pro**: Molecular Devices, v 3.1.2
- **Spectramax micro-plates spectrophotometer:** Molecular Devices, Spectramax®Plus384
- **Spectrophotometer**: NanoDrop® ND-1000 UV-Vis Spectrophotometer from NanoDrop Technologies
- **Stereo-microscope**: Nikon, SMZ1500
- **UV Transilluminator:** Biorad, Gel Doc 2000
- **UV-Vis Spectrophotometer:** Shimadzu, UV-160A

- **Vector NTI Version 9.0.0:** Invitrogen, Cat. #12605050
- **Water-bath:** Julabo, SW21

Appendix III: Plant Pedigree Summaries

In the following pages figures of plant pedigree summaries are reported for each breeding population used. Family B16 and A16 are from the same breeding population, but a summary is reported for each family separately.

Here some abbreviation listed in the pedigree summaries are reported:

TP Blk: position in Te Puke Research Centre orchard

Plant Yr: plantation year

aka: also known as, internal breeding group code

DA: Actinidia deliciosa

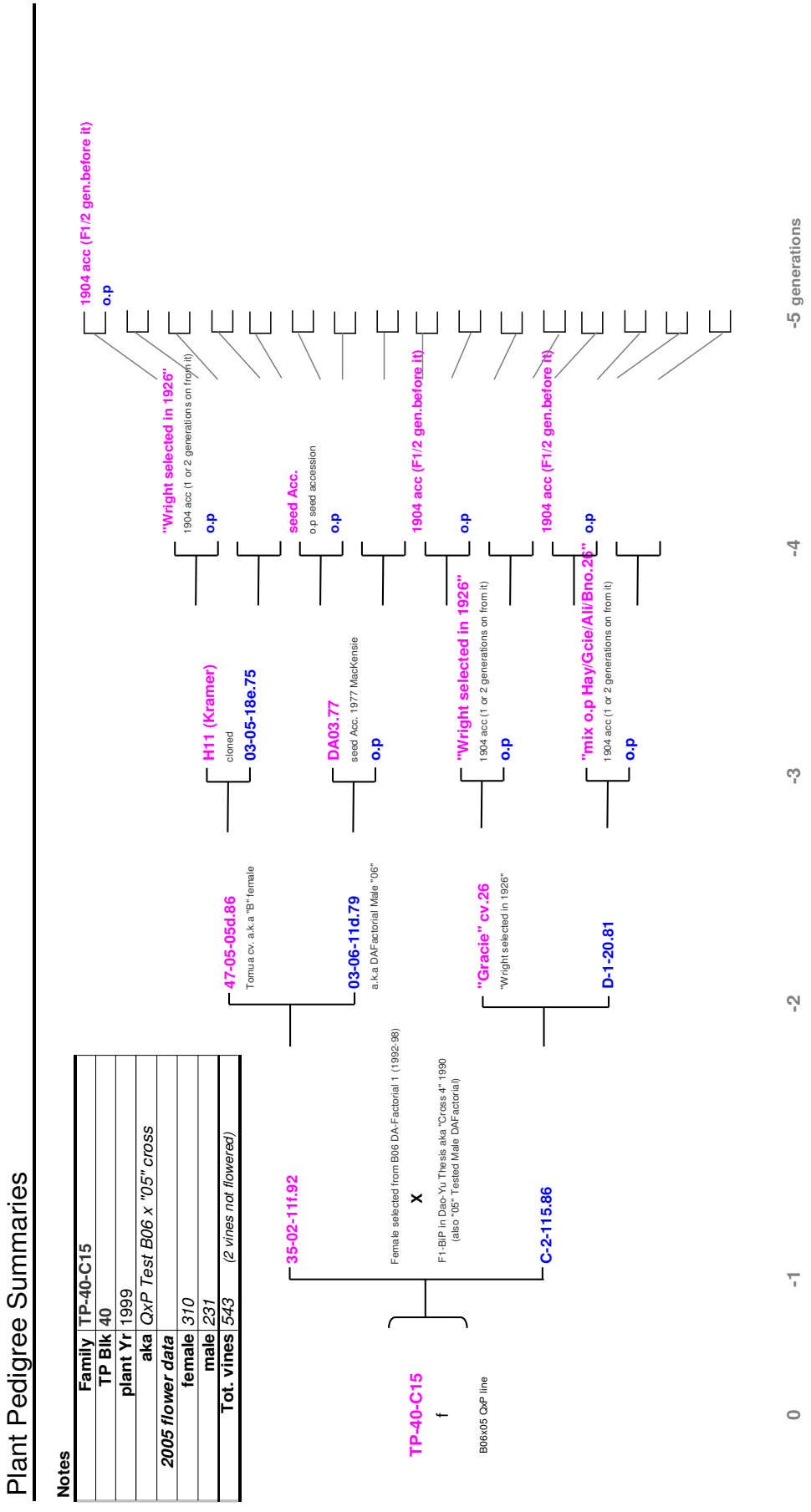


Fig. Appendix III 1: Plant Pedigree summary Family C15. Female platns are in the upper branch, whereas males are in the lower one.

Notes

Family	**TP-40-A01…A03,A05…A07,A09...A14**
TP Blk	**40**
plant Yr	1999
aka	*F2 - Male Progeny Test - ex"F05"*
2005 flower data	
female	*213*
male	*234*
Tot. vines	*451* *(4 vines not flowered)*

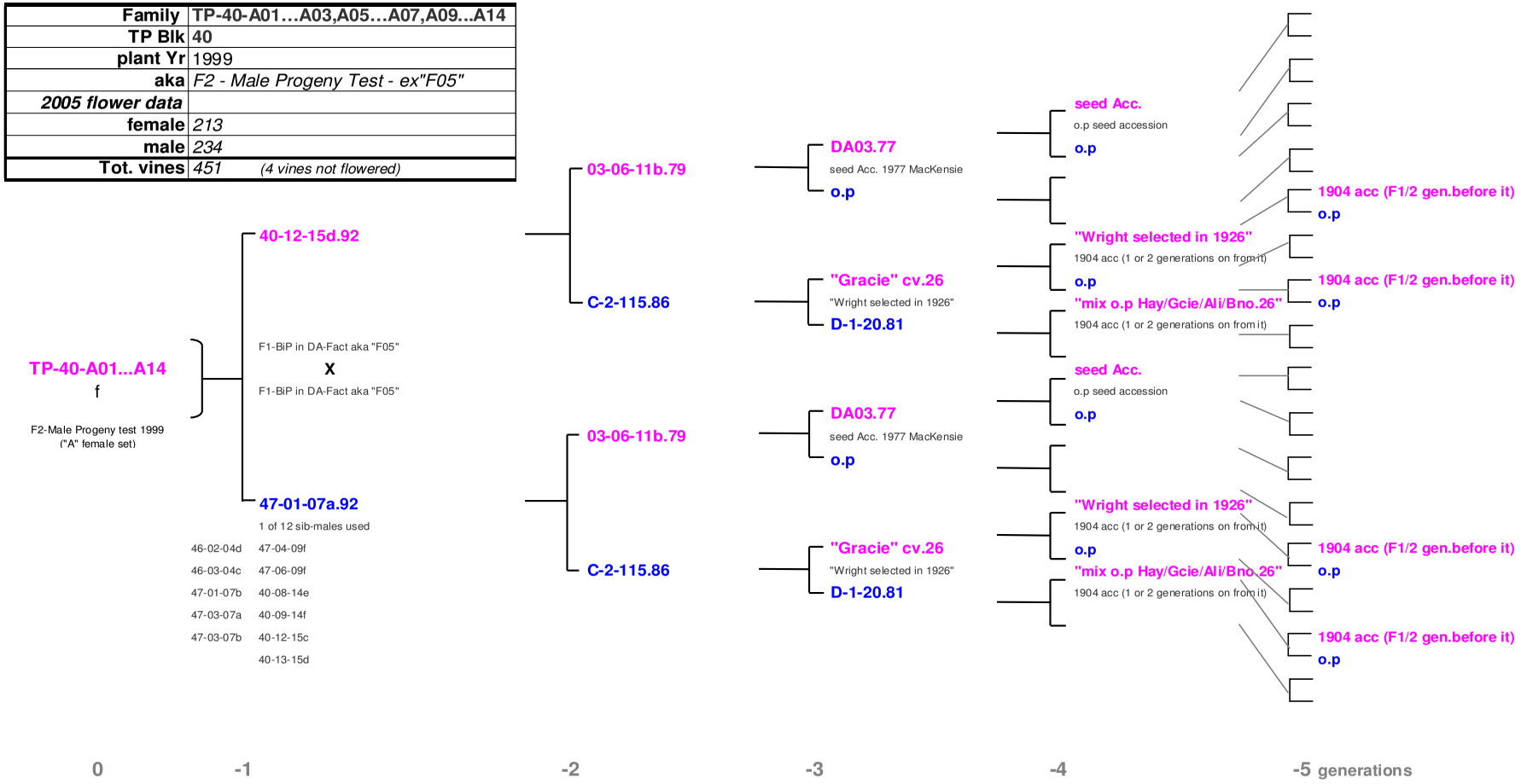


Fig. Appendix III 2: Plant Pedigree summary families A01…A03, A05…A07, A09…A14. Female platns are in the upper branch, whereas males are in the lower one.

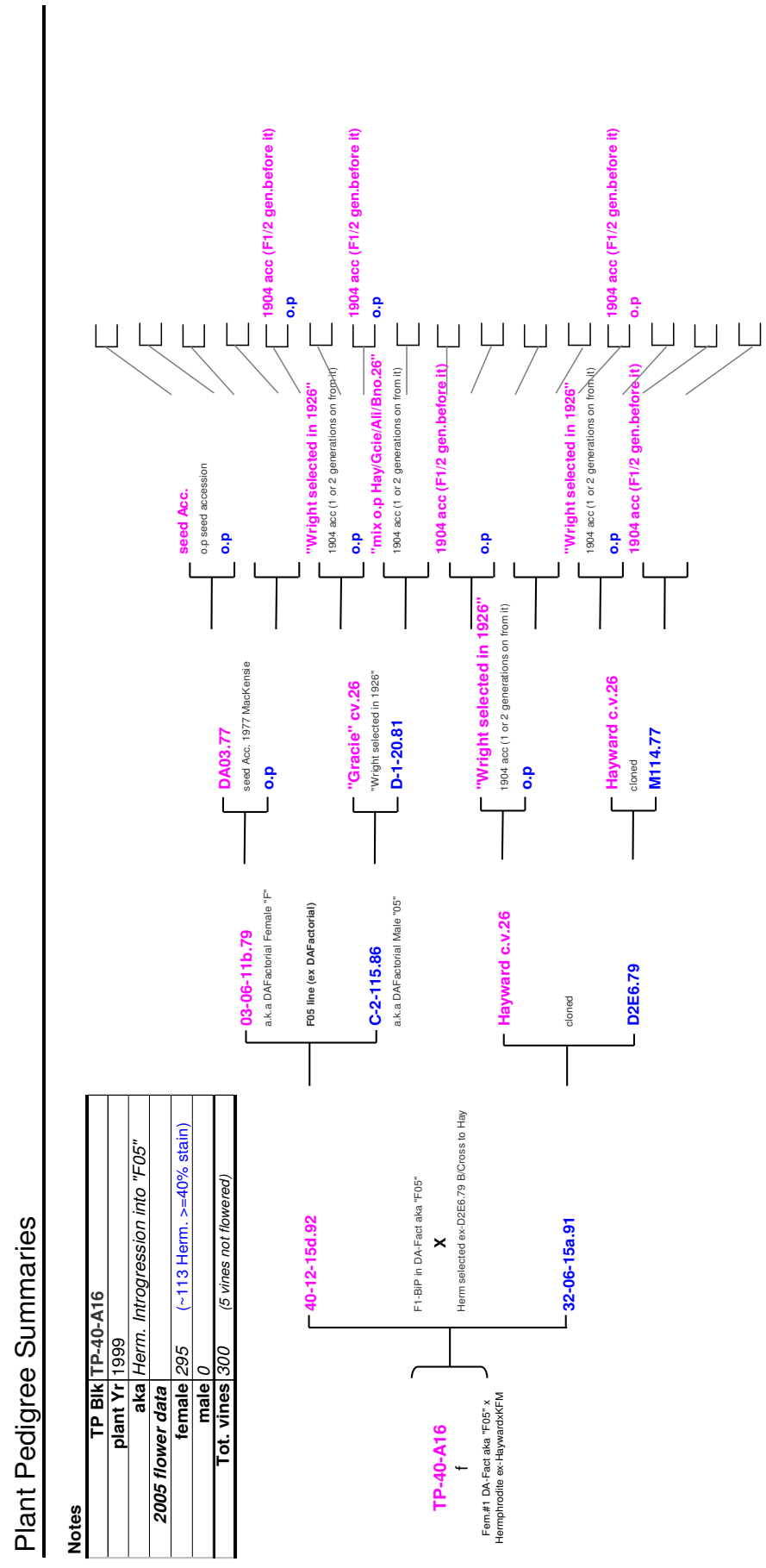


Fig. Appendix III 3: Plant Pedigree summary family A16. Female platns are in the upper branch, whereas males are in the lower one.

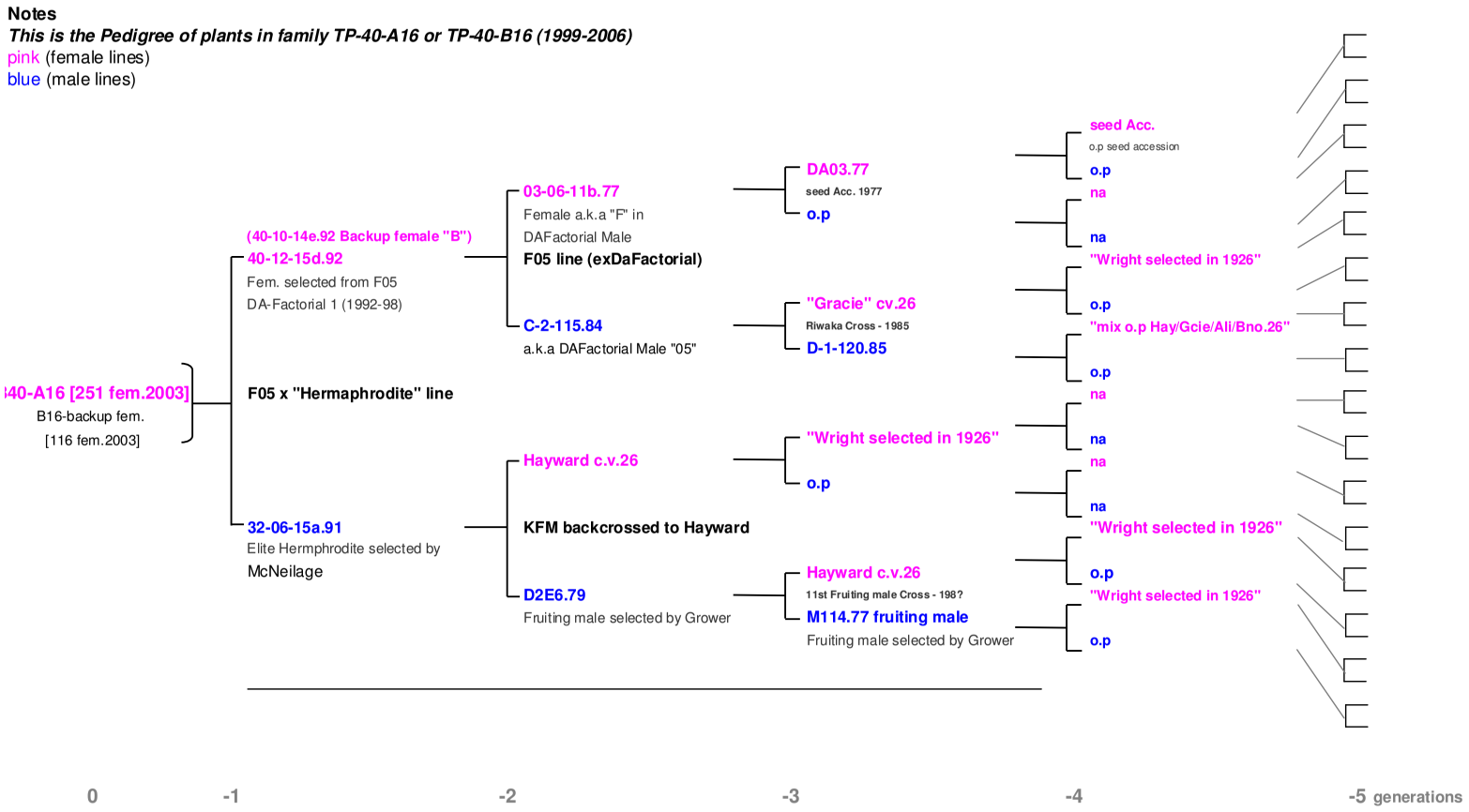


Fig. Appendix III 4: Plant Pedigree summary family B16. Female platns are in the upper branch, whereas males are in the lower one.

Appendix IV: Solutions

100 g/L - Ampicillin solution

For a 50 ml 100 g/L Ampicillin solution, weigh 5 g of Ampicillin (Calbiochem, Cat. #171254), place it in a Falcon tube and add water up to 50 ml. Dissolve completely. Filter sterilize (Maker, Cat. #). Make 1.5 mL aliquots and store in -20 ºC freezer.

Aqueous Tannic Acid Solution – 5% v/v

Formula: $C_{76}H_{52}O_{46}$

MW: 1701.20 g/mol

Dissolve 5 g of Tannic Acid in 80 mL of distilled water. Top up the volume with water.

Benzoate Buffer 20 mM, pH 4.4

Benzoic acid MW: 122.12 g/mol

Sodium benzoate MW: 144.11 g/mol

Dissolve 2.5 g/L of Benzoic Acid and 2.9 g/L of Sodium benzoate in water. Check pH, it should be 4.4, otherwise adjust with one of the component.

BSA - 2% w/v Solution

- For a 2% w/v solution, weigh 20 mg/mL of BSA (Sigma, Cat. #A3156). Weigh 2 g and dissolve in water up to 100 mL. Make 14 mL aliquots and store in -20ºC.

Chloroform:IAA - 24:1 Solution

Formulas: $CHCl_3$ (Chloroform) - $C_5H_{11}OH$ (Isoamylalcohol)

For a 500 mL solution mix 480 mL of Chloroform (Merck, Cat. #1.02445.2500) and 20 mL of IAA (Merck, Cat. #1.00979.1000) into a baked bottle.

Desalting and Equilibrating buffer (Invertases Extraction)

Make the buffer solutions fresh from stock solutions each day.

Stock solutions required are reported in Tab. Appendix IV- 1. Starting and final concentrations are also shown.

Desalting buffer contains the Protease inhibitor cocktail whereas the Equilibrating one does

not.

Tab. Appendix IV- 1: Desalting and Equilibrating buffer solutions: amounts of each reagent or solution needed are reported.

Reagents	[Stock solutions]	[Buffer concentration]	for 200 mL of buffer (mL)
MOPS solution	1 M - pH8	0.2 M	40.0
$MgCl_2$ solution	100 mM	20 mM	40.0
EDTA solution	5 mM pH 8	1 mM	80.0
Triton X-100 solution	0.5%	0.05%	20.0
BSA solution	2%	0.1%	10.0
Glycerol		3.3%	6.0
Water			4.0
	at half solution (desalting buffer) add protease inhibitors		
Complete Protease inhibitor	Roche, Cat. #11697498001		½ tablet

DTT - 0.1 M SOLUTION

Formula: $C_4H_{10}O_2S_2$

MW: 154.25 g/mol

- For 10 mL 0.1M solution weigh 0.15425 g of DTT (Gibco BRL, Cat. #15508-013). Dissolve in water. Store in fridge for a week, or aliquot and store in -20C for 1 month.

EDTA – 0.5 M PH 8 SOLUTION

Formula: $C_{10}H_{16}N_2O_8$

MW: 372.24 g/mol

For a 250 mL 0.5M solution, dissolve 46.53g of EDTA (Sigma, Cat. #E5134) in 200 mL of distilled water in a beaker. Add 5g of NaOH (Merck, Cat. 1.06498.1000). Add water up to 230 mL. Adjust pH with 1N NaOH up to 8. Bring the volume to 250mL. Autoclave if occurred. Store in the dark.

EGTA - 0.5 M PH 8 SOLUTION

Formula: $C_{14}H_{24}N_2O_{10}$

MW: 380.35 g/mol

For a 50 mL 0.5M solution, dissolve 9.51 g of EGTA (Sigma, Cat. #E0396) in 40 mL of water in a beaker. Add 2g of NaOH. Adjust pH with 5N NaOH up to 8. Bring the volume to

50mL. Autoclave if occurred. Store in the dark.

EXTRACTION BUFFER (INVERTASES EXTRACTION)

Make the buffer solution fresh from stock solutions each day.

Tab. Appendix IV- 2: Extraction buffer solution: amounts of each reagent or solution needed are reported.

Reagents	[Stock solutions]	[Buffer concentration]	for 75 mL of buffer (mL)
HEPES solution	1 M - pH8	0.2 M	15.00
$MgCl_2$ solution	100 mM	20 mM	15.00
EDTA solution	5 mM pH 8	1 mM	15.00
EGTA solution	5 mM pH 8	1 mM	15.00
Triton X-100 solution	0.5%	0.05%	7.50
BSA solution	2%	0.1%	3.75
DTT solution	0.1M	2 mM	1.50
Glycerol		3.3%	2.25
Complete Protease inhibitor	Roche, Cat. #11697498001		1 tablet
PVPP	add to each extraction	2% w/v of 3 mL buffer	60 mg

FERRIC AMMONIUM SULPHATE SOLUTION - 1% W/V

Formula: $NH_4Fe(SO_4)_2 \bullet 12H_2O$

MW: 482.19 g/mol

For a 100 mL solution, weight 1.82 g of Ferric Ammonium Sulphate (BDH GPR, Cat. #27164.4M) and dissolve in 90 mL of distilled water. Top up the volume with water.

FIXATIVE

For 100 mL of 2% Formaldehyde, 2.5% Glutaraldehyde (Merck, Cat. # 1.04239) and 25 mM Phosphate buffer, mix 20 ml of 10% fresh Formaldehyde solution, 10 ml of 25% Glutaraldehyde, 50 mL of 50 mM phosphate buffer and 20 mL of distilled water.

FORMALDEHYDE

Formula: (CH2O) n (paraformaldehyde)

Fixative, made fresh from para-formaldehyde powder, diluted for use.

For 10% w/v solution, dissolve 2.0 g of paraformaldehyde powder (Merck, Cat.

#1.04005.1000) in 20 mL of distilled water and heat to 60-65ºC under a fume hood. Add a few drops of 1.0 N sodium hydroxide until the solution becomes clear. Allow the solution to cool before use.

GLUCOSE ASSAY BUFFER

Just before use dilute 0.5 M stock solution of Imidazole Buffer 2.5-fold to 0.2 M Imidazole/3 mM $MgCl_2$ (eg. 3 mL of 0.5 M up to 7.5 mL with RO water). 7.5 mL solution is enough to fill a plate (Costar, 96 well up to 200 µL).

Add ATP (Sigma, Cat. #A-2383) and $NADP^+$ (Sigma, Cat. #N-0505) to diluted Imidazole. ATP and $NADP^+$ are stored in freezer. Weigh out appropriate amount of ATP and $NADP^+$ on 4-place balance to have 5.4 mg/mL Assay Buffer and 3.2 mg/mL Assay Buffer respectively.

HEPES BUFFER - 1M PH 8 SOLUTION

Formula: $C_8H_{18}N_2O_4S$

MW: 238.31 g/mol

- For a 250 mL 1M solution weigh 59.58g of HEPES (Sigma, Cat. #H3375). Dissolve it in 200 mL of water. Adjust pH at 8 with NaOH 6N. Top the volume up to 250 mL. Store in the dark.

IMIDAZOLE BUFFER

Formula: $C_3H_4N_2$ (Imidazole)

MW: 68.08 g/mol

For 250 mL of 0.5M solution with 7.5 mM $MgCl_2$ (use 500mM stock solution of $MgCl_2$) weigh 8.51 g of Imidazole (Sigma, Cat. #I0125) and add 3.75 mL of $MgCl_2$.

Adjust pH at 6.9 with 0.2N HCl. Store refrigerated.

LIQUID LB MEDIUM FOR BACTERIA GROWTH

For 500 mL of medium weigh:

- 20 g/l LB Broth Base (Invitrogen, Cat. #12780-052): 10g

Stir in a beaker LB powder and 350 mL of water until the solution is clear. Check the pH: must be 7. Pour in a bottle and bring the volume up to 500 mL.

LITHIUM CHLORIDE - 12 M SOLUTION

Formula: LiCl

MW: 42.39 mol/g

For a 50 mL 12 M solution dissolve thoroughly 25.43 g of Lithium Chloride (Sigma, Cat. #L9650) in 40 mL of sterile water. Top the volume up to 50 mL and filter sterilize the solution into a sterile 50 mL Falcon tube using a Minisart 20 μm filter (Sartorius, Cat. #16534K).

MAGNESIUM CHLORIDE - 0.5 M SOLUTION

Formula: $MgCl_2 \bullet 6H_2O$

MW: 203.3 g/mol

For a 0.5 M solution weigh 25.41 g of Magnesium chloride (Sigma, Cat. #M0250) and dissolve in water up to 250 mL.

MOPS BUFFER- 1 M PH 8 SOLUTION

Formula: $C_7H_{15}NO_4S$

MW: 209.3 g/mol

For 250 mL of 1M solution weigh 52.33 g of MOPS (Sigma, Cat. #M1254). Dissolve in 170 mL of water and bring the pH up to 8 with NaOH 6N. Bring the volume up to 250 mL.

Store in the dark.

PHOSPHATE BUFFER – 50 MM PH 6.8

For 500 mL of a 50 mM pH 6.8 solution mix 52.5 mL of monobasic sodium phosphate (stock solution A) and 47.5 mL of bibasic sodium phosphate (stock solution B). Store solution in refrigerator.

PINE TREE METHOD EXTRACTION BUFFER

- Use baked glassware and sterile tips. Reagent must be added in the given order (Tab. Appendix IV- 3). Wash stirring fly with ethanol and RNase free water. For a 500 mL solution pour 300 mL of RNase free water in a 1 litre baked Beaker. Add 10 g of CTAB (Merck, Cat. #1.02342.1000) and wait till the solution is clear, warming it up on a hot plate. Then add 10 g of PVP-40 K30 (Sigma, PVP40) . Wait a bit, but the solution will not be clear till you add 50 mL of Tris-HCl and 25 mL of EDTA solutions. Than add 58.44 g

of NaCl (Merck, Cat. #1.06404.0500). When you add NaCl the solution becomes very viscous. Add 0.25 g of spermidine when the solution is cold. Bring the solution to the final volume of 500 mL with RNase free water. The solution should be filtered through a sterile Nalgene Steriltop filter (Millipore, Cat. #SCG PT02RE)

Tab. Appendix IV- 3: List of reagents required for the Pine Tree extraction buffer and relative concentrations.

Reagents	[Final]
CTAB	2% w/v
PVP 40 K30 (K 29-32)	2% w/v
Tris-HCl 1 M pH 8.0 solution	100 mM
EDTA 0.5 M pH 8.0 solution	25 mM
NaCl	2 M
Spermidine	0.5 g/L
RNase FREE sterile water	Up to final volume

RNASE-FREE CHILLED ETHANOL

Absolute Ethanol (Merck, Cat. #1.00983.2500) was filter sterilized under a laminar flow hood into a sterile Falcon tube using Minisart 20 μm filter (Sartorius, Cat. #16534K).

SODIUM ACETATE BUFFER - 0.5 M PH 5

Formula: CH_3COONa (anhydrous)

MW: 82.03 g

- For 100 mL solution dissolve 4.10 g of Sodium Acetate (Sigma, Cat. #S2889) in 80 mL of distilled water. Adjust pH to 5 with glacial acetic acid (Merck, Cat. #1.00063.2500). Bring the volume up to 100 mL. Filter sterilize in baked bottle with Minisart 20 μm filter (Sartorius, Cat. #16534K).

SODIUM ACETATE BUFFER - 3 M PH 5.5

Formula: CH_3COONa (anhydrous)

MW: 82.03 g

For 50 mL solution dissolve 12.3 g of Sodium Acetate (Sigma, Cat. #S2889) in 40 mL of distilled water. Adjust pH to 5.5 with glacial acetic acid (Merck, Cat. #1.00063.2500). Bring the volume up to 100 mL. Filter sterilize in baked bottle with Minisart 20 μm filter (Sartorius, Cat. #16534K).

SODIUM PHOSPHATE BIBASIC – 50 MM SOLUTION

Formula: Na_2HPO_4

MW: 141.96 g/mol

For 500 mL of 50 mM solution dissolve 3.55 g of bibasic sodium phosphate (Merck AnalR, Cat. #10249.4C) in 450 mL of distilled water. When dissolved, bring the volume up to 500 mL. This is Phosphate buffer stock solution B, and must be refrigerated.

SODIUM PHOSPHATE MONOBASIC – 50 MM SOLUTION

Formula: $NaH_2PO_4 \bullet H_2O$

MW: 137.99 g/mol

For 500 mL of 50 mM solution dissolve 3.45 g of monobasic sodium phosphate (Merck AnalaR, Cat. #10245.4R) in 450 mL of distilled water. When dissolved, bring the volume up to 500 mL. This is Phosphate buffer stock solution A, and must be refrigerated.

SOLID LB MEDIUM FOR BACTERIA GROWTH

For 500 mL of medium (it makes 18-20 Petri dishes ϕ 65 mm) weigh:

- 20 g/l LB Broth Base (Invitrogen, Cat. #12780-052): 10g
- 1 g/100mL Bacteriological Agar (Oxoid, Cat. #LP0011): 5g

Stir in a beaker LB powder and 350 mL of water until the solution is clear. Check the pH: must be 7. Pour in a bottle, add agar and bring the volume up to 500 mL.

Autoclave it (45' at 121ºC). Cool it down to 60º-65ºC and then add the antibiotic Ampicillin (usually stock solution is 100-50 mg/mL) to a final concentration of 50 µg/mL.

Under a laminar flow hood pour 30 mL of medium in each Petri dish. Leave the medium hardener.

For a X-gal-IPTG (Isopropyl β-D-1-thiogalactopyranoside) white-blue colony discrimination add to each plate:

- 40 µL of X-gal 40 mg/mL solution to a final concentration of 20 µg/mL (Invitrogen, Cat. #15520-034)
- 50 µL of IPTG 4% 0.1 M to a final concentration of 0.3 mM (Sigma, Cat. #I6758)

Spread them on all the surface and put in the incubator at 37 ºC for 30 minutes to allow the absorption.

SSTE BUFFER

Wash fly with ethanol and rinse it with RNase free water. For 50 mL solution pour 35 mL of RNase free water in a 100 mL baked Beaker (or in a 50 mL sterile Falcon tube). Add 2.922 g of NaCl (Merck, Cat. #1.06404.0500), 0.5 mL of Tris-HCl and 0.1 mL of EDTA, and stir for 2-5 minutes. Add 0.25 g of SDS (Calbiochem, Cat. #42823) and stir other 2 minutes. Bring the solution to the final 50 mL volume and transfer the solution to a baked bottle. It must be autoclaved. Buffer's final concentration are reported in Tab. Appendix IV- 4.

Tab. Appendix IV- 4: List of reagents required for SSTE buffer and relative final concentrations.

Reagents	[Final]
NaCl	1 M
Tris-HCl 1 M pH 8.0	10 mM
EDTA 0.5 M pH 8.0 solution	1 mM
SDS (C_{12} $H_{25}O_4$ S Na)	0.5% w/v
RNase FREE sterile water	Up to final volume

SUCROSE - 0.5 M SOLUTION

Formula: $C_{12}H_{22}O_{11}$

MW: 342.3 g/mol

For 10 mL of 0.5 M solution weigh 1.71 g of sucrose (Applichem, Cat. #A2211.0500) and dissolve in 5 mL of water. Top the volume up to 10 mL.

TOLUIDINE BLUE STAINING – 0.05% (W/V) IN 20 MM BENZOATE BUFFERE PH 4.4

For a 100 mL solution weigh out 50 mg of toluidine blue and add 100 mL of Benzoate buffer (20 mM pH 4.4). Stir till toluidine blue is dissolved completely.

TRITON® X-100 - 0.5% V/V SOLUTION

Formula: $C_{14}H_{22}O(C_2H_4O)_n$

For 500 mL of 0.5% v/v Triton® X-100 solution, pour in a beaker 2.5 mL of Triton® 100-X (BDH, Cat. #306324N), add 400 mL of distilled water, stir till completely dissolved then top the volume up to 500 mL.

Appendix V: Genotype's Detail

Tab. Appendix V- 1: Anthesis dates and flower numbers per vine observed during the seasons 2004-05 and 2006-07 for the 10 genotypes.

	2004-05 Season		2006-07 Season	
Vine	**Anthesis date**	**Flowers per vine (No)**	**Anthesis**	**Flowers per vine (No)**
1	24/11	127	18/11	240
3	27/11	75	20/11	348
5	21/11	138	16/11	226
17	20/11	276	20/11	231
25	29/11	120	24/11	160
26	02/12	98	24/11	173
27	22/11	80	18/11	213
28	23/11	120	12/11	93
29	23/11	75	16/11	154
30	23/11	60	21/11	47
min	20/11	60	12/11	47
max	02/12	276	24/11	348

Appendix VI: qPCR Gene Expression's Details

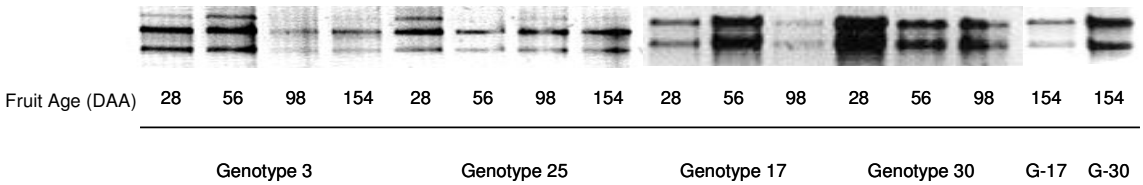


Fig. Appendix VI- 1: rRNA agarose gel of 2004-05 genotype's samples. Four time points and four genotypes were used for the experiments.

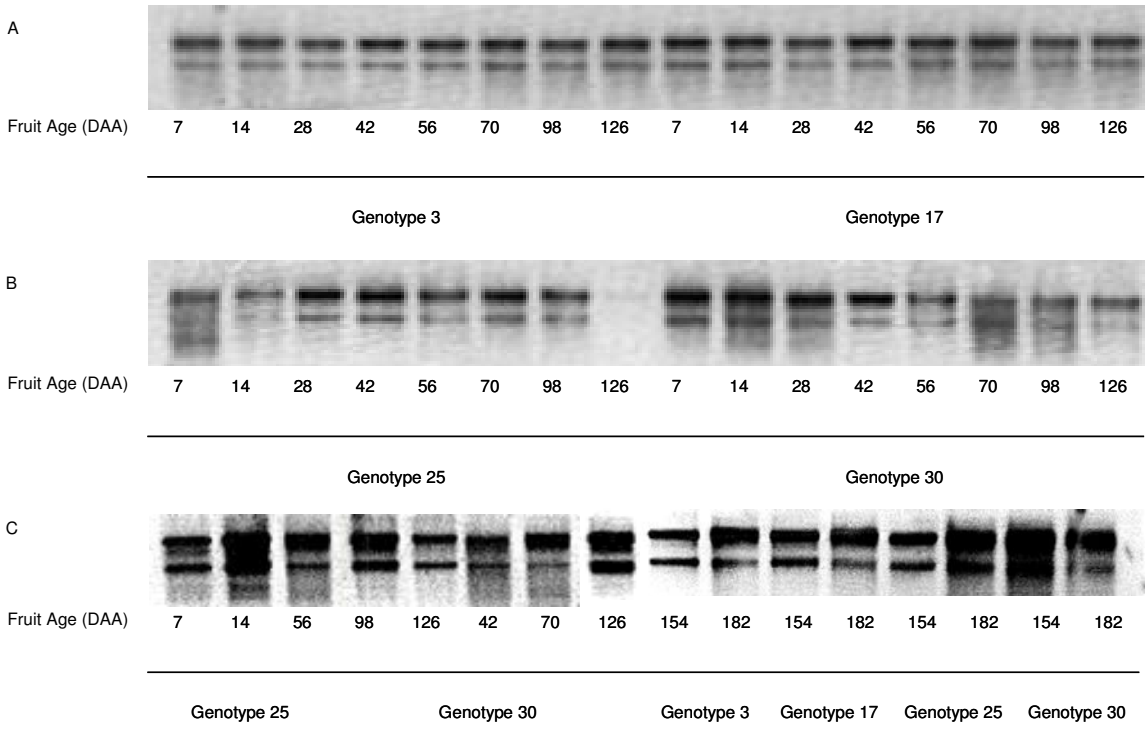


Fig. Appendix VI- 2: rRNA agarose gel of 2006-07 genotype's samples. Ten time points and four genotypes were used for the experiments. Lane B shows some missing and degraded rRNA. Extraction were repeated and rRNA are reported in Lane C, left side. Lane C right side shows last two time points for all four genotypes.

Tab. Appendix VI- 1: List of gene which ESTs were found in HortResearch Actinidia EST Database. Homologous Arabidopsis gene is also reported, as well as the EST number and the identity (Identity from *Arabidopsis* tblastn *Actinidia*).

Gene class	***Arabidopsis* gene**		***Actinidia* gene**	HR-EST	Identity
SUSY	AT3G43190	At-SUS4	SUS1	285852	85%
SUSY	AT5G20830	At-SUS1	SUS2	447973	79%
SUSY	AT4G02280	At-SUS3	SUSA	100046	82%
SUSY	AT1G73370	At-SUS6	SUS6	232862	63%
Vacuolar invertase	AT1G62660	At-FRUCT3	V-INV3	101593	70%
Vacuolar invertase	AT1G12240	At-FRUCT4	V-INV4	179044	58%
Cell wall invertase	AT3G13790	At-FRUCT1	CW-INV1	230140	66%
Cytoplasmic invertase	AT1G22650	At-A/N-INVD	A/N-INVD	270970	92%
Cytoplasmic invertase	AT5G22510	At-A/N-INVE	A/N-INVE	75715	87%
Cytoplasmic invertase	AT4G09510	At-A/N-INVI	A/N-INVI	102915	94%
Cytoplasmic invertase	AT4G34860	At-A/N-INVK	A/N-INVK	292067	92%
AGPase large subunit	AT5G19220	At-AGPL1	AGPL1	314681	84%
AGPase large subunit	AT1G27680	At-AGPL2	AGPL2	203662	79%
AGPase large subunit	AT2G21590	At-AGPL4	AGPL4	311362	90%
AGPase small subunit	AT5G48300	At-AGPS1	AGPS1	192438	92%
Protein Phosphatase	AT1G13320	At-PP2A	PP2A	312205	90%

Tab. Appendix VI- 2: List of primers used for qPCR gene expression studies.. Primers are left to right 5 prime-3 prime oriented. F: forward; R: reverse; HR: HortResearch database; SUSY: sucrose synthase; PP2A: protein phosphatase regulatory subunit 2A; DACH: days after Hydrogen cyanamide treatment; DAP: days after pollination.

Gene class	*Actinidia* gene	HR-EST	Specie and libraries	Primers
SUSY	SUS1	285852	*A. deliciosa* developing shoot buds	F: ACCACTTTTCGTGCCAGTTC R: GTCCAACGGTGTTCTTGCTT
SUSY	SUS2	447973	*A. eriantha* young fruit	F: TGCTGGAAGCAAGAACACTG R: TTTGGGGTCAAACACATCAA
SUSY	SUSA	100046	*A. deliciosa* ripe fruit inner cortex	F: CTGCCGAATTACAGGGTGTT R: GAGCAATGGTGCACTGTGTT
SUSY	SUS6	232862	*A. deliciosa* small fruit 13 DAP	F: CGGTTTCTGGGAGTTTTTCA R: CACTTGCCCAATTCTCATCA
Vacuolar invertase	V-INV3	101593	*A. deliciosa* ripe fruit inner cortex	F: TTCGATTCCAATGGTGTGTG R: GGTTTTGCACTTGCACGTAA
Vacuolar invertase	V-INV4	179044	*A. eriantha* young fruit	F: CTGGCTGTTGAAGTGAACAAGA R: TCCACAATCGAATGATCGAC
Cell wall invertase	CW-INV1	230140	*A. deliciosa* dormant buds three DAHC	F: TAGCGCTAAGAAACGGAGGA R: CTCCTTGGAATTGCCTGAAT
Cytoplasmic invertase	A/N-INVD	270970	*A. chinensis* active meristems	F: ATCTTGGCCAGTGCTTCTGT R: GTCTTTGAGCAGACGGCTTT
Cytoplasmic invertase	A/N-INVE	75715	*A. deliciosa* petal	F: CACCCTCTGGAAATTCAGTCA R: TTGTTCAGAGCTTGGATCAGG
Cytoplasmic invertase	A/N-INVI	102915	*A. deliciosa* ripe fruit inner cortex	F: ATGTGCTGATGGATGCTCAA R: TTGCCAAAGCACATCTCAGT
Cytoplasmic invertase	A/N-INVK	292067	*A. chinensis* young fruit library	F: TCGACACATTCCCGACTCTT R: GCATCTCAAAGCCATGAAGAA
AGPase large subunit	AGPL1	314681	*A. chinensis* young leaf library	F: GAGGATGCAAGAAGCAAGGA R: GGTGATATCGGCACCACTTT
AGPase large subunit	AGPL2	203662	*A. deliciosa* dormant buds one DAHC	F: ATTGGCTGCCACTCAAACA R: TTTTTGTTCTTGGCGTCCTC
AGPase large subunit	AGPL4	311362	*A. chinensis* young fruit library	F: TGGGGGCAGACTATTACCAA R: TGCGTTCTTGTCGATTATGC
AGPase small subunit	AGPS1	192438	*A. chinensis* ripe fruit	F: TGAGGGTGCAAGCTTACTTGT R: AATCTGGCACTGGCTTTTTG
Protein Phosphatase	PP2A	312205	*A. chinensis* young fruit library	F: GCAGCACATAATTCCACAGG R: TTTCTGAGCCCATAACAGGAG

Appendix VII: Microarray's Details

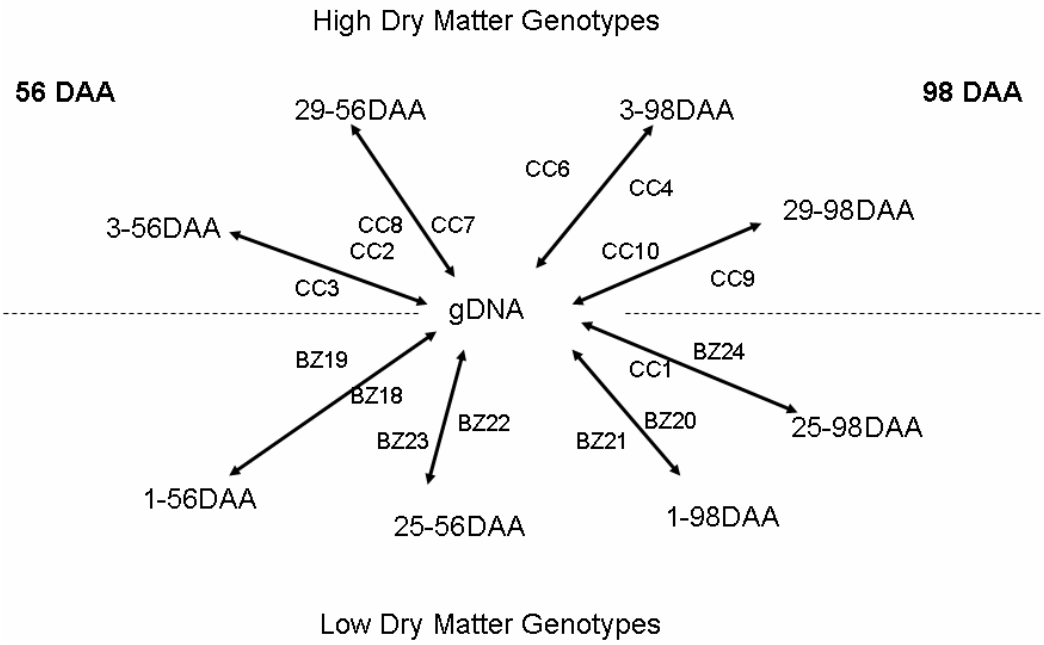


Fig. Appendix VII- 1: Microarray experimental design. Arrows indicate dye swap, numbers on arrow sides are slide's codes. Over the dashed line high dry matter genotypes are reported, whereas low dry matter genotypes are located below it.

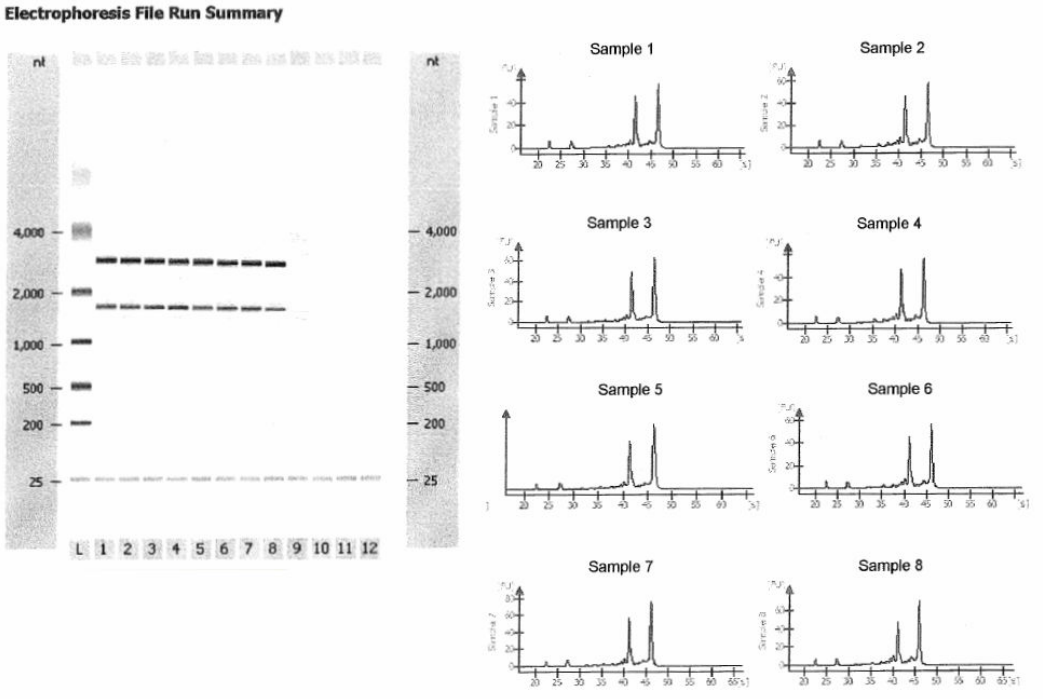


Fig. Appendix VII- 2: RNA quality detected at the Agilent Bioanalyzer for microarray experiment. Electrophoresis result is reported on the left whereas graphs of the detected band intensity are reported on the right side of the picture. Non-degraded RNA shows clean peaks and bands. Genotype 1-56 DAA (sample 1), Genotype 1-98DAA (sample 2), Genotype 3-56DAA (sample 3), Genotype 3-98DAA (sample 4) Genotype 25-56DAA (sample 5), Genotype 25-98DAA (sample 6), Genotype 29-56DAA (sample 7), Genotype 29-98DAA (sample 8).

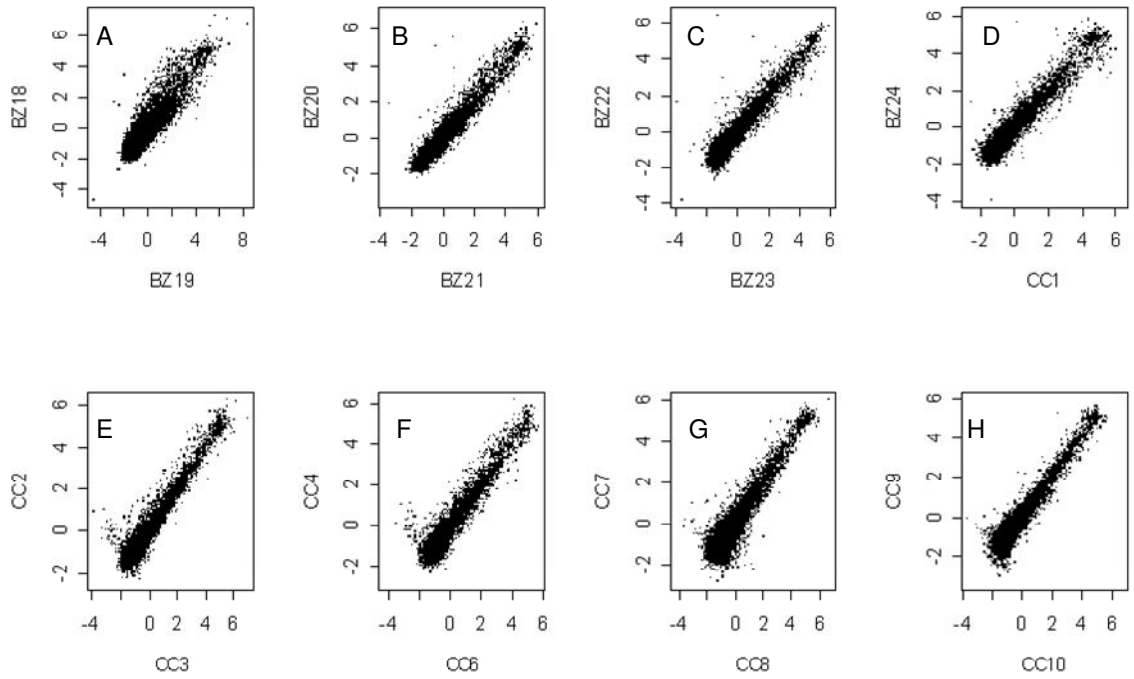


Fig. Appendix VII- 3: Dye swap. Cy3 slide spots were plotted on X axis, whereas Cy5 slide spots were plotted on Y axis. Genotype 1-56 DAA (A), Genotype 1-98DAA (B), Genotype 25-56DAA (C), Genotype 25-98DAA (D), Genotype 3-56DAA (E), Genotype 3-98DAA (F) Genotype 29-56DAA (G), Genotype 29-98DAA (H).

Tab. Appendix VII- 1: Slide codes, dye swap and number of "good spots" used for each slide (EST-Oligos).

Slide code	Cy3	Cy5	EST-Oligos (No.)
BZ19	gDNA	RNA-Genotype 1 56DAA	17,401
BZ18	RNA-Genotype1 56DAA	gDNA	17,459
BZ21	gDNA	RNA- Genotype 1 98DAA	17,259
BZ20	RNA- Genotype 1 98DAA	gDNA	17,369
BZ23	gDNA	RNA- Genotype 25 56DAA	17,433
BZ22	RNA- Genotype 25 56DAA	gDNA	17,090
CC1	gDNA	RNA- Genotype 25 98DAA	17,278
BZ24	RNA-Genotype 25 98DAA	gDNA	17,523
CC3	gDNA	RNA- Genotype 3 56DAA	16,723
CC2	RNA- Genotype 3 56DAA	gDNA	17,019
CC6	gDNA	RNA- Genotype 3 98DAA	15,971
CC4	RNA- Genotype 3 98DAA	gDNA	16,847
CC8	gDNA	RNA- Genotype 29 56DAA	15,905
CC7	RNA- Genotype 29 56DAA	gDNA	16,733
CC10	gDNA	RNA- Genotype 29 98DAA	17,337
CC9	RNA- Genotype 29 98DAA	gDNA	16,791

Tab. Appendix VII- 2: 56 DAA changes microarray FC>2. high DM upregulated genes

Oligo-EST (*Arabidopsis* gene)	FC	Description	Biological function	Molecular function
Metabolism				
273597 (At3g06860)	2.1	Cluster: Putative uncharacterized protein, ; Glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a	metabolism	catalytic activity
116321 (At4g13940)	2.1	Cluster: Adenosylhomocysteinase	one-carbon compound metabolism	adenosylhomocysteinase activity
319453 (At5g47120)	2.2	Cluster: Bax inhibitor 1	anti-apoptosis, apoptosis, regulation of apoptosis, negative regulation of programmed cell death	catalytic activity
222699 (At3g49340)	2.6	Light repressed protein A; ribosomal protein; Cluster: Sigma 54 modulation protein/ribosomal protein	proteolysis	cysteine-type peptidase activity
273607 (At1g55860)	2.2	Cluster: E3 protein	protein ubiquitination, ubiquitin-dependent protein catabolic process	ubiquitin-protein ligase activity
21705 (At5g64440)	2.4	Cluster: amidotransferase amidohydrolase, protein	unknown	amidase activity, N-(long-chain-acyl)ethanolamine deacylase activity
298924 (At1g76730)	2.4	Cluster: Hypothetical protein DKFZp469G0629	metabolism	catalytic activity
Cell compartments and components				
72903 (At3g01500)	2.8	carbonic anhydrase (carbonate dehydratase); Carbonic anhydrase, chloroplast precursor	carbon utilization	carbonate dehydratase activity zinc ion binding
310530 (At3g02570)	5.0	Mannose-6-phosphate isomerase (Phosphomannose isomerase)	carbohydrate metabolic process, embryonic development ending in seed dormancy	mannose-6-phosphate isomerase activity
300813 (At3g52940)	2.3	Cluster: reductase protein	cytoskeleton organization and biogenesis	actin binding
100258 (At5g57960)	2.9	Cluster: Small GTP binding protein domain	unknown	GTP binding
260725 (At3g59850)	2.3	Cluster: precursor ; polygalacturonase (pectinase)	carbohydrate metabolism	polygalacturonase activity
Transport				
269009 (At3g12400)	2.4	Cluster: Similarity protein	protein modification protein transport ubiquitin cycle	unknown
269760 (At2g16800)	2.3	Not nickel-transport family protein ; high-affinity nickel-transport family protein	nickel ion transport	nickel ion transmembrane transporter activity
230444 (At2g29940)	2.4	Cluster: drug resistance - Pleiotropic drug resistance protein 12	multidrug transport	ATPase activity, coupled to transmembrane movement of substances
241116 (At2g43330)	2.2	sugar transporter family protein; Putative polyol (cyclic)-H+ symporter family (tonoplast)	transport	transporter activity
Binding				
30855 (At2g25290)	2.3	not determined by homology	unknown	binding
236259 (At4g32060)	2.6	Cluster: Hypothetical protein F10N7.140 EF-hand containing proteins:Group III	unknown	calcium ion binding
316335 (At2g44110)	2.2	Cluster: protein ; MLO protein homolog 1 - calmodulin binding	cell death defense response	calmodulin binding
221214 (At5g56360)	2.2	Glucosidase 2 beta subunit precursor (Glucosidase II beta subunit)	unknown	calmodulin binding

Continued from previous page

Oligo-EST (*Arabidopsis* gene)	FC	Description	Biological function	Molecular function
Kinase				
204803 (At3g24030)	2.8	Hydroxyethylthiazole kinase (Thz kinase) (TH kinase)	thiamin biosynthesis	hydroxyethylthiazole kinase activity
232051 (At3g47580)	2.3	Cluster: protein kinase ; leucine rich repeat transmembrane protein kinase	protein amino acid phosphorylation	ATP binding protein kinase activity
291341 (At4g08480)	2.2	mitogen-activated protein kinase	protein amino acid phosphorylation	kinase activity
300307 (At1g26150)	2.1	not determined by homology	protein amino acid phosphorylation	protein kinase activity
Stress related-responsive				
191072 (At2g17840)	3.0	senescence/dehydration associated protein	response to cold, response to water deprivation, response to high light intensity, response to salt stress	unknown
179237 (At3g05390)	2.9	expressed protein; hypothetical protein	release of virus from host	unknown
241293 (At5g21430)	2.4	Cluster: OSIGBa0142I02-OSIGBa0101B20.15 protein	unknown	heat shock protein binding
312257 (At5g26990)	2.1	drought-responsive family protein	response to water deprivation	unknown
Energy transport				
322451 (At1g57770)	3.1	Cluster: COG1233: Phytoene dehydrogenase and related proteins	electron transport	oxidoreductase activity
295071 (At1g26340)	2.8	Cytochrome b5	electron transport	heme binding, transition metal ion binding
324355 (At5g05690)	2.7	Cluster: Cytochrome P450 oxidoreductase, CYP90A15 on paired cress	electron transport	heme binding iron ion binding monooxygenase activity
324338 (At3g61040)	2.6	cytochrome P450 - CYP76C7	electron transport	heme binding iron ion binding monooxygenase activity
222692 (At4g02580)	2.4	NADH-quinone oxidoreductase chain E (NADH dehydrogenase I, chain E) (NDH-1, chain E)	electron transport mitochondrial electron transport, NADH to ubiquinone	NADH dehydrogenase ubiquinone activity
320524 (At3g19270)	2.2	5-alpha-taxadienol-10-beta-hydroxylase; cytochrome P450 family protein - ABA catabolism	electron transport	heme binding iron ion binding monooxygenase activity
324579 (At2g40890)	2.2	cytochrome P450 - CYP98A	electron transport	heme binding iron ion binding monooxygenase activity
Traslational and Post-transcriptional regulators				
249455 (At1g17640)	2.5	RNA recognition motif (RRM)-containing protein; RNA binding protein Musashi homolog 1 (Musashi-1)	unknown	RNA binding nucleic acid binding nucleotide binding
315316 (At3g48500)	2.5	expressed protein; Cluster: B1065G12.12 protein	unknown	RNA binding
Transcription factor				
100090 (At3g27010)	4.3	TCP family transcription factor; P0623F08.31 gene product	anatomical structure morphogenesis	transcription factor activity

Continued from previous page

Oligo-EST (*Arabidopsis* gene)	FC	Description	Biological function	Molecular function
234958 (At4g24020)	2.9	Cluster: T19F6.16 protein ; RWP-RK domain-containing protein	regulation of transcription	DNA binding
305839 (At5g18230)	2.8	Cluster: CCR4-NOT transcription of subunit 3	regulation of transcription	transcription regulator activity
187208 (At1g28050)	2.8	zinc finger (B-box type) family protein; C2C2-CO-like TF Family	regulation of transcription	transcription factor activity DNA binding zinc ion binding
260785 (At4g17490)	2.7	Ethylene-responsive transcription factor 1 - AP2-EREBP TF Family	regulation of transcription, DNA-dependent	transcription factor activity
292502 (At4g13640)	2.5	myb family transcription factor - G2-like TF Family	double fertilization forming a zygote and endosperm	transcription factor activity DNA binding transcription factor activity
238213 (At1g28420)	2.4	Homeobox binding transcription specific DNA binding ; (transcription factor)	regulation of transcription, DNA-dependent	transcription factor activity
307391 (At3g02380)	2.2	zinc finger (B-box type) family protein - C2C2-CO-like TF Family	regulation of flower development	transcription factor activity DNA binding zinc ion binding
313472 (At1g22190)	2.2	Cluster: Putative element binding binding protein, ; AP2 domain-containing transcription factor	regulation of transcription, DNA-dependent	transcription factor activity
Expressed or Uncharcterized proteins				
194921 (At5g46850)	3.4	Cluster: Arabidopsis thaliana genomic DNA, chromosome 5, P1 clone:MSD23;	unknown	unknown
116800 (At4g13230)	2.8	Cluster: Putative uncharacterized protein	embryonic development ending in seed dormancy	unknown
311579 (At5g49210)	2.8	expressed protein	unknown	unknown
270877 (At4g32910)	2.6	Cluster: Hypothetical protein At4g32910	unknown	unknown
310329 (At1g42480)	2.5	expressed protein;	unknown	unknown
100930 (At5g24600)	2.3	expressed protein	unknown	unknown
260764 (At5g24680)	2.3	Cluster: PREDICTED: finger protein ; Cluster: Zinc finger protein C6orf113	unknown	unknown
269632 (At2g20635)	2.2	spindle checkpoint protein	unknown	unknown
310326 (At5g55860)	2.1	expressed protein; Cluster: Prefoldin	unknown	unknown
285163 (At3g59520)	2.1	Cluster: Putative uncharacterized protein	unknown	unknown
209279 (At5g41560)	2.0	expressed protein	unknown	unknown
Not determined				
27 ESTs	8.0-2.0	not determined by homology	unknown	unknown

Tab. Appendix VII- 3: 56 DAA changes microarray FC>2. high DM down-regulated genes

Oligo-EST (*Arabidopsis* gene)	FC	Description	Biological function	Molecular function
Metabolism				
237954 (At5g45960)	-5.1	GDSL-motif lipase/hydrolase family protein; family II extracellular lipase 1 (EXL1); family II extra	lipid metabolism	hydrolase activity, acting on ester bonds
260703 (At1g16470)	-3.2	Proteasome subunit alpha type 2 (Proteasome component C3) (Macropain subunit C3) (Multicatalytic end	ubiquitin-dependent protein catabolism	threonine endopeptidase activity
249723 (At4g30810)	-3.1	Cluster: serine carboxypeptidase protein	proteolysis	serine carboxypeptidase activity
321826 (At2g22240)	-3.0	Inositol-3-phosphate synthase (Myo-inositol-1-phosphate synthase) (MI-1-P synthase)	response to heat, inositol biosynthetic process, phospholipid biosynthetic process, response to high light intensity, response to hydrogen peroxide	inositol-3-phosphate synthase activity
294517 (At3g60820)	-3.0	Cluster: Proteasome subunit beta type	ubiquitin-dependent protein catabolic process	peptidase activity
308969 (At4g00230)	-2.5	subtilase family protein	proteolysis	subtilase activity
270853 (At1g72030)	-2.3	23 kDa jasmonate induced protein	metabolic process	N-acetyltransferase activity
298741 (At1g45000)	-2.2	Proteasome-activating nucleotidase (Proteasome regulatory subunit)	protein catabolic process	ATP binding
292607 (At1g12240)	-2.2	Acid beta-fructofuranosidase precursor (Acid sucrose hydrolase) (Acid invertase)	carbohydrate metabolic process	beta-fructofuranosidase activity, hydrolase activity, hydrolyzing O-glycosyl compounds
234783 (At1g15410)	-2.2	Cluster: Putative aspartate/glutamate racemase	metabolism	racemase and epimerase activity, acting on amino acids and derivatives
Secondary metabolism				
312805 (At4g22880)	-5.7	oxidoreductase, 2OG-Fe(II) oxygenase family protein; Flavonol synthase/flavanone 3-hydroxylase (FLS)	response to wounding, vacuole organization and biogenesis, anthocyanin biosynthetic process, response to jasmonic acid stimulus, proanthocyanidin biosynthetic process	leucocyanidin oxygenase activity
310182 (At5g08640)	-3.5	Leucoanthocyanidin dioxygenase (LDOX) (Leucocyanidin oxygenase) (Leucoanthocyanidin hydroxylase)	flavonoid biosynthetic process	flavonol synthase activity
Cell compartments and components				
240153 (At5g14920)	-4.0	Cluster: Gibberellin-regulated protein 1 precursor	response to gibberellin stimulus	structural constituent of cell wall
321725 (At5g05170)	-3.7	cellulose synthase, catalytic subunit; Cellulose synthase catalytic subunit [UDP-forming]	cellulose biosynthesis	cellulose synthase UDP-forming activity
125043 (AtCg00340)	-3.5	Photosystem I P700 chlorophyll a apoprotein A2 (PsaB) (PSI-B)	photosynthesis, light harvesting in photosystem I, photosynthesis, light harvesting in photosystem II	chlorophyll binding chloroplast thylakoid membrane, plastoglobule
319732 (At5g11790)	-2.5	Ndr family protein; pollen specific protein SF21	cell differentiation	unknown
293010 (At3g16110)	-2.5	thioredoxin family protein; protein disulfide isomerase	cell redox homeostasis	thiol-disulfide exchange intermediate activity
324807 (At2g45790)	-2.5	Phosphomannomutase 2 (PMM 2); phosphomannomutase (PMM)	mannose biosynthesis metabolism	catalytic activity phosphomannomutase activity

Oligo-EST (*Arabidopsis* gene)	FC	Description	Biological function	Molecular function
323194 (At1g79930)	-2.8	heat shock protein 105 kDa (Heat shock 110 kDa protein); heat shock protein	response to heat, protein folding	ATP binding
186758 (At2g44060)	-2.8	late embryogenesis abundant family protein (LEA family protein)	response to desiccation, embryonic development ending in seed dormancy	unknown
307538 (At1g74670)	-2.5	Cluster: protein ; unknown protein ; gibberellin-regulated family protein	response to gibberellin stimulus, gibberellic acid mediated signaling	unknown
305330 (At3g07230)	-2.1	Cluster: Putative wound induced basic protein	unknown	unknown
DNA silencing				
306537 (At5g15380)	-2.2	Cluster: Putative cytosine methyltransferase	DNA methylation, methylation-dependent chromatin silencing, histone H3-K9 methylation	DNA (cytosine-5-)-methyltransferase activity
Transcription factors				
286459 (At2g04240)	-3.6	zinc finger (C3HC4-type RING finger) family protein;C3H TF Family	response to osmotic stress, response to salt stress	transcription factor protein binding zinc ion binding
234876 (At3g47640)	-3.3	Cluster: Hypothetical protein; basic Helix-Loop-Helix (bHLH) TF	regulation of transcription	transcription factor activity DNA binding
240421 (At3g18380)	-3.0	expressed protein	regulation of transcription, DNA-dependent, regulation of transcription	transcription coactivator activity sequence-specific DNA binding
238689 (At3g58680)	-2.4	ethylene-responsive transcriptional coactivator; Multiprotein-bridging factor 1; Endothelial differe	transcription, response to ethylene stimulus, positive regulation of transcription	transcription coactivator activity sequence-specific DNA binding
225789 (At5g65640)	-2.3	basic helix-loop-helix (bHLH) family protein; bHLH TF Family	regulation of transcription	transcription regulator activity
324304 (At1g53320)	-2.3	F-box family protein (tubby family protein); TUB TF Family	regulation of transcription	transcription factor activity phosphoric diester hydrolase activity,
Translational and Post-transcriptional regulators				
269470 (At5g23570)	-2.7	Cluster: Emb|CAB62356.1	virus induced gene silencing, vegetative phase change, defense response to virus, RNA interference, production of ta-siRNAs	unknown
240825 (At3g26560)	-2.5	RNA helicase; pre-mRNA-splicing factor ATP-dependent RNA helicase mog-1	unknown	ATP binding ATP-dependent helicase activity
227274 (At1g54290)	-2.2	Cluster: translation initiation factor ; Protein translation factor SUI1 ; eukaryotic translation initiation factor SUI1	protein biosynthesis translational initiation	translation initiation factor activity
323046 (At3g10950)	-2.0	60S ribosomal protein L37a; 60S ribosomal protein L7 (RPL7D)	translation, ribosome biogenesis and assembly	structural constituent of ribosome
299609 (At4g27000)	-2.0	Polyadenylate binding protein 1 (Poly(A) binding protein 1) (PABP 1); RNA binding protein 47 (RBP47)	RNA binding	RNA binding nucleic acid binding nucleotide binding
Others				
312391 (At3g58040)	-4.0	Cluster: SINA2p	development ubiquitin-dependent protein catabolism	unknown

Continued from previous page

Oligo-EST (*Arabidopsis* gene)	FC	Description	Biological function	Molecular function
298010 (At5g58710)	-3.0	Cluster: Peptidyl-prolyl cis-trans isomerase	protein folding	peptidyl-prolyl cis-trans isomerase activity
259579 (At5g64350)	-2.7	Cluster: Peptidyl-prolyl cis-trans isomerase	protein folding	FK506 binding peptidyl-prolyl cis-trans isomerase activity
319382 (At4g22220)	-2.2	iron-sulfur cluster assembly complex protein; Cluster: Putative iron-sulfur cofactor synthesis protein	iron-sulfur cluster assembly	structural molecule activity
Expressed or Uncharcterized proteins				
319604 (At4g27435)	-3.2	expressed protein	unknown	unknown
288172 (At5g42090)	-2.9	expressed protein	unknown	unknown
291549 (At3g46430)	-2.8	expressed protein	unknown	unknown
90796 (At2g42610)	-2.7	Cluster: Putative protein	unknown	unknown
222667 (At2g46080)	-2.7	expressed protein	unknown	unknown
306176 (At4g32870)	-2.3	expressed protein	unknown	unknown
292989 (At1g75000)	-2.2	expressed protein	unknown	unknown
194540 (At1g05205)	-2.1	expressed protein	unknown	unknown
48364 (At1g76780)	-2.1	Cluster: determined uncharacterized protein	unknown	unknown
Not determined				
19 ESTs	-6.1/-2.1	not determined by homology	unknown	unknown

Appendix VIII: CPPU Experiment's Details

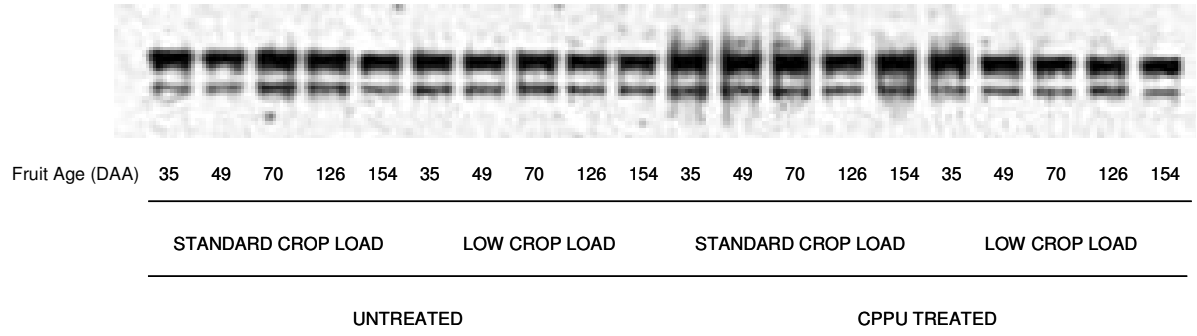


Fig. Appendix VIII- 1: rRNA agorose gel analysis for CPPU gene expression esperiment.

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