Tomato Fruit Growth

Integrating Cell Division, Cell Growth and Endoreduplication by Experimentation and Modelling

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Contents

Abstract

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Fruit size is a major component of fruit yield and quality of many crops. Variations in fruit size can be tremendous due to genotypic and environmental factors. The mechanisms by which genotype and environment interact to determine fruit size are complex and poorly understood. Genotype-by-environment interactions emerge from cellular and molecular processes underlying fruit growth.

In this thesis the basis for variations in tomato fruit size was analysed through the development of a dynamic fruit growth model integrating three fundamental fruit cellular processes: cell division, cell growth and cell endoreduplication. Experiments were carried out to understand the link between cellular processes and fruit growth and their responses to genotypic factors, contrasting fruit loads and temperature conditions.

Experimental data showed that the contribution of cell number and cell size to the genotypic variation in final fruit size depends on the timing of assimilate supply to the fruit. Genotypic variation in fruit fresh weight, pericarp volume and cell volume was linked to pericarp glucose and fructose content. Genotypic variation in cell number was positively correlated with variation in pericarp fructose content. Reduction in final fruit size of early-heated fruit was mainly associated with reduced final cell volume in the pericarp. Early heating increased the number of cell layers in the pericarp, but did not affect the total number of pericarp cells significantly. Continuously heating of a fruit reduced anticlinal (direction perpendicular to fruit skin) cell expansion more than periclinal (direction parallel to fruit skin) cell expansion.

Information derived from the experiments was incorporated into a dynamic model of fruit growth. The model describes fruit growth from anthesis until maturation and covers the stages of cell division, endoreduplication and cell growth. Model development relied on understanding and integrating biological interactions between processes at the cell, tissue and fruit scales. The model was parameterized and calibrated for low fruit load conditions and was validated for high fruit load and various temperature conditions. The model was able to accurately predict final cell number, cell mass and pericarp mass under contrasting fruit load and most of the temperature conditions. Model sensitivity analysis showed that variations in

final fruit size are mainly associated with variations in parameters involved in the dynamics of cell division. Among these parameters, cell division duration had the strongest influence on final cell number and pericarp mass.

The model can be used to carry out virtual experiments with treatments that are difficult or impossible to test experimentally and allows for predicting and analysing fruit growth responses to genotype-by-environment interactions.

This thesis has contributed to closing the gap between genotype and phenotype related to tomato fruit growth. An integral and coherent development of models at relevant levels of plant organization can further help to close this gap.

Keywords: cell division, cell growth, cell endoreduplication, fruit growth, genotype, G×E interaction, model, tomato.

1. General introduction

Variation in fruit size

The fruit is the harvestable part of many horticultural crops. In these crops, fruit size is an important aspect of crop yield and quality. Variations in fruit size can be tremendous among different crop species, but also among cultivars from the same species. This is especially evident in tomato where large differences in fruit size are observed between varieties ranging from the small-fruited cherry tomato to the large-fruited beef tomato. Although fruit size is primarily a genotype-related trait, variations due to intrinsic and environmental factors can be appreciable. Experimental data showed that assimilate availability and temperature strongly affect fruit growth and final fruit size in many crop species (Marcelis and Baan Hofman-Eijer, 1993; Calderón-Zavala et al., 2004; Bertin, 2005). For example, Prudent et al. (2010) found that growing tomato plants under limited sugar supply reduced their fruit weight by 47%. Sawhney and Polowick (1985) observed that the fruit size of tomato plants grown under 28/23 ^{o}C (Day/Night) was reduced by 42% compared to fruits of plants grown under 23/18 ^{o}C (Day/Night). Temporal fluctuations in environmental conditions during fruit growth may also cause variations in final fruit size (Calderón-Zavala et al., 2004, Adams et al., 2001). Fruit size differences between genotypes may vary widely across environments because of genotype-by-environment interactions (Prudent et al., 2010; Ortiz et al. 2007). The mechanism by which genotype and environment interact to determine fruit size remains poorly understood. This limited insight represents a real challenge in manipulating and predicting fruit yield and size. Genotype-by-environment interactions emerge from complex processes underlying fruit growth.

Cellular processes underlying fruit growth

In tomato, three cellular processes successively occur during the growth of a fruit. Before anthesis fruit growth is mainly the result of cell division. This process is temporally arrested at anthesis and resumes after a successful fertilization (Gillaspy et al., 1993). The duration of cell division after anthesis is variable in different fruit species. It ranges from 1-2 weeks in cucurbits and tomato, 3 weeks in apple and 4-7 weeks in peach (Bourdon et al., 2010). In avocado and strawberries, cell division continues for the entire period of fruit growth (Chevalier et al., 2011). For fruits in which the division phase is restricted to the first period

of fruit growth, cell division is gradually replaced by cell expansion. The cell expansion phase usually lasts the longest. During this phase, cells accumulate water and carbon resulting in an increase of up to 11000 fold their initial volume (Cheniclet et al., 2005). Cell expansion may be accompanied by endoreduplication, i.e. an increase in the ploidy level of fruit cells up to 2^8 times the haploid nuclei DNA content in tomato fruits (Bertin, 2005). Endoreduplication has been postulated to play a role in the determination of cell and organ size (Cheniclet et al., 2005), in plant adaptation to adverse environmental conditions (Barrow et al., 2006) and in cell differentiation (Chevalier et al., 2011).

In the past two decades the cellular basis for variation in fruit growth have been studied extensively (Cheng and Breen, 1992; Higashi et al., 1999; Prudent et al., 2010). For example, many authors observed that genotypic variation in final fruit size in many fruit species is related to the variation in cell division activity (Scorzal et al., 1991; Higashi et al., 1999; Bertin et al., 2003a). Other studies investigated assimilate supply and temperature effects on fruit cellular processes (Marcelis and Baan Hofman-Eijer, 1993; Bertin, 2005) and related the observed effects with fruit growth variation. Although these studies provide some insight into the link between individual cellular processes and fruit growth, they do not explain how these underlying processes taken together regulate fruit growth. To progress in our understanding of fruit yield and quality build-up, it is fundamental to integrate the description of cellular processes underlying fruit growth and their interaction with the environment into a common knowledge base system.

Dynamics of fruit growth

Fruit growth starts long before anthesis with the initiation, differentiation and development of floral primordia (Anastasiou and Lenhard, 2008). In many fruit species, growth is interrupted at anthesis and resumes after pollination (Gillaspy et al., 1993). Fruit growth relies on the presence of many hormones such as auxins, cytokinins, and gibberellins which are mainly produced by the fruit seeds (Bohner and Bangerth, 1988a). The cumulative growth of many fleshy fruits follows an "S" shaped curve (Bourdon et al., 2010). A deviation from this pattern is observed in few fruit species such as stone fruits or some berries of which growth curves follow a double sigmoid pattern (Bourdon et al., 2010). The duration of fruit growth period is variable among different fruit species and ranges from 5-8 weeks in tomato and cucumber (Marcelis and Baan Hofman-Eijer, 1993; Cheniclet et al., 2005), to 60 weeks in many citrus species (Bourdon et al., 2010). Within the same species, variation in fruit growth duration is

cultivar dependent and can be modified by the environment (Bertin, 2005; Prudent et al., 2010).

Fruit growth depends mainly on the supply of assimilate produced in the leaves. When assimilate supply is higher than fruit demand, fruits can grow at their potential rate (Van der Ploeg and Heuvelink, 2005). When supply is lower than demand, fruits compete for assimilates. For each fruit on the plant the severity of the competition depends on its spatial and temporal relationship with vegetative organs and with other generative organs (Ho, 1992). For example, in monocarpic fruit species such as apple and pear, competition between fruits and vegetative organs is less severe because fruit development starts after the completion of vegetative growth (Ho, 1992). In polycarpic fruit species such as tomato, competition for assimilates is localized between a truss and its neighbouring leaves and fruits of the same truss, which are all developing at the same time. In these plants early set fruits might inhibit the growth of later initiated ones (Marcelis et al., 1998). Competition between fruits of the same truss can be alleviated through fruit pruning (Prudent et al., 2010) or fruit set synchronization (Bohner and Bangerth, 1988b). In general competition for assimilates affects fruit growth rate more than growth duration. By contrast, temperature affects both fruit growth rate and duration (Marcelis and Baan Hofman-Eijer, 1993; De Koning, 2000). Above the minimum and below the optimum temperature threshold for fruit growth, increase in temperature increases fruit growth rate and reduces fruit growth duration (Marcelis and Baan Hofman-Eijer, 1993). The extent to which final fruit size is affected by high temperature depends on the magnitude of increase in growth rate relative to the decrease in growth duration (De Koning, 1994). When the increase in fruit growth rate compensates for the reduction in the fruit growth duration no effect of high temperature is expected on final fruit size. This situation is generally observed when assimilate supply is not limiting (Bertin, 2005). When assimilate supply is limiting the effect of high temperature on fruit growth is more severe and results in smaller fruits (Marcelis and Baan Hofman-Eijer, 1993). In many studies where high temperature effects on fruit growth were investigated, whole plants were exposed to the temperature treatments (De Koning, 1994; Bertin, 2005). In these experiments high temperatures may partly affect fruit growth indirectly via the increase in the rate of plant development resulting in more sink organs competing for assimilates (Wubs et al., 2009). To quantify direct temperature effects on fruit growth it is important to apply temperature treatments at the fruit level (Adams et al., 2001). Several studies showed that fruit sensitivity to temperature differs during fruit development in many fruit species including tomato (Adams et al., 2001), cucumber (Marcelis and Baan Hofman-Eijer, 1993) and apple

(Calderón-Zavala et al., 2004). This difference in fruit sensitivity during development is expected considering that very different underlying processes are successively involved during the development of a fruit. Understanding these underlying processes and their reaction to the environment is the first step towards the control of fruit growth and yield.

Genotype-by-environment interactions in fruit growth

Genotype-by-environment interactions are observed when variations in the performance of two or more genotypes depend on the environment to which they are exposed (Wright et al., 1996). These interactions are common in multi-environment trials used in plant breeding programs to evaluate the relative performance of plant genotypes. G×E interactions can be observed on fruit growth characteristics at different scales of organization (Bertin et al., 2010; Prudent et al., 2010). Several studies reported the occurrence of G×E interactions for fruit size in tomato and other crop species. Prudent et al. (2010) observed that fruit load-induced assimilate limitation resulted in a reduction in tomato fruit size which was more pronounced for a large-fruited genotype than for a small-fruited genotype. Ortiz et al. (2007) reported a significant genotype by temperature interaction for tomato fruit size in a study involving 15 tomato genotypes across 18 locations. G×E interactions for fruit size was also reported in other fruit species including cherry (Olmstead et al., 2007), banana (Ortiz and Vulylsteke, 1995), eggplant (Muñoz-Falcón et al., 2008) and pumpkin (El-Hamed and Elwan, 2011). G×E interactions observed at the fruit level can be associated with genotypic variation in cellular processes underlying fruit growth and their response to the environment (Marcelis and Baan Hofman-Eijer, 1993; Higashi et al., 1999; Bertin, 2005; Bertin et al., 2010).

Variations in cell division activity have been associated to genotypic variation in final fruit size in many crops including tomato (Bertin et al., 2003a), melon (Higashi et al., 1999), strawberry (Cheng and Breen, 1992), cherry (Olmstead et al., 2007) and peach (Scorzal et al., 1991). Cheniclet et al. (2005) observed that genotypic variation in tomato fruit size was partly related to variations in cell endoreduplication. Several other studies have shown that cellular processes are affected by environmental factors. Bertin (2005) found that fruit load-induced assimilate limitation reduced final cell number and cell size in tomato. Defoliation-induced assimilate limitation also reduced final cell size in chestnut (Famiani, 2000). Bertin (2005) observed that increasing air temperature from 20/20 ^{o}C to 25/25 ^{o}C (day/night) shortened the cell division period and extended the cell expansion period in tomato. In the same experiment the highest ploidy level was observed in fruits grown under the high temperature regime

(Bertin, 2005). Increasing fruit temperature reduced final cell number in cucumber fruits, but did not affect final cell size when assimilate was limiting (Marcelis and Baan Hofman-Eijer, 1993). Genotypic and environment related variations in cellular processes underlying fruit growth can be traced down at the molecular scale.

Molecular control of fruit growth

At the molecular scale, a network of interacting genes controls cell division, cell expansion and cell endoreduplication. Frary et al. (2000) identified a major quantitative trait locus (QTL) *fw2.2*, accounting for 30% difference in fruit fresh weight between domesticated and wild tomato. *Fw2.2* is hypothesized to function as a negative regulator of cell division, but its precise role is not known yet (Baldet et al., 2006). Cell cycle genes play a key role in the regulation of cell division and endoreduplication (Francis, 2007). They control the transition between the different phases of the cell cycle. The classic cell cycle consists of four phases: the first gap phase (G1 phase) during which a cell grows before committing itself into DNA synthesis (S phase). The S phase is followed by a second gap phase (G2 phase) during which the cell grows again and prepare itself for mitosis (M phase) (Francis, 2007). During mitosis a mother cell divides into two daughter cells. The cell cycle is equipped with several "check points" where cell cycle events are monitored and signals are generated specifying whether the cell cycle should be temporally or definitely arrested (Murray, 2004). The classic four phases cell cycle occurs in dividing cells. As cell division stops, some fruit cells do not immediately exit the cell cycle, but continue to grow (Gap phases) and duplicate their DNA (S phase) without mitosis. This incomplete cell cycle of which the M phase is inhibited is called endocycle or endoreduplication cycle (Chevalier et al., 2011). The regulation of the mitotic cell cycle and the endocycle depends on the activity of two special classes of proteins termed cyclins and cyclins dependent kinases (CDKs). Cyclins and CDKs formed complexes that are active at different points of the cell cycle (Francis, 2007). Many types of cyclins and CDKs with known functions have been identified in fruit tissues. For instance, A-type cyclins are involved in the progression through the S-phase, B-type cyclins regulate the G2/M transition, and D-type cyclins are involved in the control of the progression through the G1 and S phase (Bourdon et al., 2010). Some proteins in the cell act as activators of CDKs/cyclins complexes (CDK activating kinases or CAK), while others such as WEE1 kinases or KRP (Kip-related proteins) act as inhibitors (Francis, 2007). WEE1 and KRP have been proposed to be involved in the switch from mitotic cycles to endocycles, but the exact

mechanisms through which they act is still under debate (Chevalier et al., 2011). Experimental data show that the expression of cell cycle genes can be affected by environmental factors such as hormone application or sugar supply (Baldet et al., 2002; Baldet et al., 2006).

Genes that control sugar metabolism and cell wall properties have a direct effect on cell expansion. In tomato sugar is imported into fruit cells in the form of sucrose and may be transformed into glucose and fructose by invertase or into fructose and UDP glucose by sucrose synthase (Massot et al., 2010). Starch synthase converts hexoses into starch, which is temporally stored and used as a reservoir for the synthesis of hexoses (Ho, 1992). The accumulation of hexoses into fruit cells creates a gradient of osmotic potential leading to cell water import and cell expansion (Ho, 1992). Cell expansion itself partly depends on the expression of genes controlling cell wall synthesis and degradation such as expansins (Prudent et al., 2010). Genotypic variation in the expression of these genes has been reported in tomato fruit (Prudent et al., 2010). The expression of these genes can be influenced by hormone and sugar availability (Prudent et al., 2010).

Modelling processes underlying fruit growth

Understanding what determines fruit size in response to genotype and environment is challenging, as fruit growth emerges from underlying interrelated complex cellular and subcellular processes. To deepen our understanding of fruit growth it is important to well describe underlying processes involved in fruit response to genotype and environment (Génard et al., 2007). This objective motivated the development of many models of processes underlying fruit growth (Bertin et al., 2003b; Lee et al., 2004; Csikasz-Nagy et al., 2006; Bertin et al., 2007). An appealing feature of explanatory models is their ability to integrate knowledge.

Recent progresses in the understanding of the molecular control of the cell cycle has led to the development of many models of the cell cycle regulation (Novak and Tyson, 2004; Csikasz-Nagy et al., 2006; Barik et al., 2010). These models attempt to simulate protein interaction networks regulating the activity of cyclins and cyclin dependent kinases. Differential equations are used to describe the dynamics of each protein in the network. Bifurcation diagrams are used to analyse the transition between different phases of the cell cycle. The main drawback of these models is their high number of parameters (up to 90) and the difficulty to measure these parameters. A simpler and phenomenological model of cell

division in tomato fruit was proposed by Bertin et al. (2003b). In this model cell division is assumed to occur exponentially for an initial period after which the division activity declines progressively after each division cycle. The model was able to predict cell number in two contrasting tomato cultivars, but did not address environmental effects.

Expansion models have mostly been described at the fruit scale. For example Fishman and Génard (1998) used the biophysical laws governing the expansion of a cell to describe fruit growth in peach. In their model the fruit is considered as a large cell receiving water and sugar from the parent plant through xylem and phloem tissues. Thermodynamic equations are used to describe water and sugar flow into the fruit. This model was used to simulate seasonal changes in fruit fresh and dry mass under different fruit load and water stress conditions. The Fishman and Génard model was later modified to simulate the shift from symplasmic to apoplasmic sugar unloading in tomato (Liu et al., 2007) and the plastic and elastic changes of cell wall in mango fruits (Lechaudel et al., 2007). Carbon import into the fruit has also been modelled using the sink regulation concept (Marcelis et al., 1998). This concept assumes that the ability of a fruit to attract assimilates depends on its sink strength relative to the sink strength of all organs. The sink strength of an organ is defined as its potential capacity to attract assimilates. It is measured as the organ potential growth rate, i.e. its growth rate when assimilate supply is non-limiting (Marcelis et al., 1998). The sink regulation concept was used to describe organ growth in many fruit species including cucumber (Marcelis, 1994), tomato (Heuvelink, 1996), kiwifruit (Lescourret et al., 1998a), peach (Lescourret et al., 1998b) and grape (Vivin et al., 2001).

Compared to other cellular processes, the molecular mechanism underlying endoreduplication is not yet well understood, making it difficult to model this process mechanistically. This probably explains why only relatively few models describe endoreduplication. Schweizer et al. (1995) proposed a model of endoreduplication in maize endosperm. Their model assumed that the transition rate from a lower ploidy level to a higher ploidy level is linear. This assumption was later modified by Lee et al. (2004) in their model of endoreduplication in orchid flower. Lee et al. (2004) assumed that the transition from a lower to higher ploidy level decreases according to a fermi function. Their model agreed with measured data, but the large number of parameters required is a major drawback of this model. Bertin et al. (2007) proposed a simpler model of mitotic activity and enodoreduplication in tomato fruits. This model could well simulate the variation in the number of cells and ploidy levels in two tomato cultivars, but did not address environmental effects.

Although the models described above provide some insight into processes underlying fruit growth they were usually limited to describing one (Marcelis, 1994; Fishman and Génard, 1998; Bertin et al., 2003b; Lee et al., 2004) or rarely two (Bertin et al., 2007) processes and environment effects were sometimes not considered (Bertin et al., 2003b, Bertin et al., 2007). As illustrated by the following examples, cellular processes underlying fruit growth are tightly interrelated. The link between cell growth and cell division can be seen in meristematic cells of which size remains constant after many cell division cycles (Beemster et al., 2003). It has been postulated that cells need to reach a critical cell mass for DNA synthesis or mitosis to occur (Jorgensen and Tyers, 2004; Francis, 2007). In many plant tissues, cells that have started endoreduplication cannot re-enter cell division (Sugimoto-Shirasu and Roberts, 2003; Sabelli et al., 2008). A positive correlation was reported between fruit cell size and endoreduplication level (Bertin, 2005; Cheniclet et al., 2005). Modelling fruit underlying cellular processes separately does not capture these interrelationships, which might lead to inaccuracies, especially in simulating fruit growth in the presence of genotype-by-environment interactions or under fluctuating environmental conditions. In practice, the environment to which the fruit is exposed fluctuates during the development of a fruit. There is clearly a need to integrate fruit growth underlying processes in a model as such an integrated model can be used to analyse complex fruit responses to genotype-by-environment interactions and fluctuating environmental conditions.

Objective and thesis outline

The objectives of this study were 1) to develop a model of tomato fruit growth integrating cell division, cell endoreduplication and cell growth, and 2) to use the model to analyse fruit responses to fluctuating assimilate supply and temperature conditions, and genotype-by-environment interactions.

The study was divided into two sections: an experimental section and a modelling section. Results of the experimental section are presented in Chapters 2 and 3. **Chapter 2** presents our findings on the investigation of the histological and molecular basis for genotypic and fruit load-induced variation in fruit size. First, the effects of low fruit load applied at two stages of fruit development on fruit size, fruit cell characteristics, fruit sugar content and cell cycle gene expression were investigated for a set of tomato genotypes differing in their final fruit size. Subsequently, we attempted to quantify the relationships between variations in final fruit size and cell characteristics or fruit sugar levels. **Chapter 3** analyses the responses of fruit

underlying cellular processes to contrasting fruit temperatures. In this chapter we investigated the effects of increased temperature applied early or late during fruit development on fruit cellular processes. To ensure that the observed responses resulted from direct temperature effects on fruit growth, temperature treatments were applied at the fruit level.

Results of the modelling section are presented in Chapters 4 and 5. In **Chapter 4** results from previous chapters are incorporated in a dynamic model of tomato fruit growth integrating underlying fruit cellular processes. This chapter also presents the results of the model sensitivity analysis and compares model simulations under various fruit loads and temperature conditions with experimental data. **Chapter 5** presents a theoretical framework for future modelling of fruit growth.

The thesis concludes with a general discussion (**Chapter 6**). This chapter analyses the strengths and limitations of the findings presented in earlier chapters and presents suggestions for future research.

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2. Fruit growth response to fruit load and genotype

Fanwoua J, de Visser PHB, Heuvelink E, Angenent G, Yin X, Marcelis LFM, Struik PC (2012) Histological and molecular investigation of the basis for variation in tomato fruit size in response to fruit load and genotype. *Functional Plant Biology*. **39**, 754-763

Abstract

Understanding the molecular mechanisms and cellular dynamics that cause variation in fruit size is critical to control fruit growth. This study aimed at investigating how both genotypic factors and carbohydrate limitation cause variation in fruit size. We grew a parental line (*Solanum lycopersicum* L.) and two inbred lines from *Solanum chmielewskii* producing small or large fruits under three fruit loads (FL): continuously 2 fruits/truss (2&2F) or 5 fruits/truss (5&5F) and a switch from 5 to 2 fruits/truss (5&2F) 7 DAA (days after anthesis). Final fruit size, sugar content and cell phenotypes were measured. The expression of major cell cycle genes 7 DAA was investigated using quantitative PCR. The 5&5F treatment resulted in significantly smaller fruits compared with the 5&2F and 2&2F treatments. In the 5&5F, treatment cell number and cell volume contributed equally to the genotypic variation in final fruit size. In the 5&2F and 2&2F treatment, cell number contributed twice as much to the genotypic variation in final fruit size than cell volume did. FL treatments resulted in only subtle variations in gene expression. Genotypic differences were detected in transcript levels of *CycD3* (cyclin) and *CDKB1* (cyclin-dependent-kinase), but not *CycB2*. Genotypic variation in fruit fresh weight, pericarp volume and cell volume was linked to pericarp glucose and fructose content (R^2=0.41, R^2=0.48, R^2=0.11, respectively). Genotypic variation in cell number was positively correlated with pericarp fructose content (R^2=0.28). These results emphasize the role of sugar content and of the timing of assimilate supply in the variation of cell and fruit phenotypes.

Keywords: cell cycle genes, variety, histology, *Solanum lycopersicum*, assimilates.

Introduction

Fruit size is a major component of fruit yield and quality of many horticultural crops including tomato (*Solanum lycopersicum* L.). It is determined by the number of cells and size of individual cells (Gillaspy *et al.* 1993; Anastasiou and Lenhard 2008). Like other quantitative traits, both cell number and cell size are affected by interactions between environmental and genetic factors (Yin *et al.* 2004; Génard *et al.* 2007).

In most fruit types, cell division only takes place in early stages of ovary and fruit development (Gillaspy *et al.* 1993). Active cell division is already present in the ovary of

young flowers before anthesis, then the division process gradually reduces and stops at anthesis (Gillaspy *et al.* 1993). After successful pollination cell division resumes and gradually declines about one to three weeks after anthesis (Bohner and Bangerth 1988*a*; Giovannoni 2004; Bertin *et al*. 2007). Fruit growth proceeds as a result of cell expansion, leading to more than 100 fold increase in fruit size (Gillaspy *et al.* 1993). In most fruits the expansion phase is accompanied by endoreduplication, i.e. a multiplication of the genome without mitosis, or, in other words, an incomplete cell cycle resulting in an increase of the nuclear DNA (Vlieghe *et al.* 2007). In mature tomato fruit cells, the nuclear DNA content can reach values as high as 256C, i.e. 2^8 times the haploid nuclei DNA content (Tanksley 2004). A positive correlation between endoreduplication and pericarp cell size has been reported in a wide range of plants, including tomato (Kondorosi *et al.* 2000; Bertin 2005; Cheniclet *et al.* 2005; Bourdon *et al.* 2010).

Although cell expansion contributes by far the most to fruit growth, variation in final fruit size has been associated with differences in cell number in many plant species, including tomato (Bohner and Bangerth 1988*a*; Bertin *et al.* 2003; Prudent *et al.* 2010), pear (Zhang *et al.* 2006), and melon (Higashi *et al.* 1999). Several studies have shown that final fruit cell number and cell size are both affected by the availability of assimilate to the fruit (Marcelis 1993; Bertin 2005; Baldet *et al.* 2006; Prudent *et al.* 2010). Bertin (2005) found that fruit size, cell number and cell size were reduced by fruit load-induced assimilate limitation in tomato. Other studies suggested that fruit size might be related to fruit sugar content (Klann *et al*. 1996; Massot *et al.* 2010). In many fleshy fruits including tomato, fruit sugar content is the result of imported sucrose and its enzymatic conversion into glucose and fructose or transient storage in the form of starch (Ho 1992). The relationship between fruit sugar content and fruit size could be linked to the role of sugar in osmotically driven cell expansion (Doerner 2008), or to the function of sugar as signal that promotes gene expression (Riou-Khamlichi *et al.* 2000). The physiological mechanism through which assimilate supply regulates fruit size is still not well understood, but Baldet *et al.* (2006) proposed that carbohydrate controls fruit size by regulating the mitotic activity of pre- and post-anthesis ovaries.

Cell division in the fruit is under the control of key regulators of the cell cycle such as cyclins (Cycs) and cyclin-dependent kinases (CDKs) (Chevalier 2007). The CDKs associate with specific cyclins to drive cells through the entire cell cycle. Three types of cyclins (so-called A, B and D types) and four types of CDKs (so-called A, B, D and F types) have known functions associated with the cell cycle in plants (Francis 2007). B-type cyclins and CDKs have been found to be essential for mitosis to occur, while most D-type cyclins and A-type CDKs are

involved in DNA synthesis (Francis 2007). Endoreduplication is controlled by the activity of cyclins and CDKs specific for DNA synthesis (Vlieghe *et al.* 2007).

High assimilate supply to ovaries of tomato during the pre-anthesis phase can result in high transcript levels of *CycB2*, *CycD3* and *CDKB2*, many cells and large fruits (Baldet *et al.* 2006). Less attention has been paid to the response of post-anthesis cell division and expansion to assimilate supply and very few studies have addressed the quantitative contribution of both cell division and expansion to final fruit size (Bertin 2005). High assimilate supply induced by low fruit load applied after an initial period of assimilate limitation increased cucumber fruit size, fruit growth rate and cell size (Marcelis 1993). Similar results were observed for the fruit size with tomato (Heuvelink 2005; de Koning 1994), but responses at cell and gene scale were not investigated.

We hypothesize that the timing of high assimilate supply to the fruit affects final fruit size through its effects on cell number, cell volume, fruit sugar content and the expression of cell cycle genes. Moreover, we aim to quantify such effects for a set of genetically related genotypes, which differ in their final fruit size. To this end, we manipulated assimilate supply levels by using different fruit load treatments during fruit development for each genotype. The impact of these fruit load treatments on fruit size, cell number, cell volume and fruit sugar content was assessed. The differences in expression of major cell cycle genes between high and low fruit load treatments of the genotypes were examined. The relationships between the fruit sugar content and the measured phenotypes at the cell and fruit scale were analysed.

Materials and methods

Plant material

Three tomato genotypes were used in this study: a genotype with large-sized fruits (*Solanum lycopersicum* 'Moneyberg') and two inbred lines originating from a cross between Moneyberg and *Solanum chmielewskii* and producing small (g36) or large (g49) fruits, respectively. Genotypes g36 and g49 carry their main introgression of *Solanum chmielewskii* on chromosome number three and eight, respectively.

Growth conditions

Plants were sown in the first week of November 2008. Eight weeks later seedlings were transplanted into a 144 m^2 greenhouse (Wageningen 51.57N, 5.31E, the Netherlands) on stonewool slabs at a density of 2.5 plants m^{-2}. Nutrients were provided by fertigation (EC 4.5,

pH 5.6). Climatic conditions in the greenhouse were 22/18 ^{o}C day/night temperature, 400 µmol mol^{-1} CO_2, and 16-hour light daily (0.6-28.4 MJ m^{-2} d^{-1} natural light supplemented with artificial light using high pressure sodium lamps (SON-T Agro 600 Watt, Philips). Side shoots were removed once a week. Flowers were pollinated by vibrating each truss three times a week.

Experimental design and treatments

Fruit load treatments were applied starting from anthesis of flowers at the second proximal position of the third truss of the plant. We compared fruits grown under continuously low or high fruit load with fruits grown first under high fruit load during the first seven DAA (days after anthesis), then under low fruit load during the rest of the fruit growth period. So, for each genotype three fruit load treatments were applied (Fig. 1):

1. a treatment with a continuously high fruit load where five fruits were kept on each truss from anthesis until the end of fruit growth (5&5F treatment);
2. a treatment with a switch in fruit load where five fruits were kept on each truss during the first seven DAA, then all trusses were pruned to two fruits per truss for the rest of the fruit growth period (5&2F treatment); and
3. a treatment with a continuously low fruit load treatment where two fruits were kept on each truss from anthesis until the end of fruit growth (2&2F treatment).

All nine combinations between the three genotypes and the three fruit load treatments were arranged according to a completely randomized design with five replicate plants.

Observations and measurements

Flower and fruit measurements: Measurements were carried out for each plant on the second proximal fruit of the third truss of the plant. The anthesis of all flowers was recorded every day. Anthesis date was considered as the first date on which the flower opened fully. Fruits were harvested 7 DAA and at breaker stage. Fruits harvested at breaker stage were weighed and their diameters were measured using a digital calliper. Each fruit harvested 7 DAA or at breaker stage was split into two halves at the equatorial plane. Pericarp isolated from fruits harvested 7 DAA was used for the gene expression study. Pericarp isolated from fruits at the breaker stage was used for cell histology and sugar analyses.

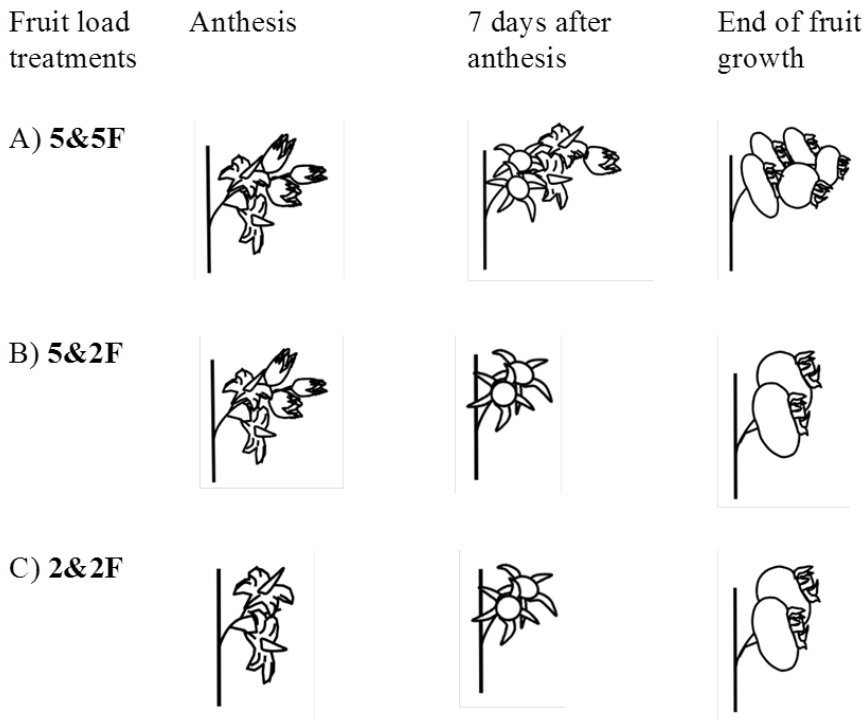

Fig. 1. Schematic representation of fruit load treatments. A) in 5&5F plants each truss was pruned to 5 fruits at anthesis of the second proximal fruit and these were maintained until the end of fruit growth period; B) in 5&2F plants each truss was pruned to 5 fruits at anthesis of the second proximal fruit, then 7 days after anthesis all trusses were pruned to 2 fruits for the rest of the fruit growth period; C) in 2&2F plants each truss was pruned to 2 fruits at anthesis of the second proximal fruit and these were maintained until the end of fruit growth period. All measurements were carried out on the second proximal fruit of the third truss.

Cell histology: Isolated pericarps were fixed overnight at room temperature in a 1 acetic acid : 2 formaldehyde : 5 ethanol solution. During fixation partial vacuum was applied to extract intercellular gases. Samples were washed and dehydrated using ethanol and embedded in Technovit 7100 (Kulzer, Wehrheim, Germany). Sections of 3 μm thick were made using metallic knives on a microtome (Leica, Rijswijk, Netherlands). Sections were stained using toluidine blue and photographed on a light microscope equipped with a colour digital camera. Images were analysed with Image J (National Institutes of Health, USA). During image analysis two tissues were distinguished in each pericarp section: the first 5 cell layers representing the exocarp and the region between the 5^{th} cell layer and the endocarp representing the mesocarp (Fig. 2A).

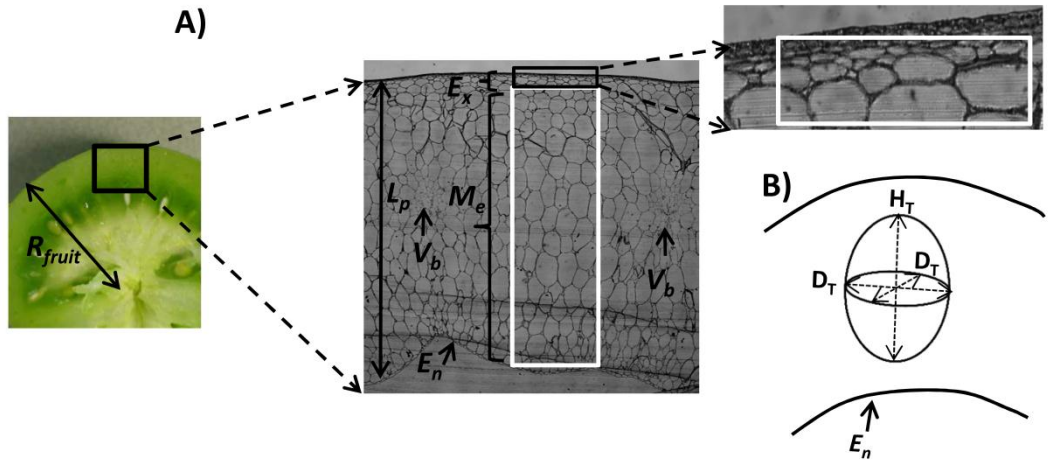

Fig. 2. Structure of tomato pericarp at breaker stage: A) the white rectangles are examples of the region within which mean cell area was estimated in the exocarp and mesocarp as detailed in the materials and methods; B) schematic representation of the ellipsoid cell shape in the pericarp. R_{fruit} is the fruit radius, L_p is the pericarp thickness, V_b is the vascular bundle, E_x is the exocarp, M_e is the mesocarp, E_n is the endocarp, H_T is the cell diameter perpendicular to the fruit skin, D_T is cell diameter parallel to the fruit skin. We assumed that cell diameter parallel to the fruit skin and the diameter in the longitudinal direction (3rd dimension) are equal.

To determine cell area a rectangle was drawn in each tissue centred in the area containing no vascular bundle (Fig. 2A). Vascular bundles and endocarp cells were excluded from the measurements because they do not contribute much to pericarp size. The mean pericarp cell area in each tissue was determined as the ratio between the area of the rectangle and the number of cells inside the rectangle. The mean cell area in each tissue was used to estimate mean cell volume assuming that tomato cells have an ellipsoid shape (Fig. 2B).

$$C_{vol,T} = \frac{4}{3} 0.5 C_{a,T} D_T \qquad (1)$$

where subscript T refers to the measured tissue, i.e. the exocarp or mesocarp, $C_{vol,T}$ is the average cell volume (in mm^3) in the exocarp or mesocarp, $C_{a,T}$ is the average cell area in the exocarp or mesocarp (in mm^2), D_T is the cell diameter in the longitudinal direction (3rd dimension of the ellipsoid) in the exocarp or mesocarp (Fig. 2B) calculated according to the formula:

$$D_T = \frac{C_{a,T}}{0.25\pi H_T} \qquad (2)$$

where H_T is the average cell diameter perpendicular to the fruit skin in the exocarp or mesocarp (in mm) (Fig. 2B). H_T was calculated by dividing the tissue thickness by its number of cell layers, π=3.14. Equation 2 assumes that cell diameters parallel to fruit skin in the transversal and longitudinal direction are equal. To check this assumption we sectioned some pericarps in the longitudinal and transversal directions and analysed cell diameter differences in both directions. Cell diameter parallel to fruit skin in both directions was not statistically different (data not shown).

The average cell volume of the pericarp was calculated as the weighted average of cell volume in the exocarp and mesocarp.

The number of cells in the exocarp or mesocarp tissue ($C_{n,T}$) was calculated by dividing the exocarp or mesocarp tissue volume, $P_{vol,T}$ by the mean tissue cell volume $C_{vol,T}$:

$$C_{n,T} = \frac{P_{vol,T}}{C_{vol,T}} \quad (3)$$

where $P_{vol,T}$ (in mm^3) is calculated approximating the tomato fruit to a sphere. In case of exocarp, the formula for $P_{vol,T}$ is:

$$P_{\mathrm{vol,T}} = \frac{4}{3}\pi[R_{\mathrm{fruit}}^3 - (R_{\mathrm{fruit}} - L_{\mathrm{E}})^3] \quad (4)$$

where R_{fruit} is the fruit radius (in mm), and L_E is the exocarp thickness (in mm). Then $P_{vol,T}$ for mesocarp is expressed as:

$$P_{\mathrm{vol,T}} = P_{\mathrm{vol}} - \frac{4}{3}\pi[R_{\mathrm{fruit}}^3 - (R_{\mathrm{fruit}} - L_{\mathrm{E}})^3] \quad (5)$$

where P_{vol} is the pericarp volume (in mm^3), which was calculated as:

$$P_{\mathrm{vol}} = \frac{4}{3}\pi[R_{\mathrm{fruit}}^3 - (R_{\mathrm{fruit}} - L_{\mathrm{P}})^3] \quad (6)$$

where L_P is the pericarp thickness (in mm).

Extraction and analysis of soluble sugars and starch: Soluble sugars were extracted from freeze-dried powdered pericarp tissue according to the method described by Hajjaj *et al*. (1998). Samples were boiled in 80% ethanol (v/v) at 80 ^{o}C for 20 minutes. The mixture was cooled down and centrifuged for 5 minutes at 25,000 *g*. The resulting supernatant was dried by evaporation at 45 ^{o}C for 2 hours in a rotavapor apparatus. The dried residue was dissolved

in 1 ml distilled water in an ultrasonic bath for 10 minutes, centrifuged for 15 minutes, diluted 20 times and analysed by high-performance liquid chromatography (HPLC) (Dionex, Sunnyvale, California, USA).

To determine the pericarp starch content, pellets obtained after soluble sugar extraction were dissolved and digested in α–amylase and amyloglucosidase solutions (Smith, 1988). The resulting glucose was analysed by HPLC.

Gene expression: RNA was isolated from fruit pericarp with Tripure Isolation Reagent kit (Roche Applied Science, Indianapolis, USA) and cleaned with RNeasy mini kit (Qiagen, Austin, USA). Isolated RNA was treated with DNAse using the DNAse kit (Invitrogen, Landsmeer, the Netherlands). Complementary DNA was synthesized using a cDNA synthesis kit (Biorad, Hercules, Canada). Quantitative PCR (qPCR) was performed on cDNA with the sets of primers defined in Table S1 [Supplementary Information] using the iQ SYBR Green Supermix kit (Biorad, Hercules, Canada) in a single colour real-time PCR detection system (Biorad, USA). The expression of three major cell cycle genes, *CycB2*, *CycD3* and *CDKB1*, was investigated. The *CycB2* gene is known to encode for a protein that regulates the transition between the second gap phase (G2 phase) and mitosis (M phase) in the cell cycle (Francis 2007). The *CycD3* gene encodes for a protein that is involved in the transition between the first gap phase (G1 phase) and the DNA synthesis phase (S phase) and is proposed to be a primary sensor of external conditions such as sugar levels (Chevalier 2007). The *CDKB1* gene encodes for a protein involved in the transition between the G2 and M phase (Chevalier 2007; Francis, 2007). Three technical replicates were run for each qPCR of a fruit. qPCR reactions were performed on four replicate fruits for each fruit load treatments seven DAA, a transition stage between the cell division and the cell expansion phase in the tomato fruit (Bohner and Bangerth 1988*a*; Giovannoni 2004). The expression of the genes at this stage should be the same between 5&5F and 5&2F treatments; so qPCR was conducted only for fruits of the 5&5F and 2&2F treatments. The levels of expression of each gene were normalized against the expression of the *TIP41* gene, a housekeeping gene in tomato (Exposito-Rodriguez *et al*. 2008). Normalized gene expression was calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Statistical analysis

The effects of fruit load and genotype on measured variables were analyzed by two-way ANOVA and *F*-tests were used to determine the statistical significance (Matlab, USA). When

significant effects were detected, Tukey's least significant difference test was used to compare means. The relationships between fruit fresh weight, pericarp volume, cell number and cell volume were analysed using linear regression. The coefficient of determination and the statistical significance of simple linear regression were used to assess the quality of relationships involving two variables. Multiple linear regression was used to study the joint effect of cellular variables (cell number and cell volume) on the phenotype at the fruit scale. We also used multiple linear regression to identify the sugar component of which the concentration was important for the variation in fruit phenotype, cell number or cell volume. When the regression model involved predictors measured in different units (e.g. cell number and cell volume), we first checked if the predictors were correlated or not. If there was no correlation between the predictors, the standardized regression coefficient was computed (Archdeacon 1994) to quantify the contribution of each predictor on the response.

Results

Effects of fruit load and genotype on fruit size, pericarp size and number of cell layers

Fruit load significantly affected fruit size ($P<0.001$). Continuously high fruit load on the truss (5&5F) resulted in smaller fruits compared to fruits subjected to continuously low fruit load (2&2F) (Table 1). Averaged over all genotypes fruit fresh weight did not differ significantly between the 5&2F and the 2&2F treatments (Table 1). Genotypic effects were large ($P<0.0001$) and consistent with expectations. No significant interaction was found between fruit load and genotype ($P=0.77$). Fruit growth duration was not affected by genotype ($P=0.80$) or fruit load treatments ($P=0.37$).

Fruit load significantly affected pericarp volume ($P<0.0001$). In the 5&5F and 5&2F treatments pericarp volume was significantly reduced compared with the 2&2F treatment (Table 1). Genotypic effects were large ($P<0.0001$) and in line with genotypic differences in fruit size. The interaction between fruit load and genotype was not significant for pericarp volume ($P=0.39$). For all other comparisons, pericarp volume responded similarly as fruit fresh weight (Table 1). Pericarp volume appeared to be linearly related to fruit fresh weight, even when all genotype × fruit load treatment combinations were pooled in one analysis (Fig. 3). Hence pericarp volume might be sufficient to describe variations in fruit fresh weight. Pericarp thickness was not affected by fruit load treatments ($P=0.13$). Averaged over all fruit load treatments pericarps of large-fruited genotypes (Moneyberg and g49) were significantly thicker compared with pericarps of the small fruited genotype (g36) (Table 1).

Table 1. Effects of fruit load treatments and genotype on final fruit fresh weight, pericarp volume, pericarp thickness, number of pericarp cell layers, exocarp cell volume, mesocarp cell volume, mean pericarp cell volume and cell number. 5&5F, 5&2F and 2&2F are continuously high, switch and continuously low fruit load treatments, respectively. Each value of fruit fresh weight and pericarp volume is the mean of 15 replicate fruits; each value at the cell scale is the mean of 2 pericarp measurements performed on 15 replicate fruits. Mon = Moneyberg.

	Fruit load treatments*			Genotype+		
	5&5F	5&2F	2&2F	g36	Mon	g49
Fruit fresh weight (g)	61.6^{a}	80.2^{b}	88.2^{b}	44.4^{a}	86.4^{b}	99.2^{c}
Pericarp volume (cm^3)	39.7^{a}	51.7^{b}	59.4^{c}	26.1^{a}	56.7^{b}	68.0^{c}
Pericarp thickness (mm)	5.6^{a}	5.9^{a}	5.9^{a}	4.2^{a}	6.5^{b}	6.7^{b}
Number of pericarp cell layers	24^{a}	24^{a}	25^{a}	21^{a}	26^{b}	26^{b}
Exocarp cell volume ($\times10^{-3}$) (mm^3)	3.5^{a}	3.6^{a}	2.9^{a}	3.1^{a}	3.3^{a}	3.7^{a}
Mesocarp cell volume ($\times10^{-3}$) (mm^3)	29.6^{a}	35.7^{a}	34.3^{a}	27.4^{a}	37.2^{b}	35.0^{b}
Mean pericarp cell volume ($\times10^{-3}$) (mm^3)	27.5^{a}	33.2^{a}	32.1^{a}	25.0^{a}	34.9^{b}	32.9^{b}
Cell number ($\times10^{6}$)	2.3^{a}	2.6^{ab}	3.1^{b}	1.9^{a}	2.8^{b}	3.3^{c}

* For the same response variable, means across fruit load treatments were not statistically different when followed by the same letter (P>0.05).

+ For the same response variable, means across genotypes were not statistically different when followed by the same letter (P>0.05).

The interaction between fruit load and genotype was not significant for pericarp thickness (P=0.88). Thicker pericarp might result from increase in pericarp cell volume and/or formation of more cell layers. The number of cell layers in the pericarp responded similarly as pericarp thickness to fruit load and genotype (Table 1).

Effects of fruit load and genotype on cell number and cell volume

Continuously low fruit load on the truss (2&2F) resulted in significantly more cells compared with the 5&5F treatment (Table 1). Pruning the truss from five fruits to two fruits 7 DAA (5&2F) did not significantly increase pericarp cell number compared with treatment 5&5F (Table 1). Differences in cell number between 5&2F and 2&2F were not statistically significant (Table 1).

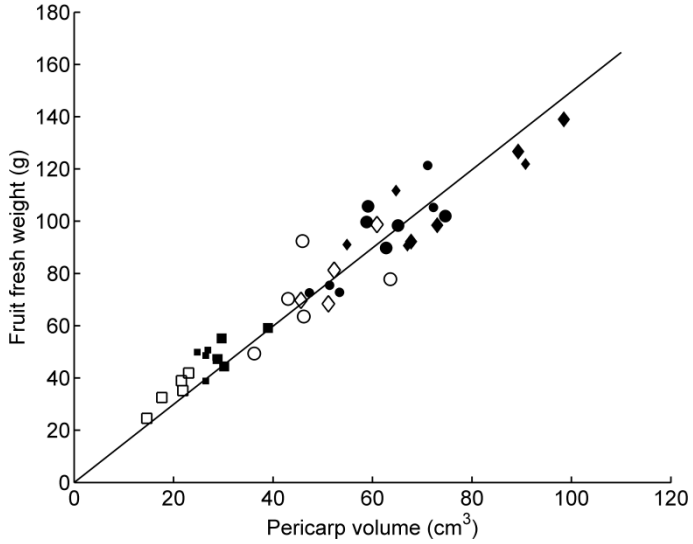

Fig. 3. Relationship between fruit fresh weight and pericarp volume. Each point is an individual fruit at the breaker stage of genotypes g36 (square), Moneyberg (circle), g49 (diamond) measured in 5&5F (open symbols), 5&2F (small, closed symbols) and 2&2F (large, closed symbols) fruit load treatments. Slope of regression is 1.5 g cm^{-3}, R^2=0.90, P<0.001.

Within each fruit load treatment cell number was 1.4-2.0 times higher for the large-fruited genotypes (Moneyberg and g49) than for the small-fruited genotype (g36). No significant interaction was found between fruit load and genotype for cell number (P=0.33). Fruit load and genotype treatments resulted in similar exocarp cell volume (Table 1). No significant interaction was found between genotype and fruit load treatments for exocarp, mesocarp and average pericarp cell volume (P>0.05). Mesocarp cell volume was affected by genotype but not by fruit load treatments (Table 1). Mean pericarp cell volume responded similarly as mesocarp cell volume to genotype and fruit load treatments (Table 1). Averaged over all fruit load treatments the small-fruited genotype had significantly smaller pericarp cells than the large fruited genotypes (Table 1). Within each fruit load treatment cells were 1.2-1.6 times larger for large-fruited genotypes (Moneyberg and g49) compared with the small-fruited genotype (g36). Differences in cell volume between genotypes were less pronounced than differences in cell number, which suggests that fruit size differences between genotypes could be primarily related to differences in cell number rather than cell volume.

Relative contribution of cell number and cell volume to variation in pericarp volume

An analysis using a general linear model, in which cell number and cell volume were included as co-variants, showed that in contrast to the effect of fruit load treatments, the effect of

genotypes on pericarp volume can be explained by genotypic variation in cell number and cell volume. Therefore, the relationship between pericarp volume and cell number and/or cell volume was analysed to quantify the relative contribution of cell number and cell volume in affecting pericarp volume for individual fruit load treatment.

In each fruit load treatment, the separate contribution of cell number and cell volume to genotypic variations in pericarp volume was first examined using simple linear regression [Supplementary Information - Fig. S1]. The coefficients of determination of the regression lines suggest that cell number accounted for variations in pericarp volume in the 5&2F and 2&2F treatments more than cell volume did [Supplementary Information - Fig. S1A and S1B]. By contrast, in the 5&5F treatment, both cell number and cell volume contributed significantly to variations in pericarp volume [Supplementary Information - Fig. S1A and S1B].

Table 2. Estimates (± standard error) and statistics of parameters of regression ($P_{vol} = \alpha C_n + ßC_{vol} + \gamma$) fitted for different fruit load treatments. P_{vol} is the pericarp volume in cm^3, C_n is the cell number ($\times 10^6$) and C_{vol} is the cell volume in mm^3. Parameter α is expressed in cm^3, $ß$ is dimensionless, γ is expressed in cm^3 .

Treatment	Parameters	Parameter estimates*	Standardized coefficient	R^2
5&5F	α	16.9 (± 2.6)	0.74	0.85
	$ß$	1183.9 (± 223.2)	0.61	
	γ	-31.2 (± 9.0)		
5&2F	α	22.2 (± 2.6)	0.92	0.87
	$ß$	902.1 (± 240.7)	0.41	
	γ	-36.8 (± 11.5)		
2&2F	α	18.5 (± 1.6)	0.90	0.94
	$ß$	1322.0 (± 257.3)	0.39	
	γ	-40.4 (± 9.7)		

* All parameters were significant ($P < 0.01$)

This relative importance of cell number and cell volume in the prediction of pericarp volume was confirmed by more compelling multiple regression analyses (Table 2).

Since there was little correlation between cell number and cell volume in all fruit load treatments [Supplementary Information - Fig. S1C], the magnitude of the standardized regression coefficient was used to measure the relative importance of each predictor (Table 2). In the 2&2F and 5&2F treatments, values of the standardized regression coefficient indicated that cell number contributed two times more than cell volume to the genotypic variation of pericarp volume. In the 5&5F treatment, genotypic variations in pericarp volume were almost equally shared between cell number and cell volume (Table 2).

Effects of fruit load and genotype on pericarp sugar and starch content

The interaction between fruit load and genotype was significant for sucrose content (P=0.01), but not for starch, fructose, glucose content and sucrose: hexoses ratio (P>0.05). The treatment with a switch in fruit load (5&2F) resulted in a significantly higher pericarp sucrose content in genotype g36 than the treatments with a constant fruit load, but not in Moneyberg and g49 (Table 3).

Table 3. Effects of fruit load treatments and genotype on pericarp sucrose content* (µg/mg dry weight). Each value is the mean of 5 replicate fruits at the breaker stage.

Fruit load treatments	Genotypes		
	g36	Moneyberg	g49
5&5F	5.1[a]	3.9[a]	5.1[a]
5&2F	7.9[b]	4.8[a]	3.8[a]
2&2F	4.7[a]	4.9[a]	4.2[a]

* Means per fruit load and genotype combination were not statistically different when followed by the same letter (P>0.05).

Fruit load treatments did not affect starch, glucose, fructose content and the sucrose: hexoses ratio (P>0.05). Averaged over all fruit load treatments, glucose and fructose content were significantly higher in the large-fruited genotypes (Moneyberg, g49) compared with the small-fruited genotype (g36) (Table 4). By contrast starch content was highest in the small-fruited genotype (g36) compared with the large-fruited genotypes (Moneyberg, g49) (Table 4). Sucrose: hexoses ratio was also highest in the small-fruited genotype (g36) compared with the large-fruited genotypes (Moneyberg, g49).

Table 4. Effects of fruit load treatments and genotype on pericarp glucose, fructose and starch content (µg/mg dry weight), sucrose: hexoses ratio (%) and expression of *CycB2*, *CycD3* and *CDKB1* gene. Each value of sugar content is the mean of 15 replicate fruits at the breaker stage. Gene expression values are mRNA relative abundance normalized to *TIP41* (a housekeeping gene in tomato). Each value of mRNA abundance is the mean of three technical replicates performed on 12 fruit replicates for each fruit load treatment or on 8 fruit replicates for each genotype. 5&5F, 5&2F and 2&2F are continuously high, switch and continuously low fruit load treatment respectively. DAA = days after anthesis; Mon = Moneyberg.

		Fruit load treatments*			Genotype+		
		5&5F	5&2F	2&2F	g36	Mon	g49
Pericarp sugar content of mature fruits	Glucose	191[a]	188[a]	199[a]	176[a]	207[b]	195[b]
	Fructose	200[a]	198[a]	208[a]	180[a]	218[b]	209[b]
	Starch	3.1[a]	7.2[a]	9.2[a]	14.2[b]	3.5[a]	1.8[a]
	Sucrose: hexoses	1.2[a]	1.5[a]	1.1[a]	1.7[b]	1.1[a]	1.1[a]
Relative mRNA abundance 7 DAA	*CycB2*	0.9[a]	-	1.3[a]	1.3[a]	1.1[a]	0.9[a]
	CycD3	0.9[a]	-	0.7[a]	1.2[b]	0.7[ab]	0.5[a]
	CDKB1	1.3[a]	-	1.4[a]	1.9[b]	1.2[a]	0.9[a]

* For the same response variable, means across fruit load treatments were not statistically different when followed by the same letter (P>0.05).

+ For the same response variable, means across genotypes were not statistically different when followed by the same letter (P>0.05).

To identify which of these four non-structural carbohydrate components were the most relevant for the genotypic variation in fruit or cell phenotype, we attempted to relate phenotypes (i.e. fruit fresh weight, pericarp volume, cell number and cell volume) to non-structural carbohydrate contents. Phenotypic measurements and pericarp non-structural carbohydrate contents of individual fruits were related in multiple regression functions involving all four non-structural carbohydrate components (phenotype = a×starch + b×sucrose + c×glucose + d×fructose + e, where a, b, c, and d are regression coefficients and e is a constant). The number of regressors in the model was gradually reduced by backward elimination. For fruit fresh weight, backward elimination resulted in a model with glucose and fructose as regressors (R^2=0.41; P<0.02). The model with glucose and fructose as regressors also best described pericarp volume (R^2=0.48; P<0.0001) and cell volume (R^2=0.11; P<0.03).

For cell number, backward elimination resulted in a model with only fructose as regressor. Cell number was positively correlated to pericarp fructose content (R^2=0.28; P<0.001).
Negative correlations were found between sucrose: hexoses ratio and fruit fresh weight (R^2=0.14; P<0.01), pericarp volume (R^2=0.21; P=0.001) and cell number (R^2=0.19; P=0.002). Cell volume was not correlated with sucrose: hexoses ratio (P=0.6).

Effects of fruit load on expression of cell cycle genes

We investigated whether high or low fruit load had an effect on the expression of cell cycle genes in the three genotypes. Therefore the expression of three major cell cycle genes, *CycB2*, *CycD3* and *CDKB1*, was analyzed. For all genes investigated no significant interaction between fruit load and genotype was found (P>0.05). Variations in the expression levels of *CycB2*, *CycD3* and *CDKB1* were subtle and not significantly enhanced by the continuously low fruit load (2&2F) treatment (Table 4). No genotypic differences were detected in the expression of *CycB2* (P=0.51). By contrast significant differences in the expression of *CDKB1* (P=0.01) and *CycD3* (P=0.04) were found among the three genotypes. Surprisingly, the highest transcript levels of *CycD3* and *CDKB1* were found in the small-fruited genotype (g36), which were about two times higher than transcript levels in large-fruited genotypes (Moneyberg and g49) (Table 4). The overall transcript level of *CDKB1* was almost twice as high as that of *CycD3*, but did not differ significantly from that of *CycB2* (Table 4; analysis not shown).

Discussion

Low fruit load increases individual fruit weight in tomato (Bertin 2005; Baldet *et al.* 2006). In our experiment continuously low fruit load (2&2F) effectively resulted in a strong increase in individual tomato fruit fresh weight and pericarp volume compared with the continuously high (5&5F) fruit load treatment (Table 1). At the cell scale, continuously low fruit load treatment increased cell number compared with the continuously high fruit load treatment (Table 1). Similar observations were made by Bertin (2005) and Baldet *et al.* (2006) on tomato fruits. Continuously low fruit load treatment did not significantly increase cell volume (Table 1). This result disagrees with many studies which showed that high assimilate supply increased cell size in tomato fruits (Bertin 2005; Bohner and Bangerth 1988*b*). These authors also observed that within a tomato truss distal fruits are more sensitive to assimilate limitations than proximal fruits (Bohner and Bangerth 1988*b*; Bertin 2005). For example,

Bohner and Bangerth (1988*b*) found that plant defoliation resulted in a decrease in cell number and cell size that was more pronounced for distal than for proximal fruits. In this experiment, competition between individual fruits for assimilates was probably more severe than in our experiment. Bertin (2005) observed that low fruit load increased cell number in distal fruits, but not in proximal fruits. Prudent *et al*. (2010) found that cell size measured on proximal fruits was not significantly affected by fruit load treatments in one of the two tomato genotypes they investigated. In our experiment fruit size differences among genotypes were more related to cell number than to cell volume. These results collectively indicated that cell number played a more important role than cell volume in determining fruit size in our experiment. Genotypic or fruit load-induced variations in final tomato fruit size have been mainly associated with differences in cell number (Bertin *et al.* 2003; Baldet *et al.* 2006; Prudent *et al.* 2010).

In our experiment fruit load did not affect pericarp starch, glucose or fructose content (Table 4). A wide range of responses of pericarp starch and sugar content to fruit load treatments has been reported in tomato (Prudent *et al*. 2009; 2010; Do *et al.* 2010; Massot *et al.* 2010). For example, Massot *et al.* (2010) found that low fruit load increased pericarp hexose content while Prudent *et al*. (2009) observed that fruit load reduction did not affect pericarp sugar content. Differences in starch and sugar responses to fruit load treatments might be caused by several interacting factors including cultivar differences, the stage of fruit development or plant growing conditions (Luengwilai *et al*. 2010). Fruit sugar content is the net result of carbohydrate import and utilization. If a higher carbohydrate supply is expected to increase fruit sugar content, a higher utilization would result in the opposite effect. Hence in our experiment the absence of fruit load effects on pericarp sugar content could be explained by the fact that the effects of higher assimilate supply in the 2&2F mature fruits were counteracted by a higher sugar utilization, which resulted in a similar sugar content as found for the 5&5F treatment.

Our results showed that the genotypic variation in final fruit fresh weight and pericarp volume was related to fruit glucose and fructose content (R^2=0.41 and R^2=0.48, respectively). Variation in fruit size has been associated with changes in fruit sugar content in many plant species including tomato (Klann *et al*. 1996; Massot *et al*. 2010), avocado (Richings *et al.* 2000) and muskmelon (Kultur 2001). At the cell scale, genotypic variation in cell volume was related to pericarp fructose and glucose content (R^2=0.11), while the genotypic variation in cell number was positively correlated with pericarp fructose content (R^2=0.28). Since the expression of cell cycle genes is sensitive to hexose availability (Riou-Khamlichi *et al.* 2000),

our results suggest that differences in cell division and final fruit size among genotypes might be partly linked to their differences in sugar metabolism. Kwon and Wang (2011) observed that increased cell number in transgenic tobacco plants overexpressing *CycD3* gene was related to high invertase activity and high hexose levels. In plant cells, invertase is responsible for the enzymatic conversion of sucrose into fructose and glucose (Ho 1992) and might play an important role in the endogenous levels of hexoses necessary to enhance the cell cycle machinery (Kwon and Wang 2011). Genotypic variations in the activity of invertase was reported in tomato fruits (Elliott *et al*. 1993; Prudent *et al*. 2010). Genotypic differences in carbohydrate metabolism in our experiment were suggested by the higher sucrose: hexoses ratio observed in the small-fruited genotype compared with the large-fruited genotypes (Table 4). Besides its role in the cell cycle machinery, the sucrose: hexoses ratio was proposed to be involved in osmotically driven cell expansion (Doerner 2008). In our experiment sucrose: hexoses ratio was correlated with cell number, but not with cell volume.

In our study gene expression levels of the small fruited genotype (g36) were similar (*CycB2*) to, or higher (*CycD3* and *CDKB1*) than, those of the large fruited genotypes (Moneyberg, g49) (Table 4). Variations in the expression levels of *CycB2*, *CycD3* and *CDKB1* genes were only subtle and not significantly affected by fruit load treatments (Table 4). Small changes in expression levels over a long period of fruit development could lead to substantial effects on fruit growth. Baldet *et al.* (2006) and Joubès *et al.* (2000) have shown that the expression of the *CycB2* and *CycD3* genes measured at five time points before or after anthesis increased when tomato fruits were subjected to high sugar supply. In another study Baldet *et al*. (2002) observed that darkness-induced assimilate limitation reduced the expression of eight cell cycle genes measured at five time points during tomato fruit growth. In our experiment gene expression was investigated only once during fruit development. At this time point (7 DAA) each fruit could be in a slightly different phase of the fruit developmental program. More expression analyses at different time points during fruit development could give a better view on the expression levels and patterns associated with the treatments.

Lowering fruit load from five fruits per truss at anthesis to two fruits per truss 7 DAA (the 5&2F treatment) increased the final fruit weight and pericarp volume by up to 30% compared with the 5&5F treatment (Table 1). Similar observations were made on cucumber (Marcelis 1993) and tomato (de Koning 1994; Heuvelink 2005). Since fruit growth duration was not affected by fruit load treatments in our experiment, increase in final fruit size must have been related to an increase in fruit growth rate. Heuvelink (2005) and de Koning (1994) observed that a sudden change from limiting to non-limiting assimilate supply during the development

of the tomato fruit gradually increased fruit growth rate, but did not affect its growth duration. A similar but faster response to sudden assimilate increase was reported with cucumber (Marcelis 1993). Our results suggest that the low fruit load applied from 7 DAA onwards affected fruit growth processes responsible for fruit size increase. Indeed fruit histological analyses revealed that the 5&2F treatment resulted in similar cell number as the 2&2F treatment (Table 1). One argument can be put forward to explain the effect of the treatment with the switch in fruit load on cell number. During the first 7 DAA when the fruit load was high (5 fruits/truss), genes of the cell cycle machinery such as *CycB2* and *CDKB1* that drive the entry into the M-phase (Francis 2007) were exposed to low assimilate. This probably resulted in a lower expression of these genes (Joubès *et al.* 2000; Baldet *et al.* 2006) and thus in a lower cell number. Although high fruit load did not affect the expression of cell cycle genes in our experiment, other studies involving more measurement time points early in tomato fruit growth reported a reduction in the expression of cell cycle genes under low assimilate supply (Joubès *et al.* 2000; Baldet *et al*. 2002; Baldet *et al.* 2006). Seven DAA when the truss was pruned to two fruits per truss in our experiment, assimilate supply increased. This probably resulted in enhanced expression of all cell cycle genes (Joubès *et al.* 2000; Baldet *et al.* 2006). However, since the cell division phase is almost over 7 DAA in tomato fruits (Giovannoni 2004), the enhancing effect of high assimilate supply on cell cycle genes involved in mitosis was probably present for a short period in the 5&2F treatment. This might explain the slight increase in pericarp cell number in the 5&2F treatment compared with the 5&5F treatment (Table 1).

Our data showed that genotypic variation in pericarp volume could be partly accounted for by cell number or cell volume in the 5&5F treatment (Table 2). This indicates that both cell number and cell volume were limiting to pericarp volume in this treatment. When fruits were subjected to high assimilate supply from anthesis onwards or 7 days later (5&2F and the 2&2F) cell number contributed about twice as much to the variation in final pericarp volume than cell volume did (Table 2). These results suggest that cell number rather than cell volume was more limiting in these treatments. The most likely explanation is that in the 5&2F and 2&2F treatments, high assimilate supply covers the entire period of cell expansion (Bohner and Bangerth 1988*a*) and has probably enabled cells to reach their potential size (Marcelis 1993). On the contrary, in none of our fruit load treatments was high assimilate supplied during the entire period of cell division (Gillaspy *et al.* 1993) and the potential cell number was probably never reached. Indeed even in the 2&2F treatment, high assimilate supply was imposed from anthesis onwards and pre-anthesis ovary development took place under high

fruit load. Cell division before anthesis is important in determining final cell number in tomato fruits (Bohner and Bangerth 1988*a*; Bourdon *et al.* 2010). Baldet *et al.* (2006) observed that high fruit load reduced pre-anthesis cell division and final cell number in tomato fruits. Therefore, the contribution of cell number or cell volume to fruit load-induced variations in fruit size may depend on the timing of assimilate supplied to the fruit.

Conclusions

This study aimed at investigating how both growth factors (i.e. carbohydrate limitation) and genotypic factors cause variation in fruit size. Fruit load-induced variations in fruit size were more related to cell number than to cell volume. Our results suggest that the timing of assimilate supply affects the relative contribution of cell number and cell volume to the genotypic variation in fruit size. Differences in transcript levels of cell cycle genes between genotypes could be associated with genotypic differences in the scheduling of gene expression. These results also illustrate the difficulty of assessing gene response at a single developmental stage. This study suggests a link between pericarp sugar content and genotypic variation in fruit and cell phenotypes. Measuring the activity of enzymes involved in sugar metabolism in these genotypes might help to elucidate this relationship.

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Supplementary information

Table S1. Primer sequences used to amplify gene-specific regions. For each gene the accession number and reference are given.

cDNA	Primer sequence	Accession number	References
CycB2	Forward ATTCAATCTTGGAGAGGATTAAAG Reverse GTAGCCATTTCAGCCCTATC	AJ243455	Joubès *et al*., 2000
CycD3	Forward CAAGGAGAAGGTGGAGAGGATG Reverse GGTGATGAAGTAACTGATGTAGC	AJ002590	Kvarnheden *et al*., 2000
CDKB1	Forward ATGGAGAAATACGAGAAATTGGAG Reverse ACGATGTAGAGAGAATGAGATAGC	AJ297916	Joubès *et al*., 2001
TIP41	Forward GCTGCGTTTCTGGCTTAGG Reverse ATGGAGTTTTTGAGTCTTCTGC	SGN-U321250	Exposito-Rodriguez *et al*., 2008

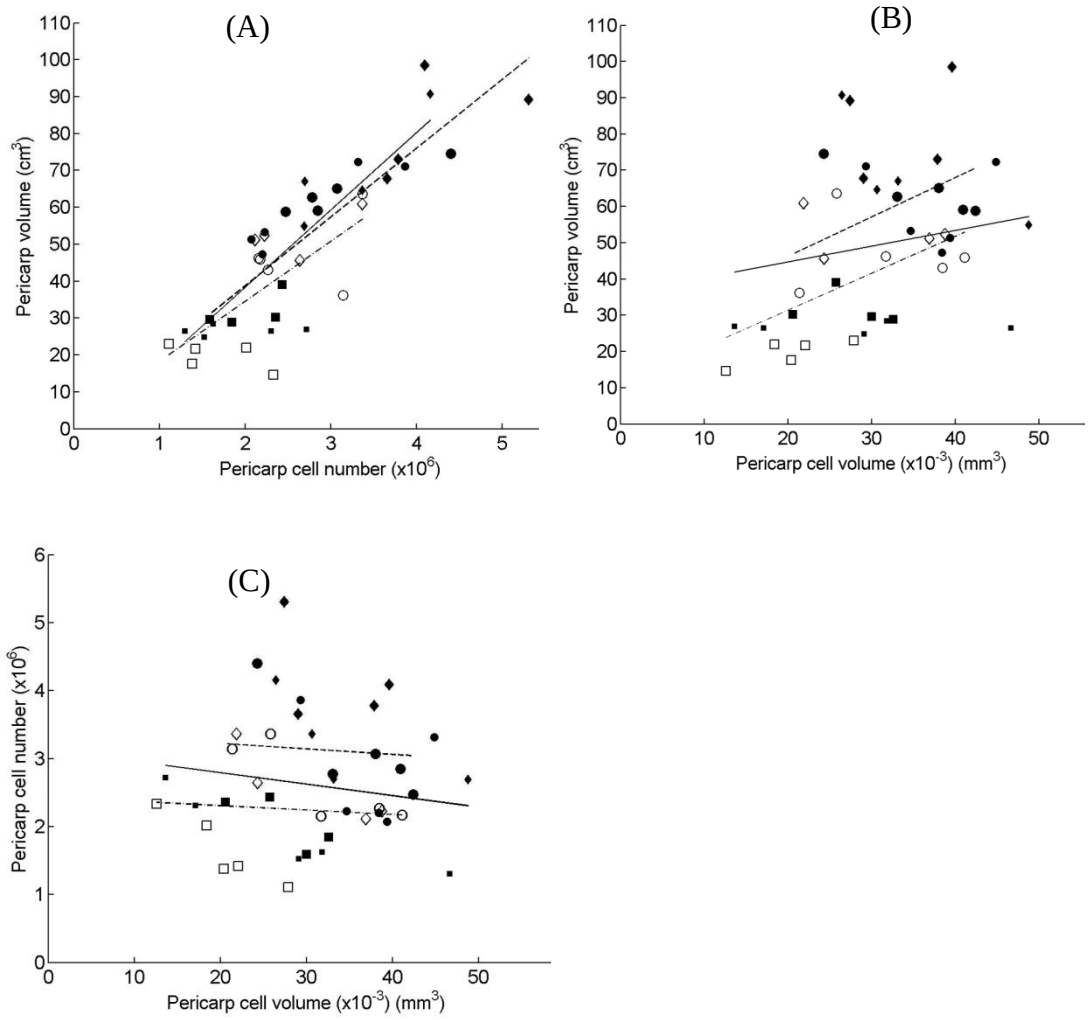

Fig. S1. Relationships between A) pericarp volume and cell number, B) pericarp volume and cell volume, C) cell number and cell volume. Each point is an individual fruit at the breaker stage of genotypes g36 (square), Moneyberg (circle) and g49 (diamond) measured in 5&5F (opened symbols and dash-dot lines), 5&2F (small symbols and continuous lines) and 2&2F (filled symbols and dashed lines) fruit load treatments. Slope (α) of regression is A) α =15.8, R^2=0.48, *P*=0.006 (5&5F); α =20.0; R^2=0.71, *P*<0.001 (5&2F) and α =18.2, R^2=0.79, *P* <0.001 (2&2F); B) α =1.08; R^2=0.31, *P*=0.04 (5&5F); α =0.50; R^2=0.05, *P*=0.43 (5&2F), and α =1.19; R^2=0.12, *P*=0.23 (2&2F), C) α=-0.006; R^2=0.006, *P*=0.80 (5&5F), α =-0.017; R^2=0.04, *P*=0.50 (5&2F), α =-0.007; R^2=0.002, *P*=0.89 (2&2F).

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3. Fruit growth response to temperature

Fanwoua J, de Visser PHB, Heuvelink E, Angenent G, Yin X, Marcelis LFM, Struik PC (2012) Response of cell division and cell expansion to local fruit heating in tomato fruit. *Journal of the American Society for Horticultural Science*. **137**, 294-301.

Abstract

To improve our understanding of fruit growth responses to temperature it is important to analyse temperature effects on underlying fruit cellular processes. This study aimed at analysing the response of tomato (*Solanum lycopersicum* L.) fruit size to heating as affected by changes in cell number and cell expansion in different directions. Individual trusses were enclosed into cuvettes and heating was applied either only during the first 7 days after anthesis (DAA) or from 7 DAA until fruit maturity (breaker stage) or both. Fruit size and histological characteristics in the pericarp were measured. Heating fruit shortened fruit growth period and reduced final fruit size. Reduction in final fruit size of early-heated fruit was mainly associated with reduction in final pericarp cell volume. Early heating increased the number of cell layers in the pericarp, but did not affect the total number of pericarp cells. These results indicates that in the tomato pericarp, periclinal cell divisions respond differently to temperature than anticlinal or randomly oriented cell divisions. Late heating only decreased pericarp thickness significantly. Continuously heating fruit reduced anticlinal cell expansion (direction perpendicular to fruit skin) more than periclinal cell expansion (direction parallel to fruit skin). This study emphasizes the need to measure cell expansion in more than one dimension in histological studies of fruit.

Keywords. Fruit temperature, *Solanum lycopersicum*, fruit size, histology, periclinal cell expansion, anticlinal cell expansion.

Introduction

Fruit growth is strongly influenced by temperature. A shorter fruit growth period as a result of an increase in temperature has been reported in several plant species including kiwi (*Actinidia deliciosa*) (Greer et al., 2003), apricot (*Prunus armeniaca* L.) (Jackson and Coombe, 1966), cucumber (*Cucumis sativus*) (Marcelis and Baan Hofman-Eijer, 1993) and tomato (De Koning, 1994). Temperature also has a direct effect on the rate of fruit growth (Van der Ploeg and Heuvelink, 2005). In general no fruit growth is expected below a certain temperature threshold (Van der Ploeg and Heuvelink, 2005), estimated to be 5.7 ^{o}C in tomato (Adams et al., 2001). Above this base temperature and below the optimum temperature threshold of 30

^{o}C (Pearce et al., 1993), increase in air temperature increases fruit growth rate, but final fruit size might be decreased (Adams et al., 2001; Sawhney and Polowick, 1985) or not affected (Bertin, 2005; Van der Ploeg and Heuvelink, 2005) due to a shorter fruit growth period. Air temperature may also influence fruit growth indirectly through other plant growth and development processes (Adams et al., 2001; Bertin, 2005). For example, within a certain temperature range, an increase in air temperature is associated with an increase in the rate of plant development resulting in more sink organs formed by the plant (De Koning, 1994). This creates a stronger competition among individual sinks for assimilates, sometimes leading to abortion of flowers or incipient fruit (De Koning, 1989; Van der Ploeg and Heuvelink, 2005; Wubs et al., 2009).

When whole plants are exposed to various temperatures, indirect temperature effects make it impossible to quantify the extent to which temperature directly affects fruit growth. To overcome this limitation it is important to apply temperature treatments locally at the fruit level (Adams et al., 2001; Gautier et al., 2005). So far most studies have investigated the effects of whole plant temperatures on fruit growth (Bertin, 2005; De Koning, 1994), probably because of the technical challenges involved in implementing local temperature treatments. Adams et al. (2001) observed that heating individual tomato trusses from 15 ^{o}C to 20 and 25 ^{o}C in transparent chambers increased absolute fruit growth rate, but this effect was compensated by a reduced growth duration resulting in no significant temperature effects on the final fruit size. By contrast Gautier et al. (2005) found that fruit heating from 18.5/15.5 to 21/18.4 ^{o}C day/night reduced tomato fruit size with more pronounced effects under limited assimilate supply. Marcelis and Baan Hofman-Eijer (1993) also showed that heating individual cucumber fruit from 17.5 to 27.5 ^{o}C reduced their final size when fruit were grown under limited assimilate supply.

Experimental data showed that the sensitivity of fruit growth to temperature is not the same during the whole fruit growth period in many plant species including cucumber (Marcelis and Baan Hofman-Eijer, 1993), apple (*Malus domestica*) (Calderón-Zavala et al., 2004) and tomato (De Koning, 2000). For instance, tomato fruit size and time to maturity were reduced when fruit were heated during the first 3 weeks after anthesis, but were not affected when heating was applied 1 or 2 weeks later (Adams et al., 2001). This difference in fruit sensitivity to temperature is not surprising considering that different processes are successively involved during the growth of a fruit (Gillaspy et al., 1993). During the first 7 to 10 DAA, fruit growth in tomato is mainly the consequence of cell division. As cell division progressively stops, individual cells expand until the fruit reaches its final size (Bohner and Bangerth, 1988). To

improve our understanding of fruit growth responses to temperature it is important to analyse temperature effects on underlying fruit cellular processes.

Many studies described the effects of temperature on fruit growth at the fruit level (De Koning, 1994; Greer et al., 2003), but more rarely at the cell level (Bertin, 2005; Marcelis and Baan Hofman-Eijer, 1993). Increasing air temperature reduced cell number and increased cell sizes in tomato fruit due to a reduced cell division period and extended cell expansion period (Bertin, 2005). The author applied temperature treatments on whole plants and local temperature responses were not measured. In cucumber increasing local fruit temperature reduced cell number, but did not affect cell size when fruit were grown under limited assimilate supply (Marcelis and Baan Hofman-Eijer, 1993). Other experiments suggest that temperature effects on cell expansion might not be the same in all expansion directions (Erwin et al., 1991; Strøm and Moe, 1997). These experiments were carried out on plant stems and leaves (Erwin et al., 1991; Strøm and Moe, 1997). To the best of our knowledge, no studies on the effects of temperature on cell expansion in different expansion directions have been reported in fruit tissue.

This study investigates the response of tomato fruit growth to local temperature applied early or late during fruit growth. The objectives were first to analyse whether the reduction in final fruit size of early or late heated fruit is related to reduction in cell number or cell size and, second to investigate temperature effects on fruit cell expansion in different expansion directions.

Materials and methods

Plant material and growth conditions

Two experiments were conducted in 2010 (Exp. 1) and 2009 (Exp. 2) to study the effects of local heating on the growth of tomato fruit. In both experiments seedlings of tomato (*Solanum lycopersicum* 'Moneyberg') were grown for 8 weeks (Exp. 1: Jan.-Feb. 2010; Exp. 2: Nov.-Dec. 2009), and then transplanted in the greenhouse on stonewool slabs at a density of 2.5 plants/m^2. Nutrient solution was prepared according to De Kreij et al. (1997) and provided by fertigation (Electrical conductivity 3.0 dS$\cdot$ m^{-1}, pH 5.6). Climatic conditions in the greenhouse were 21.5 ± 0.09 SE (standard error) ^oC (day temperature) and 18.1 ± 0.05 SE ^oC (night temperature) in Exp. 1 or 22.0 ± 0.17 SE ^oC (day temperature) and 18.5 ± 0.10 SE ^oC (night temperature) in Exp. 2, 16 h photoperiod (0.6-28.4 $MJ\cdot m^{-2}\cdot d^{-1}$ natural light supplemented with artificial light using high pressure sodium lamps (135 $\mu mol\cdot m^{-2}\cdot s^{-1}$ photosynthetic active

radiation (*PAR*), SON-T Agro, Philips, Eindhoven, The Netherlands) and 400 μmol·mol^{-1} CO_2. Flowers were pollinated by vibrating each truss three times per week. All trusses on the plants were pruned to five fruit. Side shoots were removed once per week.

Heating system

Individual trusses were heated into small cuvettes constructed from perspex (WSV Kunststoffen BV, Utrecht, The Netherlands) (Fig. 1A). Each heating cuvette consisted of a cylindrical chamber (13 cm diameter and 20 cm long) equipped with an electronic heating unit (Bausatz, Conrad Electronic SE, Hirschau, Germany); a fan (1004KL-04W-B40-B00, NMB-Minebea, Chatsworth, California) at the base of the chamber blew air into the chamber and released it via an outlet at the top. The maximum flow rate of the fan was 40 L/min. Sensors (Bausatz, Conrad Electronic SE, Hirschau, Germany) continually monitored air temperature inside and outside the chamber. The heating unit was calibrated to control the functioning of the heating block (Cirrus 25, DBK, Spartanburg, South Carolina) and to regulate the temperature of the air inside the chamber. The realised air temperature inside the chamber was 26.9 ± 0.12 SE ^{o}C (day) and 22.7 ± 0.10 SE ^{o}C (night) in Exp. 1 or 27.0 ± 0.44 SE ^{o}C (day) and 23.0 ± 0.20 SE ^{o}C (night) in Exp. 2. When cuvettes were used for the control treatment the heating unit was switched off, but not the fan, and the temperature inside the chamber was the same as the temperature outside.

Experimental design and treatments

Heating treatments were implemented 10 weeks after transplanting. For each plant at this stage the truss of which the second proximal flower was at anthesis was selected to receive one of the treatments and was enclosed into the cuvette. All other trusses on the plant were not enclosed into a cuvette.

In Exp. 1 treatments were applied to investigate the effects of continuous local heating on fruit growth. In the heating treatment, trusses were heated continuously until the breaker stage of the second proximal fruit which was reached 46 DAA. In the control treatment, trusses were also enclosed into cuvettes until the breaker stage of the second proximal fruit, which was reached 54 DAA.

In Exp. 2, treatments were applied to investigate the effects of local heating at two different stages of fruit growth on final fruit phenotype. We studied the effects of heating fruit during the first 7 DAA, the effects of heating fruit from 7 DAA until fruit maturation and the interaction between these two heating schemes. In the interaction, we tested whether the

effects of fruit heating applied during the first 7 DAA on a response variable was affected by the heating treatment applied from 7 DAA until breaker stage. Four treatments were applied as described in Fig. 1B. Treatments were applied to experimental units, each of which consisted of six plants (Exp. 1) or one plant (Exp. 2). Experimental units were arranged according to a completely randomized design with five replicates.

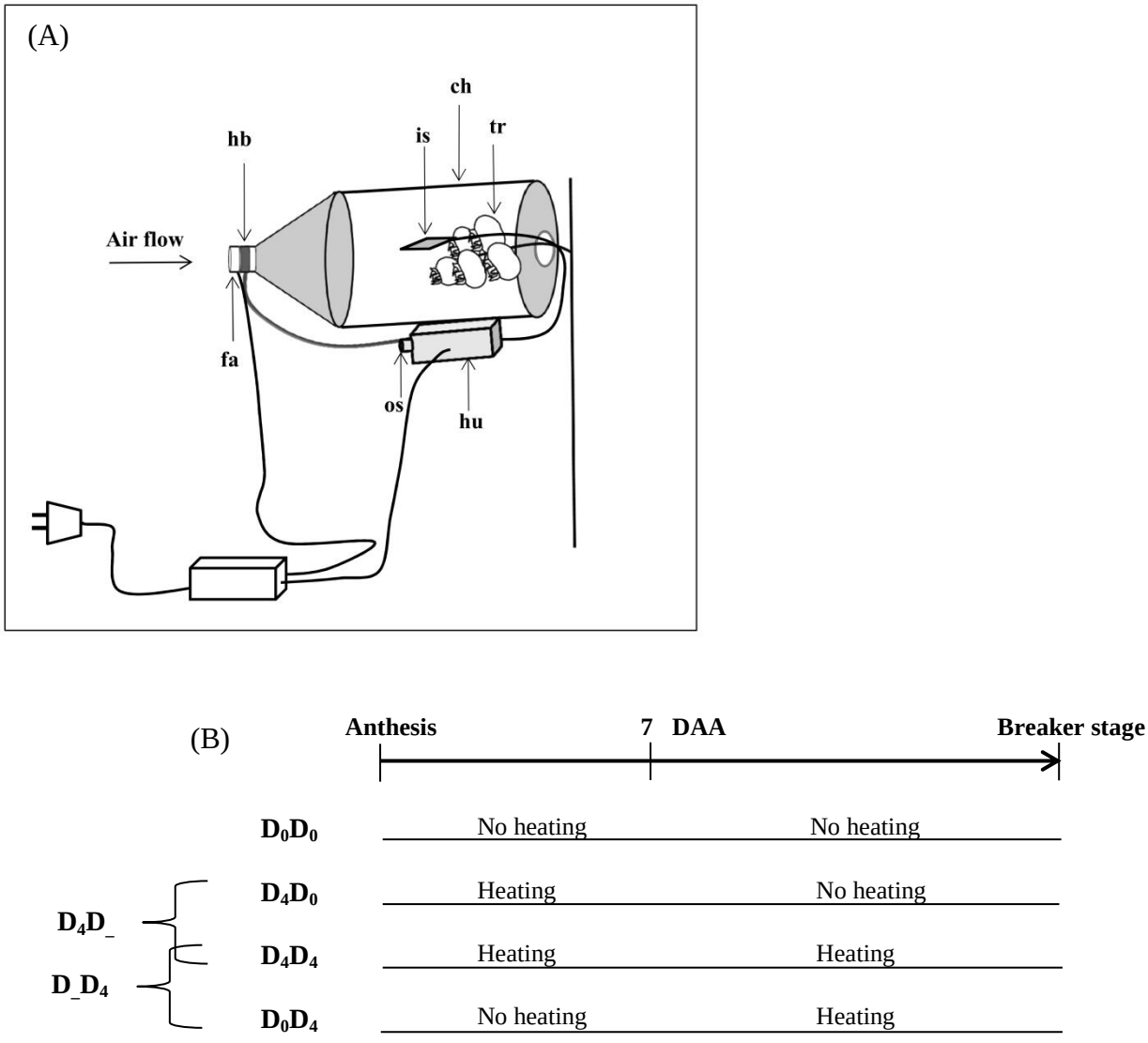

Fig. 1. (A) Schematic representation of the heating system: hb = heating block, is = inside temperature sensor, ch = chamber, tr = tomato truss, hu = heating unit, os = outside temperature sensor, fa = fan. (B) Scheme of tomato fruit heating treatments in Exp. 2: in D_0D_0 treatment fruit were not heated from anthesis until breaker stage, in D_4D_0 fruit were heated from anthesis until 7 days after anthesis (DAA) and not heated from 7 DAA until breaker stage, in D_4D_4 fruit were heated from anthesis until breaker stage, in D_0D_4 fruit were not heated from anthesis until 7 DAA and heated from 7 DAA until breaker stage.

Observations and measurements

Flower and fruit measurements: In each experimental unit, observations and measurements were carried out on one fruit located at the second proximal position of the selected truss. For all selected trusses, observations of anthesis were recorded before trusses were enclosed into the cuvettes. Anthesis was considered as the first day on which a flower opens fully. In Exp. 1, the second proximal fruit in each selected truss was harvested at a specific thermal time (calculated as the temperature sum above a base temperature of 5.7 °C (Adams et al., 2001)), i.e., 0, 69, 129, 232, 374 ^{o}Cd after anthesis, and at breaker stage (reached at 881 ^{o}Cd after anthesis). This corresponded to 0, 2, 5, 11, 18 and 46 DAA in the heating treatment or to 0, 3, 7, 15, 25 and 54 DAA in the control treatment. In Exp. 2 the second proximal fruit in all cuvettes was harvested at breaker stage which was reached at 853 ^{o}Cd after anthesis, and corresponded to 55, 52, 46 and 45 DAA respectively in the D0D0, D4D0, D0D4 and D4D4 treatments (Fig. 1B).

Harvested fruit were weighed and their diameters in the horizontal plane measured using a digital calliper (Schneider-klein, Suki-international, Landscheid, Germany). Average fruit diameter was calculated after estimating fruit diameter in the vertical direction calculated from the relationship between fruit diameter in the vertical and horizontal directions, estimated in cv. 'Moneyberg' (N=58) (Fig. S1). Each harvested fruit was split into two halves at the equatorial plane, the pericarp was isolated and used for cell histology.

Cell histology: For each harvested fruit, four pericarp samples were fixed overnight at room temperature in a 1 acetic acid : 2 formaldehyde : 5 ethanol solution. Sections of 3 μm thick were stained using toluidine blue and photographed on a light microscope (Eclipse 50i, Nikon Instruments Europe, Kingston, UK) equipped with a colour digital camera. Images were analysed with image J (National Institutes of Health, Bethesda, Maryland). During image analysis two tissues were distinguished in each pericarp section: the first five cell layers (including the cuticle) representing the exocarp and the region between the 5th cell layer and the endocarp representing the mesocarp (Fig. 2A). To determine cell area a rectangle was drawn in each tissue centred in the area containing no vascular bundle (Fig. 2A).

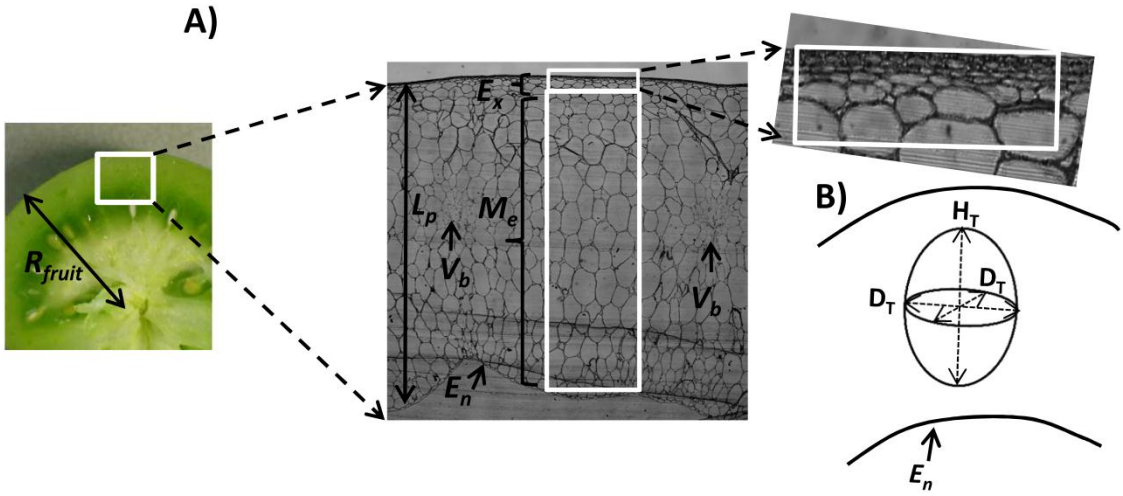

Fig. 2. Structure of tomato pericarp at breaker stage: A) the white rectangles are examples of the region within which mean cell area was estimated in the exocarp and mesocarp as detailed in the materials and methods; B) schematic representation of the ellipsoid cell shape in the pericarp. R_{fruit} is the fruit radius, L_p is the pericarp thickness, V_b is the vascular bundle, E_x is the exocarp, M_e is the mesocarp, E_n is the endocarp, H_T is the cell length (anticlinal direction i.e., perpendicular to the fruit skin), D_T is cell width (periclinal direction i.e., parallel to the fruit skin).

Vascular bundles and endocarp cells were excluded from the measurements because they do not contribute much to pericarp size. The mean pericarp cell area in each tissue was determined as the ratio between the area of the rectangle and the number of cells inside the rectangle. The mean cell area in each tissue was used to estimate mean cell volume assuming that tomato cells have an ellipsoid shape (Fig. 2B).

$$C_{vol,T} = \frac{4}{3} 0.5 C_{a,T} D_T \qquad (1)$$

where subscript T refers to the measured tissue, i.e., the exocarp or mesocarp, $C_{vol,T}$ is the average cell volume (in millimeters^3) in the exocarp or mesocarp, $C_{a,T}$ is the average cell area in the exocarp or mesocarp (in millimetres ^{2}), D_T is the cell width in the 3rd dimension of the ellipsoid (Fig. 2B) calculated according to the formula:

$$D_T = \frac{C_{a,T}}{0.25 \pi H_T} \qquad (2)$$

where H_T is the average cell length (in the anticlinal direction i.e., perpendicular to the fruit skin) in the exocarp or mesocarp (in millimetres) (Fig. 2B). H_T was calculated by dividing the tissue thickness by its number of cell layers, π=3.14. Eq. [2] assumes that cell width (in

the periclinal direction i.e., parallel to fruit skin) in 2-dimensional plane equals cell width in the 3rd dimension of the ellipsoid. To check this assumption we sectioned some pericarps in the longitudinal and transversal directions and analysed cell length and width differences in both directions. Cell width in both directions was not statistically different (data not shown).

The average cell volume of the pericarp was calculated as the weighted average of cell volume in the exocarp and mesocarp.

The number of cells in the exocarp or mesocarp tissue ($C_{n,T}$) was calculated by dividing the exocarp or mesocarp tissue volume, $P_{vol,T}$ by the mean tissue cell volume $C_{vol,T}$:

$$C_{n,T} = \frac{P_{vol,T}}{C_{vol,T}} \quad (3)$$

where $P_{vol,T}$ (in millimeters^3) is calculated approximating the tomato fruit to a sphere. In case of exocarp, the formula for $P_{vol,T}$ is:

$$P_{\mathrm{vol,T}} = \frac{4}{3}\pi[R_{\mathrm{fruit}}^3 - (R_{\mathrm{fruit}} - L_{\mathrm{E}})^3] \quad (4)$$

where R_{fruit} is the fruit radius (in millimetres), and L_E is the exocarp thickness (in millimetres). Then $P_{vol,T}$ for mesocarp is expressed as:

$$P_{\mathrm{vol,T}} = P_{\mathrm{vol}} - \frac{4}{3}\pi[R_{\mathrm{fruit}}^3 - (R_{\mathrm{fruit}} - L_{\mathrm{E}})^3] \quad (5)$$

where P_{vol} is the pericarp volume (in millimeters^3), which was calculated as:

$$P_{\mathrm{vol}} = \frac{4}{3}\pi[R_{\mathrm{fruit}}^3 - (R_{\mathrm{fruit}} - L_{\mathrm{P}})^3] \quad (6)$$

where L_P is the pericarp thickness (in millimetres).

Statistical analysis

The effects of heating treatments on variables measured on fruit at the same growth stage were analysed by ANOVA and F-tests were used to determine the statistical significance (Matlab, The Mathworks, Natick, Massachusetts). The relationships between fruit fresh weight, pericarp volume, cell number and cell volume were analysed using linear regression. The coefficient of determination and the statistical significance of the simple linear regression were used to assess the quality of the relationships.

Results

Effects of continuous heating on fruit and cell characteristics during fruit growth

Continuous heating significantly reduced final fruit fresh weight (P = 0.002), fruit diameter (P = 0.004) and fruit volume (P = 0.003) in Exp. 1. In Exp. 2, final fruit size of continuously heated fruit was also reduced (P = 0.02), but the effects were less pronounced than in Exp. 1 (Fig. 3A, 3B). The time course of fruit growth measured in Exp. 1 showed that reduction in final fruit size was mainly accounted for by the effect of heating on fruit growth duration.

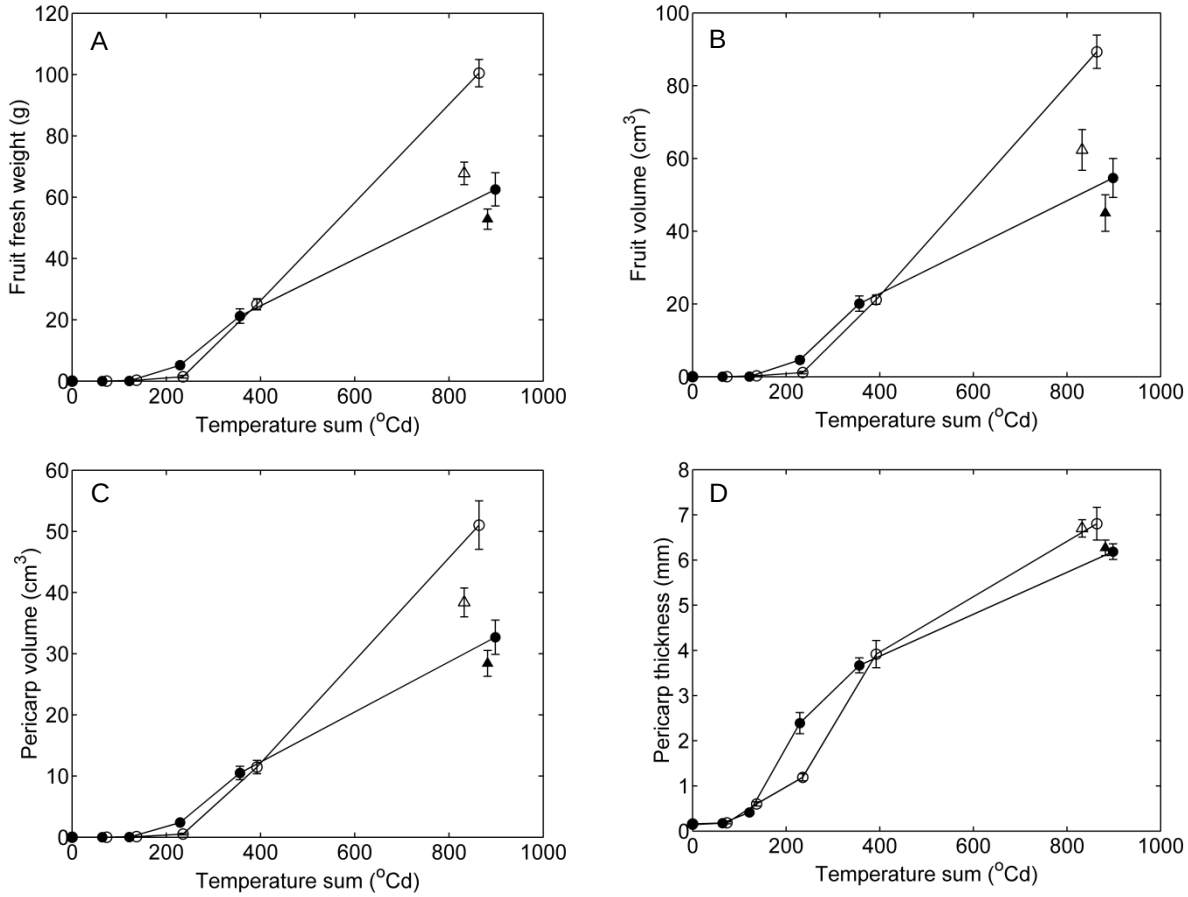

Fig. 3. Time course of (A) tomato fruit fresh weight, (B) fruit volume, (C) pericarp volume and (D) pericarp thickness during fruit growth (Exp. 1; circles). Each point is the mean (± standard error) of three to five replicate fruit heated continuously (closed symbols) or non-heated (open symbols). The last data points were measured from fruit at the breaker stage, at which data were also available from Exp. 2 (triangles) and were presented for comparison between the two experiments.

Heating initially increased fruit growth rate (Figs. 3A, S2A), but this increase was more than compensated by a drastic reduction in fruit growth duration of 8 ± 1.6 d (P = 0.010) in the

heating treatment compared with the control (Fig. S2A). When fruit size was plotted against temperature sum, final fruit size was reached at approximately the same temperature sum (881 ± 17 ^{o}Cd) in heated and non-heated fruit (Fig. 3, 3B). The time course of pericarp volume growth responded similarly to continuous fruit heating as fruit fresh weight (Fig. 3C). Final pericarp volume was reduced by 36% (Exp. 1) or 26% (Exp. 2) in heated fruit. In contrast, fruit heating did not significantly affect final pericarp thickness (P = 0.18), although during fruit growth increase in pericarp thickness started earlier in the heating treatment compared with the control (Figs. 3D, S2D). At the same temperature sum, pericarp thickness was similar in heated and non-heated fruit except at 232 ^{o}Cd (Fig. 3D).

From anthesis until maturity cell volume increased approximately 11,000 fold in both heated and non-heated fruit. Continuously heating the fruit significantly reduced their final pericarp cell volume in Exp. 2 (Figs. 4A, S3A). In Exp. 1, increase in cell volume started earlier in heated fruit compared with non-heated fruit (Figs. 4A, S3A), but this did not result in significant differences in final volume of pericarp cells (P = 0.79). A similar pattern was observed with exocarp and mesocarp cells where heating treatment did not significantly affect their final volume either (P > 0.65; data not shown). Interestingly, although heating did not affect final pericarp cell volume in Exp. 1, its effect was not the same in all cell expansion directions. At 232 ^{o}Cd cell length was larger in heated fruit than in non-heated fruit (P = 0.003; Fig. 4B). After this point, cell expansion in the anticlinal direction (i.e., increase in cell length) was faster in non-heated fruit than in heated fruit (P = 0.07 at 374 ^{o}Cd; Fig. 4B) leading to a significant reduction in final cell length in heated fruit compared with non-heated fruit (P = 0.001). The effects of heating on periclinal cell expansion (i.e., increase in cell width) did not result in significant differences in final pericarp cell width (P = 0.35).

The generation of new cell layers occurred from anthesis onwards at similar rate in heated and non-heated fruit (Fig. 4D). In non-heated fruit the maximum number of cell layers was reached at 129 ^{o}Cd. However, new cell layers continued to be formed up to 232 ^{o}Cd in heated fruit (Fig. 4D), which led to significantly more cell layers in matured heated fruit compared with non-heated fruit (P = 0.01). In Exp. 2 the increase in the final number of cell layers in continuously heated fruit was less pronounced than in Exp. 1.

Cell division occurred at a higher rate in the pericarp of non-heated fruit compared with heated fruit (Figs. 4E, S3E). In both treatments no more cells were produced after 232 ^{o}Cd (Fig. 4E). Matured heated fruit had 3.8 (± 0.66) $\times 10^{6}$ (Exp. 1) or 2.8 (± 0.22) $\times 10^{6}$ (Exp. 2) cells compared with 4.8 (± 0.34) $\times 10^{6}$ (Exp. 1) or 3.1 (± 0.25) $\times 10^{6}$ (Exp. 2) cells in non-heated fruit.

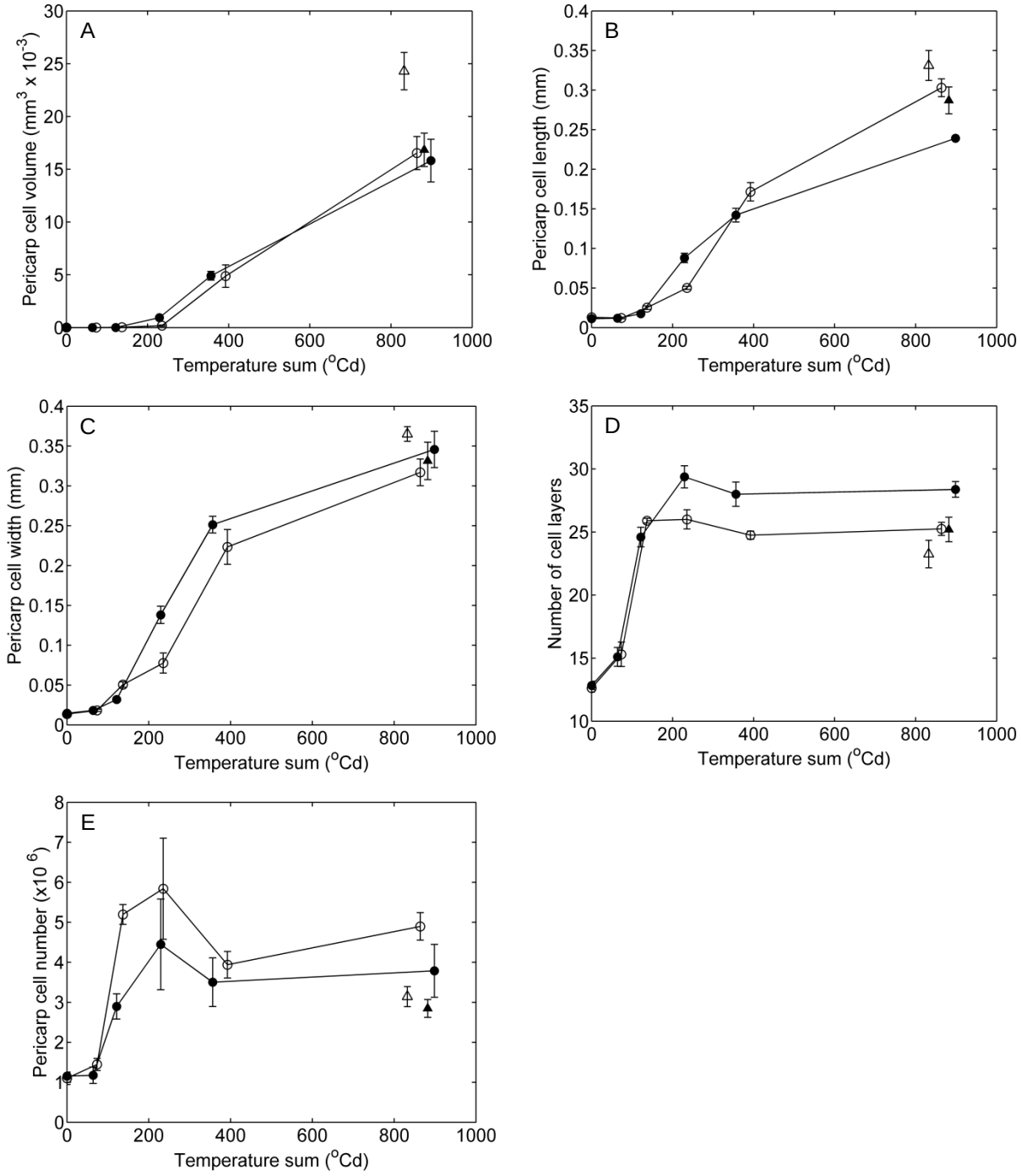

Fig. 4. Time course of (A) tomato fruit pericarp cell volume, (B) pericarp cell length (anticlinal direction), (C) pericarp cell width (periclinal direction), (D) number of cell layers and (E) pericarp cell number during fruit growth (Exp. 1; circles). Each point is the mean (± standard error) of three to five replicate fruit heated continuously (closed symbols) or not heated continuously (open symbols). The last data points were measured from tomato fruit at the breaker stage, at which data were also available from Exp. 2 (triangles) and were presented for comparison between the two experiments.

Effects of heating applied during the first 7 days after anthesis and from 7 days after anthesis onwards on final fruit characteristics

For all fruit and cell characteristics measured, no interaction was detected between heating during the first 7 DAA and heating from 7 DAA onwards. Therefore main effects were tested. Heating fruit during the first 7 DAA reduced final fruit fresh weight by 12%, fruit volume by 21% and pericarp volume by 16% compared to the situation where no heating was applied during the first 7 d (Table 1). Heating fruit from 7 DAA onwards had no significant effects on final fruit fresh weight, fruit volume and pericarp volume ($P > 0.07$).

Table 1. Effects of fruit heating from anthesis until 7 days after anthesis (DAA) ($D_4D\_$) and of fruit heating from 7 days after anthesis onwards (D_D_4) on final tomato fruit and cell phenotypes. In the D0D_ treatment, fruit were not heated from anthesis until 7 DAA; in the D_D0 treatment, fruit were not heated from 7 DAA until breaker stage. Each value is the mean of 8-10 replicate fruit (Exp. 2).*

	Heating from anthesis until 7 DAA			Heating from 7 DAA until maturity		
	Not Heated (D0D_)	Heated (D4D_)	*P*-value	Not Heated (D_D0)	Heated (D_D4)	*P*-value
Fruit fresh weight (g)	64.2	56.2	**0.05**	63.7	56.7	0.09
Fruit volume (cm^3)	61.2	48.3	**0.01**	57.0	52.5	0.28
Pericarp volume (cm^3)	36.6	30.7	**0.01**	35.7	31.6	0.08
Pericarp thickness (mm)	6.4	6.5	0.77	6.7	6.2	**0.02**
Number of cell layers	23.8	26.1	**0.02**	25.1	24.8	0.70
Average cell volume ($\times 10^{-3}$ mm^3)	22.3	16.3	**0.02**	20.0	18.5	0.54
Cell volume exocarp ($\times 10^{-3}$ mm^3)	1.6	1.5	0.68	1.6	1.5	0.81
Cell volume mesocarp ($\times 10^{-3}$ mm^3)	23.6	17.1	**0.02**	21.2	19.6	0.54
Cell number ($\times 10^6$)	3.21	3.19	0.96	3.34	3.06	0.51
Pericarp cell width (mm)	0.36	0.33	0.17	0.34	0.34	0.99
Pericarp cell length (mm)	0.31	0.28	0.07	0.30	0.29	0.19

* For all response variables measured, no significant interaction was observed between the effects of heating from anthesis until 7 DAA and heating from 7 DAA until breaker stage.

Final pericarp thickness was not affected by early heating but was statistically significantly reduced by 8% when fruit were heated from 7 DAA onwards (Table 1). Early heating significantly increased the number of cell layers, but not the total number of cells in the

pericarp (Table 1). Heating fruit from 7 DAA onwards did not affect the final number of cell layers nor the total number of cells (Table 1). Average pericarp cell volume was significantly decreased by 27% in fruit heated during the first 7 DAA (Table 1).

The effect of heating applied from 7 DAA onwards on pericarp cell volume was not statistically significant (Table 1). Mesocarp cell volume responded similarly to heating treatments as average pericarp cell volume did (Table 1). The effects of heating treatments on final exocarp cell volume were not statistically significant (Table 1).

Effects of fruit heating on the correlation between fruit and histological variables in mature fruit

We investigated whether heating fruit during the first 7 DAA or from 7 DAA onwards had an influence on the correlations between fruit and histological characteristics. We used simple linear regression to analyse the relationships between fruit weight, pericarp volume, pericarp cell number and pericarp cell volume in different heating treatments. In all heating treatments, pericarp volume linearly correlated to fruit fresh weight (Fig. 5A). Regression analysis was carried out between pericarp volume and other histological traits. In none of the heating treatments cell volume significantly correlated with pericarp volume ($P > 0.25$) (Fig. 5B). Positive correlations were noted between cell number and pericarp volume for fruit heated during the first 7 DAA ($P = 0.01$), but not for fruit heated from 7 DAA onwards ($P = 0.07$; Fig. 5C). Negative correlation coefficients were found between cell number and cell volume for fruit heated during the first 7 DAA ($P = 0.01$), but not for fruit heated from 7 DAA onwards ($P = 0.16$; Fig. 5D).

Discussion

In agreement with the literature (De Koning, 1994; Gautier et al., 2005; Greer et al., 2003) continuous fruit heating in our experiments reduced fruit growth duration and final fruit size. Surprisingly, the effects of continuous heating on final fruit size were more pronounced in Exp. 1 than in Exp. 2 (Fig. 3A). Since fruit temperature was similar in both experiments the reasons for this difference in the magnitude of effects remain unclear. In our experiments we used moderate temperature range, far below the temperatures responsible for heat stress in tomato fruit (Pearce et al., 1993). We showed that in this temperature range, fruit size reduction was mostly due to heating during the first week of fruit growth (Table 1).

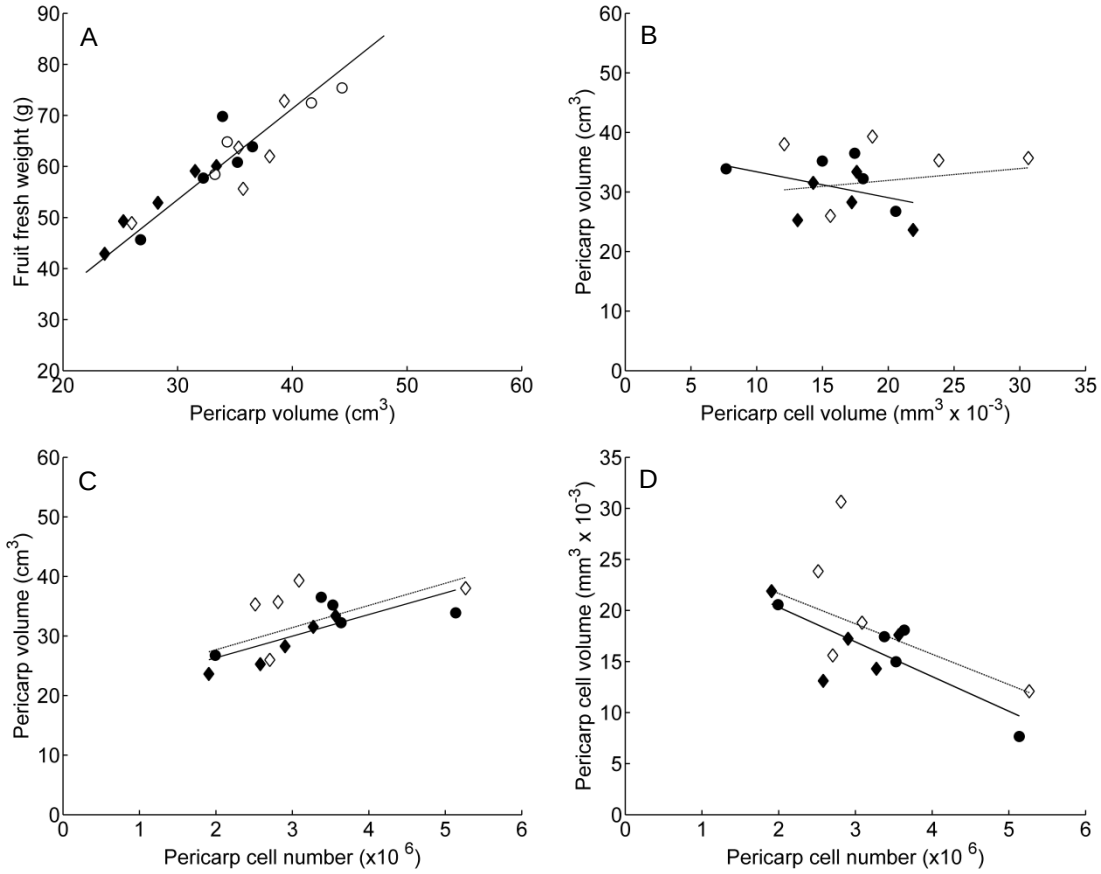

Fig. 5. Correlations between (A) tomato fruit fresh weight and pericarp volume, (B) pericarp volume and pericarp cell volume, (C) pericarp volume and pericarp cell number, and (D) pericarp cell volume and pericarp cell number. Regression in (A) is fitted over all data points. Each point is an individual fruit at the breaker stage of fruit heated from anthesis to 7 days after anthesis (DAA) (D4D_: closed symbols and solid line), fruit heated from 7 DAA until maturity (D_D4: diamond and dotted line) and fruit not heated from anthesis until maturity (D_0D_0: open circles). The slope (α) of regression is (A) α=1.8 g·cm^{-3}, R^2=0.82, P < 0.001; (B) α=-0.43 cm^3·mm^{-3}, R^2=0.16, P = 0.26 (D4D_) and α=0.20 cm^3·mm^{-3}, R^2=0.04, P = 0.58 (D_D4); (C) α=3.63 cm^3, R^2=0.59, P = 0.01 (D4D_) and α=3.72 cm^3, R^2=0.36, P = 0.07 (D_D4); (D) α=-3.39 mm^3, R^2=0.61, P = 0.01 (D4D_) and α=-2.97 mm^3, R^2=0.22, P = 0.17 (D_D4).

This result is consistent with the findings of De Koning (1994) and Adams et al. (2001) who observed that tomato fruit are more sensitive to heating applied during the first weeks of fruit growth than to heating applied later. Similar observations were reported by Marcelis and Baan Hofman-Eijer (1993) in cucumber fruit.

The linear correlation found between fruit fresh weight and pericarp volume in all our heating treatments suggests that pericarp volume might be sufficient to describe variations in fruit fresh weight. In our experiment, final pericarp thickness was not affected by heating

treatments (Fig. 3D). Pericarp thickness is the consequence of two underlying cellular processes: cell expansion and the generation of new cell layers. The absence of heating effects on final pericarp thickness was the result of the opposite effects of heating on cell expansion and on cell layer generation.

The onset of cell expansion was advanced in heated fruit. This early cell expansion probably explains the initial increase in fruit growth rate observed in heated fruit in our experiment. Several authors reported an increase in the growth rate of young fruit exposed to high temperatures (Bertin, 2005; Marcelis and Baan Hofman-Eijer, 1993). Bertin (2005) observed that growing tomato plants under high temperatures (25/25 ^{o}C) advanced the onset of cell expansion in tomato fruit, but did not alter cell expansion rate. In the same experiment, she reported that final cell volume was increased by high temperature (Bertin, 2005), which contrasts with our results where final cell volume was either reduced (early heating or continuous heating (Exp. 2)) or not significantly affected (continuous heating (Exp. 1) or heating from 7 DAA onwards) by heating treatments. This discrepancy might be linked to the low fruit assimilate supply in our experiments induced by high fruit load (5 fruit/truss) compared with the high assimilate supply in Bertin's experiment induced by low fruit load (2 fruit/truss). Indeed Gautier et al. (2005) showed that the effects of fruit heating were more pronounced when tomato fruit were grown under limited assimilate supply. In cucumber fruit, Marcelis and Baan Hofman-Eijer (1993) observed that high temperature increased final cell size when fruit were grown under non-limiting assimilate supply, but temperature did not affect final cell size when assimilate supply was limiting.

In our experiments the effects of continuous fruit heating on final cell length was more pronounced than on cell width (Fig. 4B, 4C). This suggests that heating might not affect periclinal and anticlinal cell expansion in a similar way. In kidney bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* L.) stems, Ikeda et al. (1999) observed that cell elongation rates and durations were very different in the cell length and cell width directions. The direction of cell expansion in plants is mainly determined by the orientation of cellulose microtubules and microfibrils (Wasteneys and Ambroise, 2009). In the internode of pea (*Pisum sativum* L.) seedlings, Akashi and Shibaoka (1987) observed that transverse and longitudinal microtubules responded differently to low temperature treatment and hormone application. Exposing potato (*Solanum tuberosum*) stolons to gibberellins resulted in the transversal orientation of cell microtubules and microfibrils (Prat, 2010). In tomato fruit, high temperatures are known to affect the production of plant hormones such as gibberellins (Sasaki et al., 2005) and could indirectly influence the orientation of cell microtubules and microfibrils, and thus the

orientation of cell expansion in our experiments. High temperature increased cell length but did not affect cell width in stems of *Campanula isophylla* (Strøm and Moe, 1997) and *Lilium longiflorum* (Erwin et al., 1991). These results together with our data underscore the need in histological studies to measure cell expansion in more than one dimension in order to reflect the dynamics of cell expansion in plant organs.

In our experiments the final number of cell layers was increased in heated fruit. The formation of new cell layers in tomato pericarp mainly occurs early in fruit growth (Fig. 4D; Cheniclet et al., 2005) and, as expected, the final number of cell layers in our experiment was sensitive to heating applied during the first week of fruit growth (Table 1). The number of cell layers in the pericarp is an indication of periclinal cell division (i.e., division plane parallel to fruit skin) activity (Cheniclet et al., 2005). Our results suggest that heating prolonged periclinal cell division as new cell layers continued to be generated until 232 ^{o}Cd in heated fruit compared with 129 ^{o}Cd in non-heated fruit. In a tomato fruit, the total number of cells in the pericarp results not only from periclinal cell divisions, but also from anticlinal cell divisions (i.e., division plane perpendicular to fruit skin) and randomly oriented cell divisions (Cheniclet et al., 2005). With increasing fruit temperature, a decrease in cell division period and final fruit cell number is usually found (Bertin, 2005; Marcelis and Baan Hofman-Eijer, 1993). Continuous fruit heating also shortened the cell division period in our experiment (Fig. S3E), but not to the extent that reduction in final cell number was statistically significant. Early heating had no effect on final pericarp cell number (Table 1). This might be due to the fact that in our experiment, early heating was applied only during the first week of fruit growth. During this period heating mainly affected periclinal cell division which occurs during the first week of fruit growth, whereas anticlinal and randomly oriented cell division continued for a longer period in the pericarp. A longer heating period early in fruit growth would probably affect all types of cell division in the pericarp and final cell number. In this study, final fruit size reduction of early heated fruit was not related to the reduction in cell number, but to the reduction in final cell volume (Table 1).

In contrast with the duration of cell layers production which was prolonged in continuously heated fruit, we observed that the duration of cell division in the whole pericarp was shortened by continuous heating (Fig. S3E). These contrasting effects suggest that periclinal cell division and other types of cell divisions might respond differently to temperature and might be regulated differently. In tomato fruit Cheniclet et al. (2005) observed that periclinal cell division was completed 5 DAA, whereas other types of cell divisions continued up to 20 DAA. Similar observations were reported by Joubès et al. (1999) and Cong et al. (2002). They

showed that the major quantitative trait locus *fw2.2*, which controls fruit size in tomato, is involved in the regulation of anticlinal and randomly oriented cell divisions, but not periclinal cell divisions (Cheniclet et al., 2005; Cong et al., 2002). The regulation of periclinal cell division is not well understood yet.

In our experiment final pericarp volume was positively correlated with cell number but not with cell volume within each heating treatment (Fig. 5). This suggests that within each heating treatment, variation in pericarp volume was mainly accounted for by cell division. Bertin (2005) found that the size of tomato fruit grown under similar temperature regimes was positively correlated with cell number. The negative correlations noted between cell number and cell volume in our experiment agreed with Bertin's findings and suggest that pericarp cells could be viewed as a population of competing sinks.

Conclusions

This study aimed at analysing the response of tomato fruit size to heating as affected by changes in cell number and cell expansion in different directions. Our results showed that reduction in final fruit size of early-heated fruit was mainly associated with reduction in final pericarp cell volume. This study also suggests that in the tomato pericarp, periclinal cell divisions respond differently to temperature than anticlinal and randomly oriented cell divisions. The effects of fruit heating on the time course of cell length and cell width suggest that high temperature reduces expansion in the anticlinal direction more than in the periclinal direction. Our results emphasize the need to measure cell expansion in more than one dimension in histological studies of fruit.

Supplementary information

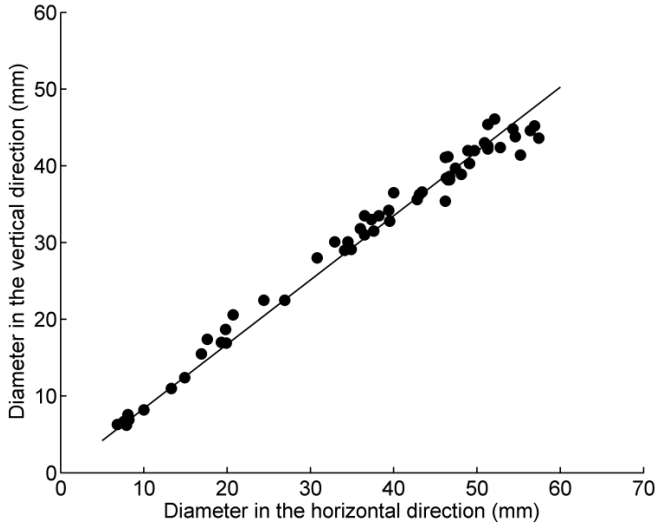

Fig. S1. Relationship between tomato fruit diameter measured in the horizontal and vertical axis in tomato (cv. 'Moneyberg'). The slope of the regression line forced through the origin is α=0.84, R^2=0.98. Data were collected in 2011 from an independent experiment (N=58).

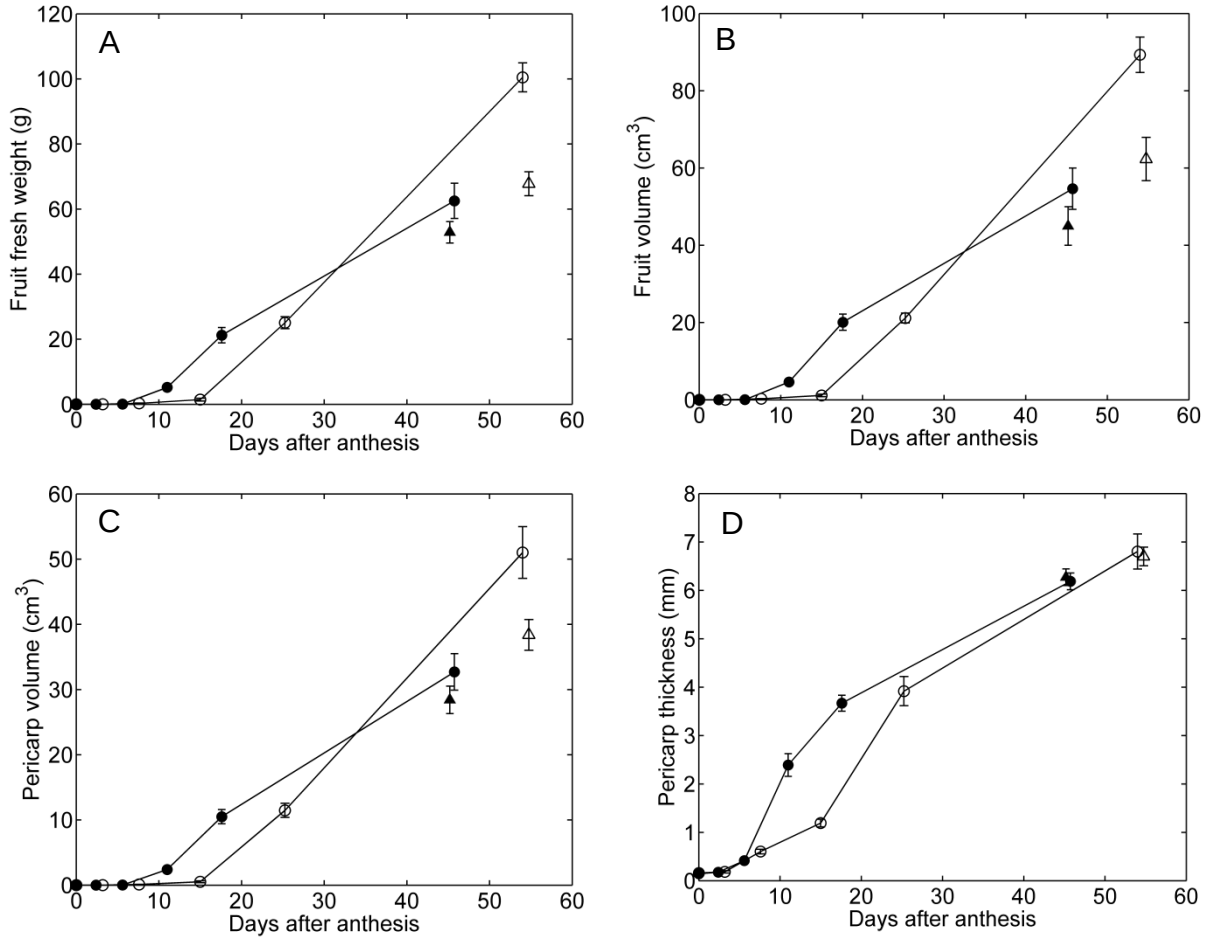

Fig. S2. Time course of (A) tomato fruit fresh weight, (B) fruit volume, (C) pericarp volume and (D) pericarp thickness during fruit growth (Exp. 1; circles). Each point is the mean of three to five replicate fruit heated continuously (closed symbols) or non-heated (open symbols). Vertical bars represent standard errors of the mean. The last data points were measured from fruit at the breaker stage, at which data were also available from Exp. 2 (triangles) and were presented for comparison between the two experiments.

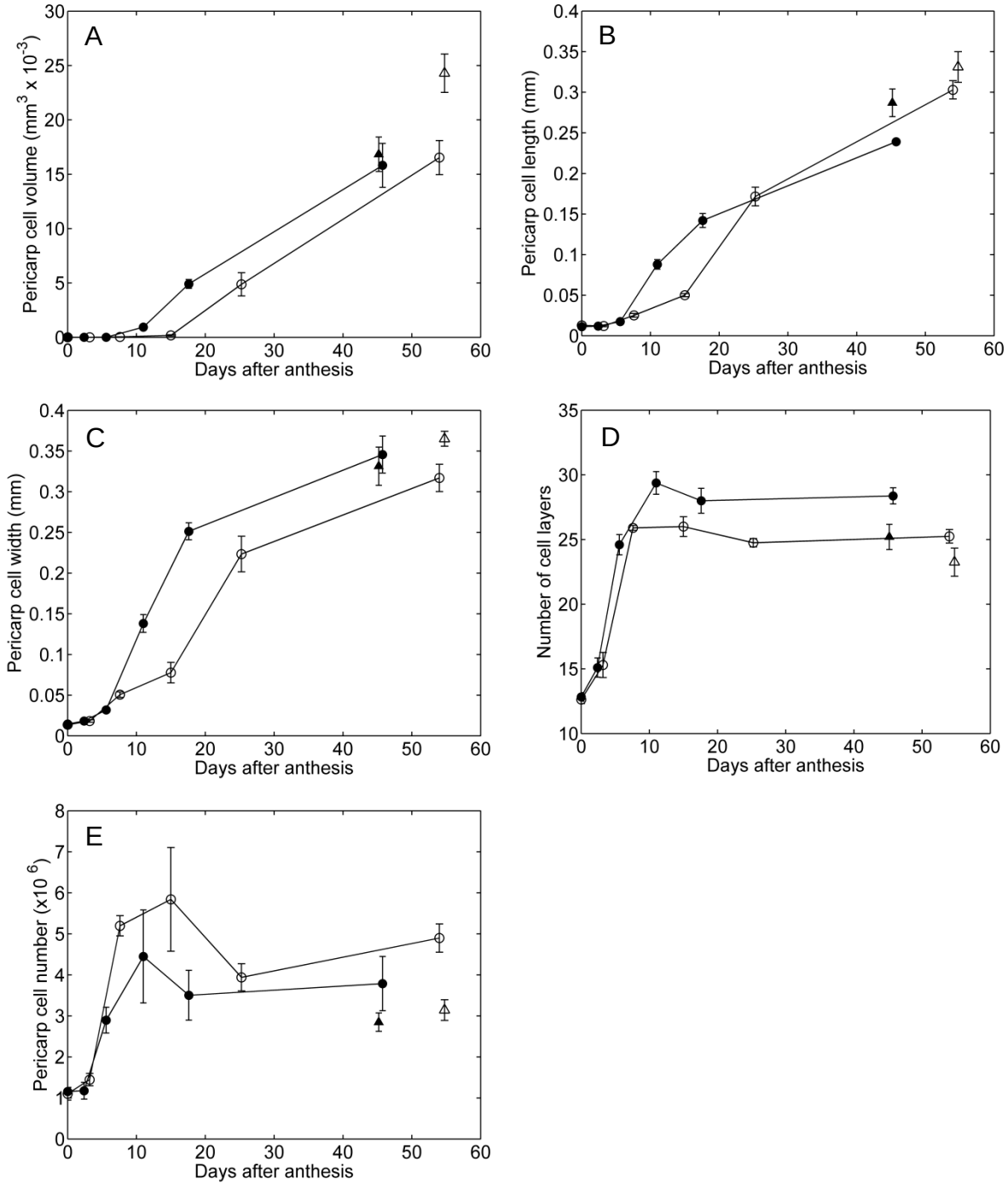

Fig. S3. Time course of (A) tomato fruit pericarp cell volume, (B) pericarp cell length (anticlinal direction), (C) pericarp cell width (periclinal direction), (D) number of cell layers and (E) pericarp cell number during fruit growth (Exp. 1; circles). Each point is the mean of three to five replicate fruit heated continuously (closed symbols) or non-heated (open symbols). Vertical bars represent standard errors of the mean. The last data points were measured from fruit at the breaker stage, at which data were also available from Exp. 2 (triangles) and were presented for comparison between the two experiments.

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4. A dynamic model of tomato fruit growth integrating cell division, cell growth and endoreduplication

Submitted as

Fanwoua J, de Visser PHB, Heuvelink E, Yin X, Struik PC, Marcelis LFM. A dynamic model of tomato fruit growth integrating cell division, cell growth and endoreduplication.

Abstract

In this study a model of tomato fruit growth integrating cell division, cell growth and endoreduplication was developed. The fruit is considered as a population of cells grouped in cell classes differing in their initial cell age and cell mass. The model describes fruit growth from anthesis until maturation and covers the stages of cell division, endoreduplication and cell growth. The transition from one stage to the next is determined by predefined cell ages expressed in thermal time. Cell growth is the consequence of sugar import from a common assimilate pool according to the sink/source concept. Cell growth rate increases with increasing cell ploidy. Cell division or endoreduplication occur when cells exceed a critical threshold cell mass: ploidy ratio. The model was parameterized and calibrated for low fruit load conditions and was validated for high fruit load and various temperature conditions. The model was able to accurately predict final cell number, cell mass and pericarp mass under various contrasting fruit load and most of the temperature conditions. The framework developed in this model opens the perspective to integrate information on molecular control of fruit cellular processes into the fruit model and to analyse gene-by-environment interaction effects on fruit growth.

Keywords: model, fruit growth, tomato, cell division, cell growth, cell endoreduplication

Introduction

The control of fruit size is complex. This complexity emerges from the cumulative and interactive effects of successive genetically and environmentally controlled, diverse cellular processes during fruit development. In most fleshy fruits, growth starts with intense cell division (Gillaspy *et al.* 1993). After the first weeks of fruit growth cell division gradually declines and rapid cell growth starts to occur (Gillaspy *et al.* 1993; Bertin *et al.* 2003a). During this period individual cells accumulate water and carbon resulting in spectacular increase in cell volume (more than 10,000-fold in tomato (*Solanum lycopersicum* L.) mesocarp cells) and fruit volume (Cheniclet *et al*. 2005). In many fleshy fruits, cell growth is

accompanied by an increase in cell DNA content through the process of endoreduplication, i.e. an incomplete cell cycle where cells continue to increase their DNA content (S-phase) without mitosis (M-phase) (Bourdon *et al*. 2010). At the subcellular scale, cell division, cell growth and endoreduplication are controlled by networks of multiple genes of which expression is sensitive to the environment (Baldet *et al*. 2006; Chevalier 2007). The regulation of fruit size is further complicated by the interrelationships between fruit cellular processes. For example, a positive correlation between cell size and the number of endoreduplication cycles was reported in some plant organs, including tomato fruits (Kondorosi *et al.* 2000; Bertin 2005; Cheniclet *et al*. 2005; Bourdon *et al*. 2010). Several authors observed that most cells that start endoreduplication are not able to re-enter mitosis (Sugimoto-Shirasu and Roberts 2003; John and Qi 2008; Sabelli *et al*. 2008). Experimental data show that cell growth is strongly linked with cell cycle regulation and suggest that there is a critical cell size for endoreduplication or cell division to occur (Nasmyth 1979; Jorgensen and Tyers 2004; Francis 2007).

Models have proven to be powerful tools to understand the behaviour of complex systems (Struik *et al.* 2005). In order to unravel the complexity of fruit size, ecophysiological models describing underlying fruit growth processes have recently been proposed (Liu *et al*. 2007; Génard *et al*. 2007; Martre *et al*. 2011). Bertin *et al.* (2003b) proposed a simple phenomenological model of cell proliferative activity in growing tomato fruits under constant environmental conditions. Their model assumes an initial phase of exponential cell proliferation after which the proliferative activity declines as division proceeds. This model was able to predict differences in cell number between two tomato cultivars, but did not address environmental effects (Bertin *et al*. 2003b). In the last two decades, progress in the understanding of molecular control of the cell cycle inspired the development of several models of cell cycle regulation (Tyson *et al.* 2002; Novák and Tyson 2004; Csikász-Nagy *et al*. 2006; Barik *et al*. 2010; Roodbarkelari *et al*. 2010). These models attempt to predict the behaviour of cells based on differential equations describing the network of protein interactions. The main drawbacks of these models are their complexity and the difficulty to measure model parameters, limiting the possibilities for their application at the tissue and fruit scales (Bertin *et al*. 2007; Martre *et al*. 2011).

Most expansion models describe fruit growth at the organ scale. A typical example is the peach (*Prunus persica* L.) model of Fishman and Génard (1998) in which the fruit is considered as one big cell and thermodynamic equations are used to describe water and carbon accumulation in the fruit. This model was used to predict fruit load and tree water

status effects on the fresh and dry mass of peach fruits (Fishman and Génard 1998). The model was further modified to describe plastic and elastic changes of cell wall in mango (*Mangifera indica* L.) fruits (Lechaudel *et al*. 2007) and the switch from symplasmic to apoplasmic phloem unloading in growing tomato fruits (Liu *et al*. 2007). Carbon accumulation in the fruit has also been modelled using the sink regulation concept (Marcelis *et al.* 1998). This concept assumes that carbon import into a fruit depends on the fruit sink strength relative to the sink strength of all other organs. In these models an organ sink strength is defined as the potential capacity of the organ to accumulate assimilates and is quantified by the organ's potential growth rate (Marcelis *et al*. 1998). The sink strength approach has been used to model assimilate import in several fruit species including cucumber (*Cucumis sativus*) (Marcelis 1994), tomato (Heuvelink 1996), kiwifruit (*Actinidia deliciosa*) (Lescourret *et al*. 1998a), peach (Lescourret *et al.* 1998b) and grapevine (*Vitis vinifera* L.) (Vivin *et al.* 2002).

In comparison to the number of expansion and cell division models, only few models of endoreduplication have been developed so far. A mathematical model was proposed to describe endoreduplication in maize (*Zea mays* L.) endosperm (Schweizer *et al*. 1995) and later in orchid flowers (Lee *et al*. 2004). The model of Lee *et al*. (2004) assumes that the potential for endoreduplication decreases for cells with higher ploidy levels. Model predictions agreed well with experimental data, but the high number of relevant parameters is a major disadvantage of this model. The same holds for two modelling studies on cell cycle regulation including endoreduplication (Csikász-Nagy *et al*. 2006; Roodbarkelari *et al*. 2010). A simpler model describing mitotic activity and endoreduplication in tomato fruits was proposed by Bertin *et al*. (2007). Their model was able to predict the number of cells and ploidy levels in two contrasting tomato cultivars, but environmental effects were not considered.

An interesting feature of the Bertin *et al*. (2007) model is that it integrates the arrest of mitosis with the onset of endoreduplication. Although experimental data suggest that underlying fruit cellular processes are interrelated, integrating two or more cellular processes has been rarely done in fruit ecophysiological models. An attempt to integrate cell division and cell growth was made by Beemster *et al*. (2006) in their model of *Arabidopsis* leaf growth. Their model assumes that a critical ratio between cell size and cell DNA content triggers the synthesis of cyclins and CDKs regulating the S and M phases. This model was used to simulate the effects of an overproduction of cell cycle inhibitor on cell division and expansion, but endoreduplication was not presented (Beemster *et al.* 2006). Even though it is difficult to integrate all physiological processes in one model, several authors agree that models

integrating the main underlying processes would improve our understanding of the emerging properties of a complex system (Génard *et al.* 2007; Yin and Struik 2010). In the case of fruit growth, such models would also open the way to analyse complex fruit environment responses (Liu *et al.* 2007; Martre *et al.* 2011). In order to achieve this goal, Génard *et al.* (2007) proposed to describe each process underlying fruit growth in a simple way.
The objectives of this study were 1) to develop a model of tomato fruit growth integrating cell division, cell growth and endoreduplication, and 2) to use the model to analyse assimilate supply and temperature effects on fruit growth at the cell and fruit scale.

Model description

The model describes the growth of a tomato fruit pericarp from anthesis until maturation, thus covering the period of cell division, cell endoreduplication and cell expansion. In our previous studies we showed that there is a linear relationship between pericarp size and fruit size (Fanwoua *et al*. 2012a, b). In the model the pericarp consists of a population of cells grouped into q cell classes (Fig. 1). Cell classes are defined based on the mass and age of cells at the beginning of the simulation. This definition accounts for the variability already present at anthesis in many fleshy fruits including tomato (Bertin 2005; Baldet *et al.* 2006). The distributions of initial cell mass and age across cell classes are assumed to follow a normal distribution. Cells do not move from class to class during fruit development and within each class all cells have the same mass, age and behaviour. Cell age is expressed in degree-hours and calculated as follows:

$$CellAge_n(i) = CellAge_n(1) + \sum_{t=1}^{i} deghour\,(t) \qquad (1)$$

where, $CellAge_n(i)$ is the age of cells in class n ($1 \leq n \leq q$) at step i in degree-hours (°Ch), and $CellAge_n(1)$ is the age of cells in class n at the beginning of the simulation. The second expression in equation (1) integrates hourly values of *deghour*, i.e. the differences between hourly temperature and the base temperature *Tb* (*Tb*=5.7 °C, Adams *et al.* 2001).

In each cell class, cells are able to increase their mass (cell growth), increase their number (cell division), or increase their ploidy level (cell endoreduplication) (Fig. 2). Each of these processes are closely linked to cell age as described below.

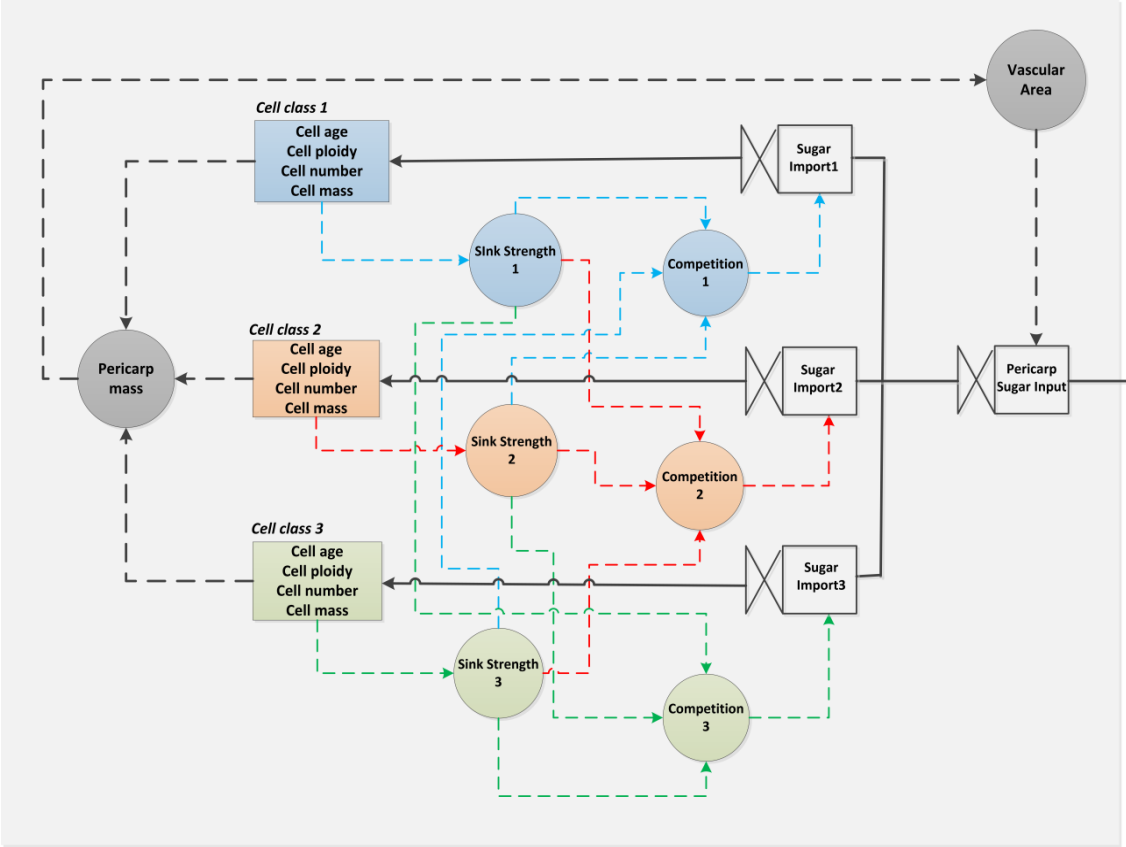

Fig. 1. General scheme of the fruit model structure showing sugar distribution between different cell classes in the fruit. Rectangles represent state variables in a cell class, valves represent rates, circles are model parameters, solid arrows represent sugar flow, dashed arrows indicate information flow. For simplicity, we represent only three cell classes for this illustration.

Cell growth

Cell growth involves the increase in total cytoplasmic mass (Génard *et al.* 2007) and is the consequence of resource exchange between the cell and the rest of the plant. At the fruit level, growth has often been modelled using the source/sink concept (Marcelis *et al.* 1998). We applied this approach to the cell scale.

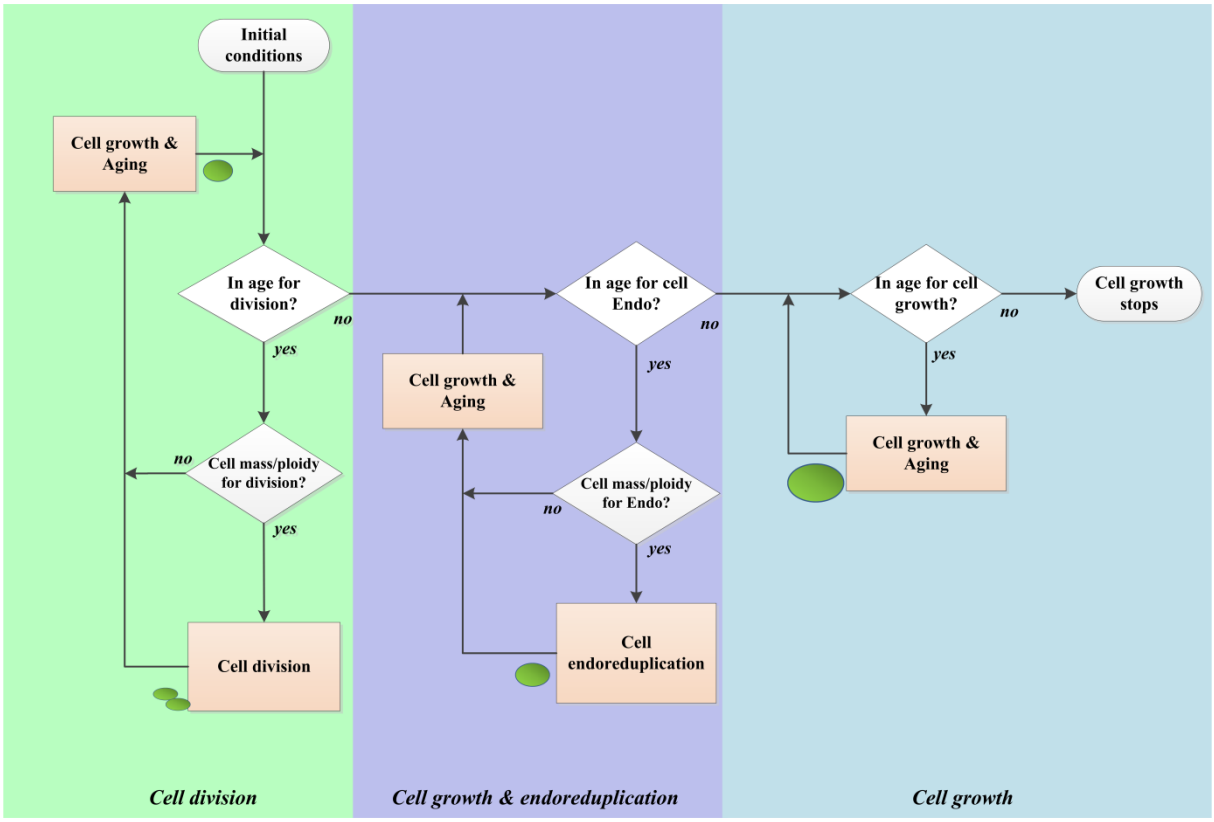

Fig. 2. Schematic representation of the decision for an average cell to grow, divide or endoreduplicate (Endo) within a cell class in the fruit model. Rectangles represent cellular processes, diamonds are decision rules, solid arrows show progression through the scheme.

For each time step, the cell growth rate and cell mass in each class are calculated:

$$CellMass_n(i) = ActualCellgrowthrate_n(i) * TimeStep + CellMass_n(i-1) \quad (2)$$

where *TimeStep* is the model time step (1 hour), $CellMass_n$ is the average cell mass in class n (mg), and $ActualCellgrowthrate_n$ is the actual growth rate of each cell in class n (mg h^{-1}) and is calculated as the ratio between the actual amount of sugar imported by class n ($ActualClassSugarImport_n$, mg h^{-1}) and the number of cells in class n ($ClassCellNumber_n$).

$$ActualCellgrowthrate_n(i) = ActualClassSugarImport_n(i) / ClassCellNumber_n(i) \quad (3)$$

Calculation of $ClassCellNumber_n$ is described in the cell division section. The actual amount of sugar imported by class n depends on the pericarp sugar input ($PeriSugarInput$, mg h^{-1}) and should not exceed the sink strength or potential growth rate of class n ($ClassSinkStrength_n$, mg h^{-1}).

$$ActualClassSugarImport_n(i) = \min[(PeriSugarInput\ (i) * ClassCompetition_n(i), ClassSinkStrength_n(i)] \quad (4)$$

Pericarp sugar input ($PeriSugarInput$) depends on the pool of sugar available for the fruit ($SugarPool$, mg), the contact surface area between the vascular and pericarp tissue ($VascArea$, mm^2) and the respiration coefficient ($RespCoef$).

$$PeriSugarInput\ (i) = \aleph * SugarPool * VascArea\ (i-1) * (1 - RespCoef) \quad (5)$$

where $\aleph$ is a constant proportionality factor ($h^{-1}\ mm^{-2}$). The pool of sugar available for the fruit ($SugarPool$) and the respiration coefficient ($RespCoef$) are assumed to be constant. The contact surface area between the vascular tissue ($VascArea$) depends on the area of the pericarp tissue according to an empirical relationship determined experimentally (see later).

In equation 4, $ClassCompetition_n$ describes the competitive ability of cells in class n to import sugar relative to that of all other cell classes. This variable accounts for the interaction between fruit cells (Bertin 2005). As pointed out by Génard *et al*. (2007), "a tissue cannot be considered as being a simple juxtaposition of independent cells". In the model $ClassCompetition_n$ is calculated by dividing the sink strength of class n ($ClassSinkStrength_n$) by the total sink strength of all classes.

$$ClassCompetition_n(i) = \frac{ClassSinkStrength_n(i)}{\sum_{n=1}^{q} ClassSinkStrength_n(i)} \quad (6)$$

Sink strength of a class n is the product of the potential growth rate of individual cells in class n (*PotCellGrowthRate*$_n$, mg h^{-1}) and the number of cells in class n (*ClassCellNumber*$_n$).

$$Class\_SinkStrength_n(i) = PotCellGrowthRate_n(i) * ClassCellNumber_n(i) \quad (7)$$

For dividing cells, we assumed that the potential growth rate ($PotCellGrowthRate_n$) during one division cycle length (*CycleLength*) is a constant (*RateDiv*) estimated experimentally. For non-dividing cells, $PotCellGrowthRate_n$ depends on cell ploidy ($CellPloidy_n$) and cell age ($CellAge_n$) following a Richards growth rate equation (Hunt 1982).

$$PotCellgrowthrate_n(i) = \frac{a*c*E}{d} * (1+E)^{-\left(\frac{1}{d}+1\right)} * deghour(i) * f(CellPloidy_n(i)) \quad (8)$$

where $E = e^{b-c*(CellAge_n(i)-CellAge\_division)}$, parameters *a* (mg), *b*, *c* (mg mg^{-1} $^oCh^{-1}$) and *d* characterize the potential growth of a diploid cell in the fruit and *CellAge_division* (^{o}Ch) is the

total time a cell spends in the division phase. The function $f(CellPloidy_n)$ was determined experimentally and describes the fold changes in potential cell growth rate per unit change in cell ploidy. Calculation of $CellPloidy_n$ is described in the endoreduplication section.

Cell division

The cell cycle machinery is equipped with several "checkpoints" where cell cycle events are monitored and signals generated, which determine whether the cell cycle should proceed or be temporally or definitively arrested (Murray 2004). This probably explains why DNA synthesis and mitosis are each preceded by a gap phase (G1 and G2 phases) during which a cell needs to grow before committing itself to the next cycle event (Francis 2007). For example, in several organisms it has been postulated that a cell must reach a critical cell size per unit DNA content for DNA synthesis (S phase) or mitosis to occur (Zetterberg *et al.* 1995; Csikász-Nagy *et al*. 2006). To account for this role of cell size and DNA content in cell division, we assumed in the model that cell division occurs if cell mass: ploidy ratio in a class n ($DNACellMassRatio_n$) reaches a critical threshold value ($DNACellMassTresholdDiv_n$).

$$DNACellMassRatio_n(i) = \frac{CellMass_n(i)}{2*CellPloidy_n(i)} \quad (9)$$

In the model, $DNACellMassTresholdDiv_n$ increases with increasing cell cycle number ($CycleNumber_n$), so that the potential of cells to divide decreases with increasing cell cycle number.

$$DNACellMassThresholdDiv_n(i) = DNACellMassThresholdDiv0 * (\rho_1 * (1 + CycleNumber_n(i))^{\rho_2}) \quad (10)$$

where $DNACellMassThresholdDiv0$ is the threshold cell mass: ploidy ratio just before the first cell division cycle, ρ_1 and ρ_2 are constant parameters (ρ_1 and ρ_2 are unitless).

Division events are accompanied by the doubling of the number of cells and halving of mass per cell in a class.

$$If \quad DNACellMassRatio_n(i-1) \geq DNACellMassThresholdDiv_n$$

$$Class\_CellNumber_n(i) = 2 * Class\_CellNumber_n(i-1) \quad (11)$$

$$CellMass_n(i) = 0.5 * CellMass_n(i-1) \quad (12)$$

The model assumes a predefined age above which a cell should exit cell division, *CellAge_division* (^{o}Ch) (Fig. 2). It has been observed in most fruits, including tomato, that the

cell division period is restricted to the first weeks of fruit development and can be modified by the environmental factors like temperature (Bertin 2005).

Cell endoreduplication

Endoreduplication is a modified cell division cycle where cells continue to increase their DNA content (S phase) without cell division (Chevalier *et al*. 2011). It has been observed that most cells that start endoreduplication are no longer able to divide (Sugimoto-Shirasu and Roberts 2003; Sabelli *et al*. 2008). In the model endoreduplication can only occur in cells that have exited the cell division phase and their age is below a threshold value *CellAge_endo* (Fig. 2). The "decision" of a cell to endoreduplicate follows a similar mechanism as described above for cell division. Endoreduplication occurs if cell mass: ploidy ratio ($DNACellMassRatio_n$) reaches a threshold value ($DNACellMassTresholdEndo_n$) (Fig. 2). Endoreduplication events are characterized by the doubling of cell ploidy.

$$If \quad DNACellMassRatio_n(i) \geq DNACellMassTresholdEndo_n(i)$$
$$CellPloidy_n(i) = 2 * CellPloidy_n(i-1) \qquad (13)$$

We assumed that the threshold cell mass: ploidy ratio for endoreduplication to occur in a class n ($DNACellMassTresholdEndo_n$) is not constant, but increases with increasing cell cycle number ($CycleNumber_n$) and cell ploidy. Thus a cell with a higher cell cycle number (number of divisions or number of endocycles) or with a higher ploidy needs to grow larger in order to endoreduplicate.

$$DNACellMassTresholdEndo_n(i) = DNACellMassTresholdEndo0 * \left(\mu1 * CycleNumber_n{}^{\mu2}(i) + \mu3 * endocount_n(i) * e^{(\mu4 * endocount_n(i) * Ploidy_n(i)_n)}\right) \qquad (14)$$

where $DNACellMassTresholdEndo0$ is the threshold cell mass: ploidy ratio just before the first endoreduplication cycle, $endocount_n$ is the number of endocycles performed by cells in class n, and μ_1, μ_2, μ_3 and μ_4 are constant parameters (μ_1, μ_2, μ_3 and μ_4 are unitless).

Materials and methods

Experimental data

To parameterize and validate our model, we used our recent experimental data (Fanwoua *et al*. 2012a, b) and those published in other literature (e.g. Nafati *et al*. 2011). We give below brief information about the experiments of Fanwoua *et al*. (2012a, b).

Three experiments were conducted in a glasshouse in Wageningen (51.57N, 5.31E, the Netherlands) using a large-fruited tomato (*Solanum lycopersicum*) cv. Moneyberg of which final fruit fresh weight is approximately 80 g.

The experiments were conducted to investigate the effects of fruit load (Experiment 1) and temperature (Experiment 2, Experiment 3) on tomato fruit growth. In Experiment 1 and Experiment 2, plants were grown at 22/18 ^{o}C day/night temperature. In Experiment 1, three fruit load treatments were applied on all trusses starting from the anthesis of flowers at the second proximal position of the third truss: a continuously low fruit load of 2 fruits per truss (2&2 fruits/truss), a continuously high fruit load of 5 fruits per truss (5&5 fruits/truss) and a switch from high (5 fruits per truss) to low fruit load treatment (2 fruits per truss) seven days after anthesis (5&2 fruits/truss). Each treatment was replicated five times. The second proximal fruit of the third truss in each treatment was harvested at anthesis, 7 DAA (days after anthesis) and at breaker stage (detailed in Fanwoua *et al*. 2012a).

In Experiment 2 all trusses were pruned to five fruits. Trusses flowering at the same moment were selected and enclosed in small transparent cuvettes (diameter: 13 cm, length: 20 cm). Heated air was continuously blown in the cuvette to heat the trusses by 4-5 ^{o}C compared to the greenhouse air temperature (22/18 ^{o}C day/night). Heating was applied either only during the first 7 DAA (D4&D0), from 7 DAA until fruit maturity (breaker stage) (D0&D4) or both (D4&D4). In the control treatment (D0&D0) trusses were also enclosed into cuvettes but the heating systems was switched off, so that the temperature inside the cuvette was the same as the greenhouse air temperature. Each treatment was replicated five times. The second proximal fruit of the third truss in each treatment was harvested at breaker stage for measurement (detailed in Fanwoua *et al*. 2012b).

Experiment 3 aimed at investigating the effect of continuous heating on fruit growth. Greenhouse air temperature was 21/18 ^{o}C day/night. All trusses were pruned to five fruits. Trusses flowering at the same moment were enclosed in small cuvettes as described in Experiment 2. Heating was applied continuously from anthesis until breaker stage. In the control treatment trusses were also enclosed in cuvettes but not heated. Each heating

treatment was replicated five times. The second proximal fruit in each selected truss was harvested at a specific thermal time (calculated as the temperature sum above a base temperature of 5.7°C (Adams *et al*. 2001)), i.e. 0, 69, 129, 232, 374 ^{o}Cd after anthesis, and at breaker stage. This corresponded to 0, 2, 5, 11, 18 and 46 DAA in the heating treatment or to 0, 3, 7, 15, 25 and 54 DAA in the control treatment (detailed in Fanwoua *et al*. 2012b).

In all three experiments, fruit diameter, fruit mass, and pericarp mass were measured. Pericarps isolated from the fruits were fixed and embedded in Technovit 7100 (Kulzer, Wehrheim, Germany) (detailed in Fanwoua *et al*. 2012a). Sections of 3 μm thick were made, stained and photographed on a light microscope (Eclipse 50i, Nikon Instruments Europe, Kingston, UK). Images obtained were analysed in Image J (National Institutes of Health, Bethesda, Maryland) to measure pericarp volume, cell volume, cell number according to the method described by Fanwoua *et al*. (2012a). Cell mass was calculated as the ratio between pericarp mass and cell number. Image J was also used to analyse the distribution of individual cell volumes (converted into cell mass) in the pericarp of young tomato fruits at anthesis. From this distribution the mean, standard deviation, minimum, maximum cell mass at anthesis were estimated.

Model simulation and sensitivity analysis

Model simulation and sensitivity analysis were carried out in Matlab (The Mathworks, Natick, Massachusetts). The sensitivity of final model output to changes in parameter values was analysed. For each parameter and output investigated, model sensitivity was quantified by the normalized sensitivity coefficient:

$$\text{Sensitivity Coefficient}=(\Delta Y/Y)/(\Delta P/P) \quad (15)$$

where $\Delta P/P$ is the relative change in model parameter value, $\Delta Y/Y$ is the relative change in model output. Model output were: final pericarp cell mass, pericarp cell number, pericarp cell ploidy and pericarp mass.

Results

Model parameter estimation

Model parameters were either estimated from measurements on tomato fruits cv. Moneyberg as described in the materials and methods or derived from the literature. Table 1 presents a summary of model parameters estimated experimentally or derived from the literature. The

value of few model parameters could not be estimated experimentally or found in the literature. For each of these parameters model calibration was carried out by comparing model output obtained for several values of the parameters with experimental data of final cell and pericarp characteristics measured under low fruit load treatment (Experiment 1). Table 2 presents a summary of model parameters estimated by model calibration.

Simulations started at anthesis. At this stage the average number of cell layers in the pericarp was measured to be 12 and used as the number of cell classes in the model.

Four parameters described the initial conditions within each cell class: the initial cell number n_0, initial cell mass $mass_0$, initial cell age age_0, and initial cell ploidy $ploidy_0$. The initial cell number was estimated as the measured number of cells in the tomato pericarp at anthesis (1.13×10^6 cells) distributed equally among the 12 cell classes ($n_0=0.94\times10^5$ cells). The distribution of initial cell mass across cell classes was assumed to follow a normal distribution with mean μ_{m0} and standard deviation σ_{m0}. Measurements performed on tomato pericarp at anthesis resulted in estimates of μ_{m0} and σ_{m0} of 7.65×10^{-8} mg and 8.56×10^{-9} mg, respectively. The distribution of cell age across the different cell classes was also assumed to follow a normal distribution with mean μ_{age0} and standard deviation $\sigma_{age0.}$ Cell age at anthesis could not be measured directly from our experiments. In a population of tomato pericarp cells Bertin *et al*. (2003a) observed 80% of 2C cells and 20% of 4C cells at anthesis and few 8C cells 2 days after anthesis. These data suggest that in the tomato fruit at anthesis, few cells have just stopped cell division. Based on the observations of Bertin *et al*. (2003a) we assumed that in the tomato pericarp at anthesis the oldest cells have just reached the threshold age for a cell to exit the cell division phase (*CellAge_division*) and the youngest cells have just been produced by the meristem and have an age of zero. Maximum cell age at anthesis was assumed to be equal to *CellAge_division* (Table 1) and for a normal distribution it is known that 99% of the probability density lies between mean minus 3 times standard error, and mean plus 3 times the standard error. This enables the estimation of μ_{age0} and σ_{age0} (μ_{age0} = 2745.6 ^{o}Ch and σ_{age0} = 915.2 ^{o}Ch). The initial cell ploidy was assumed to be 2C or 4C if cell age was below or above the threshold cell age for cell division, respectively.

Six parameters were involved in the dynamics of cell division: the duration of one complete cell cycle, *CycleLength*, the constant *RateDiv* representing the potential growth rate of a cell in the division phase, the threshold cell mass: ploidy ratio that triggers the first cell division, $DNACellMassTresholdDiv0$, the parameters (ρ_1, ρ_2) describing the variation in $DNACellMassThresholdDiv_n$ after the first division cycle and the threshold cell age above which a cell should exit the cell division phase, *CellAge_division*.

Table 1. List of parameters estimated experimentally or derived from the literature. In Experiment 1 parameter estimation was based on 5 replicate fruits from the continually low from load treatment. Parameter estimation in Experiment 3 was based on 3-5 replicate fruits.

Parameter description	Symbol	Value	Source
Number of cell classes	q	12	Experiment 1
Initial conditions			
Mean initial cell dry mass	μ_{m0}	$7.65*10^{-8}$ mg	Experiment 1
Standard deviation initial cell dry mass	σ_{m0}	$8.56*10^{-9}$ mg	Experiment 1
Mean initial cell age	μ_{age0}	2745.6 ^{o}Ch	Experiment 1
Standard deviation initial cell age	σ_{age0}	915.2 ^{o}Ch	Experiment 1
Initial cell number	n_0	$1.13*10^{6}$	Experiment 1
Base temperature	*Tb*	5.7 ^{o}C	Adams *et al*. (2001)
Cell division			
Cell cycle length	*CycleLength*	892.3 ^{o}Ch or 2.6 days	Experiment 1
Potential growth rate of dividing cells	*RateDiv*	$8.2*10^{-10}$ mg/h	Experiment 1
Age above which cells cannot divide	*CellAge_division*	16 days or 384 h	Experiment 3
Threshold cell mass: ploidy ratio that triggers the first division cycle	*DNACellMassTresholdDiv0*	$2.5*10^{-8}$	Chevalier *et al*. (2011), Experiment 1
Cell endoreduplication			
Threshold cell mass: ploidy ratio that triggers the first endocycle	*DNACellMassTresholdEndo0*	$3.2*10^{-8}$	Kononowicz *et al*. (1992), Experiment 1
Age above which cells can no longer endoreduplicate	*CellAge_Endo*	40 days or 960 h	Nafati *et al*. (2011)
Cell growth			
Age above which cells can no longer grow	*CellAge_growth*	60 days or 1440 h	Experiment 3
Parameter in the Richards function	*a*	$1.32*10^{-4}$ mg	Experiment 1
Parameter in the Richards function	*b*	-7.24	Van der Ploeg and Heuvelink (2005)
Parameter in the Richards function	*c*	$2.7*10^{-4}$ mg mg^{-1} ^{o}Ch^{-1}	Van der Ploeg and Heuvelink (2005)
Parameter in the Richards function	*d*	10^{-4}	Van der Ploeg and Heuvelink (2005)
Fruit sugar input	$SugarPool$	6.3 mg	Van der Ploeg and Heuvelink (2005)
Respiration coefficient	$RespCoef$	0.3	Ho *et al*. (1987)

Table 2. List of parameters estimated by model calibration. Model calibration was based on data measured from 5 replicate fruits in the low fruit load treatment (Experiment 1).

Description	Parameter*	Value
Parameter describing variation in $DNACellMassTresholdDiv_n$	ρ_1	0.584
Parameter describing variation in $DNACellMassTresholdDiv_n$	ρ_2	0.815
Parameter describing variation in $DNACellMassTresholdEndo_n$	μ_1	0.1
Parameter describing variation in $DNACellMassTresholdEndo_n$	μ_2	0.4
Parameter describing variation in $DNACellMassTresholdEndo_n$	μ_3	1.2
Parameter describing variation in $DNACellMassTresholdEndo_n$	μ_4	0.1
Constant proportionality factor	γ	0.0928 h^{-1} mm^{-2}

* ρ_1, ρ_2, μ_1, μ_2, μ_3, and μ_4 are unitless.

The numbers of cells at anthesis and 7 DAA in the pericarp of fruits grown under low fruit load were used to calculate *CycleLength* (*CycleLength* = 892.3 ^{o}Ch). Considering that cell mass is doubled during one *CycleLength* enabled us to estimate the potential growth rate of dividing cells *RateDiv*. Parameter *RateDiv* was estimated experimentally to be 0.378 *10^{-9} mg h^{-1}. $DNACellMassTresholdDiv0$ was estimated as the ratio between cell mass and cell ploidy at the G2/M transition. We assumed that in a population of dividing cells, the largest cells correspond to cells at the G2/M transition. Cell mass at G2/M transition was thus estimated as the average mass of the largest cells in the pericarp at anthesis. $DNACellMassTresholdDiv0$ was calculated as the ratio between the average mass of the largest cells at anthesis and cell ploidy at the G2/M transition (i.e. 4C) (Chevalier *et al.* 2011), ($DNACellMassTresholdDiv0$ = 0.225*10^{-7}). Parameters ρ_1 and ρ_2 were estimated by model calibration. *CellAge_division* was calculated experimentally as the temperature sum above which no more cell division occurs in the pericarp (*CellAge_division* 5491 ^{o}Ch).

Five sets of parameters described the dynamics of cell growth and endoreduplication after the division phase: the parameters of the Richards function describing the growth rate of a diploid cell (*a*, *b*, *c* and *d*), the threshold cell mass: ploidy ratio just before the first endoreduplication

cycle, $DNACellMassTresholdEndo0$, parameters describing the variation in $DNACellMassTresholdEndo_n$ during fruit development (μ_1, μ_2, μ_3 and $\mu 4$), the fold increase in potential cell growth rate per unit increase in cell ploidy, *f(ploidy)*, and the age above which cell endoreduplication and cell growth stops, *CellAge_endo* and *CellAge_growth*, respectively.

Parameters defining the potential growth rate of a diploid cell (*a*, *b*, *c* and *d*) were estimated assuming that potential cell growth follows a similar growth curve as potential fruit growth reported for tomato (Van der Ploeg and Heuvelink 2005). ($a = 13.2*10^{-5}$ mg; $b = 7.6*10^{-8}$; $c = 0.27*10^{-3}$ mg mg^{-1} ^{o}Ch^{-1}; $d = 0.1*10^{-3}$).

$DNACellMassTresholdEndo0$ was estimated as the ratio between cell mass and cell ploidy at the G1/S transition. It is well established that before the first endoreduplication cycle cells are diploid (ploidy=2C) at G1/S transition (Chevalier *et al.* 2011). Measurement of cell mass at G1/S transition is not straightforward and could not be directly carried out from our experiments. To estimate cell mass at G1/S transition, we assumed that cell growth rates during the G1 and G2 phases are similar. This assumption implies that the proportion of time a cell spends in the G1 phase (compared to the sum of time spent in G1 and G2) equals the proportion of mass contributed by the G1 phase (compared to the sum of mass due to G1 and G2 i.e. cell mass at G2/M transition). Kononowicz *et al.* (1992) observed that tobacco cells spend 63.8% of time in G1 phase based on the sum of time spent in G1 and G2 phases. Cell mass at G1/S transition was estimated as 63.8% of cell mass at G2/M transition ($DNACellMassTresholdEndo0 = 0.33*10^{-7}$).

f(ploidy) was deduced from a relationship between cell size and endoreduplication index described by Nafati *et al*. (2011) as shown in Fig. 3. Based on known duration of endoreduplication (Bertin *et al*. 2003a; Nafati *et al*. 2011) and cell growth in tomato pericarp, we adopted the values 13728 ^{o}Ch and 20592 ^{o}Ch for *CellAge_endo* and *CellAge_growth*, respectively.

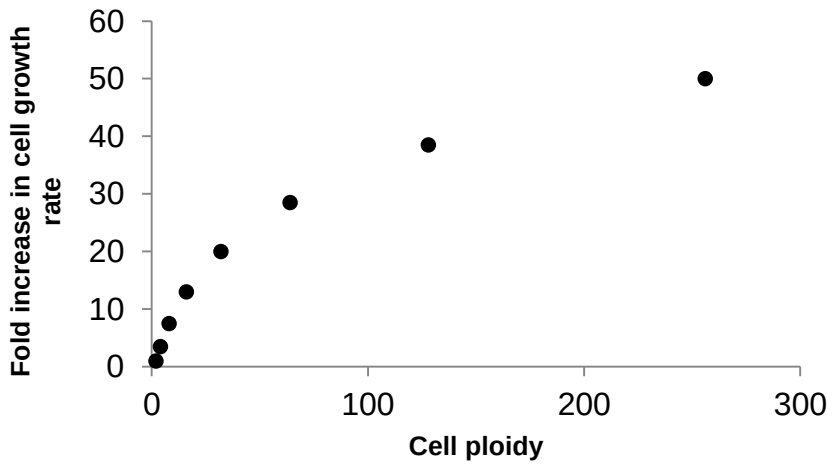

Fig. 3. Relationship between fold increase in cell growth rate and increase in cell ploidy (estimated from Nafati *et al*. 2011).

The relationships between firstly, pericarp mass and pericarp area, and secondly, pericarp area and vascular area estimated experimentally are shown in Fig. 4.

Variation in environmental conditions was defined by two parameters: the pool of sugar available for the fruit, *SugarPool*, and the fruit local temperature, *TempFruit*. *SugarPool* was estimated from the maximum growth rate of tomato fruit under non-limiting assimilate conditions (Van der Ploeg and Heuvelink 2005) and including the respiration costs (*SugarPool* = 6.3 mg). The constant proportionality factor γ was estimated by calibrating the model to the situation of non-limited assimilate supply ($\gamma = 0.0927$ h^{-1} mm^{-2}). Hourly greenhouse temperature measured during the experiment was used in the model as *TempFruit*. Since the initial cell age and cell mass in the model were derived from a normal distribution each model run could result in a different output. For this reason simulation results were presented as the average of 70 model runs, which correspond to the number of runs at which the average model output remained stable.

Model sensitivity analysis

The sensitivity of final model output to 10% increase or 10% decrease in model parameters was analysed. Simulations were carried out under the conditions of non-limited fruit sugar supply and standard temperature (22/18 ^{o}C day/night). Final cell ploidy level was not influenced by a 10% change in model parameter values. Influence of model parameters on final cell ploidy was observed only after changes in parameter values larger than 30%.

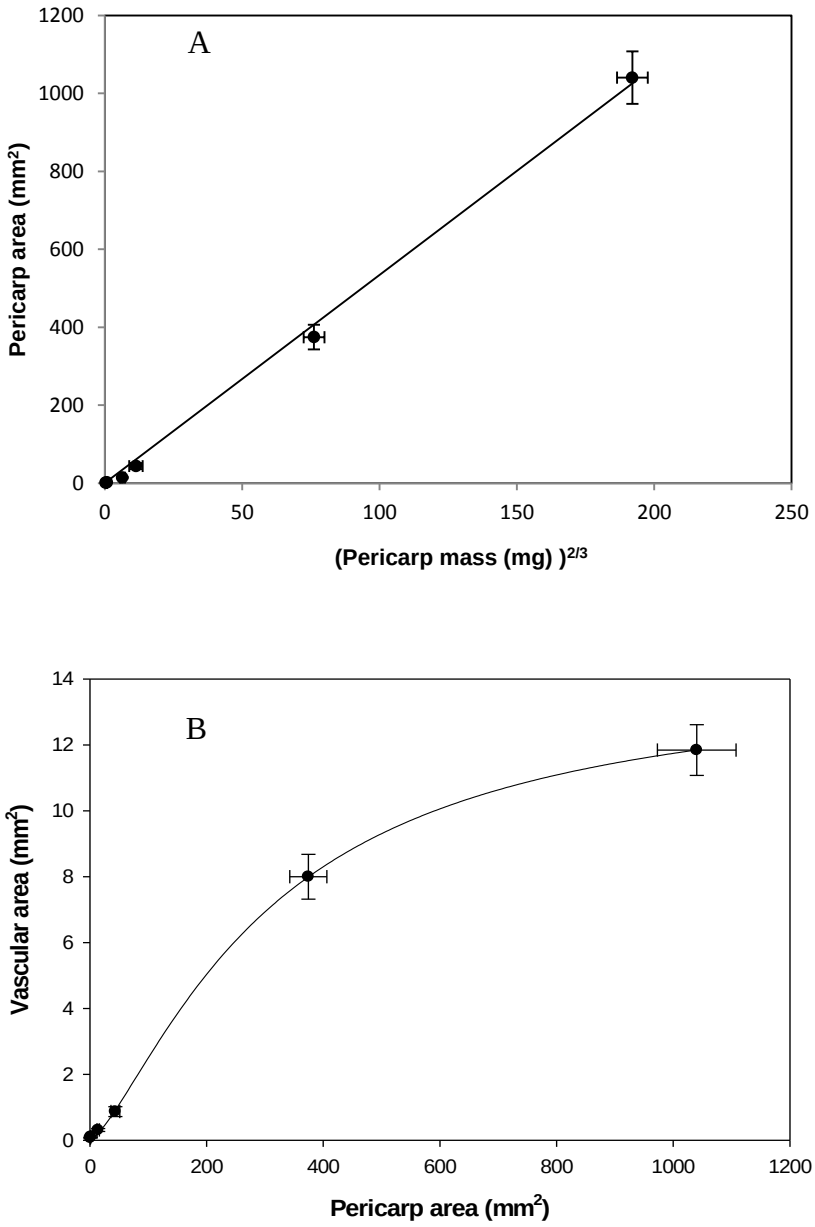

Fig. 4. Relationship between A) pericarp area and a transformed expression of pericarp mass and B) vascular area and pericarp area. Each point represents the mean of measurements on 3-5 tomato fruits. The slope of the linear regression in A) is α=5.34, P<0.0001, R^2=0.99; the equation fitted in B) is $y=a_1/(1+(x/a_2)^{a3})$, a1=14.07, a2=306.7, a3=-1.36, P<0.0001, R^2=0.99. (Data source: Experiment 3).

When these large perturbations were applied, final pericarp cell ploidy was positively influenced by the duration of cell growth period (*CellAge_growth*) and parameter *b* of the Richards function (Equation 8), but was negatively influenced by two parameters describing the variation in the threshold cell mass: ploidy ratio (μ_3 and μ_4) for endoreduplication (Equation 14) (data not shown).

Table 3 shows the sensitivity coefficients of final cell mass, final cell number and final pericarp mass to 10% increase or decrease in parameter values. Final cell mass was largely influenced by three parameters of the Richards function (*a*, *b*, *c* of Equation 8), the duration of cell growth period (*CellAge_growth*) and the duration of cell division period (*CellAge_division*). The sensitivity coefficients of final cell mass to a change in the value of these parameters were on average larger than 0.3. A 10% increase or decrease in parameter *a* resulted in a proportional change in final cell mass (Sensitivity coefficient = 1; Table 3). The sensitivity coefficients of parameter *b*, *c* and *CellAge_growth* on final cell mass were larger for a 10% decrease than a 10% increase in parameter value. An increase in the duration of the cell division period (*CellAge_division*) negatively influenced final cell mass with a slightly larger effects for a 10% increase than for a 10% decrease in parameter value.

Final pericarp cell number was largely influenced by seven parameters of which sensitivity coefficients were higher than 0.3 (Table 3): the duration of cell division (*CellAge_division*), the threshold cell mass: ploidy ratio for the first division cycle (*DNACellMassTresholdDiv0*) and two parameters describing its variation over time (ρ_1, ρ_2), the cell cycle length (*CycleLength*), the initial pericarp cell number (n_0) and parameter *b* of the Richards function (Equantion 8). Final pericarp cell number was positively influenced by *CellAge_division* and n_0. The effect of *CellAge_division* on final cell number was more than proportional to the change in the value of this parameter with larger effects when its value was increased by 10% (Table 3). A 10% increase or decrease in n_0 resulted in a proportional change in final pericarp cell number (Table 3).

The effects of *DNACellMassTresholdDiv0* and of ρ_1 and ρ_2 on final pericarp cell number were exactly the same. These three parameters negatively influenced final cell number with larger effects when their value was decreased by 10%. *CycleLength* also negatively influenced the final pericarp cell number with larger effects when its value was decreased by 10%. Final cell number was decreased by 6.2% when the value of parameter *b* was increased by 10%, but was not affected by a 10% decrease in the value of *b*.

In general, if a change of parameter value strongly affected final pericarp cell number, it had a weaker and opposite effect on final cell mass (Table 3). For each model parameter analyzed, the additive combination of its effects on final cell mass and final cell number was equivalent to its effects on pericarp mass. Final pericarp mass was largely influenced by the duration of cell division period (*CellAge_division*), the parameters describing the threshold cell mass: ploidy ratio threshold (*DNACellMassTresholdDiv0*, ρ_1 and ρ_2), the cell cycle length (*CycleLength*), parameter *a* of the Richards function and the initial cell number (n_0).

Table 3. Sensitivity coefficients of final simulated pericarp cell mass, pericarp cell number and pericarp mass after 10% increase or decrease in model parameter values. Simulations were carried out under the conditions of non-limited fruit sugar supply and standard temperature (22/18 ^{o}C day/night).

Parameter	Cell mass (mg)		Cell number		Pericarp mass (mg)	
	10%	**-10%**	**10%**	**-10%**	**10%**	**-10%**
CycleLength	0.001	0.028	-0.625	-2.500	-0.624	-2.465
q	0	0	0	0	0	0
a	1.019	1.022	0	0	1.019	1.022
b	0.189	1.225	-0.625	0	-0.448	1.225
c	0.420	0.604	0	0	0.420	0.604
d	0.104	0.132	0	0	0.104	0.132
n0	-0.002	-0.001	1.0	1.0	0.998	0.999
DNACellMassTresholdDiv0	0.001	0.218	-0.625	-5.625	-0.624	-5.284
DNACellMassTresholdEndo0	-0.023	-0.022	0	0	-0.023	-0.022
CellAge_division	-0.435	-0.172	5.625	1.250	4.945	1.100
CellAge_growth	0.305	0.882	0	0	0.305	0.882
CellAge_Endo	0	0	0	0	0	0
γ	0.001	0.010	0	0	0.001	0.010
SugarPool	0.001	0.010	0	0	0.001	0.010
ρ_1	0.001	0.218	-0.625	-5.625	-0.624	-5.284
ρ_2	0.001	0.219	-0.625	-5.625	-0.624	-5.283
μ_1	-0.001	0	0	0	-0.001	0
μ_2	-0.046	-0.040	0	0	-0.046	-0.040
μ_3	-0.065	-0.050	0	0	-0.065	-0.050
μ_4	-0.001	-0.001	0	0	-0.001	-0.001

Model validation: analysis of fruit growth characteristics in response to assimilate supply

The model was calibrated for the situation of non-limited fruit sugar supply. The predictive quality of the model was evaluated for the situation of limited fruit sugar supply by comparing model predictions with final pericarp characteristics measured on fruits grown in Experiment 1 under continuously high fruit load of 5 fruits per truss (5&5 fruits/truss) or under high fruit load (5 fruits per truss) during the first seven days after anthesis and low fruit load (2 fruits per truss) for the rest of fruit growth duration (5&2 fruits/truss). To simulate the effects of sugar limitation on fruit growth, the pool of sugar available for the fruit *SugarPool* was reduced to 62.5%. Assuming a fruit load-induced assimilate limitation, the percentage of reduction of *SugarPool* was calculated as the ratio between the amount of dry matter

partitioned into individual tomato fruits of a five-fruits truss and a two-fruits truss. This calculation was based on the known proportion of dry matter partitioned into a tomato truss (i.e. 70%, (Heuvelink 1996)) under standard fruit load (i.e. 7 fruits/truss for Moneyberg, Prudent *et al*. 2009).

Fig. 5 depicts the time course of cell mass, cell number, cell ploidy and pericarp mass simulated by the model under continuously non-limited (2&2 fruits/truss) or limited (5&5 fruits/truss) sugar supply, or under limited sugar supply during the first seven days after anthesis and non-limited supply for the rest of fruit growth (5&2 fruits/truss). Continuously limited sugar supply during fruit growth did not affect cell growth duration, but reduced cell growth rate resulting in a decrease in final pericarp cell mass compared to the situation where sugar supply was not limiting (Fig. 5A). Simulated final pericarp cell mass under continuously limited sugar supply agreed with cell mass measured experimentally in the 5&5 fruits/truss treatment (Table 4). When sugar limitation was imposed only during the first seven days after anthesis, the model did not predict a reduction in cell growth, which remained potential (Fig. 5A). This prediction agreed well with measured final cell mass in the 5&2 fruit load treatment which was similar to cell mass measured in the 2&2 fruit load treatment (Table 4).

Endoreduplication was slightly delayed when sugar supply was continuously limiting, but this delay did not result in differences in the final ploidy levels between the three sugar supply schemes (Fig. 5B).

Limited sugar supply resulted in a reduction in the final number of pericarp cells (Fig. 5C). This effect was more pronounced when sugar limitation was imposed continuously than when sugar limitation was applied only during the first seven days after anthesis (Fig. 5C). Model prediction of final number of pericarp cells in the 5&5 fruits/truss and 5&2 fruits/truss treatments was not statistically different from experimental data (Table 4). Sugar limitation-induced reduction in final cell number was directly linked to the reduction of cell division duration as sugar supply did not affect cell division rate (Fig. 5C).

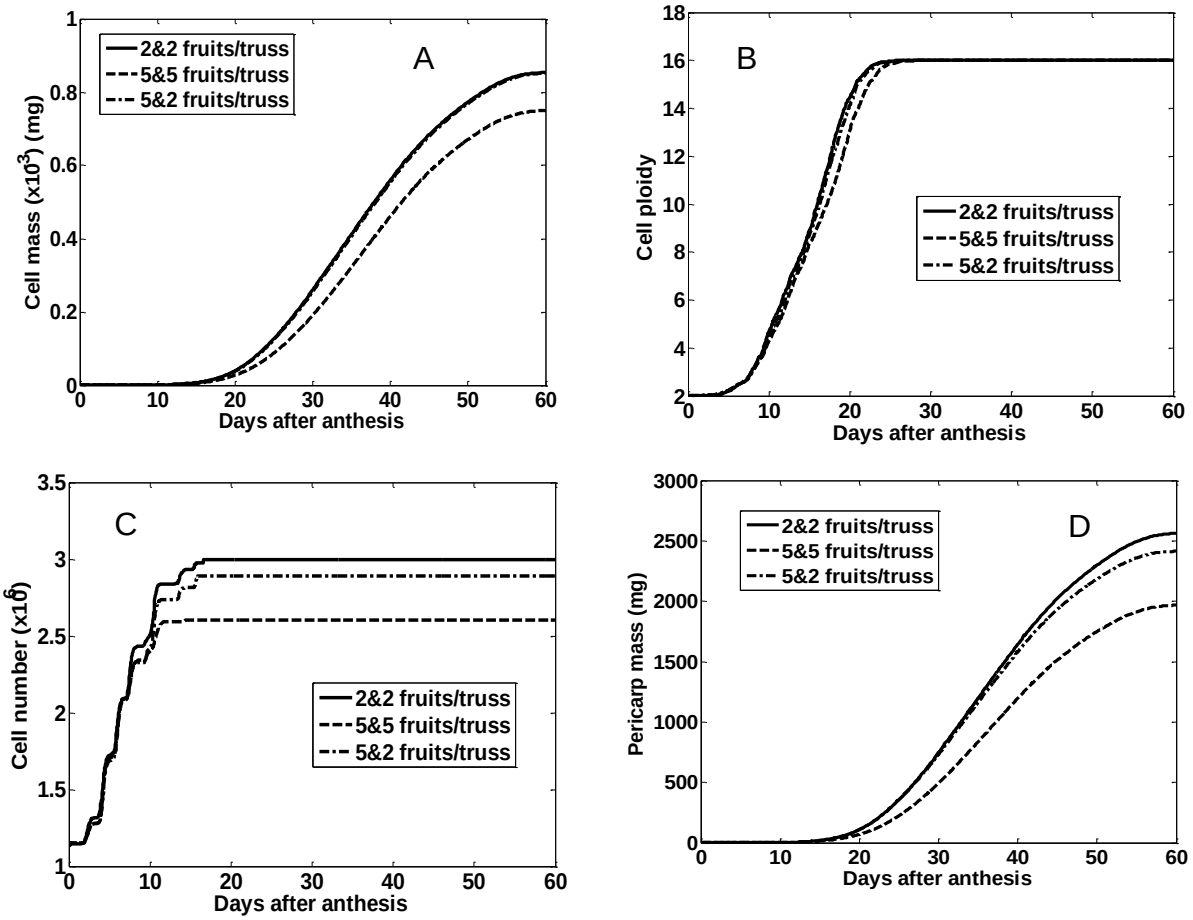

Fig. 5. Dynamics of simulated A) cell mass, B) cell ploidy, C) cell number, and D) pericarp mass during tomato fruit growth in the treatments with continuously low fruit load (2&2 fruits/truss, solid line), continuously high fruit load (5&5 fruits/truss, dashed line) and a switch from high to low fruit load 7 DAA (5&2 fruits/truss, dash-dotted line). Each line represents the mean of 70 model runs.

Limited sugar supply induced a decrease in final simulated pericarp mass (Fig. 5D). This response was more severe when sugar was continuously limiting than when sugar limitation was imposed only during the first seven DAA. Model prediction of final pericarp mass in the 5&5 fruits/truss and 5&2 fruits/truss was not significantly different from experimental data (Table 4). Sugar limitation did not affect pericarp growth duration, so that the reduction of final pericarp mass could be attributed to a lower pericarp growth rate (Fig. 5D).

Table 4. Measured and simulated final pericarp characteristics in the treatments with continuously low fruit load (2&2 fruits/truss), continuously high fruit load (5&5 fruits/truss) and a switch from high to low fruit load (5&2 fruits/truss). Each measured value is the mean ± standard error of the mean of 4-5 fruits at breaker stage in Experiment 1. Each simulated value is the mean of 70 model runs at day 60.

	Treatments	**Measured**	**Simulated**
Cell number ($\times 10^6$)	2&2F	3.11±0.34	3.00
	5&5F	2.62±0.26	2.60
	5&2F	2.73±0.36	2.89
Average cell mass ($\times 10^{-3}$) mg	2&2F	0.87±0.14	0.85
	5&5F	0.75±0.12	0.75
	5&2F	0.88±0.02	0.85
Pericarp mass ($\times 10^3$) mg	2&2F	2.63±0.07	2.56
	5&5F	1.87±0.19	1.97
	5&2F	2.37±0.27	2.41

Model validation: analysis of fruit growth characteristics in response to temperature

Hourly fruit temperatures recorded from the four fruit heating treatments (D0&D0, D4&D4, D4&D0, D0&D4) in Experiment 2 were input in the model to simulate temperature effects on fruit growth. For these simulations we used the initial cell number measured in Experiment 2 ($n_0 = 1.15*10^5$ cells), which was slightly higher than the value measured in Experiment 1. Fig. 6 shows the time course of simulated cell mass, cell ploidy, cell number and pericarp mass in each heating treatment. The largest final cell mass were simulated when fruits were not heated continuously (D0&D0) or when heating was applied only during the first seven days after anthesis (D4&D0) (Fig. 6A). In these two heating treatments cell growth lasted the longest. Continuously heating fruits (D4&D4) increased cell growth rate, but this increase was more than compensated by the reduction in cell growth duration resulting in a reduction in final simulated cell mass (Fig. 6A). A shorter cell growth duration was also responsible for the reduction in final simulated cell mass of fruits heated from 7 DAA until maturity (Fig. 6A). Comparison between model predictions and experimental data revealed that the model overestimated the final cell mass in the D0&D0 and D4&D0 treatments (Table 5). Reduction in final cell mass in the D4D0 treatment was underestimated by the model. The model predicted similar final cell mass in the D4D4 and D0D4 treatments, which agreed with the values measured experimentally (Table 5).

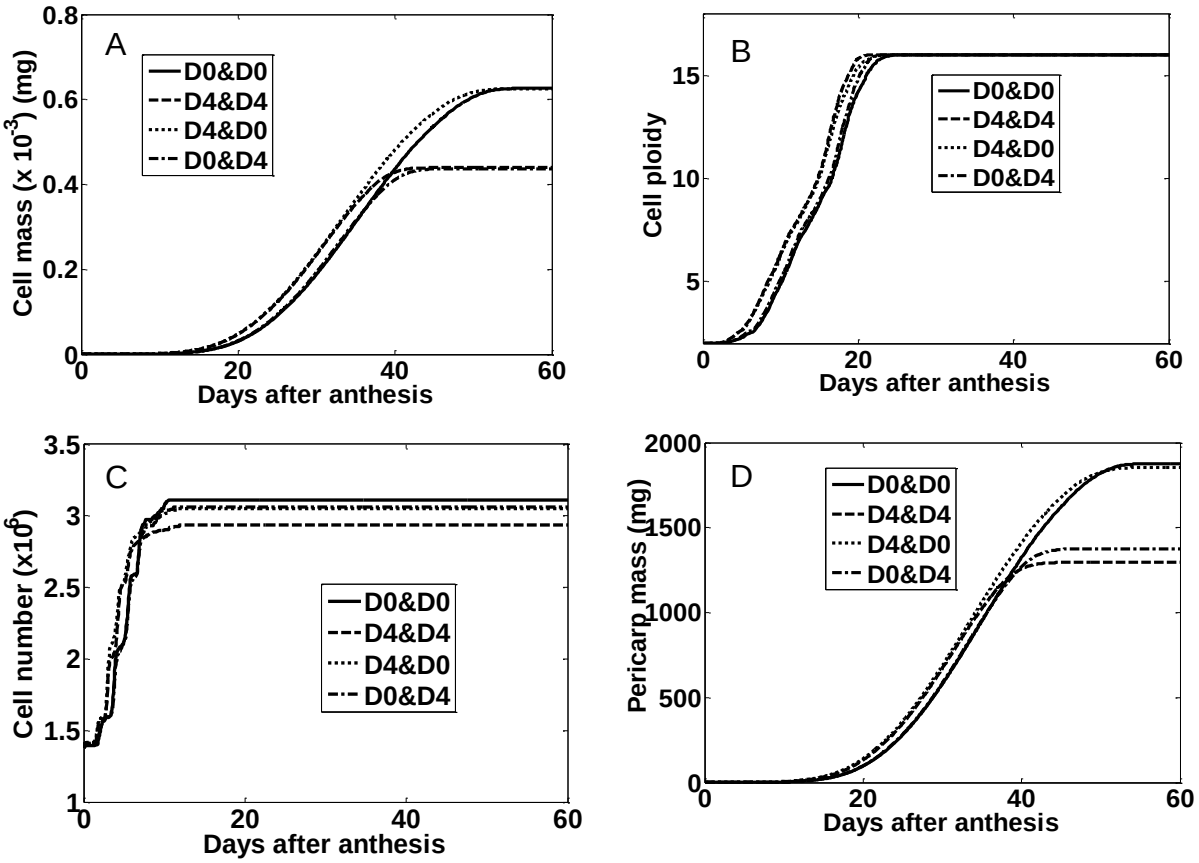

Fig. 6. Dynamics of simulated A) cell mass, B) cell ploidy, C) cell number, and D) pericarp mass during tomato fruit growth in the treatments without fruit heating (D0&D0, solid line), with heating applied continuously from anthesis till breaker stage (D4&D4, dashed line), with heating applied only during the first seven days after anthesis (D4&D0, dotted line), and with heating applied only from 7 DAA till breaker stage (D0&D4, dash-dotted line). Each line represents the mean of 70 model runs.

Endoreduplication rate was increased in fruits heated continuously (D4&D4) or heated only during the first seven days after anthesis (D4&D0). In these treatments the increase in endoreduplication rate was compensated by a short endoreduplication duration. This resulted in a similar simulated final ploidy level as in the D0&D0 and D0&D4 treatments.

Heating fruits continuously (D4&D4) increased cell division rate, but this increase was more than compensated by a reduction in cell division duration which resulted in the decrease of simulated final cell number (Fig. 6C). When fruits were heated only during the first seven days after anthesis (D4&D0), cell division rate was also increased but cell division duration was slightly prolonged, which led to slightly more cells in matured D4&D0 fruits compared to fruits in the continuously heating treatment (Fig. 6C).

Table 5. Measured and simulated final pericarp characteristics in the treatments without fruit heating (D0&D0), with heating applied continuously from anthesis till breaker stage (D4&D4), with heating applied only during the first seven days after anthesis (D4&D0), with heating applied only from 7 DAA till breaker stage (D0&D4). Each measured value is the mean ± standard error of the mean of 4-5 fruits at breaker stage in Experiment 2. Each simulated value is the mean of 70 model runs at day 60.

	Treatments	**Measured**	**Simulated**
Cell number ($\times 10^{6}$)	D0&D0	3.14±0.09	3.11
	D4&D4	2.85±0.29	2.94
	D4&D0	3.53±0.50	3.05
	D0&D4	3.28±0.51	3.06
Cell mass ($\times 10^{-3}$) mg	D0&D0	0.57±0.04	0.63
	D4&D4	0.47±0.03	0.44
	D4&D0	0.47±0.04	0.63
	D0&D4	0.49±0.06	0.44
Pericarp mass ($\times 10^{3}$) mg	D0&D0	1.80±0.10	1.88
	D4&D4	1.32±0.08	1.29
	D4&D0	1.58±0.11	1.85
	D0&D4	1.51±0.11	1.37

The model simulated the largest final number of cells in continuously non-heated fruits (D0&D0) and in fruits where heating was applied only from 7 DAA until fruit maturation (D0&D4). In these two treatments the high final cell number was due to a prolonged cell division period (Fig. 6C). Comparison between 2009 experimental data and model simulations shows that in all heating treatments the model accurately predicted final number of pericarp cells. Heating treatments did not significantly affect final measured perimarp cell number.

Model simulations of pericarp growth under continuous fruit heating (D4&D4) showed an increase in the pericarp growth rate (Fig. 6D). This increase was relatively small and did not compensate for the reduction in pericarp growing period. This resulted in the decrease in final pericarp mass. A short growing period was also responsible for the reduction in simulated final pericarp mass in fruits heated from 7 DAA until fruit maturation (D0&D4) (Fig. 6D). The largest final simulated pericarp masses were observed in the treatments with the longest pericarp growth durations (D0&D0 and D4&D0). Final pericarp masses predicted by the model in continuously non-heated fruits (D0&D0) and fruits heated continuously (D4&D4) were within the range of values measured experimentally (Table 5). The reduction in final

simulated pericarp mass was underestimated in the D4&D0 treatment and overestimated in the D0&D4 treatment. In the D4&D4 treatment the model predicted 31% reduction in final pericarp mass compared with 26% measured experimentally.

Discussion

Our dynamic model of fruit growth integrates three fundamental cellular processes responsible for the variation in fruit size: cell division, cell growth and endoreduplication. It describes variation in cell number, cell mass and cell ploidy in tomato fruit pericarp from anthesis until maturation. In the model cell division or endoreduplication are triggered by a threshold cell mass: ploidy ratio (Fig. 2). This assumption implies a link between cell growth and cell division. Several authors suggested that cell growth and the progress through the cell cycle might be closely related (Nasmyth 1979; Jorgensen and Tyers 2004; Francis 2007). This link between cell growth and cell division can be clearly seen in meristematic tissues where cell size remains fairly constant despite having divided several times (Beemster *et al.* 2003). At the sub-cellular scale cell division and endoreduplication are controlled by a complex network of genes. The main components of this network are CDKs and Cyclins of which concentrations determine whether the cell duplicates its DNA (S phase) or divides (M phase) (Chevalier *et al.* 2011). Csikasz-Nagy *et al*. (2006) proposed that the rate of synthesis of cyclins involved in the triggering of DNA synthesis or cell division might be proportional to cell mass. This hypothesis suggests an indirect link between cell division and cell growth.

In our model the threshold cell mass: ploidy ratio that triggers cell division and endoreduplication increases with the number of cycles or the cell ploidy level. This implies that the capacity of a cell to perform the next division or endoreduplication cycle decreases as cell ploidy or cell cycle number increases. Experimental data confirm that the rate of cell division and endoreduplication decreases during fruit development (Bertin 2005; Nafati *et al.* 2011). In their model of orchid flower, Lee *et al*. (2004) used an empirical function decreasing with time to describe the reduction of endoreduplication rate during organ development. A similar function was used by Bertin *et al*. (2007) to describe the decrease in cell division rate during the development of tomato fruits. Bertin *et al*. (2007) proposed that during fruit development, the proportion of endoreduplicating cells might decrease with an increase in the number of cell cycles and in the number of endoreduplication cycles already performed by the cell, although these hypotheses were not implemented in their model. The down-regulation of endoreduplication might be related to a longer S-phase required to

duplicate larger DNA sizes, a down-regulation of S-phase CDKs or the build-up of S-phase inhibitors during fruit development (Bertin *et al*. 2007; Bourdon *et al.* 2010). More studies are necessary to understand the control of endoreduplication during fruit development.

The arrest of cellular processes in our model is determined by a predefined cell age. This simplification was based on the experimental observation that cellular processes are restricted to specific periods during fruit development (Bertin 2005; Nafati *et al*. 2011). In reality the molecular mechanism controlling the arrest of cellular processes is complex and not yet well understood. It is generally accepted that the arrest of cell division and the onset of endoreduplication require the inhibition of specific regulators of the M-phase or mitosis inducing factors (MIF) (Chevalier *et al*. 2011). The role of these MIF in the transition from cell division to endoreduplication is suggested by their temporal expression pattern during fruit development. For example CDKB1, CycA3, CycA2, CycD3 are highly expressed in dividing cells and down-regulated at the onset of endoreduplication (Inzé and de Veylder 2006). In tomato, it was observed that endoreduplication and fruit maturation are associated with the maximum expression of KRP1 and KPR2 genes, respectively (Bourdon *et al*. 2010). Our fruit growth model could be extended by associating the onset/arrest of cellular processes with the expression profile of these genes.

Model sensitivity results showed that decreasing the value of some parameters by 10% had different magnitude of effects on the output than decreasing their value (Table 3). Such effects could be related to non-linear relationships between these parameters and the output variable. Surprisingly final cell ploidy was only sensitive after large perturbations in model parameters. This suggests that endoreduplication might be a more stable process compared to cell growth and cell division. The sensitivity of final cell mass to parameters describing cell growth was in line with our expectations (Table 3). Final pericarp cell mass was also influenced by the duration of cell division (Table 3). Its negative effect on final cell mass was probably an indirect effect resulting from the positive influence of cell division duration on cell number. A large cell number in the pericarp could lead to a stronger competition between cells for assimilates, and smaller final cell mass. Many examples in the literature illustrate the fact that an increase or decrease in cell division duration can have the opposite effects on final cell size (Higashi *et al.* 1999; Bertin 2005).

In general final pericarp mass was strongly sensitive to parameters involved in the dynamics of cell division. Experimental data showed that genotypic or environmentally related variations in fruit size are mainly associated with variations in cell division activity in many fruit species including tomato (Bertin *et al*. 2003a), melon (*Cucumis melo* L.) (Higashi *et al*.

1999), strawberry (*Fragaria ananassa* Duch.) (Cheng and Breen 1992), cherry (*Prunus avium* L.) (Olmstead *et al.* 2007) and peach (Scorzal *et al.* 1991). Cell division duration had the largest influence on final cell number and final pericarp mass. Its influence on final cell number was about 10 times larger than on final cell mass (Table 3). The effects of this parameter on final pericarp mass could thus be accounted for by its large effects on cell number. In melon Higashi *et al*. (1999) observed that differences in final cell number and fruit size between a large and a small-fruited melon cultivar was caused by differences in cell division duration. Similar results were reported in pear (*Pyrus pyrifolia*) (Zhang *et al*. 2006), peach (Yamaguchi *et al*. 2002) and tomato (Bertin *et al.* 2003a). Besides the duration of cell division period, final cell number was sensitive to all other parameters involved in the dynamics of cell division in the model (Table 3). The negative effects of cell cycle length and the positive effects of the initial cell number was in line with our expectations. The sensitivity of final pericarp cell number and final pericarp mass to the initial cell number highlight the importance of pre-anthesis cell division in the determination of final cell number and fruit size. Baldet *et al.* (2006) showed that fruit-load induced variations in pre-anthesis cell division and cell number at anthesis were important in determining the final cell number and fruit size in tomato. Similarly, variations in the final cell number and fruit size at the proximal and distal positions of a tomato truss have been associated with differences in cell number at anthesis (Bertin *et al*. 2003a).

We simulated the effects of limited sugar supply to the fruit by reducing the common sugar pool to 62.5%. A similar approach was used by Fishman and Génard (1998) in the peach fruit model and later by Liu *et al*. (2007) in the tomato fruit model. In these models the common sugar pool was reduced by 50% to simulate the effects of high fruit load on fruit growth. Our calculation of the reduction of pool of sugar (62.5%) took into account the fraction of assimilates partitioned to individual fruits under low and high fruit load conditions. This calculation was validated by the comparison between model output and measurements. The model was able to accurately predict final cell mass, cell number and pericarp mass for the continuously high fruit load treatment (5&5F) and for the treatment with a switch from high to low fruit load (5&2F). The reduction of simulated cell growth rate, cell division duration and pericarp growth rate under limited assimilate supply agreed well with the observations of Bertin (2005).

Globally the model simulated reasonably well the final cell number, cell mass and pericarp mass under several contrasting temperature conditions (Table 3). The model predicted that heating increases the rate and decreases the duration of cell division, cell growth and pericarp

growth. The magnitude of increase in the rate of these processes relative to the decrease in their duration determined the final pericarp and cell phenotypes. This is in agreement with the responses of cell division, cell expansion and fruit growth described by other authors (Marcelis and Baan Hofman-Eijer 1993; Bertin 2005).

Comparison between simulated and measured mean endoreduplication levels could not be directly carried out because we did not have enough experimental data. Bertin (2005) observed that the mean ploidy level measured during tomato fruit growth follows a sigmoidal function. The time course of endoreduplication emerging from our model also followed a sigmoidal pattern (Fig. 5B). Bertin (2005) reported a higher final mean C-value (24C) than the value predicted by our model (16C) (Fig. 5B; Fig. 6B); however it is worth mentioning that this represents a difference of less than one endocycle. Interestingly high fruit load delayed endoreduplication in Bertin's experiment but did not affect the final ploidy level, which agree with our simulations. These results together support the hypothesis that endoreduplication might set the potential for cell growth, but actual cell growth might be determined by the amount of assimilate available for the cell.

Concluding remarks

The model presented here uniquely integrates three main underlying processes during fruit growth from anthesis until fruit maturation: cell division, cell growth and endoreduplication. It demonstrated the importance of cell division in the variation of final fruit size. The model was able to generate acceptable predictions of fruit and cell phenotypes under various contrasting fruit load and temperature conditions. This shows that the present model has the potential to predict fruit growth under various environmental conditions and can be used to carry out *in silico* experiments on fruits. The model does not consider sugar transformations, nor does it take into account water relations within the fruit. One perspective is to incorporate these aspects of fruit growth into the fruit model, as this would improve our understanding of fruit quality build-up and its interaction with the environment. The present model could also be extended by incorporating information on the molecular control of fruit cellular processes. This can be done by relating some model parameters with the expression profile of specific genes. Such a model could be used to gain more insight into the gene-by-environment interaction effects on fruit growth.

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5. A theoretical framework for future modelling of fruit growth

Adapted from

de Visser PHB., Kromdijk W, Okello RC, Fanwoua J, Struik PC, Yin X, Heuvelink E Marcelis LFM (2012) Explaining tomato fruit growth by a multi-scale model on regulation of cell division, cell growth and carbohydrate dynamics. *Acta Horticulturae* **957**, 167-172.

Abstract

A multi-scale approach to model tomato fruit growth is proposed, in order to account for the interaction between gene functioning and growth conditions, and, ultimately, to explain the fruit phenotype of various genotypes in diverse growth environments. There is particular focus on: (I) cell division regulated by cell cycle genes, (II) cell expansion as influenced by polyploidy resulting from endoreduplication and carbohydrate and water dynamics. The growth processes at gene, cell, tissue, fruit and plant scale have been identified and included in the model. Sub-populations of cells differing in age are considered to act as sinks competing for carbohydrates. The key cell cycle genes of tomato could be incorporated into an existing model of the gene regulatory network of the cell cycle. This model can be modified to simulate endoreduplication. Moreover, the modelled cell cycle process can be made sensitive to temperature and assimilate supply. Fruit sugar and water import can be linked to plant sugar and water status. Using the multi-scale approach in future fruit models can contribute in improving the understanding and prediction of G×E interactions for fruit growth and quality.

Keywords: gene regulatory network, cell dynamics, assimilate supply, sucrose

Introduction

Until now, most fruit growth models used experimentally determined, empirical relationships between environment and growth. Only a few models explain fruit growth as a result of processes occurring at lower hierarchical scales. For tomato, Liu et al. (2007) explained fruit growth by water and sucrose uptake and for turgor driven expansion regarding the fruit as one big cell. The model of Bertin et al. (2007) explained fruit growth on the basis of cell dynamics. This model did not explain cell division from biological underlying processes but merely described this by an empirical function, fitted to the data. Creating a more realistic growth model requires incorporating basic insight into processes related to cell division, cell expansion, endoreduplication and cell fate. These cell dynamics follow from processes that occur across several organizational levels, starting at the gene level and

probably ending at the fruit and plant levels. Incorporating these organizational levels would facilitate the simulation and prediction of gene – environment interactions explicitly, in a more mechanistic way than the G×E approach that uses Quantitative Trait Loci and treats underlying genetic processes as a black box (Quilot et al., 2005; Yin et al., 2004). Such a multi-scale model that considers several organizational levels could tackle questions as to what extent tomato fruit sink strength is determined by the developmental, genetic programme of cell and tissue formation, and how much control is exerted by environmental factors during growth. *Grosso modo*, many of the mechanisms explaining tomato fruit growth can be subdivided into two categories:

- Up-scaling of sub cellular gene related processes to the organ level to unravel the basis for differences in fruit yield among genotypes.
- Exploring possible mechanisms of temperature and carbohydrate effects on fruit growth by including effects on cell division and cell growth.

Although decades of research on tomato have generated much knowledge, only a small part of the relevant processes is quantitatively known, potentially hampering model development. Yet, a provisional model could show to what extent the current advances in genetic and physiological knowledge can already predict fruit growth with a bottom-up approach, and identify the gaps in knowledge. A mechanistic modelling approach is proposed, which captures the integrated behaviour of cell division, cell and tissue expansion, endoreduplication, as well as transport and metabolism of water and carbohydrates.

The modelling consists of separate models at five different aggregation levels (Fig. 1): A. the gene, B. the cell, C. the tissues, D. the fruit, and E. the plant level. Besides, a platform that exchanges information between models is needed.

Gene regulatory network of the cell cycle

An ordinary differential equation (ODE) model on the gene regulated cell cycle could be used to derive cell division events. A division is initiated when the checkpoint of the end of the so-called gap phase 2 is reached. For this, the existing generic model of the eukaryotic cell cycle (Tyson and Novak, 2001) could be adapted for tomato. Although a gene network can be very complex, examples in literature show that it might not be necessary to model the complete network (Beemster et al., 2006; Welch et al., 2003). The model could simulate the expression of major genes involved in the cell cycle as observed for tomato (see, e.g., Czerednik et al., 2012) (Fig. 2). The model could also be extended with a module on endoreduplication using

the mechanisms of the endocycle in Arabidopsis (Magyar et al., 2012). The switch to endoreduplication is determined by passing a threshold of cyclin built-up. The cell mass affecting cyclin concentrations and thus exerting its influence on the checkpoints in the cell cycle, is itself again affected by processes at other scales and therefore, is an important variable in interactions between models.

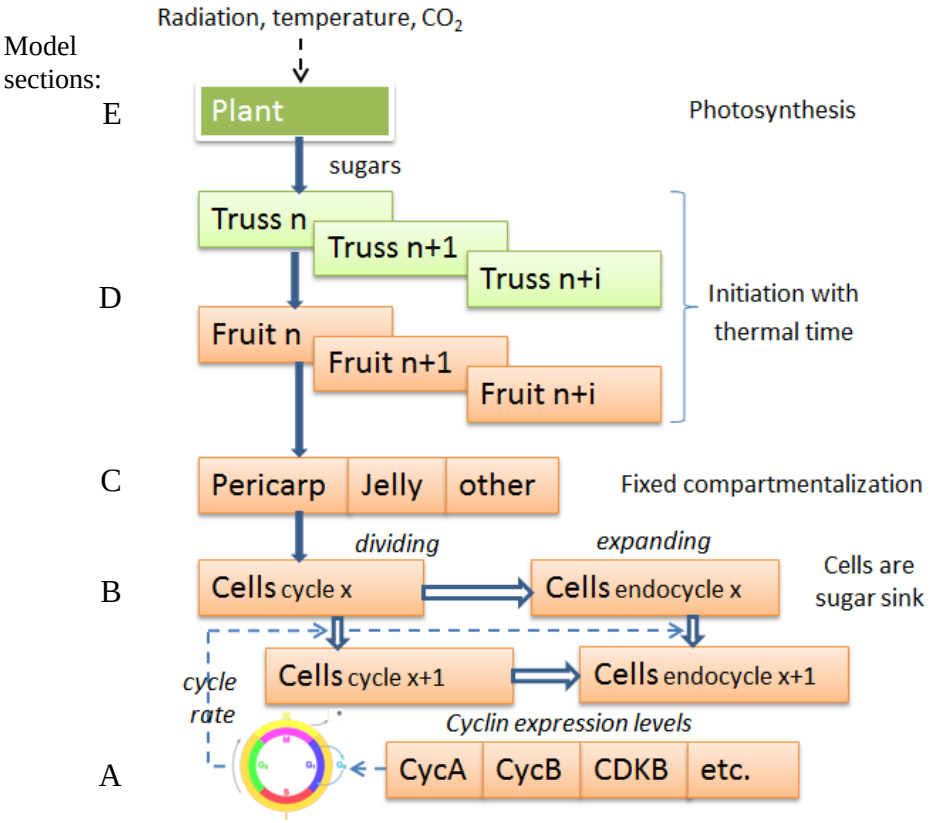

Fig. 1. State variables at the different aggregation levels, dealt with in model sections A to E (see main text). The thick solid arrows between levels indicate sugar flow. The open arrows indicate shift from cell division to expansion phase. The dividing and endoreduplicating cells are the sink for sugars from photosynthesis.

Cell

In the model, fruit cells are initiated at the moment of anthesis, i.e. directly after pollination, and all existing cells obtained a specific identity. The mechanistic model only considers two cell types: undifferentiated proliferating cells and specialized parenchyma cells that expand. At anthesis a number of cell classes on the basis of their age in thermal time is assumed. The model keeps track of cell numbers and size within each age class (Chapter 4). Thus, for each age class a calculation at gene level (A) and cell level (B) that is representative of all cells in

the age class is performed. In the fruit model presented in Chapter 4, the onset/arrest of cellular processes was determined by predefined cell ages. Here the gene regulatory network determines the occurrence of cell cycle events (Fig. 2), the number of cycles during the cell division period, and the onset and duration of the endoreduplication phase. Endoreduplication is limited to parenchyma cells in the pericarp and jelly tissue. Cell expansion is the results of cell water and sugar import. Cell water import is described using thermodynamic equations according to the method proposed by Fishman and Génard (1998) for peach fruit and later adapted by Liu et al. (2007) on tomato fruit. In these models the whole fruit was considered as a big cell (Fishman and Génard, 1998; Liu et al., 2007).

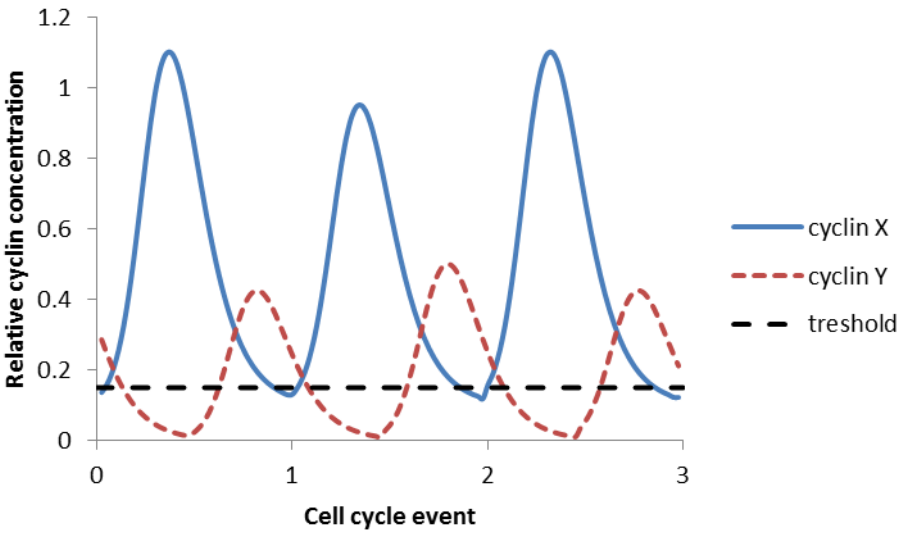

Fig. 2. Illustration of the oscillations of cyclin concentrations in the cell cycle model. A cell cycle event is initiated when cyclin X drops below a given threshold concentration level. Thus, the time period between two divisions depends on cyclin dynamics.

Water import into the cells is calculated based on the differences in hydrostatic and osmotic potential between the cells and fruit vascular tissues. In the Liu et al. (2007) model, a constant proportion of soluble sugar was assumed to contribute to fruit osmotic potential. This last assumption could be modified and changes in cell soluble sugar content could be described using simple rules capturing the main reactions involved in carbohydrate metabolism during fruit growth. Only essential chemical processes for cell growth can be dealt with in the model, given the extreme complexity of the cell's biochemistry. Sucrose is assumed to be the sole form of imported sugar by cells prior to hydrolysis into hexoses (fructose and glucose) by sucrose synthase (SuSy). The hexoses are an energy and carbon source for growth of cell

organelles, growth and maintenance respiration. Hexoses are also transported into the vacuole where they act as precursors during starch formation (Ho, 1996). Finally, most of the hexoses are transformed into starch (Schaffer and Petreikov, 1997; N'tchobo et al., 1999). Starting at around 20 days after anthesis, starch is degraded to form soluble sugars. Simple approximation of these processes could be assumed by keeping enzyme concentrations constant. Enzyme reactions can be calculated with Michaelis-Menten kinetics. Temperature influence on modelled processes could be incorporated by an Arrhenius equation. In the peach model of Fishman and Génard (1998), sugar was imported by active transport, by mass flow or by passive diffusion. Later, Liu et al. (2007) introduced a parameter describing the decrease in cell wall permeability to sugar to account for the shift from symplasmic to apoplasmic transport of sugar in tomato (Ruan and Patrick, 1995). The simulation of sugar import in a population of cells should take into account the competition between fruit cells for sugar (Bertin, 2005). This can be implemented using the relative sink strength approach (Marcelis et al., 1998). This approach implies that sugars are allocated to a cell class according its sink strength relative to that of all other classes. The sink strength of a cell class depends on its cell number (Chapter 4). Ultimately, sugar, water and temperature driven cell size increase should result in a new mitosis or endocycle if a specific threshold size is attained, and under the condition that the checkpoint for end of gap phase (level A) has been reached.

Tissues

The model should be able to mechanistically calculate the size, i.e. volume, of two tissues (jelly and pericarp) only, using for each tissue models of level A and B. The size of other tissues (columella, seed, vascular bundles, endocarp, exocarp) is estimated on the basis of the size of these two tissues. Tissue growth limitations are apparent at the fruit scale. Constraints with respect to growing space for different tissues, physical pressure of surrounding tissue and rigidness of the skin could be simulated in a future 3D model version. The import of water is directed through vascular bundles, situated in the middle of the pericarp. Water conductivity of these bundles is derived from their surface area in cross sections of the pericarp, as measured in fruits of different development stages.

Fruit

At a truss usually five fruits are held which differ in age. From level C for each fruit the sink strength per time step is calculated from the total sink strength of different tissues. Different

fruits are assumed to compete for assimilates arriving from the phloem in the supporting truss rachis. Moreover, all trusses on the plant compete for sugars residing in one central pool, and receive sugars in proportion to their sink strength relative to total plant sink strength (relative sink strength approach following Marcelis et al., 1998). Phloem sugar supply is assumed constant within the day and calculated from sugar supply at plant level on a daily basis (see level E). Simulated fruit sugar and water content could be converted into fruit organoleptic qualities such as sweetness following the approach of Génard et al. (2003) or acidity as described in the model of Wu et al. (2007). Fruit sugar and water import should be linked to sugar and water status of the plant. Xylem and phloem flows are described based on the differences in hydrostatic and osmotic potential between the fruit and the plant stem.

Plant

A mechanistic tomato plant model, based on Marcelis et al. (2009), could be used to calculate assimilation and dissimilation of carbohydrates (CH_2O). The plant is described in terms of biomass in the fractions leaves, stems, fruits and roots. The growing leaf biomass is translated into area according to a seasonally changing specific leaf area. Photosynthetically active radiation (PAR) is captured by the leaf canopy quantified by LAI and following a Lambert-Beer type negative exponential decay with depth. Absorbed PAR is converted into CH_2O using the Farquhar-von Caemmerer-Berry model (Farquhar et al., 1980; Qian et al., 2012). The hydrostatic and osmotic water potential in the plant stem could be described based on the model proposed by De Swaef (2011).

Platform

The platform should manage the tasks of the different models and invoke a computation at a specific aggregation level if a modelling process needs the outcome to proceed. The platform and all the models at different aggregation levels can be programmed in for instance Matlab.

Conclusions

In the current model framework the cell clearly has a pivotal role in fruit growth. The different cell types create the upper threshold of the fruit size, because (1) the number of vascular cells determine the supply capacity of sugars and water, (2) the number of pericarp

cells together with (3) the maximum cell size determines the fruit sink size, (4) the competition between cells for sugars determines the slope of the sugar concentration gradient and the size of the adjacent cells. Yet, such cell related constraints again are a result of internal cell physiological mechanisms, e.g. activity of enzymes that control sink strength.

The theoretical model presented here involves five hierarchical scales influencing fruit growth. It contains the most relevant processes that are reported in the literature to drive fruit growth. Implementing these processes into future fruit models will contribute in improving the understanding and prediction of G×E interactions for fruit growth and quality.

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6. General discussion

This study attempted to unravel the complexity of tomato fruit growth through the development of a dynamic model of fruit growth integrating cell division, cell growth and cell endoreduplication. The development of such an integrated model should rely on a good understanding of fruit growth at different scales and the biological relationships between the different scales. Therefore, several experiments were carried out to understand the link between cellular processes and fruit growth and their responses to genotypic factors, contrasting fruit loads and temperature conditions. Information derived from the experiments was partly used to parameterize and validate the model. This chapter discusses the strength and practical applications of the results presented in this thesis. It also proposes possibilities and needs for future research.

Experimental investigation of the response of cell division, cell growth and endoreduplication to genotypic variation, fruit load and temperature

One of the hypotheses tested in this study was that genotypic factors, assimilate supply and temperature influence fruit growth through effects on cell division, cell expansion or both (Chapter 2, 3). In our experiments we did not detect significant genotype by environment (G×E) interactions for all final fruit characteristics measured at the cell and organ levels. The absence of G×E interactions in this study could be associated with the fact that we used tomato genotypes of similar genetic background, displaying similar responses to changes in the environment despite their phenotypic differences in final fruit size. Many authors reported significant G×E interaction effects on fruit growth in tomato and other fruit species (Asins et al., 1994; Eduardo et al., 2007; Prudent et al., 2010). Understanding what cause of G×E interactions is fundamental to control fruit growth. Although no significant G×E interaction was observed in our experiments, the fruit model developed in this study was constructed to simulate and analyse G×E interactions (see Model applications).

Cell division In Chapter 2 we investigated the relationship between cell division and fruit growth in three tomato genotypes related genetically but differing in their final fruit size. Genotypic variation in final fruit size was mainly accounted for by variation in final pericarp cell number (Chapter 2). Other studies showed that differences between large and small

fruited genotypes were mainly associated with variation in cell division in many crop species including tomato (Bertin et al., 2003), melon (Higashi et al., 1999), strawberry (Cheng and Breen, 1992), cherry (Olmstead et al., 2007) and peach (Scorzal et al., 1991). In the literature a longer cell division duration is usually associated with a high final cell number in large fruited genotypes (Higashi et al., 1999). We observed that genotypic variation in final cell number was positively correlated to the variation in pericarp fructose content (Chapter 2). Tomato fruit imports sugar in the form of sucrose, which is later converted into fructose and glucose. In plant tissues, high hexose levels might have a positive effect on cell division by promoting the expression of cell cycle genes (Kwon and Wang., 2011). Our results suggest that variations in sugar metabolism and endogenous fructose levels could partly account for the genotypic variation in final cell number and fruit size. High assimilate supply increased the final number of cells in the pericarp (Chapter 2). This result agrees with several other studies (Baldet et al., 2006; Bertin 2005; Bohner and Bangerth 1988). Baldet et al. (2006) showed that high assimilate supply induced by low fruit load treatment increases the expression of cell cycle genes and final cell number in tomato.

In this study temperature treatments did not affect the total number of cells in the pericarp, but increased the number of cell layers (Chapter 3). We proposed that periclinal cell division which is responsible for the generation of new cell layers in the pericarp might respond differently to temperature than anticlinal and randomly oriented cell divisions. Other authors suggested that periclinal and other types of cell division in the pericarp might be regulated differently (Joubès et al., 1999; Cong et al., 2002; Cheniclet et al., 2005). Although temperature treatments did not affect the number of pericarp cells in this study, intra-treatment variation in fruit size was mainly associated with variation in cell number. Similar observations were made by Bertin et al. (2003) and Bertin (2005). Intra-treatment variations between fruits can be minimized through a better control of non-experimental factors that can affect cell division. Alternatively, increasing the number of replicate fruits would help in detecting treatment effects.

Cell growth In this study cell growth partly accounted for the differences between large and small fruited tomato genotypes. When assimilate was limiting during the entire period of fruit growth, both cell number and cell volume contributed equally to the genotypic variation in final fruit size (Chapter 2). When assimilate was not limiting during the entire (or most part of) fruit growth, cell volume contributed only half to the genotypic variation in final fruit size compared to cell number (Chapter 2). This observation probably emerges from the successive

scheduling of cellular processes underlying fruit growth, implying that different cellular processes are affected by environmental conditions to which the fruit is exposed during its development. We proposed that the timing of assimilate supply affects the relative contribution of cell number and cell size to the variation in final fruit size (Chapter 2).

Reduction in final fruit size caused by high temperatures was associated with the reduction in final cell volume when fruits were heated early during fruit growth (Chapter 3). Our results showed that the effects of high temperature on cell expansion in the fruit pericarp might not be the same in all expansion directions. Similar observations were reported in other plant organs (Akashi and Shibaoka, 1987; Strøm and Moe, 1997; Prat and Davies, 2010). In plant cells, the orientation of cellulose microtubules and microfibrils, which determines the direction of cell expansion has been shown to be influenced by organ temperature (Prat and Davies, 2010). Our results emphasize the need in histological studies to measure cell expansion in more than one dimension in order to reflect the dynamics of cell expansion in plant tissues.

Cell endoreduplication Although endoreduplication was not directly quantified in our experiments, its responses to genotypic variation, assimilate supply and temperature have been investigated experimentally by other authors (Bertin et al., 2003; Bertin, 2005; Cheniclet et al., 2005). Cheniclet et al. (2005) studied the genetic variability of endoreduplication in a population of 20 tomato genotypes differing in their final fruit sizes. They observed large genotypic variability in cell ploidy of matured fruit pericarps. In general high mean pericarp cell ploidy was associated with large-fruited genotypes. However, in the same study, low mean pericarp cell ploidy was also encountered in a few large fruited genotypes. Such conflicting results further emphasize the need to investigate the molecular mechanism regulating the process of endoreduplication.

Only a limited number of studies investigated the effects of assimilate supply and temperature on the process of endoreduplication. Fruit load-induced assimilate limitation slightly delayed the endoreduplication process, but did not affect the final average cell ploidy in tomato pericarp (Bertin et al., 2003; Bertin, 2005). Bertin (2005) observed that growing tomato fruits under 25/25 ^{o}C day/night temperature regime increased their final pericarp cell ploidy compared with fruits grown under 25/20 ^{o}C day/night temperature. However, in the same experiment, fruits grown under 20/20 ^{o}C day/night temperature had a higher pericarp cell ploidy than fruits grown under 25/20 ^{o}C day/night temperature. Barrow et al. (2006) argued

that endoreduplication, as a mechanism for the plant to cope with adverse environmental conditions, may be increased under low or high temperature conditions.

Modelling the interactions between processes in a multi-scale fruit model

The phenotype of a fruit emerges from complex interrelated underlying processes at the molecular, cellular, tissue and organ scales. Although it is hard to include all underlying processes in a fruit model, integrating the main underlying processes would improve our understanding of the emerging fruit phenotype (Génard et al., 2007). In this study we focused mainly on modelling processes at the cell scale. Cellular processes are directly linked to organ growth and occur at organizational level intermediate between the molecular and tissue/organ scale. De Vos et al. (2012) proposed to begin the construction of a multi-scale mechanistic model of organ growth at the cellular scale, as this would make it easier to further link processes at the molecular and tissue/organ scale. In this work, three main cellular processes underlying fruit growth were considered: cell division, cell endoreduplication and cell growth (Chapter 4). Model development relied on understanding and integrating biological interactions between processes at the cell scale, but also the relationships between the processes at the cell scale and the tissue scale following to the bottom-up approach, and the feedback relationships between different processes. Fig. 1 summarizes the inter-relationships between processes at the cell and tissue scale considered in the model.

At the cell scale the relationship between cell growth and cell division was incorporated in the model by assuming that a critical cell mass: ploidy ratio triggers cell division. This assumption was based on the experimental observation that cells need to reach a certain size for division to occur (Beemster et al., 2003). A similar mechanism was used to describe the relationship between cell growth and endoreduplication. Through a feedback relationship endoreduplication also affected cell growth. The effect of endoreduplication on cell growth was introduced in the model by assuming that cell growth rate is increased with increasing cell ploidy. This assumption was based on the positive correlations between cell size and cell ploidy in fruit cells reported in the literature (Kondorosi et al., 2000; Bertin, 2005; Cheniclet *et al*., 2005; Nafati et al., 2011). In the model cell division influenced endoreduplication in two ways: first, a cell could start endoreduplication only after it has exited the cell division phase; second, the number of cell division cycles a cell experienced during the division phase affected its potential to continue endoreduplication.

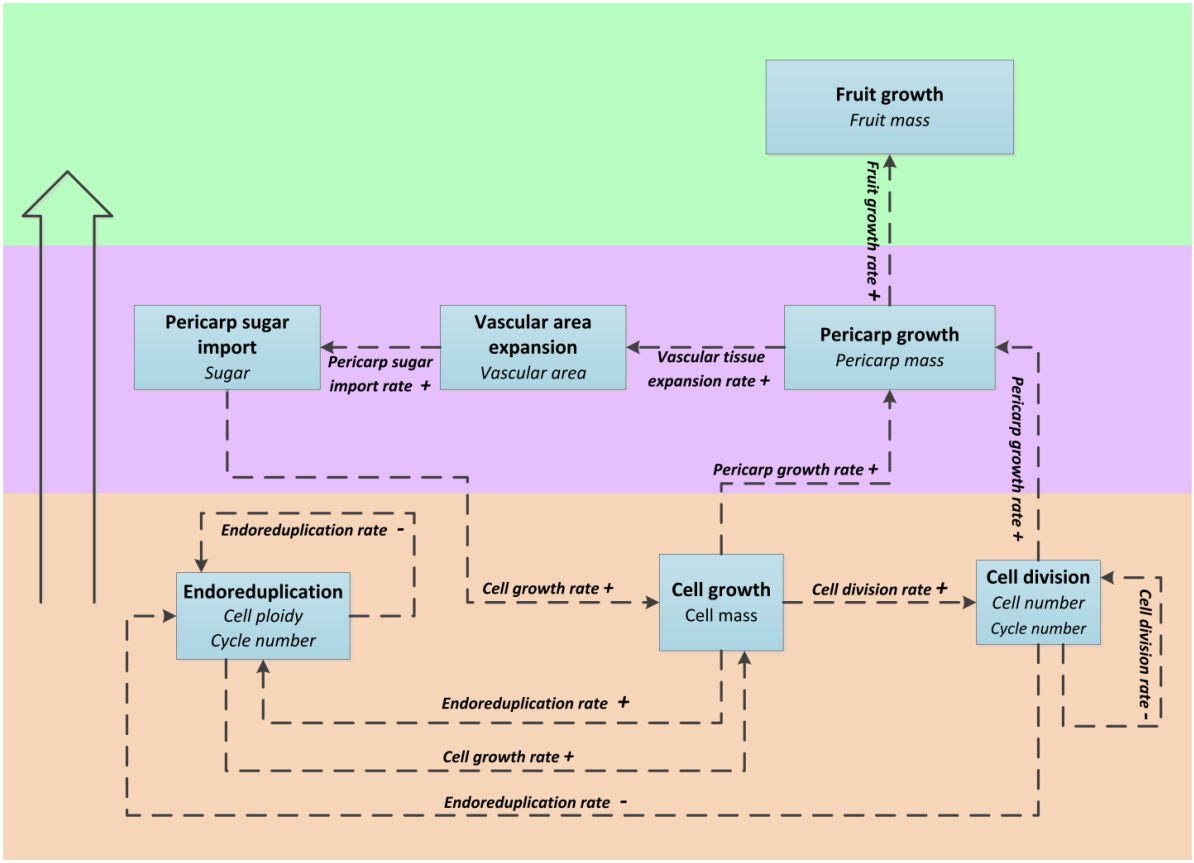

Fig. 1. Schematic representation of intra-scale and inter-scale interactions between processes in the fruit model. The symbols + and – represent positive and negative relationships, respectively.

The number of cell division cycles also negatively influenced cell division rate. Following the bottom-up approach, cell number and cell mass together determined pericarp mass, thus affecting the area of the vascular tissue (Chapter 4) and pericarp sugar import (Fig. 1). A similar approach was used to model leaf growth (Beemster et al., 2006) and flowering time (Welch et al., 2003) in Arabidopsis. Our fruit model also involved feedback relationships between the tissue scale and the cell scale. The amount of sugar imported by the pericarp tissue directly influenced cell growth, and indirectly affected cell division and cell endoreduplication (through threshold cell mass: ploidy ratio) (Fig. 1). Although the model was developed and parameterized for the pericarp tissue, model predictions can be easily scaled up to the fruit level. In our experiments we observed a linear relationship between pericarp size and fruit size. This relationship was stable across different genotypes, assimilate supply levels (Chapter 2) and temperature conditions (Chapter 3). Strong correlations between pericarp tissue size and fruit size have also been reported by other authors (Mariguele and Silva, 2010; Wetzstein et al., 2011).

The validation of our fruit model demonstrated how integrating processes underlying the functioning of biological systems can improve model predictive power (Baldazzi et al., 2012). Although the fruit model was parameterized for the conditions of constant low fruit load and normal temperature conditions, it could generate accurate predictions of final cell and tissue characteristics under various contrasting and fluctuating fruit load and temperature conditions (Chapter 4).

Model applications

The model developed in this study may serve as a research tool to carry out virtual experiments. Treatments that are difficult or impossible to apply in reality may be easily tested *in silico*. For example in Chapter 2, we applied three fruit load treatments (2&2 fruits/truss, 5&2 fruits/truss and 5&5 fruits/truss) to investigate the effects of low and high fruit loads applied early or late during fruit growth on final fruit and cell phenotype. In theory, a fourth fruit load treatments should be added in this experiment, where low fruit load (2 fruits/truss) is applied early and high fruit load (5 fruits/truss) is applied late during fruit growth (2&5 fruits/truss). This fourth treatment is impossible to implement on real plants, but its effects can now be tested using the model. Fig. 2 shows the effects of all four fruit load treatments (including the fourth treatment 2&5 fruits/truss) on cell number, cell mass, cell ploidy and pericarp mass. The effects of 2&5 fruits/truss were similar to those of 5&5 fruits/truss for all simulated tissue and cell characteristics except for cell number, which was slightly increased in the 2&5 fruits/truss treatment.

The model can be used for testing fruit growth responses under more fluctuating environmental conditions than those used for model validation (Chapter 4). In practice, the environment to which the fruit is exposed may fluctuate due to a vertical gradient in climatic factors in the greenhouse, seasonal changes, cultivation practices, etc. The model may be used to predict fruit growth under fluctuating environments. In this way, the model may serve as a tool to support decisions related to cultivation practices and climate manipulations.

The model sensitivity analysis presented in Chapter 4 indicated clearly which cellular parameters are most influential on fruit phenotype. We found that final fruit size is largely influenced by parameters affecting the cell division process (Chapter 4). Among these parameters, the duration of cell division period had the strongest influence on final fruit phenotype (Chapter 4). Such analysis may be useful in plant breeding programmes through the identification of the most influential traits needed for the development of new varieties.

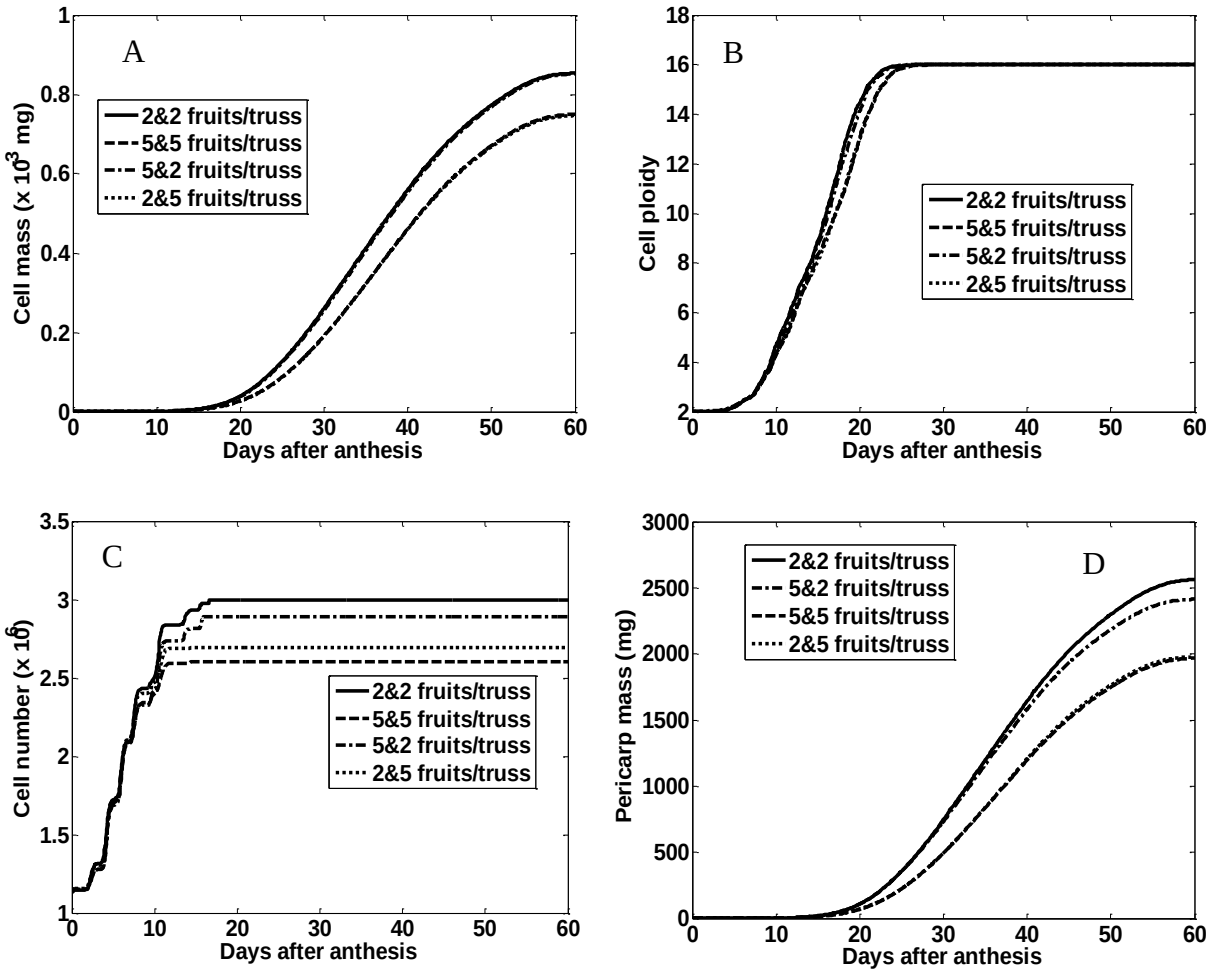

Fig. 2. Dynamics of simulated A) cell mass, B) cell ploidy, C) cell number, and D) pericarp mass during tomato fruit growth in the treatments with continuously low fruit load (2&2 fruits/truss, solid line), continuously high fruit load (5&5 fruits/truss, dashed line), a switch from high to low fruit load 7 daa (5&2 fruits/truss, dash-dotted line) and a switch from low to high fruit load 7 daa (2&5 fruits/truss, dotted line). Each line represents the mean of 70 model runs.

The model allows for testing and analysing genotypic responses to changes in the environment. Such analysis may provide valuable insights into G×E interactions on fruit growth as illustrated by the following example. We simulated the responses of a large and a small-fruited virtual tomato genotype grown under high (5 fruits/truss) and low (2 fruits/truss) fruit load conditions (Table 1). The two genotypes were assumed to differ only in their cell division duration, which was twice as long for the large-fruited genotype as for the small-fruited genotype (Bertin et al., 2009). In this case, the virtual small-fruited genotype had higher final cell mass than the large-fruited genotype (Table 1). The effect of high fruit load

Table 1. Model predictions of final cell mass, cell number, pericarp mass of large and small virtual tomato genotypes under high (5 fruits/truss) and low (2 fruits/truss) fruit load treatment. Cell division duration was assumed to be 18 days in the large fruited genotype and 9 days in the small fruited genotype. Each value is the mean of 70 model runs.

	Large fruited genotype		**Small fruited genotype**	
	2 Fruits/truss	5 Fruits/truss	2 Fruits/truss	5 Fruits/truss
Final cell mass ($\times 10^{-3}$ mg)	0.8165	0.6278	0.9056	0.8445
Final cell number ($\times 10^{6}$)	4.0872	3.7620	1.3172	1.2150
Final pericarp mass ($\times 10^{3}$ mg)	3.3024	2.2959	1.1930	1.0275

was more pronounced in the large-fruited genotype reducing its final pericarp mass by 30% compared with only 14% reduction in the small-fruited genotype (Table 1). Similar interactions between fruit load and genotype were observed in an experiment with two tomato genotypes differing in their final fruit size (Prudent et al., 2010). Considering how fruit load affected processes at the cell scale in each virtual genotype helps in explaining the interaction between fruit load and genotype observed on pericarp mass. Interestingly high fruit load reduced final cell number by approximately 8% in both genotypes (Table 1). However, in absolute terms the large-fruited genotype had three times more cells than the small-fruited genotype (Table 1). This implies assuming that assimilate supply was non limiting under 2 fruits/truss, that under high fruit load, competition between cells for assimilate was more severe in the large-fruited genotype than in the small-fruited genotype. This resulted in the larger reduction in final cell mass (23%) and thus pericarp mass in the large fruited genotype compared with cell mass (7%) and pericarp mass reduction in the small fruited genotype (Table 1).

The example presented above may be extended to analyse G×E interactions in more genotypes across a much wider environmental conditions. In plant breeding, genotypes are generally tested across several locations and seasons to evaluate their adaptation and stability in different environments. The model may assist in evaluating breeding lines, and in analysing G×E interactions across several environments, thus helping in saving time and efforts.

The model developed in this study was parameterized and validated for tomato fruits. However, the approaches used in the model to describe the dynamics of fruit growth at the tissue and cell scales are relatively generic and may serve as a basis to integrate fruit underlying processes in other fruit species. For example the model simulated sugar

distribution among different cell classes using the sink/source approach (Chapter 4). This approach is not limited to tomato as it is well accepted that in many crop species individual fruit cells compete for assimilates (Marcelis and Baan Hofman-Eijer, 1993). Likewise the generic mechanism of a critical cell mass: ploidy ratio was used to model cell division and endoreduplication events (Chapter 4). Thus the present model can be adapted to predict fruit and cell phenotypes and their responses to environmental and genotypic factors in many other fruit crops. Although model structure may be applied to other fruit species, model parameters certainly need to be estimated for different species and even different cultivars from the same species.

Future research directions

In the model we assumed that the arrest of fruit cellular processes occurs after a predefined temperature sum is reached (Chapter 4). Although this assumption was based on the experimental observations that fruit underlying cellular processes are restricted to specific periods of fruit growth (Chapter 3), it ignores the fact that genetic and hormonal factors play an important role in the regulation of cellular processes. The arrest of fruit cellular processes is controlled by a complex network of interacting genes. The exact mechanism through which these genes act to trigger the arrest of cellular processes is still under debate. However the temporal expressions of some cell cycle genes during fruit development suggest that they might play a role in the control of cellular process arrest (Baldet et al., 2006). Molecular control of cellular process arrest could be incorporated in the present model by relating parameters describing the duration of cellular processes to expression pattern of these cell cycle genes (Chapter 5). This relationship could not be implemented in this study as our gene expression study was limited to only one time point during fruit growth (Chapter 2). Future experiments could investigate the link between the duration of cellular processes and the expression of cell cycle genes measured at several time points during fruit growth. It should be especially important to investigate this link for the duration of the cell division period as model sensitivity analysis revealed that this parameter has the largest influence on final fruit phenotype. Doing so should give more insight into the G×E interactions not only at the cellular scale (as already demonstrated by the current model), but also at the gene scale.

Our model does not consider sugar transformation and water relations within the fruit. These are important aspects of fruit growth as they directly affect fruit quality. Fruit sugar composition depends on sugar supply, enzymatic sugar transformation and sugar dilution

owing to fruit growth. Future model improvements might involve incorporating water relations and fruit sugar transformation into the fruit model, as this would improve our understanding of fruit quality build-up (Chapter 5).

The present model was developed and validated for a single tomato fruit. We assumed that there is a constant sugar pool available for the fruit during its development (Chapter 4). In a tomato crop, the amount of sugar imported by an individual fruit depends on leaf assimilate production, and competition between fruits of the same truss and other sink organs on the plant. A next step could be to scale-up the fruit model to the truss and plant scale to include actual plant assimilate production and the competition between plant organs for assimilate. One way of doing so would be to link the fruit model with a crop growth model (Chapter 5). In this aggregated model, the crop model should supply the fruit model with the actual assimilate production computed based on the plant photosynthetic capacity and the environmental conditions (e.g. light, temperature, CO_2) prevailing in the greenhouse. Competition between sink organs could be incorporated in the model using the sink/source approach similar to that used in the fruit model to described sugar distribution between cell classes (Chapter 5).

Linking the fruit model to a crop model should also make it possible to simulate direct and indirect temperature effects on fruit growth. The model developed in this study was parameterized partly using data collected from fruits subjected to local fruit temperature treatments. In practice, whole plants are exposed to the greenhouse climate, and temperature affects fruit growth not only directly, but also indirectly via other plant growth and developmental processes. For example, high air temperatures are known to increase plant development rate resulting in the formation of many sink organs on the plant, creating a strong competition among individual organs for assimilates (Van der Ploeg and Heuvelink, 2005; Wubs et al., 2009). Linking a crop model to the fruit model would contribute in incorporating these indirect temperature effects into the fruit model.

Conclusions

This study demonstrated how integrated multi-scale models can be used to predict and understand complex fruit responses to G×E interactions and fluctuating environmental conditions. The success of this modelling approach depends on careful experimentation aiming at providing a good understanding of the biological processes at different scales and their responses to the environment. The ability of the model to predict fruit phenotype based

on cellular processes underlying fruit growth has several applications in research, plant breeding and cultivation. We hope that more insights into fruit growth and more applications will be identified as information at the molecular and plant scales are integrated into the fruit model.

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Summary

Fruit size is a major component of fruit yield and quality of many horticultural crops including fresh tomato. Variations in fruit size can be tremendous due to genotypic and environmental factors. Experimental data show that assimilate availability and temperature strongly affect fruit growth and final fruit size in many crop species. The mechanisms by which genotype and environment interact to determine fruit size remain poorly understood. This limited insight represents a real challenge in manipulating and predicting fruit yield and size. Genotype-by-environment interactions emerge from complex processes underlying fruit growth. Cell division, cell growth and cell endoreduplication are three fundamental cellular processes responsible for fruit growth. The cellular basis for variation in fruit growth has been studied extensively. Although these studies provide some insights into the link between individual cellular or molecular processes and fruit growth, they do not explain how these underlying processes taken together regulate fruit growth. To improve our understanding of fruit yield and quality, it is fundamental to integrate the description of fruit cellular processes and their interaction with the environment into a common knowledge base system.

The aims of this study were 1) to develop a model of tomato fruit growth integrating cell division, cell endoreduplication and cell growth, and 2) to use the model to analyse fruit responses to fluctuating assimilate supply and temperature conditions, and genotype-by-environment interactions. Experiments were carried out to understand the link between cellular processes and fruit growth and their responses to genotypic factors, contrasting fruit loads and temperature conditions. Information derived from the experiments was partly used to parameterize and validate the model.

In Chapter 2 we investigated the histological and molecular basis for genotypic and fruit load-induced variation in fruit size. We grew three genetically related tomato genotypes differing in their final fruit size under three fruit load treatments: continuously 2 fruits/truss (2&2F) or 5 fruits/truss (5&5F) and a switch from 5 to 2 fruits/truss (5&2F) 7 days after anthesis. The 5&5F treatment resulted in significantly smaller fruits than in the 5&2F and 2&2F treatments. In the 5&5F treatment cell number and cell volume contributed equally to the genotypic variation in final fruit size. In the 5&2F and 2&2F treatments, cell number contributed twice as much to the genotypic variation in final fruit size than cell volume did. Fruit load treatments resulted in only subtle variations in gene expression. Genotypic differences were detected in transcript levels of *CycD3* (cyclin) and *CDKB1* (cyclin-dependent-kinase), but not

in those of *CycB2*. Genotypic variation in fruit fresh weight, pericarp volume and cell volume was linked to pericarp glucose and fructose content (R^2=0.41, R^2=0.48, R^2=0.11, respectively). Genotypic variation in cell number was positively correlated with pericarp fructose content (R^2=0.28). These results emphasize the role of sugar content and of the timing of assimilate supply in the variation of cell and fruit phenotypes.

In Chapter 3 we analysed the responses of cell division and cell expansion to fruit heating applied early or late during fruit growth. To ensure that the observed responses resulted from direct temperature effects on fruit growth, temperature treatments were applied at the fruit level. Individual trusses were enclosed into cuvettes and heating was applied either only during the first 7 days after anthesis or from 7 days after anthesis until fruit maturity (breaker stage) or both. Heating fruit shortened fruit growth period and reduced final fruit size. Reduction in final fruit size of early-heated fruit was mainly associated with reduction in final pericarp cell volume. Early heating increased the number of cell layers in the pericarp, but did not affect the total number of pericarp cells. These results indicate that in the tomato pericarp, periclinal cell divisions (division plane parallel to fruit skin) respond differently to temperature than anticlinal (division plane perpendicular to fruit skin) or randomly oriented cell divisions. Late heating only decreased pericarp thickness significantly. Continuously heating fruit reduced anticlinal cell expansion (direction perpendicular to fruit skin) more than periclinal cell expansion (direction parallel to fruit skin). This study emphasizes the need to measure cell expansion in more than one dimension in histological studies of fruits in order to reflect the dynamics of cell expansion. Within each temperature treatment, variation in final fruit size correlated with variation in cell number. This implies that, intra-treatment variations between fruits can be minimized through a better control of non-experimental factors that can affect cell division.

In Chapter 4, results from the previous chapters were incorporated into a dynamic model of tomato fruit growth integrating cell division, cell growth and cell endoreduplication. In the model the fruit was considered as a population of cells grouped in cell classes differing in their initial cell age and cell mass. The model describes fruit growth from anthesis until maturation and covers the stages of cell division, cell growth and cell endoreduplication. Within each cell class, the transition from one stage to the next is determined by predefined cell ages expressed in thermal time. Cell growth is the consequence of sugar import from a common assimilate pool into each cell class according to the sink/source concept. Import of assimilate depends on the contact surface area between the vascular and pericarp tissue. Cell growth rate increases with increasing cell ploidy. Cell division or endoreduplication events

occur when cells exceed a critical threshold of the cell mass: ploidy ratio. The ability of cells to divide or endoreduplicate decreases with increasing number of cell division cycles or cell endocycles. The model was parameterized and calibrated for low fruit load conditions and was validated for high fruit load and various temperature conditions. The model sensitivity analysis showed that parameters which strongly affected final pericarp cell number had a weaker and opposite effect on final cell mass. Variations in final fruit size were mainly associated with variations in parameters involved in the dynamics of cell division. Among these parameters, cell division duration had the strongest influence on final cell number and pericarp mass. Final cell ploidy appeared to be sensitive only after large perturbations in model parameters. This implies that endoreduplication might be a more stable process than cell division and cell growth. The model was able to accurately predict final cell number, cell mass and pericarp mass under continuously high fruit load and under a switch from high to low fruit load during fruit development in tomato. The model could also generate acceptable predictions of fruit and cell phenotypes in most of the contrasting temperature conditions.

Chapter 5 presents a theoretical framework for future modelling of fruit growth. A multi-scale approach to model tomato fruit growth is proposed, in order to account for the interaction between gene functioning and growth conditions, and, ultimately, to explain the fruit phenotype of various genotypes in diverse growth environments. There is particular focus on: (I) cell division regulated by cell cycle genes, (II) cell expansion as influenced by polyploidy resulting from endoreduplication and carbohydrate and water dynamics. The growth processes at gene, cell and tissue, fruit and plant scale are included in the theoretical model. Sub-populations of cells differing in age are considered to act as sinks competing for carbohydrates. The key cell cycle genes of tomato could be incorporated into an existing model of the gene regulatory network of the cell cycle. This model could be modified to simulate endoreduplication. Moreover, the modelled cell cycle process could be made sensitive to temperature and assimilate supply. Implementing these processes into future fruit models will contribute in improving the understanding and prediction of fruit growth and G×E interactions

Chapter 6 discusses the strengths and limitations of the present study and proposes possibilities for further research. This study demonstrated how an integrated multi-scale model can be used to predict and understand complex fruit responses to fluctuating environmental conditions. The success of this modelling approach depends on careful experimentation aiming at a good understanding of biological processes at different scales and their responses to the environment. The ability of the model to predict fruit phenotype from

fruit cellular processes has several applications in research, plant breeding and cultivation. The model can be used to carry out virtual experiments with treatments that are difficult or impossible to test experimentally. The model may serve as a tool to support decision related to cultivation practices and climate manipulations. The model allows for predicting and analysing G×E interactions and may assist in evaluating breeding lines, across several environments, thus helping in saving time and efforts. The approaches used in model development are relatively generic and may serve as a basis to integrate fruit underlying processes in other fruit species.

A next step could be to integrate information on molecular control of fruit cellular processes into the fruit model, and to analyse G×E interactions not only at the cellular scale (as already demonstrated by the current model), but also at the gene scale. Other model improvements might involve incorporating water relations and fruit sugar transformation into the fruit model, as this would improve our understanding of fruit quality build-up. The fruit model could be scaled-up to the truss and plant levels to include actual plant assimilate production and the competition between plant organs for assimilates. Scaling-up to the plant level would contribute in incorporating indirect temperature effects into the fruit model. It is our hope that more insights into fruit growth and more applications will be identified as information at the molecular and plant scales are integrated into the fruit model.

This thesis has contributed to closing the gap between genotype and phenotype related to tomato fruit growth. An integral and coherent development of models at relevant levels of plant organization can further help to close this gap.

Samenvatting

Vruchtgrootte is een belangrijk aspect van vruchtproductie en –kwaliteit in veel tuinbouwgewassen waaronder tomaat. De vruchtgrootte kan door genotypische en klimaatverschillen enorm variëren. Experimenten laten zien dat assimilatenvoorziening en temperatuur het meest bepalend zijn voor de vruchtgroei en de uiteindelijke vruchtgrootte in de meeste gewassen. Er is weinig bekend over de mechanismen waarmee de interactie tussen genotype en milieu tot een specifieke vruchtgrootte leidt. Dit beperkte inzicht betekent dat het gevolg van een teeltmethode op productie en vruchtgrootte lastig te voorspellen is. Genotype-milieu interacties komen voort uit een complex van onderliggende processen. Celdeling, celgroei en endoreduplicatie zijn drie fundamentele, cellulaire processen die de vruchtgroei bepalen. Dit celniveau als basis voor vruchtgroei is recentelijk uitgebreid onderzocht. Hoewel die studies enigszins aangeven wat de mogelijke link is tussen processen op cel of moleculair niveau en groei, is er nog geen totaalbeeld hoe deze processen samen uitwerken op orgaanniveau. Voor een beter inzicht in vruchtgroei en –kwaliteit is het noodzakelijk om cellulaire processen en hun interactie met milieufactoren te integreren binnen een kennissysteem.

De doelen van deze studie waren 1) het ontwikkelen van een model voor de groei van de tomaat op basis van celdeling, endoreduplicatie en celexpansie, en 2) met het model de groeirespons op variaties in assimilatenvoorziening en temperatuur, en de genotype-milieu interacties te analyseren. Experimenten met verschillende genotypen, plantbelastingen en vruchttemperaturen zijn uitgevoerd om de reacties op celniveau te toetsen. Informatie uit de proeven is benut voor parametrisatie en validatie van het model.

In Hoofdstuk 2 is de histologische en moleculaire basis onderzocht van genotypische verschillen en effecten van plantbelasting, i.e. aantal vruchten per tros. Drie genotypen die verschillende vruchtgrootten voortbrengen werden bij 3 plantbelastingen onderzocht: continue 2 vruchten per tros (2&2F) of 5 vruchten per tros (5&5F), en een wijziging vanaf 7 dagen na vruchtzetting van 5 naar 2 vruchten per tros (5&2F). De 5&5F vruchten waren significant kleiner dan die in de overige behandelingen. In de 5&5F behandeling droegen celaantal en celvolume in gelijke mate bij aan de genotypische variaties in vruchtgrootte. In de 5&2F en de 2&2F behandeling droeg het celaantal dubbel zo veel bij aan de variatie in vruchtgrootte tussen genotypen dan celvolume. Plantbelasting gaf slechts subtiele verschillen in genexpressie. Genotypische verschillen zijn gevonden voor transcriptieniveau's van CycD3

(cycline D3) en CDKB1 (cycline afhankelijke kinase B1), maar niet in die van CycB2. Genotypische variatie in vrucht versgewicht, pericarp volume en cel volume waren met een R2 van resp. 0.41, 0.48 en 0.11 enigszins gerelateerd aan glucose- en fructosegehalte in het pericarp weefsel. Genotypische variatie in cel aantal was positief gecorreleerd met pericarp fructose gehalte (R2=0.28). Deze resultaten benadrukken de rol van suikergehalten en de timing van de assimilatenvoorziening in de variatie in cel- en vruchtfenotype.

In Hoofdstuk 3 is de reactie van celdeling en celexpansie op vruchtverwarming in een vroeg en een laat ontwikkelstadium van vruchtgroei onderzocht. Lokale verwarming die zich slechts beperkt tot de te onderzoeken vruchten werd gerealiseerd door gebruikmaking van cuvettes die de tros geheel omsloten. Verwarming werd toegepast in de vroege ontwikkeling, i.e. de eerste 7 dagen na anthesis, of alleen in de late ontwikkeling, i.e. vanaf 7 dagen na anthesis tot oogst (‘breaker’ stadium), of in de gehele groeiperiode. Verwarming verkleinde de groeiperiode tot breaker stadium en verlaagde het eindgewicht van de tomaat. De kleinere vrucht bij verwarming in het vroege groeistadium leek verband te houden met een verkleind celvolume in het pericarpweefsel; daarnaast resulteerde deze behandeling in een vergroot aantal cellagen in het pericarp, maar dit vergrootte het aantal cellen in de pericarp niet. Deze resultaten geven aan dat in het pericarp de periclinale celdelingen (delingsvlak evenwijdig aan vruchtepidermis) anders reageren dan de anticlinale (delingsvlak loodrecht op vruchtepidermis) of random georiënteerde delingen. Late verwarming had alleen significant effect op verkleining van de pericarp dikte. Verwarming gedurende de hele groeiperiode reduceerde anticlinale celexpansie meer dan de periclinale expansie. Deze studie geeft daarmee aan dat voor een juiste beoordeling van cel expansie deze in meer ruimtelijke dimensies moet worden onderzocht. De variatie in vruchtgrootte binnen elke temperatuurbehandeling correleerde met het aantal waargenomen cellen. Dit geeft aan dat vermindering van invloeden op celaantal, die los staan van de specifieke behandeling, van belang is om een duidelijker behandelingseffect met minder variatie tussen vruchten in een behandelde tros te realiseren.

In Hoofdstuk 4 zijn de resultaten van voorgaande hoofdstukken verwerkt in een dynamisch model van de vruchtgroei van tomaat, met medeneming van deling, groei en endoreduplicatie van cellen. Het model beschouwt de vrucht als een populatie van cel groepen of cel klassen die verschillen in initiële leeftijd en gewicht. De vruchtgroei wordt beschreven vanaf anthesis tot einde van de groei, waarbij de cellen achtereenvolgens de fasen deling, groei en

endoreduplicatie doorlopen. Binnen elke cel klasse wordt de transitie naar de volgende fase gerealiseerd na het bereiken van een bepaalde temperatuursom, gerekend vanuit initiatie van de cellen. De geschatte potentiële groei per cel klasse geeft een suikervraag weer per tijdstap. Het totaal van suikers per vrucht wordt verdeeld over de aanwezige cel klassen volgens hun relatieve sinksterkte, hetgeen dan resulteert in de actuele groei per cel klasse. Import van suikers door de vrucht hangt af van het contactoppervlak tussen pericarp en transportweefsel. Celgroei neemt toe bij toenemende ploïdie. Deling of endoreduplicatie wordt voltrokken als de celmassa/ploïdie ratio boven een grenswaarde komt. Het vermogen van een cel klasse om te delen of te endoredupliceren vermindert naarmate er meer van dergelijke delingscycli hebben plaatsgevonden. Het model is geparametriseerd en gecalibreerd voor een situatie met lage plantbelasting (weinig vruchten aan de plant) en gevalideerd voor een hoge plantbelasting en diverse temperatuurbehandelingen. De gevoeligheidsanalyse toonde aan dat modelparameters die van grote invloed waren op cel aantal in de pericarp juist een zwak maar tegengesteld effect hadden op de cel massa. Variatie in uiteindelijke vruchtgrootte werden vooral bepaald door parameters die betrokken zijn bij de dynamiek van celdeling. Daarbij hadden de parameters voor duur van de delingsperiode veel effect op de uiteindelijke aantallen en gewichten van pericarp cellen. Voor variatie in ploïdie aan eind van groei moesten de betrokken modelparameters fors gewijzigd worden. Endoreduplicatie lijkt daarmee een veel stabieler proces dan cel deling en groei. Het model kon het uiteindelijke cel aantal, de cel massa en de massa van de pericarp goed voorspellen voor situaties met hoge plantbelasting en bij een plotselinge wijziging van hoge naar lage plantbelasting gedurende de groei. Ook voor een aantal temperatuurbehandelingen werden cel en vrucht fenotype redelijk voorspeld.

Hoofdstuk 5 behandelt een theoretisch raamwerk voor toekomstige modellering van vruchtgroei. Een multi-schaal model wordt benut voor simulatie van de interactie tussen genwerking en groeicondities, om uiteindelijk het fenotype van vruchten van verschillende genotypen te verklaren. Het model kent 2 kernregels: (I) cel deling regulatie door celcyclus genen, (II) cel expansie regulatie door endoreduplicatie en water- en suikerhuishouding. Groeiprocessen op niveau van gen, cel, weefsel, vrucht en plant zijn geïntegreerd. Subpopulaties van cellen van gelijke leeftijd worden gezien als afzonderlijke sinks die onderling concurreren om suiker. In een bestaand model van gengereguleerde celcyclus werden de celcyclus genen vervangen door degene die bekend waren voor tomaat, en werd dit model uitgebreid met endoreduplicatie. Daarnaast werd het celcyclus model gevoelig gemaakt

voor temperatuur en suikeraanbod. Deze modelbenadering kan in de nabije toekomst bijdragen aan een verder inzicht en voorspelling van vruchtgroei en GxE interacties.

Hoofdstuk 6 bediscussieert de sterkten en zwakten van de huidige studie en doet voorstellen voor toekomstig onderzoek. Deze studie demonstreert hoe een geïntegreerd, multi-schaal model bruikbaar is om complexe reacties van vruchtgroei op omgevingsinvloeden te begrijpen en te voorspellen. Het succes van deze modelbenadering hangt af van een zorgvuldige experimentele toetsing van biologische processen op verschillende aggregatieniveaus en hun reactie op omgevingscondities. Het vermogen van het huidige model om het vrucht fenotype te voorspellen op grond van cellulaire processen kan tot toepassing leiden in onderzoek, plantenveredeling en teeltstrategie. Het model kan gebruikt worden voor virtuele experimenten die in werkelijkheid moeilijk of niet uitvoerbaar zijn. Het model kans als beslissingsondersteunende tool benut worden ten behoeve van teeltingrepen en klimaatregeling. Het model kan binnen zijn validatiedomein GxE interacties voorspellen en analyseren en kan helpen bij het evalueren van de prestatie van nieuwe genotypen in een specifiek groeiklimaat om zo tijd en kosten te besparen. De modelbenadering aangaande integratie van onderliggende processen is relatief generiek en kan aldus bruikbaar zijn voor vruchtgroei bij andere gewassen.

Een volgende mogelijke stap is om informatie van moleculaire controle van cellulaire processen in het model te incorporeren, en om GxE interacties niet alleen op celniveau maar ook op gen niveau analyseren. Andere relevante modelverbeteringen zijn de simulatie van de waterhuishouding en van suikertransformaties, zodat ook simulatie van inhoudsstoffen en meer uitgebreide kwaliteitsaspecten mogelijk wordt. Daarnaast is gewenst om het vruchtmodel op te schalen naar tros- en plantniveau zodat de effecten van de actuele assimilatenvoorziening en van de competitie tussen plantorganen op orgaangroei expliciet in het model aanwezig zijn. Opschaling zou ook de effecten van temperatuur op orgaangroei in het competitieverband tussen en binnen de trossen kunnen verrekenen. De hoop is dat de verwachte kennistoename, met name op het moleculaire gebied, verder in het model benut gaat worden.
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PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- Cellular and molecular processes underlying fruit growth

Post-graduate courses (10.6 ECTS)

- Mathematics and biology; Agro ParisTech (2007)
- The art of modelling; Wageningen University (2009)
- Advanced statistics-generalized linear models; Wageningen University (2009)

Laboratory training and working visits (4.5 ECTS)

- Fruit modelling; INRA Avignon, France (2007)

Deficiency, refresh, brush-up courses (2.1 ECTS)

- Matlab fundamentals and programming techniques (2007)
- Basic statistics (2007)

Competence strengthening / skills courses (4.4 ECTS)

- Techniques for writing and presenting a scientific paper; Wageningen University (2007)
- Science, the press and general public; Wageningen University (2009)
- Advanced course guide to scientific artwork; Wageningen University (2009)
- Career perspective; Wageningen University (2010)

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Discussion groups / local seminars / other scientific meetings (6.3 ECTS)

- Frontier literature in plant physiology (2007-2011)
- Systems biology day (2009)

International symposia, workshops and conferences (3.5 ECTS)

- Systems biology conference; Stanford (2009)
- Systems biology conference; Edinburg (2010)

Lecturing / supervision of practical ‘s / tutorials (0.9 ECTS)

- Research methods; 1 day (2009)
- Research methods; 2 days (2011)

Supervision of 2 MSc students; 20 days

- Effects of temperature, fruit load and gene expression in a tomato crop
- Effects of fruit load on fruit growth, cell number, cell size and gene expression of four tomato genotypes

List of publications

Fanwoua J, de Visser PHB, Heuvelink E, Yin X, Struik PC, Marcelis LFM. A dynamic model of tomato fruit growth integrating cell division, cell growth and endoreduplication. Submitted.

Fanwoua J, de Visser PHB, Heuvelink E, Angenent G, Yin X, Marcelis LFM, Struik PC (2012) Histological and molecular investigation of the basis for variation in tomato fruit size in response to fruit load and genotype. *Functional Plant Biology*. 39, 754-763.

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About the author

Julienne Fanwoua was born on the 26th February 1976 in Mbo (Khoung-khi) Cameroon. After obtaining a "Baccalaureat C" (Mathematics/Physics) in 1996, she joined the Faculty of Agronomy of the University of Dschang (Cameroon) where she studied crop production. In 2001, she conducted her thesis research entitled: "Effects of nitrogen and phosphorus starter fertilizers on nitrogen fixation and yield of soybean (*Glycine max*) in South-Cameroon" at the International Institute for Tropical Agriculture (IITA-Cameroon). After obtaining her "Ingénieur Agronome" degree in 2002, she worked as research assistant at the Plant Breeding Department of IITA-Cameroon. In 2003 she joined the Faculty of Horticulture of the Leibniz Universität Hannover (Germany), where she obtained a MSc degree in International Horticulture in 2005. Her MSc thesis research was entitled "Modelling the structural development of tomato using L-systems". From 2005 to 2007 she worked as research assistant at the Institute of Vegetable and Fruit Science (Leibniz Universität Hannover, Germany). From June 2007 she was employed as research associate by Wageningen University (The Netherlands) to conduct a PhD research focussing on explaining tomato fruit growth based on fruit underlying cellular processes using experimentation and modelling. The project was executed under the supervision of the Centre for Crop Systems Analysis and Horticultural Supply Chains group of Wageningen University, and Wageningen UR Greenhouse Horticulture. This thesis is the outcome of this research.

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