Multi-level analysis of the impact of temperature and light on tomato fruit growth

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Abstract

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Tomato fruit growth commences with an increase in cell number followed by cell expansion. Upon cessation of cell division, a spectacular increase in nuclear DNA content without cell division (endoreduplication) occurs, which is positively correlated with cell size. Evaluation of the relative importance of each cellular process during fruit growth is important for attempts aimed at manipulating fruit size.

In this thesis, the genetic and physiological basis for the differences in fruit size between cultivars and their response to fruit temperature was studied. In addition, the effects of darkness, white, blue, and red light around the fruits on tomato fruit growth were investigated. Temperature or light treatments were applied at the fruit level in all experiments in order to separate plant and fruit level responses. Fruit phenotype was assessed at whole fruit, cell and gene level. Expression patterns of 20 different genes encoding regulators of cell division, endoreduplication or cell expansion were analysed. Besides the experimental work, a literature review of the role of light in the regulatory networks of cell division, endoreduplication and cell expansion was conducted. Results from experiments were then placed into context of other studies in order to identify processes that drive fruit growth.

Experiments showed that differences in fruit size between cultivars can result from differences in both cell number and cell size. Increased cell number in the larger fruited cultivar was corroborated by an increase in the expression of three cell division promoters (*CDKB2, CycA1* and *E2Fe*) and a decrease in the expression of an inhibitor (*fw2.2*) of cell division. The observed smaller fruit size in heated compared with non-heated fruits appeared to stem from a reduction in cell size even when cell number tended to increase. The expression of three promoters (*CDKB1, CDKB2*, and *CycA1*) and one inhibitor (*fw2.2*) of cell division increased when fruits were heated. However, the expression of genes encoding proteins known to regulate endoreduplication and cell expansion did not corroborate observations on cell size in the temperature experiment. Fruits subjected to different light treatments did not differ in either fruit size or carbohydrate content. However, cell division was strongly stimulated at the expense of cell expansion by light. This thesis shows that cell

division is stimulated by light irrespective of the organ under consideration while endoreduplication and cell expansion responses are organ specific. It is proposed that light effects on cell division, endoreduplication and cell expansion stem from either degradation of transcription factors or inhibitory competition between transcription factors for promoter regions of target genes. It is also argued here that the commonly observed positive correlation between cell number and fruit size does not imply a causal relationship. In addition, the thesis argues that fruit growth is dependent on cell-autonomous and non-cell-autonomous regulatory mechanisms as well as a global coordinator, the target-of-rapamycin and, consequently, the increase in fruit size follows the neo-cellular theory of fruit growth.

This thesis provides clues on the link between gene expression and cell and fruit level observations. It also provides in depth knowledge on the role of environmental factors on the regulation of cell division, endoreduplication and cell expansion. Further studies at the level between genes and the cells will be necessary to quantify the relationship between gene expression and cell and fruit phenotype.

Keywords: cell division, endoreduplication, cell expansion, cyclin, cyclin dependent kinase, growth theory, systems biology.

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Chapter 1

General introduction

The tomato crop

The tomato (*Solanum lycopersicum* L.) crop originated in the sub-tropical Andean Mountains in Western South America (Chile, Ecuador and Peru) but it appears to have been first domesticated in Mexico (Rick, 1995; Kimura and Sinha, 2008). It has become an important vegetable crop all over the world with production being conducted in open fields and greenhouses. Yields of up to 10 kg m^{-2} per growing season have been reported for processing tomato in open field production systems in Italy (Pane et al., 2013). In the Netherlands, the average annual yield for round tomato is 66 kg m^{-2} in greenhouses without artificial lighting (Vermeulen, 2013). With supplementary lighting, yields of 90 – 100 kg m^{-2} per year have been achieved. Increase in tomato yields over the years is attributed to improvements in production systems and the development of better yielding cultivars. Notable improvements in the production system include the introduction of greenhouses with high light transmittance, carbon dioxide dosing, artificial lighting, high wire training systems, longer cultivation periods per crop, soilless culture and climate control computers (Higashide and Heuvelink, 2009).

Tomato production systems are also associated with high energy costs and a negative impact on the environment due to carbon dioxide emission. In order to improve energy use efficiency, more fruit have to be produced with less external energy input. This can be achieved for example by lowering set points for heating, use of new greenhouse designs, reduction in the use of artificial lighting or the development of cultivars whose yield can still be optimal with low external energy input. An understanding of how tomato fruit responds to changes in environmental conditions can be a starting point for breeding for tomato fruit that can be grown with less energy use. These responses need to be scaled down to the cell and gene level in order to provide clues on target genes for breeding programmes. The availability of vast genetic resources and genomic tools (Chevalier et al., 2014), and the recent publication of the complete genome sequence of tomato (Tomato Genome Consortium, 2012) make it an easy to study crop. The tomato fruit is generally considered a model for berry fruit (Kimura and Sinha, 2008).

The tomato fruit

Tomato fruit is popular all over the world because it can be eaten fresh or in processed form (Costa and Heuvelink, 2005). There are numerous cultivars with varying sizes, shapes, and colours. Based on size, tomato fruit can be classified as cherry and cocktail (10 – 20 g), round

(70 – 100 g), or beefsteak (180 – 1,000 g). Regardless of the size, tomato fruit can be marketed while still attached on the truss, referred to as vine or truss tomato (Costa and Heuvelink, 2005). Proximal fruit (older and closer to the main stem) are usually larger than distal fruit on the same truss (Bertin et al., 2003a). Fruit growth begins with formation of a floral meristem which later develops into a fully grown flower. Fruit set occurs after pollination and fertilization of ovules and is immediately followed by a period of intense cell division. This period lasts for about two weeks (Tanksley, 2004) but can be longer depending on the variety (Bertin et al., 2009) and temperature. The next phase of fruit growth involves increase in DNA content without cell division (endoreduplication) and cell expansion. By undergoing endoreduplication, tomato fruit exhibits ploidy levels of up to 512C (where C is the haploid DNA content; Chevalier et al., 2014). Increase in ploidy level is positively correlated with cell and fruit size (Cheniclet et al., 2005). Depending on the genotype and temperature, the cell expansion phase in tomato fruit can be up to six weeks long. During this time, cells increase in size through intake of sugars and water and can attain a size that is >30,000 times that observed at anthesis (Cheniclet et al., 2005). Cell wall loosening is also crucial for the process of cell expansion.

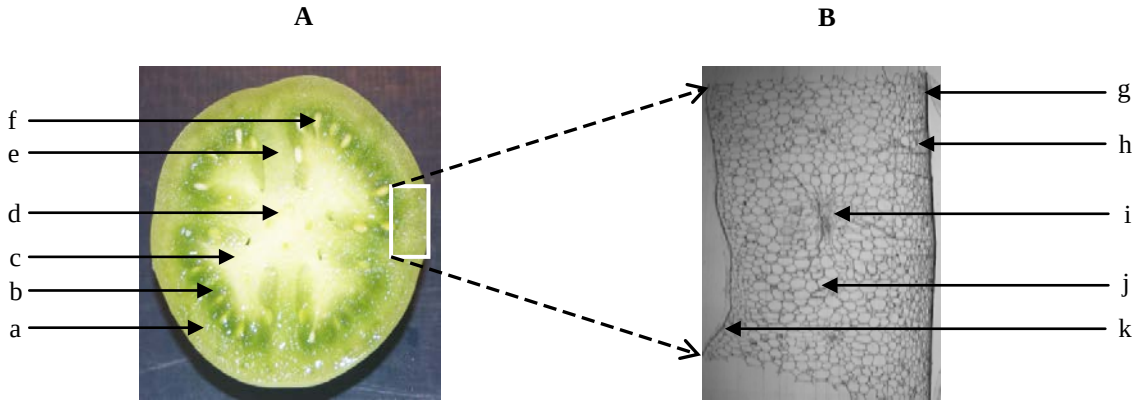

Figure 1: Anatomy of the tomato fruit showing the transverse section (A) and microscopic section of the pericarp (B). a: pericarp; b: locular tissue; c: placental tissue; d: columella; e: radial wall of pericarp; f: seed; g: epidermis; h: exocarp; i: vascular bundle; j: mesocarp; k: endocarp. The exocarp consists of approximately 5 layers of cells while the mesocarp can consist of more than 20 cell layers. The endocarp is constituted by a single layer of cells.

The tomato fruit consists of an outer epidermis, thick pericarp and seeds connected to placental tissue (Figure 1). The pericarp which is constituted by the exocarp, mesocarp, and endocarp, accounts for at least two thirds of total fruit dry weight and as such is the commonly analysed tissue in histological and genetic studies on fruit growth (Bertin, 2005; Fanwoua, 2012). The tomato fruit is made up of approximately 95% water and 5% dry matter

(Ho, 1996). Its quality is defined by physical, organoleptic and chemical properties (Causse et al., 2002). Physical traits include fruit weight, colour, firmness and elasticity while organoleptic properties are defined by taste, aroma and texture attributes. Chemical traits consist of titrable acidity, pH, and the contents of soluble solids notably carbohydrates, lycopene, carotene and other volatiles. Total soluble solids content is usually negatively correlated with fresh yield (Causse et al., 2007). The main carbohydrates in tomato fruit are sucrose, fructose, glucose and starch. Fructose and glucose (hexoses) account for approximately 50% of total fruit dry weight (Ho, 1996). These carbohydrates are imported from leaves or obtained through fruit photosynthesis (Hetherington et al., 1998). The carbohydrates are imported in the form of sucrose. Upon arrival in the fruit, sucrose is hydrolysed into hexoses which are stored as starch (Guan and Janes, 1991). Towards the end of fruit growth, starch is again hydrolysed into hexoses (Petreikov et al., 2009).

Fruit size depends on the source-sink ratio. A high source-sink ratio induced for example through fruit pruning or increase in light intensity usually leads to an increase in fruit size. Fruit on a given truss compete with each other, with other trusses as well as other vegetative plant parts for assimilates because vegetative and generative growth occur at the same time in tomato (Ho, 1992). Some genotypes, however, inherently partition more assimilates to fruit growth than others. For example, the small sized cherry tomatoes partition a smaller fraction of total plant dry matter into fruit growth compared to large fruited cultivars (Ho, 1996).

Cellular processes during tomato fruit growth

Tomato fruit growth is driven by three cellular processes: cell division, endoreduplication and cell expansion. In the first approximately two weeks after anthesis, fruit growth is largely due to increase in cell number (Tanksley, 2004). During this period, cells undergo the cell cycle consisting of; Gap 1, Synthesis (S), Gap 2 and finally mitosis (M) where one cell divides into two identical daughter cells (Figure 2; Inzé and De Veylder, 2006; Komaki and Sugimoto; 2012). The two gap phases are periods when cells expand in preparation for the next phase while S phase is characterised by doubling of nuclear DNA content. Transitions between cell cycle phases are orchestrated by Cyclin Dependent Kinases (CDK) upon dimerization with respective activator proteins; Cyclins (CYC). Unique CYC–CDK combinations confer dimer substrate specificity leading to promotion of specific cell cycle phases (Pines, 1994; Van Leene et al., 2011; Komaki and Sugimoto, 2012). Approximately 152 CDKs and 160 cyclins have been identified in plants (Dudits et al., 2007; Czerednik, 2012). Of these, seven CDKs (*CDKA1;1*, *CDKA2;1*, *CDKB1;1*, *CDKB2;1*, *CDKC;1*, *CDKC;2*, and *CDKD;1*) and eight

cyclins (*CycA1;1*, *CycA2*, *CycA3;1*, *CycB1;1*, *CycB2;1*, *CycD3;1*, *CycD3;2*, and *CycD3;3*) have been reported in tomato (Czerednik, 2012). Besides CYCs and CDKs, the cell cycle appears to be influenced by other genes whose biochemical functions have not been unravelled to date. A typical example is the fruit weight QTL at chromosome 2, number 2 (*fw2.2*; Cong et al., 2002; Lin et al., 2014). *fw2.2* accounts for as much as 30% of the variation in the fresh weight of domesticated and wild tomato species. It is also the first fruit weight QTL associated gene that was identified and cloned in tomato (Frary et al., 2000). Although the role of *fw2.2* in determining organ size is conserved in monocots and dicots, no clear cell cycle biochemical function has been attributed to it or to any of its orthologues yet (Azzi et al., 2015).

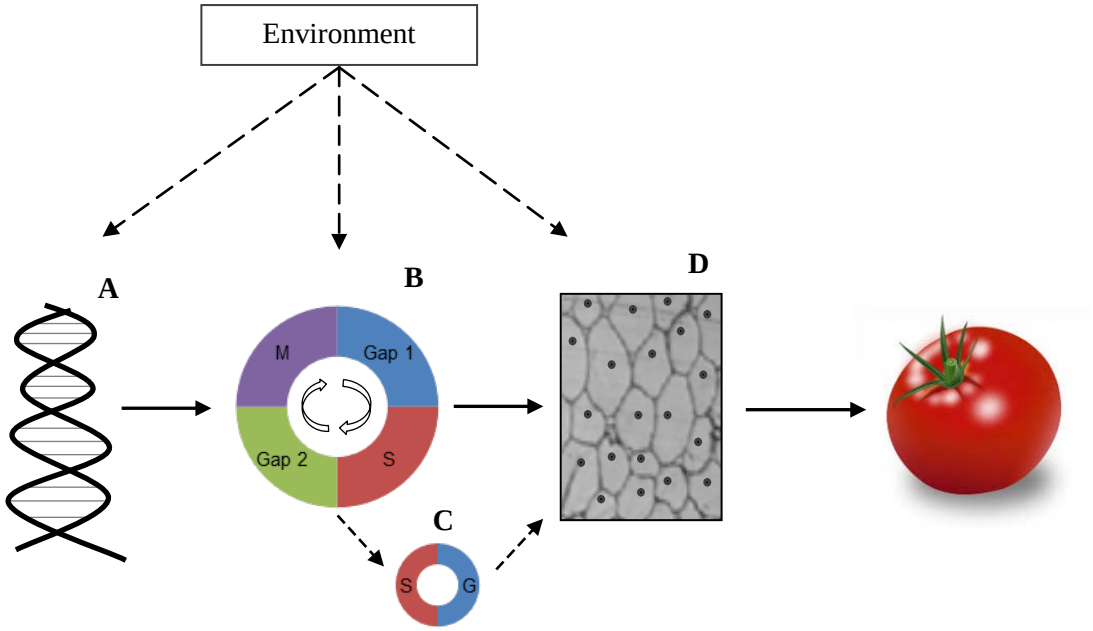

Figure 2: Schematic representation of fruit growth processes (A: gene expression; B: cell division; C: endoreduplication; D: cell expansion) for which environmental effects were studied.

Upon cessation of cell division, fruit growth occurs through cell expansion in a phase that is longer, more visible and considered physiologically very important because of its large impact on final fruit size (Gillaspy et al., 1993). The magnitude of cell expansion is determined by the amount of assimilate and water import, and the extensibility of the cell wall. Increase in cell size is also associated with endopolyploidy (Chevalier et al., 2011). Endopolyploidy is a common phenomenon in eukaryotic cells that leads to increase in cell DNA content. It can occur through the fusion of nuclei, formation of multinucleate cells, endomitosis (doubling of chromosome number within the nucleus) or endoreduplication

(Chevalier et al., 2014). Unlike endomitosis, endoreduplication involves doubling of chromatid number without a change in chromosome number. Using fluorescence *in situ* hybridization, Bourdon et al. (2010) showed that endopolyploidy in tomato fruit tissues occurs through endoreduplication. It has been shown that cell size increase can be supported by endoreduplication. This appears to arise from an internal cellular balance that keeps cytoplasmic volume to nuclear DNA ratio constant as stipulated in the karyoplasmic ratio theory (Sugimoto-Shirasu and Roberts, 2003; Bourdon et al., 2012; Chevalier et al., 2014).

Endoreduplication essentially leads to nuclear DNA content increase (up to 512C) through repeated S and gap phases without cell division (Figure 2; Bergervoet et al., 1996; Chevalier et al., 2011). By inhibiting mitotic CDK activity, KIP-RELATED (KRPs) and SIAMESE/SIAMESE-RELATED (SIM/SMR) proteins prevent the S to M transition and thus promote endoreduplication and reduce cell proliferation (Verkest et al., 2005; Weinl et al., 2005; Anzola et al., 2010; Komaki and Sugimoto, 2012). WEE1 kinase is another negative regulator of CDKs and CYCs that has been reported to inhibit mitosis and promote endoreduplication (De Schutter et al., 2007). It appears to be part of a mechanism that prevents the transition to gap 2 phase in response to genotoxic stress. Regulation of endoreduplication in tomato fruit appears to be controlled in a tissue specific manner. Teyssier et al. (2008) for example showed that 80% of nuclei in locular tissue had a ploidy level of either 32C or 64C while only 10% of nuclei had a ploidy level above 64C. No nuclei within the locular tissue exhibited a ploidy level above 256C. In contrast, the authors reported ploidy levels ranging between 4C and 512C within pericarp tissue. It is not exactly clear why different ploidy levels are exhibited by different tissues of the tomato fruit but the very high ploidy levels in pericarp tissue suggest that more insight into the regulation of endoreduplication can be obtained through studies on pericarp tissue.

Genotype by environment interactions during fruit growth

The human population is steadily increasing on planet earth. The climate on planet earth is also changing as a result of human activity. In order to ensure human survival, the production of plant food sources needs to be adapted to the changing climate because many plant traits tend to vary depending on growing conditions. This adaptation can only be achieved if we understand the interaction between the genetic potential inherent in plants and the environment. A first step towards this goal as has been attempted in many studies is to investigate the effect of changes in plant genomes on phenotypes. A second step is to

understand how (a) given genotype(s) perform(s) under different climatic conditions. Research on tomato has attempted to elucidate the genetic basis of fruit growth and quality in terms of gene functions (Tanksley, 2004; Dorais et al., 2007; Czerednik et al., 2012), but knowledge on the interaction with the environment has been scarcely reported (Ortiz et al., 2007; Bertin et al., 2010; Prudent et al., 2010). Gene regulated processes can be scaled up to explain processes at cell and organ level quantitatively only if effects of growing conditions on gene expression and resulting biochemical reactions are known. Research on peach quality for example shows that including both genetically based parameters and biochemistry at the tissue level improves insight into fruit growth processes and facilitates the interpretation of genotype × environment interaction (Quilot et al., 2005).

Temperature and light are two environmental factors that can easily be manipulated during experimentation and thus good candidates for genotype × environment interaction studies. They are often studied at the plant level and treatments applied throughout plant growth. However, plant responses to the environment can be organ or development stage specific. It is, therefore, important that organ and development stage specific treatments are deployed in studies on genotype × environment interaction studies. For example, the effect of temperature on tomato fruit growth when applied only at specific stages of fruit development is not well understood. Understanding temperature effects on growth is complicated, because response of tomato fruit growth to temperature will change during its development (De Koning, 1994; Van der Ploeg and Heuvelink, 2005). Effects of temperature changes at short intervals differ from those at longer intervals (Papadopoulos and Hao, 2001) and the net effect on growth is a balance between temperature-driven development and source-limited biomass increment. These findings indicate that the temperature response is not well understood at the fruit level and should be explained by underlying processes i.e. the temperature effects on cell division and cell elongation, as was found for a simpler pattern in monocot leaves by Ben-Haj-Sahah and Tardieu (1995). The effects of light when applied at the fruit level or specific fruit development stages have not been investigated before *in vivo* grown tomato fruit. Guan and Janes (1991) showed that growth of *in vitro* grown tomato fruit was stimulated by white light. However, neither cell nor gene level responses were reported. The above findings indicate that temperature and light responses in tomato are not well understood at the fruit level and should be explained by underlying processes. For example, Temperature-promoted cell division rate may be counteracted by possible shortages in assimilate supply that either decrease division rate (Baldet et al., 2006) or cell expansion (Bertin et al., 2007). A better

understanding of temperature and light effects will indeed be attained if responses of cellular processes (division, endoreduplication and expansion) that are separated in time during fruit development are also investigated. Studies on the expression of genes encoding proteins associated with the regulation of the above cellular processes will also provide useful insight.

Systems biology of tomato fruit growth

Systems biology is an emerging field in plant science that involves the study of biological systems at the system level (Kitano, 2007). It has become increasingly important because of progress in molecular biology, genomics, computer science, modern control theory, and nonlinear dynamics theory (Kitano, 2007). Systems biology is based on the principle that biological systems are complex and cannot be fully understood by focussing on only one component of the system (Yin and Struik, 2008, 2010; Joyard and McMcormick, 2010). Identification of all components of a biological system does not mean that its functioning is understood (Kitano, 2002). The system is rather better understood when interactions among system components are also studied over time to reveal emerging properties. The systems biology approach essentially enables the organization of information on complex systems, reveals hidden properties and helps to predict behaviour under new and untested conditions (Assmann, 2010). Models have become an integral part of the systems biology approach because they are an important means to summarise information that is useful in revealing emerging properties that can be tested through experimentation thus progressing knowledge.

There have been several attempts to model fruit growth. A notable attempt is that by Fishman and Génard (1998) in which fruit growth was described in peach. The model considers the fruit as a large cell and uses thermodynamic equations to simulate water and carbohydrate uptake from the plant in order to describe the effect of fruit load and water stress on fruit fresh and dry mass. The model was later adapted for mango (Lechaudel et al., 2007) and tomato (Liu et al., 2007). Other models have deployed the source sink ratio concept in describing fruit growth in tomato (Heuvelink, 1996), cucumber (Marcelis, 1994) and peach (Lescourret et al., 1998). Models that focus on cell level processes have also been proposed and used to predict cell number in different genotypes (Bertin et al., 2003b), and under contrasting fruit loads and temperatures (Fanwoua et al., 2013) in tomato. The model by Bertin et al. (2003b) assumed exponential cell division for a defined period followed by a progressive decline in cell division activity after each division cycle. Fanwoua et al. (2013) on the other hand, considered the three processes (division, endoreduplication and expansion)

that a cell experiences during tomato fruit growth in their model. The model groups cells into classes that differ in their initial age and size and assumes that the transition from cell division to endoreduplication and cell expansion phases is cell age dependent. It further assumes that cells divide or undergo endoreduplication when they exceed a critical cell mass: ploidy ratio while cell expansion is dependent on sugar import from a common assimilate pool as defined by the cell class sink strength. Advances in modelling show that gene-regulation of the cell cycle can be modelled (Qu et al., 2003; Novak and Tyson, 2004; Barik et al., 2010; Roodbarkelari et al., 2010; Apri et al., 2014). In these models, the activity of cyclins and cyclin dependent kinases is simulated via protein interaction networks. The activity of the proteins is defined using differential equations and cell cycle phase transitions evaluated using bifurcation diagrams.

Crop yield is an example of a complex property that can be best studied following the systems biology approach. However, genomics and molecular biology are seldom used to explain emerging complex traits like yield (Struik et al., 2005). In the last decades modelling tools have been developed that are able to quantitatively explain biological processes across various levels (Marcelis et al., 1998; Van Ittersum et al., 2003; Yin and Van Laar, 2005). Yet, these models do not consider the cell level and below, and are not able to integrate genetic processes to explain the appearing phenotype. Genetic information would be much more useful if such models would allow predictions of phenotype (Fanwoua et al., 2013).Tomato is the most favourable crop species to establish such a modelling framework as there are enormous genetic and genomic resources. The tomato fruit is a complex organ constituted by different tissues and seeds (Figure 1). Different processes occur during its growth and development. Hence final fruit size stems from interactions among different tissues and processes. The tissues are made up of cells that undergo an initial period of cell division followed by cell expansion. Unravelling the extent to which cell expansion depends on cell division is important for our understanding of tomato fruit yield. Deployment of the systems biology approach can aid our understanding of tomato fruit growth. Such an approach requires observations at different levels of aggregation for example the collection and integration of data at the gene, cell, tissue and fruit level. In order to reveal the mechanism by which fruit growth is driven, the role of carbohydrates and other environmental signals in these interrelationships also need to be investigated.

Objectives and thesis outline

Attempts have been made to investigate the effect of temperature on tomato fruit growth but other than the work of Adams et al. (2001) and Fanwoua et al. (2012), treatments are seldom applied at the fruit level hence whole plant and fruit responses cannot be separated. The effect of fruit illumination on *in vivo* grown tomato fruit has also not been studied before. There are no reports of studies in which light and temperature effects on tomato fruit growth are reported simultaneously at the gene, cell, tissue as well as fruit level. In addition, it is not exactly clear whether fruit growth is driven by cell division, or endoreduplication, or cell expansion, or all three processes contribute to fruit size increase.

The overall aim of this study was to explain whole fruit growth based on gene related processes at cellular and subcellular level under different environmental (light and temperature) conditions. The specific objectives were to determine 1) the genetic and physiological basis for differences in tomato fruit size, 2) the mechanisms by which fruit growth is driven, and 3) how light and temperature modulate the drivers of fruit growth. It was hypothesized that light increases sucrose import by the fruit and that the effect depends on the type of light treatment and phase during fruit development. Increase in fruit temperature was hypothesized to accelerate growth processes, by enhancing carbon import and initially leading to more cell division and higher expansion rate than at low temperature. The increase in cell number at high temperature, however, intensifies competition for assimilates among cells. This in conjunction with shorter fruit growth duration leads to smaller final cell and fruit size. Analyses were conducted at the gene, cell, tissue and fruit level at several harvest points between anthesis and breaker stage in order to reveal multilevel interactions and the basis for emerging phenotypes.

The study involved greenhouse experiments and a synthesis of current knowledge on regulation of fruit growth. **Chapter 2**, reports on experiments in which the temperature response of two cultivars with similar vegetative growth but contrasting fruit sizes was investigated. Temperature treatments were applied at the fruit level to ensure that fruit responses could be clearly separated from whole plant responses. Detailed measurements on pericarp cell number and cell volume were conducted. In conjunction with data on carbohydrate content of the pericarp and the expression profile of 20 genes involved in cell division, endoreduplication and cell expansion regulation, cell level data were used to explain fruit size differences between the two cultivars at two different temperatures. Analysed genes were selected and grouped according to the literature on cell division and expansion in

Arabidopsis thaliana and tomato. The first group consisted of promotors of the cell cycle, i.e. genes for Cyclin Dependent Kinases (*CDKA1, CDKA2, CDKB1* and *CDKB2*), Cyclins (*CycA1, CycB2* and *CycD3;3*) and transcription factors *E2Fa-like*, *E2Fb-like* and *E2Fe-like*) (Inzé & De Veylder, 2006; Lopez-Juez *et al*., 2008). The second group consisted of inhibitors of the cell cycle such as a transcription factor (*E2Fc-like*), Kip Related Protein1 (*KRP1*), a protein kinase (*WEE1*), cell number regulator 1-like (*fw2.2*) and Phytochrome Interacting Factors (*PIF1-like(a), PIF1-like(b)* and *PIF3-like*) (Frary *et al*., 2000; Inzé & De Veylder, 2006; Lopez-Juez *et al.*, 2008; Jang *et al*., 2010). The third group consisted of *AGPaseB* and *AGPS1* that encode a small and large subunit respectively of the same enzyme; ADP Glucose Pyrophosphorylase, which promotes starch accumulation (Schaffer & Petreikov, 1997) and consequently fruit growth (Guan & Janes, 1991). **Chapter 3** discusses findings from experiments in which fruit trusses were grown in climate controlled cuvettes fitted with red, blue or white light emitting diodes. Light treatments were also applied to fruit during different periods of fruit development. It was tested whether light grown fruit are stronger sinks than dark grown fruit and if responses are dependent on the light treatment or fruit development stage. Similar measurements as in Chapter 2 were conducted and these data were used to explain fruit level observations. In **Chapter 4**, a detailed analysis of the literature on the effect of light on the regulation of cell division, endoreduplication and cell expansion is presented. Key transcription factors and photoreceptors involved in the regulation of cell division, endoreduplication and cell expansion by light are highlighted. In addition, the mechanism by which light interacts with transcription factors in different organs is discussed. **Chapter 5** addresses the question: What drives fruit growth? This question was as a result of reflection on the findings from Chapters 2 and 3 and literature on fruit growth. Possible theories of organ growth are presented and one is suggested to be applicable to tomato fruit. A global molecular regulator for fruit growth is also proposed. **Chapter 6** concludes the thesis with a discussion of the findings from earlier chapters. Strengths and limitations of the findings are discussed and placed into perspective of current knowledge of fruit growth. In addition, recommendations for future research are presented.

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Chapter 2

A multi-level analysis of tomato fruit growth in response to fruit temperature

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Abstract

Fruit phenotype is a resultant of inherent genetic potential in interaction with impact of environment experienced during crop and fruit growth. The aim of this study was to analyse the genetic and physiological basis for the difference in fruit size between a small ('Brioso') and intermediate ('Cappricia') sized tomato cultivar exposed to different fruit temperatures. It was hypothesized that fruit heating enhances expression of cell cycle and expansion genes, rates of carbon import, cell division and expansion, and shortens growth duration, whereas increase in cell number intensifies competition for assimilates among cells. Unlike previous studies in which whole-plant and fruit responses cannot be separated, we investigated the temperature response by varying fruit temperature using climate-controlled cuvettes, while keeping plant temperature the same. Fruit phenotype was assessed at different levels of aggregation (whole fruit, cell and gene) between anthesis and breaker stage. We showed that: 1) final fruit fresh weight was larger in 'Cappricia' due to more and larger pericarp cells, 2) heated fruits were smaller because their mesocarp cells were smaller than those of control fruits, and 3) no significant differences in pericarp carbohydrate concentration were detected between heated and control fruits nor between cultivars at breaker stage. At the gene level, expression of cell division promoters (*CDKB2*, *CycA1*, and *E2Fe*) was higher while that of the inhibitory *fw2*.2 was lower in 'Cappricia'. Fruit heating increased expression of *fw2.2* and three cell division promoters (*CDKB1*, *CDKB2* and *CycA1*). Expression of cell expansion genes did not corroborate cell size observations.

Keywords: sink strength, cell number, cell size, carbohydrate dynamics, systems biology

Introduction

Like all other plant growth processes, tomato fruit growth is determined by the interaction between the genetic potential and the impact of environment, experienced during crop and fruit growth (Ortiz et al., 2007: Prudent et al., 2010). Temperature is an environmental factor the influence of which on plant growth (biomass production and partitioning) and development (leaf and truss appearance and fruit growth period) has been well studied. For a review on influences of temperature on growth in tomato, see Van Der Ploeg and Heuvelink (2005). High temperatures generally enhance rates of growth and development. In tomato, the period between anthesis and fruit maturity decreases with an increase in temperature between 14 °C and 26 °C (Van Der Ploeg and Heuvelink 2005). The effect of temperature on final fruit size, however, depends on the availability of assimilates (Marcelis and Baan Hofman-Eijer 1993).

Early fruit development can be divided into three distinct phases: fruit set, cell division and cell expansion (Gillaspy, 1993). In tomato, fruit size increase in the first two weeks after fertilization is largely attributed to cell division while subsequent fruit growth results from cell expansion (Tanksley, 2004). Recent efforts to understand the effect of temperature on fruit growth have focused on the relationship between cell and fruit level observations (Marcelis and Baan Hofman-Eijer, 1993: Bertin, 2005; Fanwoua et al., 2012a). These authors have tried to unravel the role of cell division and expansion in determining final fruit size. Bertin (2005) observed an increase in pericarp cell volume with increase in temperature under non-limiting assimilate conditions in tomato, while pericarp cell number decreased without significant effects on fruit growth. In non-assimilate limited cucumber, Marcelis and Baan Hofman-Eijer (1993) noted that pericarp cell size of individually heated fruits increased without any effects on pericarp cell number. However, under assimilate-limiting conditions, these authors observed that fruit heating did not affect cell size but cell number decreased. In assimilate-limited tomato fruits, local heating showed no significant effect on pericarp cell number, but resulted in a decrease in cell volume, fruit diameter and fresh weight (Fanwoua et al., 2012a).

No earlier studies on temperature response in tomato have related fruit fresh weight growth dynamics with observations on the ensemble of gene expression, cell division, cell expansion and carbohydrate metabolism. However, a few studies on cell cycle regulation by cyclins (Cycs) and cyclin dependent kinases (CDKs) at the gene level under contrasting assimilate availability conditions have been reported in literature. Joubès et al. (2000a, 2001)

reported repression of *CDKB2;1* and *CycB2;1* in sugar depleted tomato cell cultures. Similar findings were also shown in plants subjected to extended darkness and high fruit load (Baldet et al., 2002; Baldet et al., 2006). Another cyclin gene; *CycD3;1*, that is important in driving cells into the mitotic phase of the cell cycle (Komaki and Sugimoto, 2012; Schnittger et al., 2002; Dewitte et al., 2007; Boruc et al., 2010; Van Leene et al., 2010), was shown to be positively regulated under non-limiting assimilate conditions (Baldet et al., 2002, Dewitte and Murray, 2003). In addition, Menges and Murray (2002) and Baldet et al. (2006) observed that increased assimilate availability under low fruit load conditions down-regulate the expression of the Kip Related Protein (KRP) gene *KRP1*, causing a delay in the transition from the division to the endoreduplication phase of fruit growth. Scaling up similar gene level observations to the fruit level could provide additional insight into tomato fruit growth regulation in response to temperature and create possibilities for multi-level fruit growth modelling using process parameters that reflect genetic processes (Kromdijk et al., 2014).

We investigated whether tomato fruit size reduction due to increased fruit temperature was caused by lower cell number, smaller cell size or both. Cell size was studied following independent observations on anticlinal (perpendicular to fruit skin) and periclinal (parallel to fruit skin) cell diameter. In addition, quantitative analyses of soluble carbohydrates and starch and the expression of genes involved in cell division and expansion regulation were conducted. The response to fruit heating was explored under assimilate limiting conditions in two tomato cultivars that differ significantly in fruit size (small: ‘Brioso”; intermediate: ‘Cappricia’). Heating was applied only to the fruits (in cuvettes) whereas most other studies heated the whole crop, obscuring the temperature effects on the fruit. The question whether both cultivars had similar response to temperature at fruit, tissue, cell, and gene level was raised. The hypothesis tested was that high temperature accelerates growth processes, so enhances carbon import rate and initially leads to more cell division and a higher expansion rate than at low temperature. The increase in cell number at high temperature, however, intensifies competition for assimilates among cells. This in conjunction with shorter fruit growth duration leads to smaller final cell and fruit size.

Materials and methods

Plant material and growth conditions

The experiment was conducted in a multi-span Venlo greenhouse at the Radix Serre greenhouse complex in Wageningen, the Netherlands (52° N) between January and May 2011 using two commercial cultivars ('Brioso', cocktail tomato and 'Cappricia', intermediate tomato; Rijk Zwaan B.V., De Lier, The Netherlands). 'Brioso' was grafted on 'Maxifort' (Monsanto Vegetable Seeds, Bergschenhoek, the Netherlands) while 'Cappricia' was grafted on 'Stallone' (Rijk Zwaan B.V., De Lier, The Netherlands) rootstock. Graftlings of the two cultivars were planted on Rockwool® slabs in the greenhouse on 15th January, 2011 at a spacing of 2.55 plants m^{-2}. Pollination was conducted using an electric bee and fruit pruning was done according to commercial practice for the two cultivars ('Brioso', 10 fruits, 'Cappricia', 6 fruits per truss). All young side shoots were removed early to maintain a single main stem per plant. The greenhouse had a light transmittance of 67% and was equipped with heating, misting, artificial lighting (600 W high pressure sodium lamps providing 150 µmol. m^{-2}. s^{-1}) and carbon dioxide dosing systems. Greenhouse air temperature was 20±3 °C while average day time carbon dioxide and relative air humidity were 540±45 µmol.mol^{-1} and 72±10%, respectively. Artificial lighting was automatically switched on when global radiation levels fell below 200 W.m^{-2} and off when above 250 W.m^{-2} during a 16 h photoperiod.

Treatment application

Two temperature treatments; +0 °C (control) and +6 °C (heated) relative to greenhouse air temperature were applied to fruits from one truss per plant from anthesis until breaker stage. The two temperature treatments were applied using a cuvette system consisting of a transparent and cylindrical Perspex cuvette (length: 40 cm; diameter: 13 cm; WSV Kunststoffen BV, Utrecht, The Netherlands) fitted with a funnel on one side and a Petri dish (diameter; 14 cm) held in position on the other side using cello tape. A slit (width; 1.5 cm) was made until the centre of the cuvette to provide room for the peduncle/fruit stalk. The control treatment was achieved using small 12 V ventilators (40 L min^{-1}) placed within a round opening (diameter; 2.4 cm) on the cuvette. The ventilators continuously (24 h) sucked greenhouse air into the cuvettes. To prevent fruit temperature increase due to direct heating of fruits by sunlight, a wire frame was placed around cuvettes and the upper half covered with

aluminium foil. Realized average cuvette air temperatures were: 21.4±0.8 °C (control) and 27.1±1.5 °C (heated).

Fruit heating was achieved using two heating systems: decentralized (A) and central (B). An equal number of each system was allotted to the two cultivars. System A was made up of small heating units (Cirrus 25, DBK, Spartanburg, South Carolina, USA) with a ventilator attached inside the cuvette as described in (Fanwoua et al., 2012a). Temperature control units were calibrated to maintain air temperature inside the cuvettes at 6 °C above that of greenhouse air as sensed by thermocouples. Heating system B blew heated air to cuvettes from the funnel side through inert polyethylene tubes. The heater within system B was switched on or off by a control unit similar to that of system A. Temperature and cultivar treatments were executed within the greenhouse as a two factorial completely randomized block design with six blocks and one replicate plant per block. One cuvette was attached to one truss per plant starting at truss 7 or moved to a higher truss position in case of fruit abortion. Cuvettes were attached on the day when the second (‘Cappricia’) or fourth (‘Brioso’) flower proximal to the stem had reached anthesis. Anthesis date for the next two flowers of each genotype was subsequently noted.

Measurements

Whole fruit

Observations on the whole fruit were made at nine time points from anthesis until breaker stage. Time points (0, 7, 13, 19, 25, 30, 40, 50 days after anthesis (DAA) and breaker stage) were defined based on the control treatment. A thermal time (base temperature of 10 °C; Calado and Portas, 1987) based correction was made for heated fruits to ensure that control and heat treated fruits were always evaluated at approximately the same development stage. Two fruits (fruit number 3 and 4 in ‘Cappricia’ and fruit number 5 and 6 in ‘Brioso’) were harvested from each experimental truss. Harvested fruits were wrapped in aluminium foil and immediately placed in ice before fresh weight and equatorial diameter were measured. An equatorial section was later made to split the fruit into two equal halves. Pericarp tissue from the two halves was extracted and utilized in subsequent analyses. One half of the pericarp tissue was used for histological analysis while the other half was dipped in liquid nitrogen and then stored at -80 °C in preparation for carbohydrate and gene expression analyses. Pericarp was selected as representative tissue because: 1) related studies in literature are based on

pericarp tissue, 2) pericarp mass and fruit mass are tightly correlated, and 3) it accounts for 65% of fruit dry weight throughout fruit growth in both cultivars (Figure S1).

Histology

Triangular sections were made in fresh pericarp tissue and immediately placed in a fixation solution consisting of ethanol (96%), acetic acid, formaldehyde (37%) and MQ water in the ratio 10:1:2:7 by volume, respectively. Care was taken to ensure that the largest part of all triangular sections was from the surface at which the equatorial section was made on the fruit. Air was eliminated from the tissue through vacuum application to the tissue while in fixation solution. The vacuum was created using a vacuum pump that was switched on for 15 minutes and off for 1 h. This procedure (vacuum on and off) was repeated four times and the samples left to stand in fixation solution over night at room temperature before rinsing and storage in ethanol (70%) at 4 °C.

Stored pericarp tissue sections were later infiltrated with a solution containing ethanol, 2-hydroxyethyl methacrylate (HEMA) based resin, Technovit 7100 (Kulzer, Wehrheim, Germany), benzoylperoxide hardener, and polyethylene glycol (PEG). The tissue was polymerized using a solution prepared from a combination of the infiltration solution and a second dimethylsulfoxide based hardener. Sections (3 µm thick) were cut from the largest part of the triangular section (Leica, Rijswijk, The Netherlands) and stained with 1% toluedene blue dye before microscopic analysis as reported in Fanwoua et al. (2012a). An image of one slide per sample was made using Nikon Imaging Software (NIS-Elements). With the aid of ImageJ software (National Institutes of Health, USA), pericarp thickness, number of cell layers, and number of cells (n) in a rectangular section was counted from the tissue image. Area (A) of the rectangular section was calculated from its length and width. The rectangular sections were made within the mesocarp and exocarp (first five top cell layers of the pericarp) separately. The endocarp and regions with vascular bundles in the mesocarp were not included in the counts as they were assumed not to have a significant contribution to pericarp volume (Fanwoua et al., 2012a). Mean periclinal cell diameter (D_T; diameter parallel to fruit skin), cell volume (C_V), tissue volume (T_V) and number of cells (C_N) in pericarp tissue (mescocarp or exocarp) were then derived as illustrated in Fanwoua et al. (2012a; Appendix 1) according to equations 1 to 4 with the assumption that each cell is an ellipsoid. It was assumed that cell diameter in the longitudinal direction to the fruit skin was equal to D_T.

Periclinal cell diameter (D_T) $= \left(\frac{Ca}{0.25 \times \pi \times H_T}\right)$ (Eqn 1)

Mean cell volume (C_V) $= \frac{4}{3} \times 0.5 \times D_T \times Ca$ (Eqn 2)

Tissue volume (T_V) $= (\frac{4}{3} \times \pi) \times (r^3 - (r - Tt)^3)$ (Eqn 3)

Tissue cell number (C_N) $= \frac{T_V}{C_V}$ (Eqn 4)

Where Ca = mean cell area derived by dividing area A with the number of cells within the rectangular pericarp section, H_T = mean anticlinal cell diameter (perpendicular to fruit skin; derived by dividing tissue thickness with respective number of cell layers) in respective pericarp tissue, r = fruit radius and Tt = Tissue (mesocarp or exocarp) thickness.

Pericarp cell number was derived by summing up the number of cells in mesocarp and exocarp while pericarp cell volume was considered as sum of the weighted volume of cells in the two constituent tissues of the pericarp (Eqn 5).

Pericarp cell volume $= \left(\frac{Ev}{Pv} \times Ecv\right) + \left(\frac{Mv}{Pv} \times Mcv\right)$ (Eqn 5)

Where Ev = Exocarp volume, Pv = Pericarp volume, Ecv = Mean exocarp cell volume, Mv = Mesocarp volume and Mcv = Mean mesocarp cell volume.

Carbohydrate analysis

Frozen samples of pericarp tissue were freeze dried for 72 h. Sugars were extracted from 15 mg of freeze dried tissue by adding 5 ml of 80% ethanol to each sample and incubating in a shaking water bath at 80 °C for 20 minutes. The mixture was then centrifuged (25000 rpm for 5 minutes) and 1 ml of supernatant was dried using a speed vacuum (SpeedVac SPD 2010, ThermoFisher Scientific) for 105 minutes. Dried samples were dissolved in 1 ml water using a vortex machine and an ultrasonic water bath and again centrifuged for 5 minutes. Ten-fold diluted samples were then analysed using high performance anion exchange chromatography (HPAEC). Glucose, fructose and sucrose analysis was conducted on a Dionex system (GS50 pump, PED-2 detector) equipped with a CarboPac1 (250 x 4 mm) column eluted with 100 mM sodium hydroxide.

Samples for starch analysis were obtained from the precipitate from the ethanol extract, which was resuspended in 3 ml of 80% ethanol, centrifuged for 5 minutes and again the

supernatant discarded. This process was repeated 4 times to ensure that all the sucrose, glucose and fructose were washed out of the solid material before drying for 20 minutes using the speed vacuum. Starch within the resulting pellet was enzymatically broken down into glucose by adding 2 ml of a thermo stable α-amylase (Serva 13452) and incubated for 30 minutes in a shaking water bath at 90 °C followed by 1 ml amyloglucosidase (Fluka 10115; 0.5 mg ml^{-1} in 50 mM citrate buffer; pH 4.6) and 15 minutes incubation in a shaking water bath at 60 °C. A 1 ml sample of hydrolysed starch was centrifuged for 5 minutes, diluted 10 times and its glucose content determined through HPAEC analysis as described above except that the CarboPac1 (250 x 4 mm) column was eluted with 100 mM sodium hydroxide and 12.5 mM sodium acetate.

RNA extraction, cDNA synthesis and relative gene expression analysis

Samples for gene expression analysis were collected from green fruits harvested at development stages corresponding to approximately 125, 213, 287 and 459 °C day from both cultivars and temperature treatments. These development stages were assumed to be representative of the cell division phase and initial stages of the cell expansion phase of fruit growth. Tissue from two of six replicates per treatment were pooled to form three biological replicates from which total RNA was extracted using an InviTrap® Spin Plant RNA Mini Kit (STRATEC Molecular GmbH, Berlin, Germany). Isolated RNA was DNase treated with DNAase I (Invitrogen, Breda, The Netherlands) according to the protocol and quantitative RNA concentration measurements performed using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies**,** Delaware, USA). RNA quality was checked on gel and single stranded complementary DNA (cDNA) synthesized from 650 ng of total RNA using the TaqMan® Reverse Transcription Reagents kit (Roche Molecular Systems, Branchburg, USA). Real time quantitative PCR was performed using the iQ™ SYBR® Green Supermix (Bio-Rad, Veenendaal, The Netherlands). Primer pairs are indicated in supporting information (Table S1). Respective gene expression values were normalized using SAND (Czechowski et al., 2005) as a reference gene and relative expression was calculated following the $2^{-\Delta\Delta C_T}$ method (Livak and Schimittgen, 2001).

A total of 20 genes consisting of promoters and inhibitors of cell division, endoreduplication or cell expansion were studied. Cell cycle promoters included: Cyclins (*CycA1*, *CycB2*, and *CycD3;3*), cyclin dependent kinases (*CDKA1*, *CDKA2*, *CDKB1*, and *CDKB2*) and transcription factors (*E2Fa-like*, *E2Fb-like*, and *E2Fe-like*) while inhibitors of

the cell cycle were made up of a transcription factor (*E2Fc-like*), Kip Related Protein1 (*KRP1*), a protein kinase (*WEE1*), cell number regulator 1-like (*fw2.2*) and Phytochrome Interacting Factors (*PIF1-like(a), PIF1-like(b)* and *PIF3-like*). Promoters of cell expansion included *AGPaseB* and *AGPS1* that encode the small and large subunits respectively of an enzyme (ADP Glucose Pyrophosphorylase) involved in starch synthesis. Only one cell expansion inhibiting gene (*E2Ff*) was studied. Putative orthologs (denoted *–like*) or co-orthologs (denoted *–like(a) or –like(b)*) of respective *Arabidopsis* genes in tomato were derived through phylogenetic analysis. The above genes were specifically investigated because they or their encoded proteins had been reported in earlier studies on either cell division, endoreduplication or cell expansion (Guan and Janes, 1991; Frary et al., 2000; Joubes et al., 2000a; Joubes et al., 2000b; Baldet et al., 2002; Cong et al., 2002; De Witte and Murray, 2002; Kosugi and Ohashi, 2002; Liu et al., 2003; Baldet et al., 2006; Schaffer and Petreikov, 2007; Bertin et al., 2009; Prudent et al., 2010; Czerednik et al., 2012; Fanwoua et al., 2012b).

Statistical analysis

All data collected was analysed by Analysis of Variance (ANOVA). Fruit samples collected before breaker stage were grouped based on the same thermal time before conducting ANOVA tests. Outliers were defined at two levels and removed from the final analyses: within truss (at least 20% smaller than the largest fruit on the truss) and within averages of all trusses belonging to the same treatment. Truss averages whose residual was +/- 3 × standard error of observations within the same treatment were considered outliers. A four parameter Gompertz function (Eqn 1; De Koning, 1994; Wubs et al., 2012) was fitted to fruit fresh weight data, following:

$$Y = A + C \times \exp(-\exp(-B \times (X - M))) \quad \text{(Eqn 6)}$$

Where Y = Fruit fresh weight (g), A = Lower asymptote, B = Slope of the curve (g d^{-1} or g °C d^{-1}), C = Upper asymptote, M = Thermal time or day after anthesis (DAA), and X = Thermal time (°C d) or DAA at the inflection point. Estimated parameters were then applied to the derivative of the Gompertz function (Eqn 7) to obtain the growth rate per day. For curves fitted against thermal time, daily growth rates were obtained by multiplying Eqn 7 with the difference between the treatment average temperature and base temperature.

$$FGR = [C \times \exp\{-\exp[-B(X - M)]\} \times B \times \exp[-B(X - M)]] \quad \text{(Eqn 7)}$$

Where FGR = Fruit fresh weight growth rate (g d^{-1}) and B, C, M and X are as defined in Eqn 6. Relative growth rate (RGR) was calculated for each interval between two consecutive harvests according to equation 8, where W1 and W2 are average fruit fresh weights and t1 and t2 are the average time points in days after anthesis of the fruit at the two consecutive harvests.

$$\mathrm{RGR} = \left(\frac{\ln \mathrm{W2} - \ln \mathrm{W1}}{\mathrm{t2} - \mathrm{t1}}\right) \qquad \text{(Eqn 8)}$$

Results

Whole fruit

Fruit fresh weight and diameter at breaker stage did not show a significant interaction between temperature and cultivar (Table 1). Fruit diameter and fresh weight were both smaller in heated than in control fruits (Table 1) at breaker stage. Both cultivars had larger fruit fresh weight during most of the fruit growth period when heated, however, heated fruits were smaller than control fruits at breaker stage because of reduced growth duration (Figure 1). Fruit fresh weight growth rate as a function of the number of days after anthesis (DAA) was higher in heated fruits during the early stages of growth until approximately 25 DAA in both cultivars (Figure 2A). After this period, growth rate of heated fruits decreased to values below that of control fruits. Maximum fruit fresh weight growth rate in both cultivars was attained at approximately one third of the total fruit growth duration (Figure 2B). After this period, fruit fresh weight growth rate steadily decreased regardless of the temperature treatment in both cultivars.

Treatment differences in relative growth rate (RGR) were only observed in the early stages of fruit growth (Figures 2C and 2D). At 10 DAA (200 °C day), control fruits of ‘Brioso’ had a higher RGR compared with control fruits of ‘Cappricia’. However, between 10 and 19 DAA, RGR was generally higher in both control and heated fruits of ‘Cappricia’ compared with ‘Brioso’. Heated fruits generally had a higher RGR compared with control fruits in the early stages (10 to 19 DAA; 200 to 400 °C day) of fruit growth (Figures 2C and 2D). After this period until breaker stage, RGR did not differ between heated and control fruits.

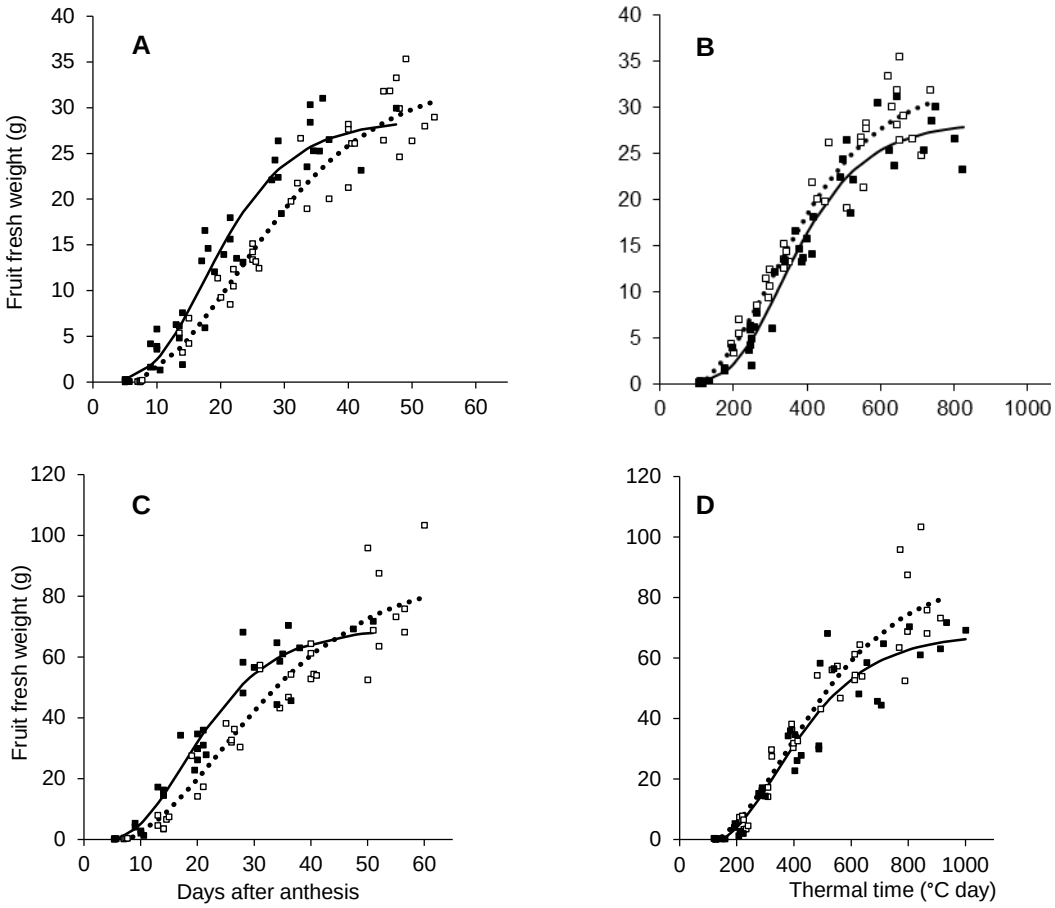

Figure 1: Effect of fruit heating on a small tomato 'Brioso' (A, B) and intermediate sized tomato 'Cappricia' (C, D) plotted as a function of the number of days after anthesis (A, C) or thermal time (B, D) in control (□) and heated (■) fruits. Breaker stage was attained at 711 and 907 °C day in 'Brioso' and 'Cappricia', respectively. Realized average cuvette air temperatures were: 21.4±0.8 °C (control) and 27.1±1.5 °C (heated). Dotted lines represent fitted curve for control fruits while continuous lines are for heated fruits. Each point is an average of two fruits from a single cuvette. All parameters of the Gompertz function were not significantly different from each other except for growth rate (higher in heated 'Cappricia' fruits) and the upper asymptote (lower in heated 'Cappricia' fruits) when fitted against number of days after anthesis.

Cell and tissue level

There was no significant interaction between cultivar and temperature for all cell and tissue level observations at breaker stage except for exocarp periclinal cell diameter (Table 1 and Supplementary Table S2). Both cultivars had similar exocarp periclinal cell diameter in the control treatment but in heated fruits, exocarp periclinal cell diameter was larger in 'Cappricia' (Table S2). On the other hand, exocarp anticlinal and mesocarp periclinal cell diameter did not differ significantly between the two cultivars. Mesocarp anticlinal cell

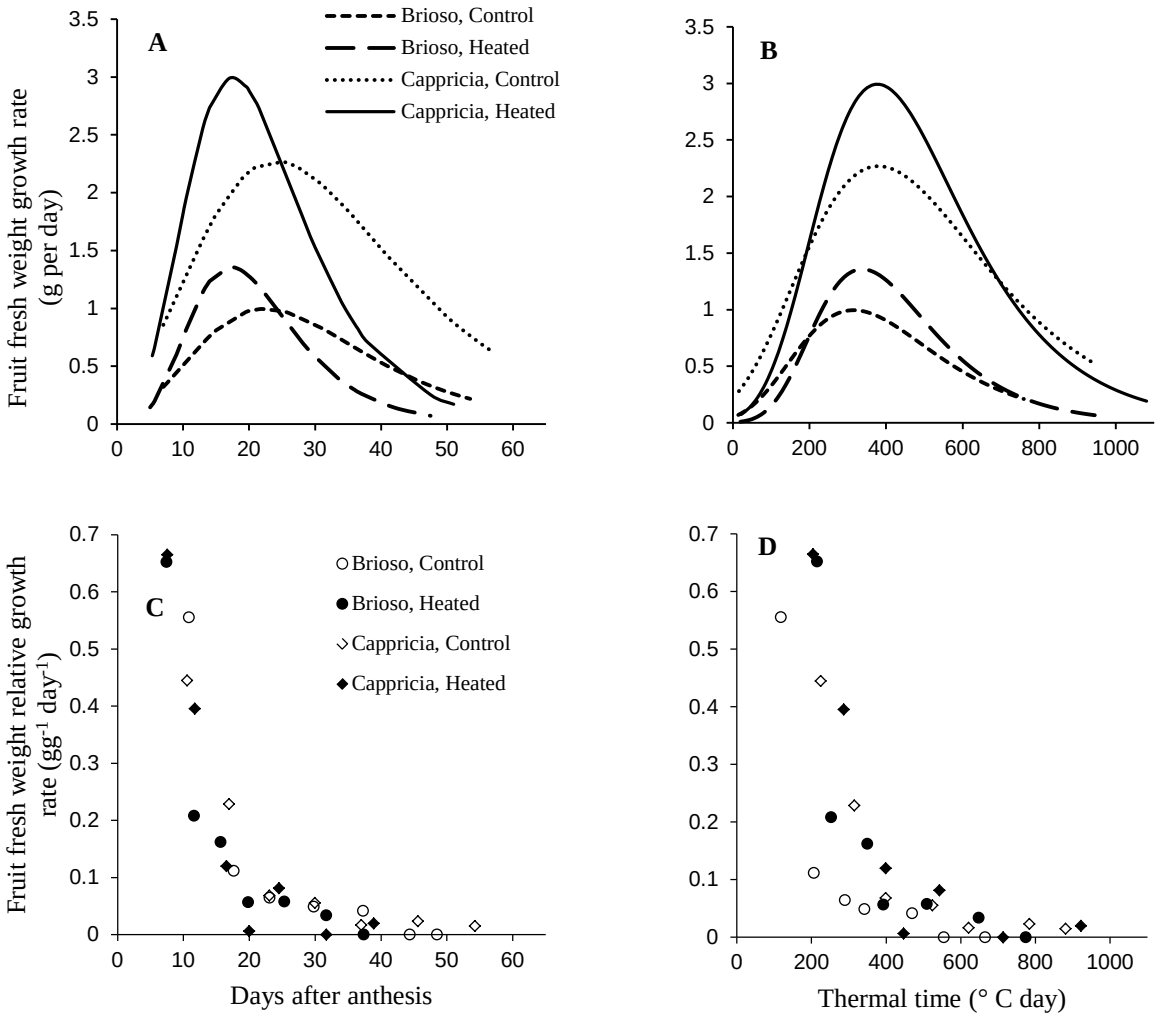

Figure 2: Fruit fresh weight growth rate for control (21.4±0.8 °C) and heated (27.1±1.5 °C) small; 'Brioso', and intermediate; 'Cappricia' sized tomato fruits fitted as a function of number of days after anthesis (A) and thermal time (B). Breaker stage was attained at 711 and 907 °C day in 'Brioso' and 'Cappricia', respectively. Individual curves in A and B are derivatives of fitted Gompertz curves for fruit fresh weight in Figure 1. Graphs C and D represent fruit fresh weight relative growth rate.

diameter, however, was significantly higher in 'Cappricia' than in 'Brioso'. This consequently led to a 40% larger pericarp cell volume in 'Cappricia' than in 'Brioso'.

The difference in average pericarp cell volume was largely due to larger mesocarp cell volume in 'Cappricia' because no significant difference in exocarp cell volume was observed between the two cultivars. The number of cells in both the exocarp and mesocarp was higher in the larger fruited 'Cappricia' than in 'Brioso'. Also consistent with fruit level and cell level observations, pericarp and pulp tissue volume were both larger in 'Cappricia' than in 'Brioso' (Table 1). Pulp volume was significantly larger in control compared to heated fruits. A similar

Table 1: Fruit phenotype (fruit and cell level) at breaker stage for control (21.4±0.8 °C) and heated (27.1±1.5 °C) 'Brioso' and 'Cappricia'. Means within the same column for the main effects of cultivar and temperature were averaged over the two temperature treatments or cultivars respectively. Comparisons are within the same row.

	Brioso	Cappricia	P value	Control	Heated	P value	Brioso		Cappricia		P value
							Control	Heated	Control	Heated	
Growth duration (days)	44.78	49.94	0.012	52.28	41.50	0.007	48.50	40.13	57.00	42.88	0.739
Fresh weight (g)	29.55	73.15	<0.001	53.51	46.21	0.055	32.22	26.22	80.11	66.19	0.397
Fruit diameter (mm)	40.13	58.61	<0.001	50.49	46.95	0.049	41.70	38.16	61.48	55.73	0.760
Pulp volume (cm^3)	12.54	33.52	<0.001	25.53	18.90	0.031	14.41	10.19	39.42	27.61	0.256
Sucrose (µg/mg)	3.39	5.16	0.233	4.67	3.72	0.482	3.80	2.88	5.76	4.55	0.821
Fructose (µg/mg)	244.9	231.2	0.053	233.2	244.3	0.175	236.9	254.8	228.6	233.8	0.348
Glucose (µg/mg)	251.9	234.1	0.054	240.3	247.2	0.806	243.8	262.1	235.9	232.3	0.135
Starch (µg/mg)	4.19	1.48	0.288	4.07	1.619	0.099	5.30	2.80	2.53	0.44	0.627
Exocarp volume (cm^3)	0.17	0.31	<0.001	0.27	0.19	0.002	0.20	0.14	0.37	0.25	0.113
Exocarp cell volume ($x10^{-5}$ mm^3)	3.75	3.87	0.472	4.37	3.18	0.021	4.44	2.89	4.28	3.47	0.103
Exocarp cell number ($x10^6$)	4.84	8.63	<0.001	6.69	6.56	0.840	4.63	5.11	9.27	8.00	0.092
Mesocarp volume (cm^3)	16.79	40.23	<0.001	29.14	26.34	0.168	18.22	15.00	42.78	37.68	0.949
Mesocarp cell volume ($x10^{-5}$ mm^3)	513.8	667.9	0.011	679.2	481.9	0.005	602.8	402.6	774.8	561.1	0.838
Mesocarp cell number ($x10^6$)	3.56	6.29	<0.001	4.29	5.46	0.066	3.25	3.94	5.60	6.98	0.424
Mesocarp cell layers	20.94	23.48	0.070	20.69	23.77	0.030	20.03	22.08	21.50	25.46	0.723
Pericarp volume (cm^3)	16.96	40.54	<0.001	29.42	26.53	0.160	18.42	15.14	43.15	37.92	0.959
Pericarp cell volume ($x10^{-5}$ mm^3)	508.6	662.7	0.010	672.6	478.2	0.005	596.4	398.9	768.0	557.5	0.834
Pericarp cell number ($x10^6$)	8.40	14.92	<0.001	10.99	12.01	0.323	7.89	9.04	14.86	14.98	0.369

trend was also observed in pericarp volume although the difference was not statistically significant (Table 1). At the cell level, it was observed that the volume of cells in the exocarp and mesocarp was significantly smaller in heated than in control fruits (Table 1). Exocarp anticlinal cell diameter was also 15% bigger in control fruits but the difference was just non-significant (P = 0.058). Exocarp cell number did not differ between the two temperature treatments. However, there were 30% more cells in the mesocarp of heated fruits although the difference was just not significant (P = 0.066; Table 1). There were also two extra cell layers in the mesocarp of heated fruits.

Carbohydrates

Interaction between temperature and cultivar was not significant for fruit carbohydrate concentration. The starch concentration peak at about 300 °C day matched with the period of maximum growth rate while sucrose, fructose and glucose concentration peaked shortly after the period of maximum growth rate in both genotypes and temperature treatments (Figure 3). Thereafter, sucrose and starch concentration decreased while fructose and glucose concentrations were high and did not change considerably throughout the remaining period of fruit growth. The two cultivars generally had similar sucrose concentration except at the beginning (300 °C day) of fruit growth where it was higher in 'Cappricia' (Figure 3A). Between 300 °C day and 550 °C day, sucrose concentration was significantly higher in heated than in control fruits (Figure 3E). After this period, the difference in sucrose concentration between the two temperature treatments was not significant.

Compared with 'Brioso', fructose and glucose concentrations were significantly higher in 'Cappricia' at the beginning (300 °C day) of fruit growth but the difference became insignificant at later stages. At breaker stage, fructose and glucose concentrations in 'Brioso' were 7% (P = 0.053) and 10% (P = 0.054) respectively higher than in 'Cappricia' although the differences were just not statistically significant (Table 1). In general, heated fruits seemed to exhibit a higher fructose concentration but this was only significant midway and just before the end of fruit growth (P = 0.004 and 0.002, respectively; Figure 3F). The effect of heating on glucose concentration was only significant at about 530 °C day. At this stage, heated fruits had a higher glucose concentration (P = 0.029; Figure 3G). Starch concentration was similar in both cultivars throughout fruit growth except at 300 °C day where it was higher in 'Brioso' (P = 0.012; Figure 3D). Pericarp starch concentration seemed to be lower when fruits were heated, however differences were generally not statistically significant (Figure 3H).

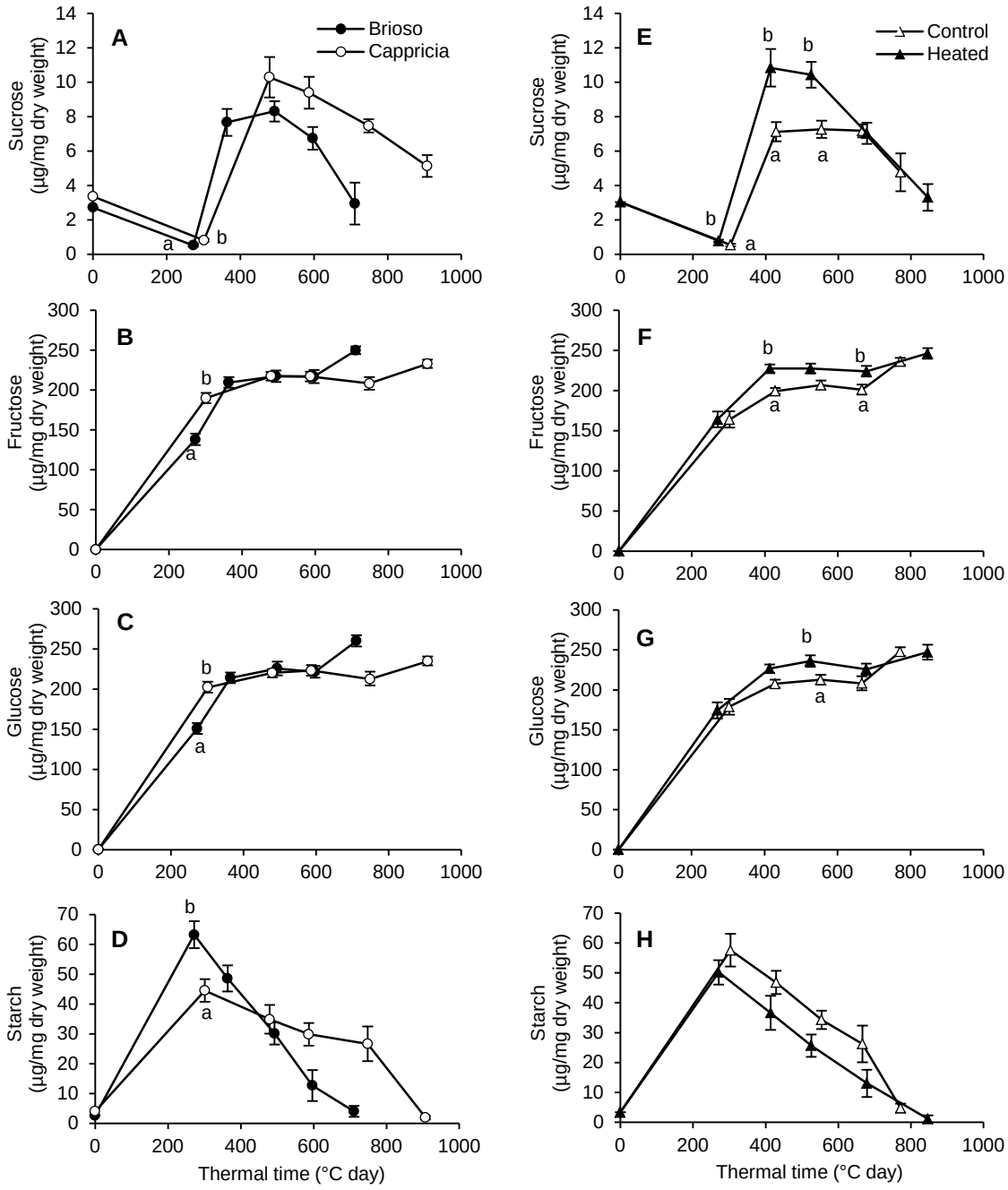

Figure 3: Sucrose (A), Fructose (B), Glucose (C), Starch (D), Sucrose (E), Fructose (F), Glucose (G) and Starch (H) content in the pericarp of small; 'Brioso' (•), and intermediate; 'Cappricia' (○) sized, Control (Δ; 21.4±0.8 °C) and Heated (▲; 27.1±1.5 °C) tomato fruits harvested at different stages from anthesis till breaker stage. Breaker stage was attained at 711 and 907 °C day in 'Brioso' and 'Cappricia', respectively. Means were averaged over the two cultivars or temperature treatments. Each point represents an average of n = 8 to 12 fruits except for the harvest stage corresponding to 0 °C day (anthesis stage) where n= 20 but analyzed as a single sample. Bars represent standard errors. Points followed by different letters significantly differ from each other while those not marked by a letter do not differ from each other. Comparisons are either between the two cultivars or temperature treatments at the same development stage.

Gene expression analysis

There was variation in expression patterns for different genes in the two cultivars. Some genes were constitutively expressed while the expression of other ones either increased or decreased with fruit age (Figure 4 and Supplementary Figures S2 and S3). Expression of *AGPS1*, *CDKA1*, *CycD3;3*, and *WEE1* was not significantly different between the two cultivars or temperature treatments at any harvest stage analysed (Figure 4). The expression of two cell cycle promoters (*CDKA2* and *CycB2*) and three cell cycle inhibitors (*PIF1-like(a)*, *PIF1-like(b)*, and *PIF3-like*) was also not significantly different between the two cultivars at both temperature treatments in fruits harvested during the cell division period (125 and 213 °C day; Figure 4). On the other hand, expression of two cell cycle promoters (*CDKB1* and *CycA1*) and one cell division inhibitor (*fw2.2*) was significantly increased by fruit heating during the period of cell division. *CDKB2* expression was stimulated by fruit heating only at 125 °C day. Compared to 'Brioso', fruits of 'Cappricia' had a higher expression of *CDKB2*, *CycA1*, *E2Fa-like*, *E2Ff-like*, *fw2.2*, and *KRP1* in at least one harvest stage of the cell division phase (125 and 213 °C day; Figure 4). On the contrary, expression of *E2Fb-like* and *E2Fc-like* was higher in 'Brioso' at 213 °C day. The expression level of the cell expansion promoter *AGPaseB* (213 °C day) and cell division promoter *E2Fe-like* (125 °C day) significantly depended on the cultivar × temperature interaction. Expression of *AGPaseB* in 'Brioso' did not respond to heating while that in 'Cappricia' decreased at 213 °C day when fruits were heated (Figure 4). Fruit heating did not alter the expression level of *E2Fe-like* in 'Cappricia' at 125 °C day while that in 'Brioso' decreased significantly at high temperature.

The expression of *E2Fa-like*, *E2Fb-like*, *E2Fc-like*, *E2Fe-like*, *E2Ff-like*, and *KRP1*, all differing between treatments in the division phase, did not differ between cultivar and or temperature treatments at the onset of cell expansion (287 and 459 °C day; Figure 4). However, fruit heating stimulated the expression of *CDKB1* and *fw2.2* and decreased *CDKA2* expression in at least one harvest stage during the onset of cell expansion. 'Cappricia' fruits had a higher expression of *AGPaseB* and *CycB2* than 'Brioso' fruits at 287 and 459 °C day, respectively. On the contrary, *PIF3-like* expression was higher in 'Brioso' fruits at 287 °C day (Figure 4). A significant cultivar × temperature interaction was observed in the expression of *ADPaseB*, *CDKB2*, *CycA1*, *PIF1-like(a)*, and *PIF1-like(b)* during at least one of the stages during cell expansion (Figure 4). Fruit heating stimulated the expression of *CDKB2* (459 °C day), *CycA1* (287 °C day), and *PIF1-like(b)* (287 °C day) only in 'Brioso'. *PIF1-like(a)*

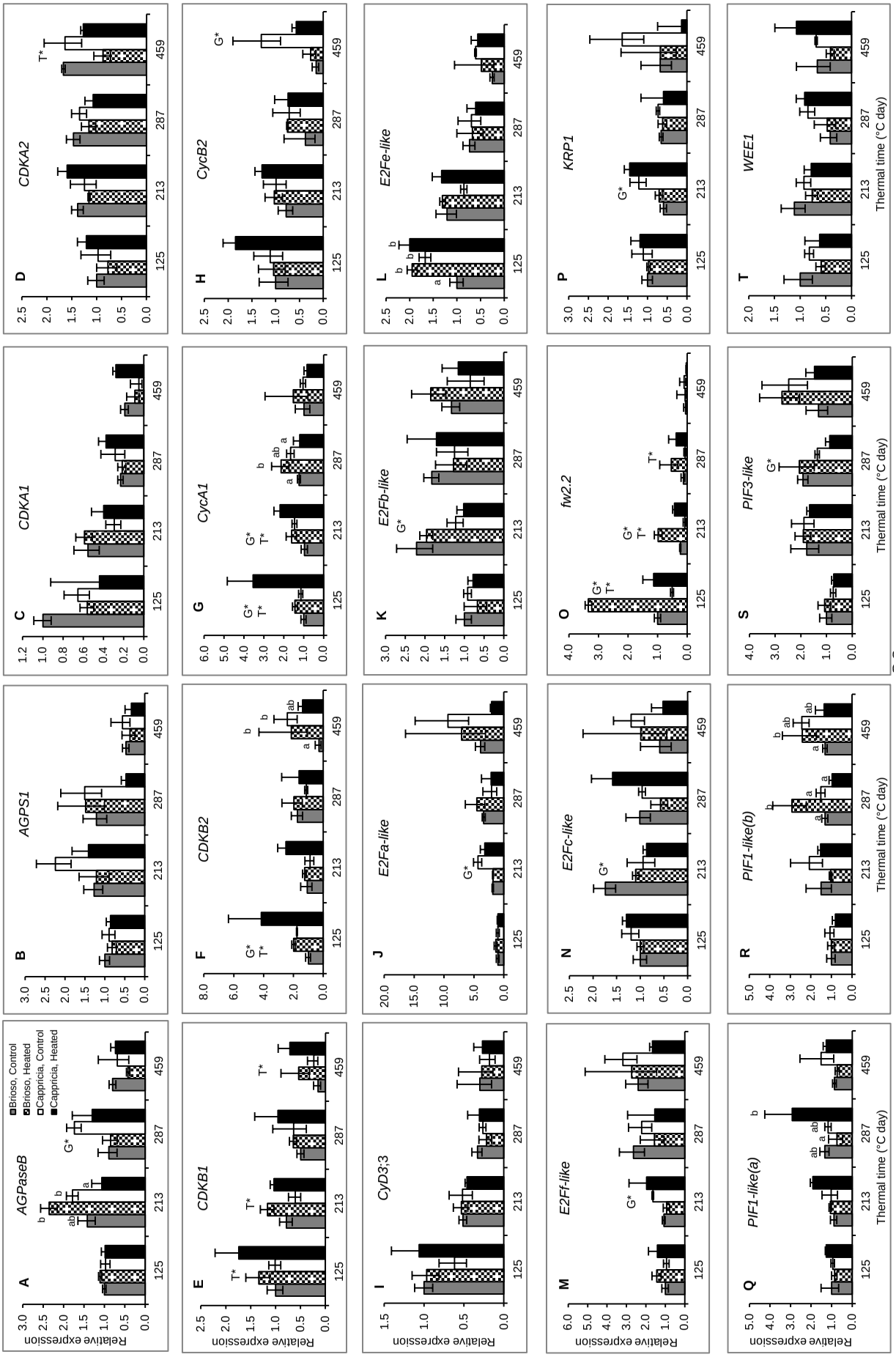
A AGPaseB
B AGPS1
C CDKA1
D CDKA2
E CDKB1
F CDKB2
G CycA1
H CycB2
I CyD3;3
J E2Fa-like
K E2Fb-like
L E2Fe-like
M E2Ff-like
N E2Fc-like
O fw2.2
P KRP1
Q PIF1-like(a)
R PIF1-like(b)
S PIF3-like
T WEE1
Brioso, Control
Brioso, Heated
Cappricia, Control
Cappricia, Heated
Relative expression
Thermal time (°C day)
125
213
287
459
G*
T*

Figure 4: Relative gene (A – B: promoters of cell expansion; C – L: promoters of cell division; M: inhibitor of cell expansion; N – T: inhibitors of cell division) expression in pericarp tissue of 'Brioso' and 'Cappricia' under control (21.4±0.8 °C) and heated (27.1±1.5 °C) conditions during the first four harvest stages when fruits were green. Breaker stage was attained at 711 and 907 °C day in 'Brioso' and 'Cappricia' respectively. Bars with different letters are indicative of means that significantly differ from each other (P = 0.05) at harvest stages where a significant cultivar × temperature interaction was found. Significant main treatment effects are indicated with an asterisk (G* = cultivars differ significantly; T* = significant temperature effect). Neither letters nor G* and T* have been indicated wherever there was no significant interaction or main treatment effect. Averages are based on three replicates and each was a pooled sample of pericarp tissue from two fruits. Means are relative to 'Brioso' under control temperature treatment. Bars are indicative of standard errors.

expression at 287 °C day was stimulated by fruit heating only in 'Cappricia' while the two cultivars showed opposite responses in *PIF1-like(b)* expression to fruit heating at 459 °C day. The expression level of *AGPaseB* did not respond to fruit heating in 'Brioso'; however, heated 'Cappricia' fruits had lower expression levels than control fruits.

Discussion

The aim of this study was to explore the mechanism for fruit size reduction at high temperature by relating observations at the fruit level with those at tissue, cell and gene level. The physiological basis for fruit size differences between an intermediate ('Cappricia') and small ('Brioso') sized tomato cultivar grown at two fruit temperatures was studied. Temperature treatments were aimed at introducing variation in fruit phenotype such that the basis for genetic and environmental variation in fruit size could be investigated simultaneously. Temperature treatments were applied at truss level, to avoid indirect effects caused by changes in the temperature of other plant parts. The organ-specific nature of the temperature treatments in this study was useful for studying organ level responses but not applicable in current commercial tomato production systems. Observations below the fruit level were restricted to pericarp tissue because it accounts for the largest proportion of fruit dry weight. For both cultivars, the pericarp contributed at least 65% of fruit dry weight during fruit development (Supporting information Figure S1).

Why do the two cultivars differ in fruit size?

A comparison of cultivar growth duration and rate effects on fruit fresh weight showed that the difference in fruit size between the two cultivars could be attributed to differences in growth rate (Table 1). The larger fruited 'Cappricia' took 8 days more (1.2 times longer) than

'Brioso' to reach breaker stage but at the moment of peak growth rate (inflection point), it grew approximately twice as fast as 'Brioso' (Figure 2). De Koning (1994) also concluded that fruit weight differences between a round ('Calypso') and beefsteak ('Dombito') tomato could be explained mainly by growth rate differences around the inflection point. Observations on relative growth rate (RGR) however, only tallied with fruit fresh weight differences between the two cultivars between 10 and 19 DAA (Figure 2C and D). At the cell level, large fruit size in 'Cappricia' was consistent with larger pericarp cell number and volume relative to 'Brioso' (Table 1). The difference between the two cultivars in pericarp cell number was 32% larger than in pericarp cell volume. This observation agrees with the conclusion by (Bertin et al., 2009) that quantitative trait loci (QTL) for fruit size were linked more to cell division than cell expansion processes.

Why were heated fruits smaller?

Temperature generally increases plant organ growth and development rate. Our observations under assimilate limitation show that locally heated tomato fruits grew faster than control fruits but over a 22% shorter period (9 days less) leading to smaller sized fruits at breaker stage (Figures 1A, 1C and Table 1). Similar findings were also reported by Adams et al. (2001) and Fanwoua et al. (2012a) in locally heated tomato fruits. The observed similarity in temperature response at the fruit level by 'Brioso' and 'Cappricia' in our study also corroborates the conclusion by Van Der Ploeg and Heuvelink (2005) of limited variation in temperature response among tomato cultivars.

An interesting observation was that the difference in fruit size between heated and control fruits appeared late during fruit development (Figures 1B and 1D). This was probably because of the positive effect of high temperature on fruit fresh weight growth rate and RGR (Figures 2A, 2B, 2C and 2D) during the early stages of development (till about 20 DAA or 400 °C day). It is possible that this boost in growth rate pushed the high temperature fresh weight growth curve closer to that of control fruits (Figures 1B, 1D and 2B) in the early stages of fruit development. Fresh weight growth rate decreased later during development in both heated and control fruits although the decrease seemed bigger in heated 'Cappricia' fruits (Figure 2B). RGR on the other hand consistently decreased in both cultivars as fruits matured, a trend similar to the one reported by Monselise et al. (1978). Control fruits were, therefore, bigger at breaker stage because of the longer growth duration and lower reduction in growth

rate late during fruit development compared to that in heated fruits. Higher maintenance respiration rate in heated fruits could also be another contributing factor.

These results suggest that increase in fruit temperature could bring about increase in fruit size if applied only at a time when fruits are ontogenetically programmed to experience increase in growth rate. Other authors, however, have proposed that high temperature during early fruit development has a larger effect on reducing fruit size than later high temperature treatments (De Koning, 1994; Bertin, 2005). Fruit growth modelling could be a useful tool in clarifying temperature effects at different development stages. Fanwoua et al. (2013) have simulated the effect of heating at different stages of development on tomato pericarp mass. Their simulations indeed showed that heating fruits only in the first 7 DAA results in a significantly higher pericarp mass compared to heating of fruits from 7 DAA until breaker stage.

The relationship between temperature effects at cell and fruit level

In agreement with results reported by other authors, exposure of fruits to high temperature in the current study led to a decrease in fruit size. It was our objective to explore the basis of this decrease in fruit size at the cell level. Our results revealed that the 24% decrease in heated fruit fresh weight was associated with a 40% decrease in mesocarp volume and a 29% increase in number of mesocarp cells although the difference in mesocarp cell number was just not significant (P = 0.066; Table 1). We assumed that fruit size would be determined mainly by mesocarp cell growth dynamics since the mesocarp constituted 99% of pericarp volume in both cultivars. This suggests that the negative effect of heating on fruit size was dominated by the negative effect on mesocarp cell volume rather than the positive effect on mesocarp cell number. In two separate experiments conducted with round tomato, Fanwoua et al. (2012a) also observed that continuous fruit heating decreased pericarp cell volume in one experiment while no significant effects were observed in the other. The observed tendency towards increase in mesocarp cell number at high temperature could be a result of shortening of the cell division cycle as also noted by Tardieu and Granier (2000) in many plant species grown at high temperature.

An important question was whether temperature mediated cell volume decrease occurred proportionately in all directions. Fanwoua et al. (2012a) also highlighted the need to observe cell expansion dynamics in more than one direction. Their observations revealed that continuous heating of tomato fruits relative to control at 20 °C decreased cell expansion in the

anticlinal more than in the periclinal plane. Our results on the contrary showed a similar decrease in both anticlinal and periclinal cell diameter when fruits were heated (Table S2). This could suggest that cell volume decrease in different planes as a result of temperature increase can be uniform or larger in one of the planes depending on the cultivar.

Cell number and volume as a consequence of carbohydrates

Variations in temperature can alter the rate of assimilate import (Linck and Swanson, 1960; Greiger, 1966; Moorby et al., 1974). Assimilate partitioning to fruits also seems to be genotype dependent. Ho (1996) concluded that cherry tomato partition a lower fraction of plant dry matter into fruits compared to cultivars with larger sized fruits. Leaf and stem measurements conducted on plants at the end of our experiment (data not shown) support this observation. The smaller fruited 'Brioso' had 18% more vegetative dry weight and 25% more leaf area compared to 'Cappricia'. One of the aims of the current study was to relate observations on cell number with those on the concentration of carbohydrates. It is likely that the increase in cell division in 'Cappricia' was stimulated by the higher hexose concentration compared to that in 'Brioso' during the cell division phase (until 300 °C day; Figures 3B and 3C). The tendency towards higher sucrose and hexose concentration in heated fruits at the beginning of fruit growth (until about 600 °C day) also supports this argument since mesocarp cell number and layers tended to be higher in heated than in control fruits (Table 1 and Figures 3E, 3F, and 3G).

Cell volume is a function of both carbohydrate and water content. The accumulation of carbon and water during the cell expansion phase causes an 11 000 fold increase in initial cell size in tomato (Cheniclet et al., 2005). Mesocarp cell volume was lower at high temperature and also in 'Brioso'. It is not clear why the slightly higher fructose and glucose concentration in 'Brioso' did not result in a larger cell volume compared to that in 'Cappricia' (Table 1). A possible explanation could be that small and large cell sized genotypes inherently maintain different carbohydrate concentrations. Low cell volume at high temperature could not be explained by carbohydrate concentrations since no significant effects of temperature treatments were detected on the concentration of all carbohydrates analysed at breaker stage. Gautier et al. (2008) also found no significant effect of increasing fruit temperature (21 °C to 26 °C) on final hexose content during ripening of harvested mature green tomato fruits.

Gene expression

Genetic regulation of tomato fruit growth has been a subject of interest in many studies in the past (Baldet et al., 2006; Bertin et al., 2009; Prudent et al., 2010; Fanwoua et al., 2012b; Czerednik et al., 2012). Fanwoua et al. (2012b) studied the expression of cell cycle genes in fruits of inbred lines obtained from a cross between *Solanum lycopersicum* L. 'Moneyberg' and *Solanum chmielewskii*. Surprisingly, they observed higher expression of *CDKB1* and *CycD3* in pericarp of the smaller fruited (g36) than in the large fruited genotype (g49). They reported no significant difference in *CycB2* expression between these two genotypes**.** In agreement with the findings of Fanwoua et al. (2012b), large fruit size in the current study tallied with increase in cell number. However, *CycB2* expression was higher in 'Cappricia' although the difference was only statistically significant at 459 °C Days. On the contrary, the current study showed no significant differences in expression of *CDKB1* or *CycD3;3* between 'Brioso' and 'Cappricia' (Figure 4). Higher expression of cell cycle promoters (*CDKB2, CycA1*, *E2Fa-like,* and *E2Fe-like*) in 'Cappricia' during early fruit growth tallied with the higher number of cells observed in 'Cappricia' fruits. Surprising, expression of the cell cycle promoting transcription factor: *E2Fb-like* was higher in 'Brioso' than in 'Cappricia'.

Higher expression of the cell cycle inhibitor *KRP1* during early fruit growth was an unexpected observation in 'Cappricia'. It is also surprising that no clear genotypic differences were observed in the expression of *CDKA1, CDKA2*, *CycD3;3, PIF1-like(a), PIF1-like(b),* and *WEE1*. These findings suggest that different cell division regulatory mechanisms may exist in different genotypes and hence cell number differences between any given pair of genotypes may not be as a result of the same set of genes. It is likely that posttranscriptional regulation plays an important role in instances in which gene expression profiles did not tally with cell and fruit level differences. However, transcript levels of some genes may still be consistently linked with differences in fruit size. For example, in agreement with the low cell number and small fruit size observed in 'Brioso', expression of cell division inhibitors: *fw2.2* (Frary et al., 2000), *E2Fc-like* and *PIF3-like* (287 °C day) was higher in 'Brioso'. Some genotypic differences may also arise under specific conditions, for example a decrease in expression of the cell division inhibitor *PIF1-like(b)* was observed in heated 'Cappricia' fruits.

Studies on the influence of assimilate availability on expression of key cell cycle promoting genes have shown an increasing trend in gene expression under non-limiting assimilate conditions in tomato. These studies show increase in expression of cell division

promoters: *CDKB2;1*, *CycB2;1* (Joubès et al., 2000a; Joubès et al., 2000b; Baldet et al., 2002; Baldet et al., 2006) and *CycD3;1* (Baldet et al., 2002; Dewitte and Murray, 2003) but a decrease in expression of a cell division inhibitor; *KRP1* (Menges and Murray, 2002) when assimilates were not limiting. The current study is the first attempt to unravel temperature effects on cell cycle regulatory genes in tomato fruit. Given the tendency towards a higher cell number and layers in the mesocarp of heated fruits, it is not surprizing that *CDKB1*, *CycA1* and *CDKB2* (only in some stages of fruit development) expression during early fruit growth was lower in control fruits. Higher expression of the cell division inhibitor: *fw2.2* in heated fruits was an unexpected finding. It is possible that the positive effect of heating on expression of *CDKB1*, *CDKB2* and *CycA1* together with other cell cycle promoting genes not analysed in this study outweighed the negative effects of *fw2.2* on cell division. However, high expression of *fw2.2* in heated compared to control and in 'Brioso' compared to 'Cappricia' fruits confirms the observation by Liu et al. (2003) and Cong et al. (2002) that high *fw2.2* transcript levels are negatively correlated with fruit mass. Cong et al. (2002) further observed in two tomato nearly isogenic lines (NIL) that the negative correlation between *fw2.2* transcript levels and fruit mass stemmed from high expression levels that suppress cell division over a prolonged period during fruit growth. They noted that expression of *fw2.2* peaked earlier and for a shorter period in the large fruited than in the small fruited NIL. It is likely that the difference in cell number and subsequently fruit size between the two cultivars in the current study arose from differences in expression levels since the shift in the duration of peak expression was not apparent in the two cultivars (Figure 4O).

Three genes (*AGPaseB*, *AGPS1* and *E2Ff*) associated with cell size regulation were observed in the current study. *AGPaseB* and *AGPS1* code for two subunits of ADP Glucose Pyrophosphorylase (ADPGPP) which is a key enzyme in starch biosynthesis (Schaffer and Petreikov, 2007). Our results showed no significant cultivar and temperature treatment responses in *AGPS1* expression. However, the relatively higher expression of *AGPaseB* in 'Cappricia' during cell expansion (287 °C day) agrees with the larger cell size observed in 'Cappricia' compared to 'Brioso'. It is not exactly clear why heating led to low expression of *AGPaseB* in 'Cappricia' at 213 °C day while no significant change was observed in heated 'Brioso' fruits at the same harvest stage. *E2Ff* is a repressor of genes involved in cell wall biosynthesis during cell elongation (Kosugi and Ohashi, 2002). Expression of *E2Ff* was expected to be higher in heated and 'Brioso' fruits since fruits from these two treatments had a lower cell volume compared to control and 'Cappricia' fruits. The expression of *E2Ff* was

surprisingly higher in ‘Cappricia’ at 213 °C day. These contrasting gene level observations show the complexity in regulation of cell division and cell expansion. A better understanding could be achieved with studies that combine transcriptional and posttranscriptional analyses.

Conclusion

This study aimed at understanding the physiological mechanisms for differences in fruit size of a small and intermediate sized tomato cultivar and also investigated their response to two temperature treatments. Our results show that differences in growth rate were more important than growth duration differences in determination of final fruit fresh weight differences between the two contrasting cultivars. At the cell level, it was observed that the two cultivars differed in fruit size mainly because of differences in mesocarp cell number. We however, noted that the reduction in fruit size at high temperature arose from reduction in cell volume and this occurred despite the 29% increase in cell number. At the gene level, expression of three promoters (*CDKB1*, *CDKB2* and *CycA1*) and one inhibitor (*fw2.2*) of cell division was stimulated by fruit heating early during fruit development. Larger cell number in ‘Cappricia’ compared to ‘Brioso’ tallied with higher expression of two cell cycle promoters (*CDKB2*, *CyCA1,* and *E2Fe*) and lower expression of *fw2.2*. Other than the higher expression of *AGPaseB* in ‘Cappricia’ at only one harvest stage, the expression of genes involved in promotion or inhibition of cell expansion did not tally with cell size observations in this study. The apparent mismatch between expression tendencies of some genes and cell and fruit level observations highlights the importance of downstream posttranscriptional regulatory mechanisms in fruit phenotype determination.

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Supplementary material

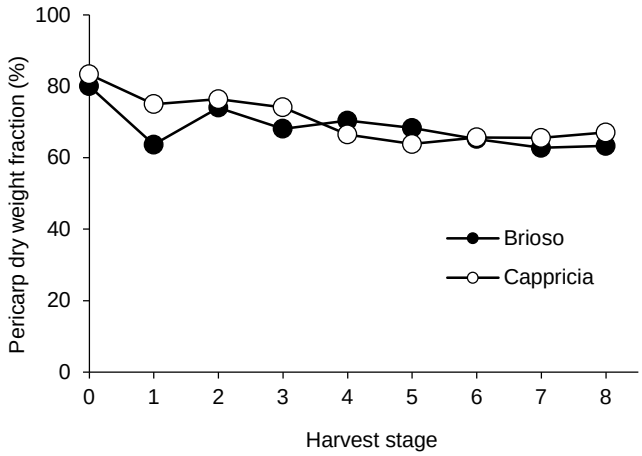

Figure S1: Pericarp contribution to total fruit dry weight expressed as a percentage. Dry weight measurements were conducted on fruits of 'Brioso' and 'Cappricia' that had been harvested at various development stages between anthesis (0) and breaker stage (8).

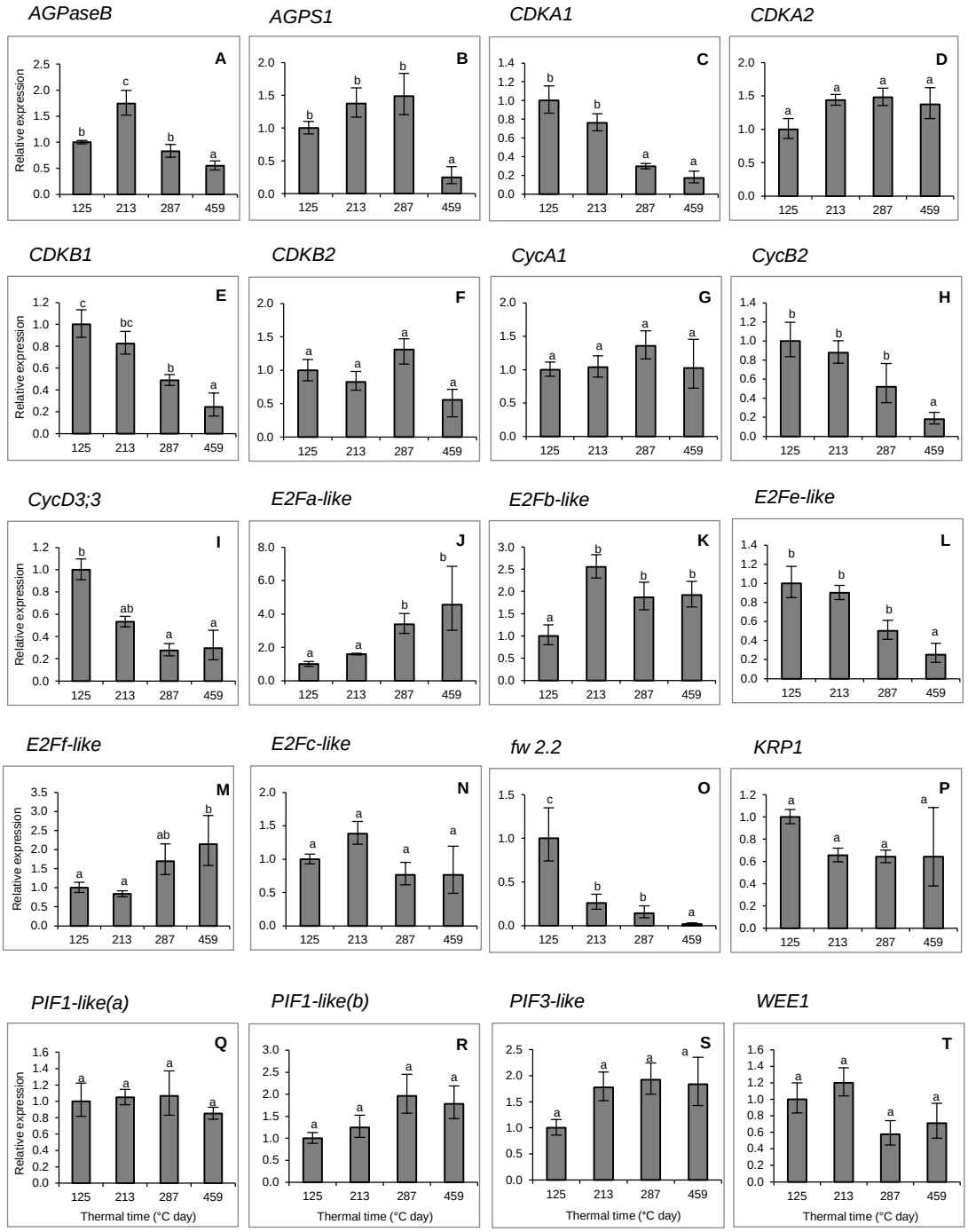

Figure S2: Relative gene (A – B: promoters of cell expansion; C – L: promoters of cell division; M: inhibitor of cell expansion; N – T: inhibitors of cell division) expression in pericarp tissue of 'Brioso' averaged over the two temperature treatments (Control; 21.4±0.8 °C and Heated; 27.1±1.5 °C) during the first four harvest stages when fruits were green (Breaker stage was attained at 711 °C day). Bars followed by different letters significantly differ from each other (P = 0.05). n = 3 at a given harvest point but a single replicate was a pooled sample of pericarp tissue from two fruits. Means are relative to the expression level at 125 °C day. Bars are indicative of standard errors.

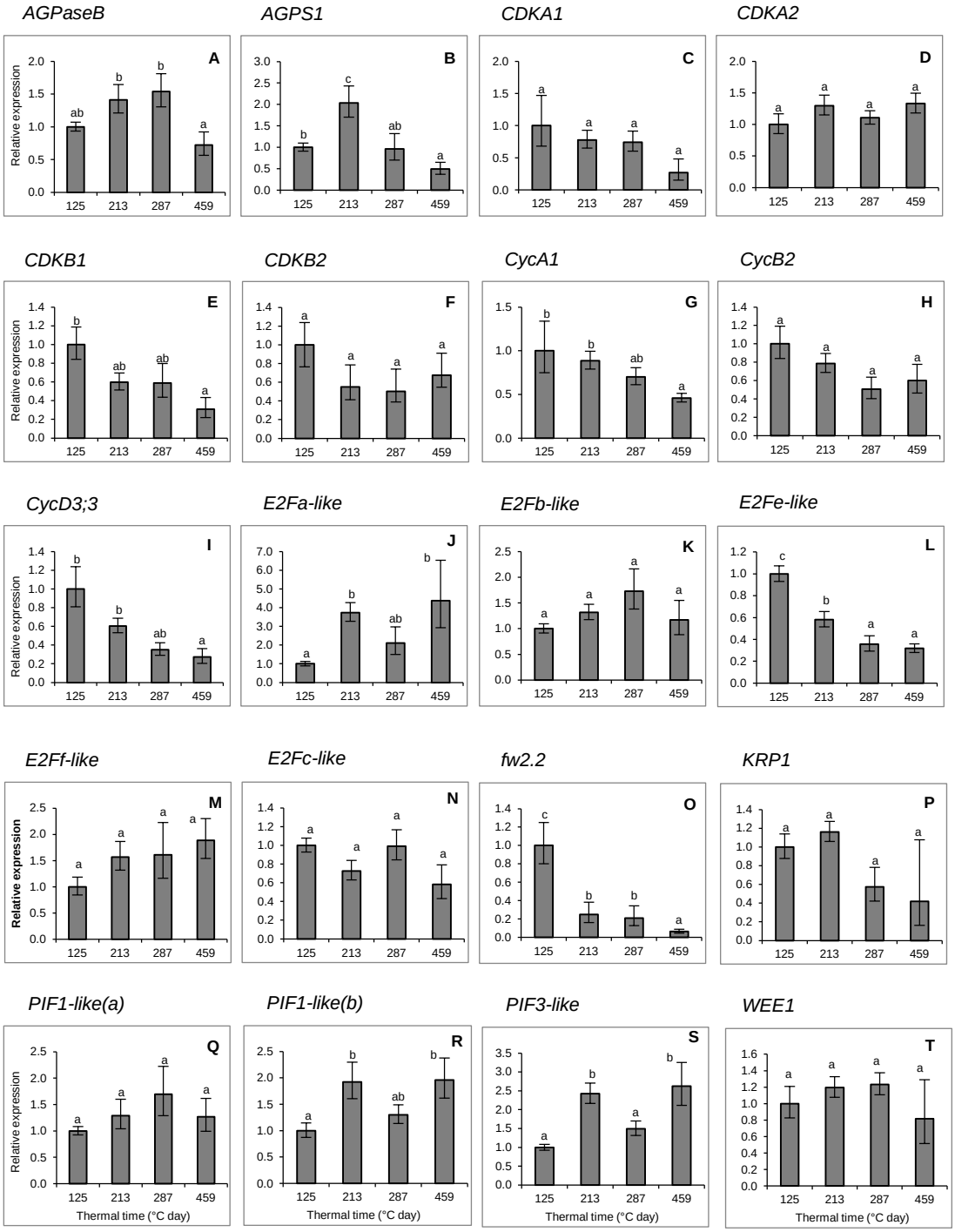

Figure S3: Relative gene (A – B: promoters of cell expansion; C – L: promoters of cell division; M: inhibitor of cell expansion; N – T: inhibitors of cell division) expression in pericarp tissue of 'Cappricia' averaged over the two temperature treatments (Control; 21.4±0.8 °C and Heated; 27.1±1.5 °C) during the first four harvest stages when fruits were green (Breaker stage was attained at 907 °C day). Bars followed by different letters significantly differ from each other (P = 0.05). n = 3 at a given harvest point but a single replicate was a pooled sample of pericarp tissue from two fruits. Means are relative to the expression level at 125 °C day. Bars are indicative of standard errors.

Table S1: Tomato gene names and primer sequences. Gene names followed by *–like* are putative orthologs of *Arabidopsis thaliana* genes identified through phylogenetic comparison of coded protein sequences for the two species. *PIF1-like(a)* and *PIF1-like(b)* are co-orthologs.

Tomato gene name	iTAG2.31	Genbank Accession Number	Forward (F) and reverse (R) primer sequences
AGPaseB	Solyc07g056140	NP_001234696	F 5'-CTGTGGTTGTTTGAGGAGCA-3' R 5'-TCCACTTTCATTGCTTGCAG-3'
AGPS1	Solyc01g109790	AAC49941	F 5'-CGCTCACACAAGAGTTTCCA-3' R 5'-CGCGATCTTTCACCCACTAT-3'
CDKA1	Solyc08g066330	Y17225	F 5'-AACCCCTGAATAGAACCAAATG-3' R 5'-GTATGTGCCGTGATTGTCTG-3'
CDKA2	Solyc12g091860	Y17226	F 5'-AAGAGAATCACTGCCCGAAG-3' R 5'-AACAGATTGGATGTCATTGGAG-3'
CDKB1	Solyc10g074720	CAC15503	F 5'-GGGATGTATTTTTGCCGAGA-3' R 5'-GAACAGCAGAGGCCAAGTTC-3'
CDKB2	Solyc04g082840	CAC15504	F 5'-ATGCTGGTAAGAGTGTATCGG-3' R 5'-CGGAGAGTAGTTGGAGGAAC-3'
CycA1	Solyc11g005090	CAB46641	F 5'-GCCAGGGAGATAATGTGAGAAG-3' R 5'-CAAACAAAGATGCTCTGCTAAGG-3'
CycB2	Solyc02g082820	CAB46645	F 5'-CTGGTGGAGGATCTGGTGTT-3' R 5'-GTCACATTGAGCAGCCTTGA-3'
CycD3;3	Solyc04g078470	CAB60838	F 5'-CTTGTTGCTGTTACTTGTCTTTC-3' R 5'-AATGGTGTTACTGGATTCATCTTC-3'
E2Fa-like	Solyc01g007760	XP_004228901	F 5'-CAAGCTGCTGACACTTTGGA-3' R 5'-TTCGCTCCTCCACTGAAAGT-3'
E2Fb-like	Solyc06g074010	XP_004242116	F 5'-TCCAGTAGGTCCTTGCTGCT-3' R 5'-TCCCCTGGTCTTGAGACATC-3'
E2Fc-like	Solyc04g081350	XP_004238619	F 5'-CACAACAGGGCCGATAGATT-3' R 5'-TTTTATGGACACCCGAGAGC-3'
E2Fe-like	Solyc03g113760	XP_004236152	F 5'-AAGCTCTTCCTCTGCACCAA-3' R 5'-CGTCTCTGGATGATGGGTCT-3'
E2Ff-like	Solyc02g087310	XP_004232256	F 5'-ACGATCCGATGGCTATGAAG-3' R 5'-AGGGACCCACTCCTCAGATT-3'
fw2.2	Solyc02g090730	AAO12185	F 5'-GGTGGTCGACTGGTCTTTGT-3' R 5'-AGGCTAGGCAATCCTGTCAA-3'
KRP1	Solyc02g090680	CAD29648	F 5'-GGAAGAAGCGTGATGGTGAT-3' R 5'-TTCCACACTGTCCTCATCCA-3'
PIF1-like(a)	Solyc07g043580	XP_004243631	F 5'-AATCAAGCAGCTGCAATGTG-3' R 5'-TGCGGTAACTGCTGAGTTTG-3'
PIF1-like(b)	Solyc09g063010	XP_004247109	F 5'-GATGCGGTTATTCCCTCTGA-3' R 5'-GTAGAAGTGGGCGTGGGATA-3'
PIF3-like	Solyc01g102300	XP_004230368	F 5'-ATGGGATTTGGGTTGGGTAT-3' R 5'-AGTCCTTGACCAGGATGTGC-3'
SAND	Solyc03g115810	XP_004235972	F 5'-TTGCTTGGAGGAACAGACG-3' F 5'-GCAAACAGAACCCCTGAATC-3'
WEE1	Solyc09g074830	CAJ56085	F 5'-AAGAGCCAGCCAATTGAAGA-3' R 5'-GGTCCATCATTGCCTTGAGT-3'

Table S2: Fruit phenotype (tissue and cell level) at breaker stage for control (21.4±0.8 °C) and heated (27.1±1.5 °C) 'Brioso' and 'Cappricia'. Means of cultivars or temperature within a row followed by different letters differ significantly (P <0.05).

	Brioso[1]	Cappricia[1]	P value	Control[2]	Heated[2]	P value	Brioso		Cappricia		P value
							Control	Heated	Control	Heated	
Exocarp thickness (mm)	0.070 **a**	0.070 **a**	0.888	0.075 **a**	0.065 **a**	0.058	0.074	0.066	0.076	0.065	0.597
Exocarp anticlinal cell diameter (mm)	0.014 **a**	0.014 **a**	0.888	0.015 **a**	0.013 **a**	0.058	0.015	0.013	0.015	0.013	0.597
Exocarp periclinal cell diameter (mm)							0.075 **b**	0.064 **a**	0.073 **ab**	0.071 **b**	0.012
Mesocarp thickness (mm)	5.03 **a**	6.96 **b**	<0.001	5.79 **a**	6.10 **a**	0.769	5.04	5.02	6.73	7.18	0.368
Mesocarp anticlinal cell diameter (mm)	0.242 **a**	0.299 **b**	<0.001	0.281 **b**	0.255 **a**	0.020	0.253	0.227	0.316	0.283	0.603
Mesocarp periclinal cell diameter (mm)	0.098 **a**	0.095 **a**	0.921	0.106 **b**	0.087 **a**	0.003	0.106	0.087	0.105	0.086	0.718
Pericarp thickness (mm)	5.09 **a**	7.03 **b**	<0.001	5.86 **a**	6.17 **a**	0.795	5.11	5.09	6.81	7.25	0.371

[1] Means within the same column have been averaged over the two temperature treatments
[2] Means within the same column have been averaged over the two cultivars

Appendix 1: Schematic representation of parameters used in histological calculations as adapted from Figure 2 in Fanwoua et al. (2012a), reproduced below.

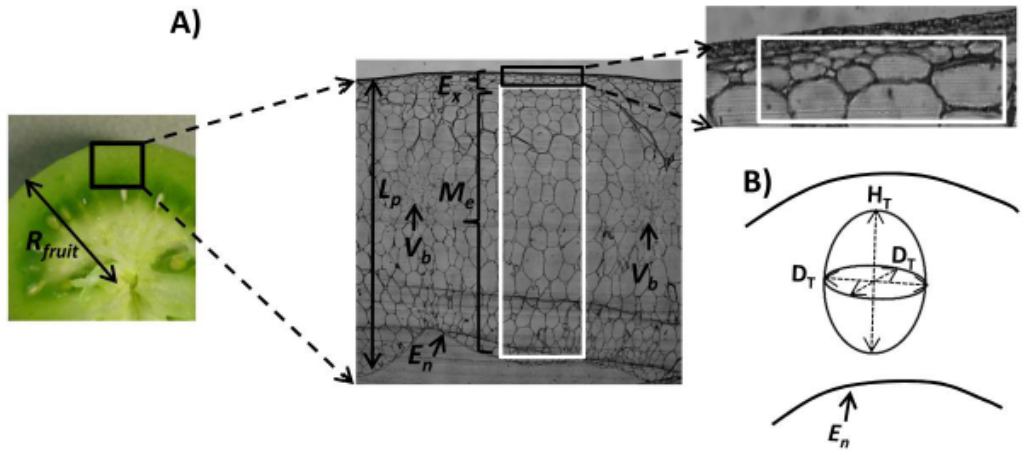

Fig. 2. Structure of tomato pericarp at breaker stage: A) the white rectangles are examples of the region within which mean cell area was estimated in the exocarp and mesocarp as detailed in the materials and methods; B) schematic representation of the ellipsoid cell shape in the pericarp. R_{fruit} 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.

Chapter 3

Fruit illumination stimulates cell division but has no detectable effect on fruit size

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Abstract

Light affects plant growth through assimilate availability and signals regulating development. The effects of light on tomato fruit growth were studied using cuvettes with light-emitting diodes providing white, red or blue light to individual tomato trusses for different periods during the day. Hypotheses tested were as follows: 1) light-grown fruits are stronger assimilate sinks than dark-grown fruits, and 2) responses depend on light treatment provided, and fruit development stage. Seven light treatments (dark, 12 h white, 24 h white, 24 h red, 24 h blue, dark in the first 24 days after anthesis (DAA) followed by 24 h white light until breaker stage, and its reverse) were applied. Observations were conducted between anthesis and breaker stage at fruit, cell, and gene level. Fruit size and carbohydrate content did not respond to light treatments while cell division was strongly stimulated at the expense of cell expansion by light. The effects of light on cell number and volume were independent of the combination of light color and intensity. Increased cell division and decreased cell volume when fruits were grown in the presence of light were not clearly corroborated by the expression pattern of promoters and inhibitors of cell division and expansion analyzed in this study implying a strong effect of posttranscriptional regulation. Results suggest the existence of a complex homeostatic regulatory system for fruit growth in which reduced cell division is compensated by enhanced cell expansion.

Keywords: light emitting diodes, partitioning, cyclin, cyclin dependent kinase, cell expansion

Introduction

Tomato (*Solanum lycopersicum*) is an important horticultural crop and model plant for berry fruits (Kimura and Sinha, 2008). Its fruit consists of pulp containing seeds within a thick pericarp. The latter develops from the ovary wall following successful fertilization (Gillaspy et al., 1993) and accounts for at least two thirds of total fruit dry weight (Ho and Hewitt, 1986). Most pericarp cells contain large vacuoles and are morphologically similar to leaf palisade cells (Gillaspy et al., 1993). The photosynthetic activity of these cells with chloroplasts satisfies up to 15% of the fruit's assimilate demands (Hetherington et al., 1998). The rest of the fruits' assimilates are imported as sucrose from the leaves. On reaching the fruit, sucrose is hydrolyzed into glucose and fructose by invertase (Yelle et al., 1988; Frommer and Sonnewald, 1995). Assimilate storage also occurs in the fruit, mainly as starch. The rate of starch accumulation increases during initial fruit growth up to a peak following a trend similar to that for the fruit growth rate (Ho et al., 1983; Ho, 1984) and then decreases to undetectable levels at breaker stage. Sucrose import decreases significantly after the period of peak starch accumulation rate and is accompanied by the breakdown of starch into hexoses (glucose and fructose). Accumulation of hexoses increases fruit osmotic potential leading to water uptake and increase in fruit size (Kaldenhoff et al., 2008). Some wild tomato species accumulate sucrose instead of starch during initial fruit development (Yelle et al., 1988; Yelle et al., 1991).

The amount of sucrose imported by a given fruit depends on its competitive ability to attract assimilates, i.e. its sink strength (Farrar, 1993; Marcelis, 1996). Fruit sink strength is determined by size and metabolic activity of the fruit (Farrar 1993; Marcelis, 1996). Environmental factors including temperature and light also affect fruit sink strength. In an *in vitro* experiment, Guan and Janes (1991) observed 45% more import of sucrose over a period of 10 hours, in 2-week-old tomato fruits grown in white light (18 h) than in dark-grown fruits. They attributed this increase in sucrose import to a surge in starch accumulation through ADP Glucose Pyrophosphorylase activation by light. They also noted 65% more dry weight in light-grown than in dark-grown fruits after 42 days. Carbohydrates are important signaling molecules in many biological processes (Hanson and Smeekens, 2009; Wang and Ruan, 2013). A typical example is the stimulation of cell division through a sucrose dependent increase in expression of cyclins: *CycD3;1* involved in cell cycle regulation (Planchais et al., 2004) and CycD4;1 in root pericycle cells during lateral root formation (Nieuwland et al., 2009).

Light conditions influence endoreduplication i.e. the increase in a cell's DNA content without cell division. Gendreau et al. (1997, 1998) concluded that the elongation of etiolated hypocotyls of dark grown Arabidopsis thaliana was caused by an extra endocycle. Berckmans et al. (2011) further proposed that activation of the endocycle repressor DEL1/E2Fe by its transcription factor E2Fb in the presence of light prevents the occurrence of more endocycles. In the dark, E2Fc is said to inhibit the expression of *DEL1* by occupying its promoter region. In another study with blue and red light, Dougher and Bugbee (2004) concluded that an increase in the blue light fraction decreases internode length of soybean through inhibition of cell division while, oppositely, leaf area decreased because of reduced cell expansion. In contrast, they observed leaf area increase in lettuce when the fraction of blue light was increased. This increase in leaf area was attributed to an increase in both cell division and expansion.

To the best of our knowledge, no earlier studies have investigated the effect of light treatments of tomato fruits *in vivo* on their growth. The study by Guan and Janes (1991) showed that lighting of *in vitro* grown tomato fruits increased their sink strength and thereby their sugar content and size. In commercial tomato production, trusses are typically shaded by the leaves and the light environment is changed in both quality and quantity. The aim of this work, therefore, was to study light effects on tomato fruit growth without exposing vegetative organs to the light treatments. Such treatments impact fruit growth with minimum effect on whole plant responses. It was hypothesized that light increases sucrose import by the fruits and that the effect depends on the type of light treatment and phase during fruit development. Light treatments were allotted to trusses enclosed in climate controlled cuvettes from anthesis to breaker stage. Observations at the fruit level were related with those at cell and gene level in order to provide a mechanistic explanation of resulting fruit phenotypes.

Materials and methods

Plant material and growth conditions

Two experiments were conducted with *Solanum lycopersicum* L. cv. 'Komeett' (Monsanto Vegetable Seeds, Bergschenhoek, the Netherlands) grafted on 'Maxifort' (Monsanto Vegetable Seeds, Bergschenhoek, the Netherlands). Experiment I was conducted in summer 2011 and Experiment II from spring to summer 2012 in the same greenhouse in Bleiswijk, the Netherlands. Planting date (4 weeks after germination) for both experiments was December

23rd of the preceding year. Plants were grown on Rockwool® slabs in a 500 m^2 Venlo glasshouse with a light transmittance of 67% and no artificial light installation. Average carbon dioxide level during the day in Experiment I was 560 ppm, relative air humidity was on average 80% while average 24 h temperature was 19 °C. During Experiment II, average day-time carbon dioxide level, relative air humidity and average 24 h temperature were 634 ppm, 76% and 19 °C, respectively. Planting density was 2.55 plants m^{-2} but plants were maintained on a high wire system with one or two stems per plant leading to a density of 3.83 stems m^{-2}. Experimental plants had only one stem. All trusses were pruned to three fruits per truss at 32 (Experiment I) or 25 (Experiment II) weeks after planting to ensure that fruits would grow under unlimited assimilate supply. Thereafter, all trusses were pruned to three flowers per truss at anthesis of the first flower. One week later, treatment cuvettes were attached to trusses pruned at this early stage.

Treatment application

In Experiment I, three light treatments were applied to the fruits: 24 h dark (dark), 24 h white light (24 h) and control. The control consisted of fruits growing under natural light conditions and photoperiod. Experiment II also had three light treatments as in Experiment I except that the control was replaced by 12 h white light (12 h; lights were switched on between 07:00 and 19:00). In addition, four other treatments were included: 24 h blue (B), 24 h red (R) light, darkness during the first 24 days after anthesis (DAA) followed by 24 h white light until breaker stage (DL) and 24 h white light in the first 24 DAA and then darkness until breaker stage (LD). Average 24 h greenhouse air temperature during the first 24 DAA and between 24 DAA and breaker stage for the DL and LD treatments were 20.15±0.17 °C and 20.56±0.44 °C respectively. In both experiments, treatments were applied using a completely randomized design to a single truss per plant using transparent Perspex cuvettes (length: 40 cm; diameter: 13 cm; WSV Kunststoffen, Utrecht, The Netherlands). There were six replicate plants per treatment. Each cuvette had a funnel attached to the front and was covered at the back with a transparent Petri-dish (diameter: 14 cm). The Petri-dish had a 1.5 cm wide slit made from its edge to the center. A 12 V ventilator (40 l min^{-1}) was attached on top of a 2.4 cm wide round opening on the Petri-dish to continuously replenish air inside the cuvette with that from the greenhouse and thus ensure similarity in cuvette and greenhouse air temperature (Figure 1).

Control treatment cuvettes in Experiment I had an external iron frame with aluminum foil attached 5 cm above and to only half of the cuvette surface to prevent direct sunlight from heating the fruit. Dark treatment cuvettes on the other hand had their entire surfaces covered

with a layer of black plastic followed by aluminum foil. A flexible tube (length: 42 cm; diameter: 1 cm) covered with black plastic and aluminum foil, like the cuvette, was attached to the funnel side of the cuvette. The tube was bent to face the greenhouse floor and its bottom covered with black cloth to ensure that external light did not enter the cuvette. The 24 h treatment cuvettes were similar to dark treatment cuvettes except that there was a layer of aluminum foil directly on the cuvette surface before the black plastic layer. In addition, a slit (length: 5 cm; width: 1.5 cm) was made through all the aluminum and black plastic layers and a strip of light emitting diodes (LEDs; 88Light, Dordrecht, The Netherlands) of similar dimension attached on the upper side of the cuvette. The inner aluminum foil improved light distribution through reflection of light from the LEDs in all directions within the cuvette. The LEDs provided white light with two major peaks around 450 nm and 560 nm (Supplementary material Figure S1). PAR measured with a single channel Jaz spectrometer (Ocean Optics, Duiven, The Netherlands) at the cuvette center was 66 µmol m^{-2} s^{-1}. Blue and red light treatment cuvettes were prepared just like the 24 h white light cuvettes but the white LEDs were replaced with monochromatic 30 µmol m^{-2} s^{-1} blue or 15 µmol m^{-2} s^{-1} red LED (Supplementary material Figure S1).

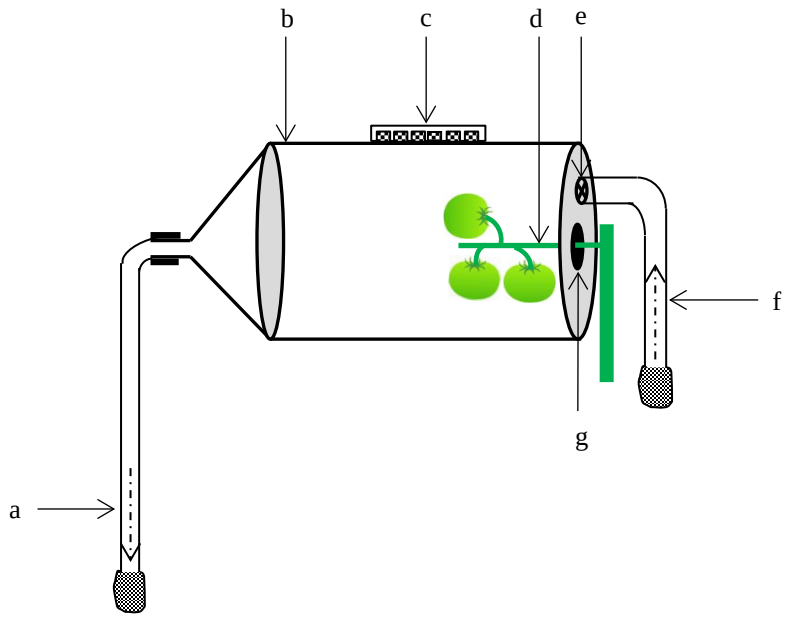

Figure 1: Schematic representation of the cuvette system used to illuminate trusses (a = air outlet pipe covered with black cloth at exit point; b = Perspex cuvette; c = strip of light emitting diodes; d = peduncle with 3 fruits; e = ventilation fan; f = air inlet pipe covered with black cloth at the entry point; g = Terostat VII® seal around peduncle). Dotted arrows indicate the direction of air flow.

In order to attach cuvettes, Petri-dishes were taped on to trusses across the 1.5 cm wide slit at anthesis of the first flower on the truss. Terostat VII® (Henkel Teroson GmbH,

Heidelberg, Germany) was used to cushion and seal the gap between the peduncle and slit in the Petri-dish. The Petri-dishes were covered with the same layer material as described for the cuvettes. For the dark and light treatments, a black polyethylene tube (length: 31 cm; diameter: 2.5 cm) covered with aluminum foil and bent 90° at the first 8 cm was then attached to the Petri-dish opening to which a ventilator was attached using Terostat VII®. The other end of the tube was covered with black cloth (Figure 1) ensuring that the 24 h dark cuvettes remained completely dark while insignificant amounts of light from 24 h light treatments could escape to the outside. Cuvettes hanging on strings attached to the trellis system were then connected on to the Petri-dishes using cello tape. Cuvettes were vibrated by hand to enhance pollination once a day for the next five days. Cuvettes were attached at truss position 27 to 30 (Experiment I) or 20 to 23 (Experiment II). There was variation in experimental truss position because cuvettes were moved to a higher position in situations where trusses were damaged or less than two fruits had set on the truss. Fruit set was observed through a window in the cuvette that could be sealed without compromising the treatments. Trusses below the experimental truss were harvested when mature (breaker stage) and this ensured that there were always seven other trusses on the plant at the time when cuvettes were attached.

Air temperature from eight cuvettes of each treatment was measured after the end of Experiment I over a period of 45 h. Half (4) of the cuvettes from which air temperature measurements were conducted for each treatment were placed 1.2 m below the crop canopy while the other half were located 1.2 m below the top cuvettes. Air temperature inside the 24 h light cuvettes from Experiment I was on average 0.5 °C and 1 °C higher than in the dark and control cuvettes, respectively. For Experiment II, cuvette air temperature was measured during the experiment over a period of 45 days. The 24 h light treatment cuvettes were 1.3 °C and 0.3 °C warmer than dark and control treatment cuvettes respectively. Air temperature of 24 h white light cuvettes was 0.9 °C and 1.1 °C higher than that of red and blue light cuvettes respectively.

Measurements

Fruit level

In Experiment I, the first and second proximal fruits were harvested at 24 DAA or when fruits had just turned red over the entire fruit surface (breaker stage). Harvests at breaker stage were conducted after evaluation through a window (capable of being completely sealed) on the

surface of cuvettes. In Experiment II, the first, second and third proximal fruits were harvested at 6, 12, 18, 24, 35, and 45 DAA, and breaker stage. Fruits were wrapped in aluminum foil and placed in ice immediately after harvest before fresh weight and horizontal plane diameter (measured with a digital caliper) measurements were conducted on fruits 1 and 2 (Experiment I) or fruits 1, 2, and 3 (Experiment II). Fruit dry weight was estimated based on dry matter content of half of fruit 1 (Experiment I) or whole fruit 1 (Experiment II). The dry weight was measured after oven drying at 70 °C for 24 h followed by 105 °C for 48 h. After fresh weight and diameter measurements, an equatorial section was made to split fruit samples into two equal halves. Pericarp tissue from each half was separated from locular tissue and seeds. One half of the pericarp from a given fruit was used in histological measurements while the other half was again split into two samples for carbohydrate and gene expression analyses. Samples for carbohydrate and gene expression analyses were stored at -80 °C within 1.5 h after harvest. Pericarp tissue was selected for more detailed analyses because it accounts at least 65% of fruit dry weight.

Histological, carbohydrate and gene expression analyses

Histological, carbohydrate and gene expression analyses were conducted using pericarp tissue. Samples for histological analysis were harvested at breaker stage and fixed in a solution containing ethanol (96%), acetic acid, formaldehyde (37%) and MQ water and later embedded following the Technovit protocol (Kulzer, Wehreim, Germany) as described in Fanwoua et al. (2012a) and Chapter 2. Slides (3 µm thick) were prepared, stained using 1% toluedene blue dye and images were made using a microscope and NIS-Elements software (Nikon Instruments, New York, USA). The images were analyzed with ImageJ software (National Institutes of Health, Maryland, USA) and pericarp thickness, number of cell layers, longitudinal cell diameter, mean cell volume, tissue volume, tissue cell number and weighted pericarp cell volume determined as described in Fanwoua et al. (2012a) and Chapter 2.

Carbohydrate analysis was conducted using samples from fruits harvested at breaker stage. Sucrose, fructose, glucose, and starch concentrations were determined by high performance anion exchange chromatography (HPAEC) as described in Chapter 2. Gene expression analysis was conducted with samples harvested at 6, 12, 18, and 35 DAA in Experiment II. Three biological replicates per treatment and harvest stage were obtained by pooling pericarp tissue from 2 of the 6 replicate plants. Total RNA extraction, DNase treatment, RNA qualitative and quantitative measurements and real time quantitative PCR were conducted with primer pairs as described in Chapter 2. A total of 20 genes involved in

either cell division or cell expansion regulation were studied. Putative tomato orthologs of *A. thaliana* genes were identified through bi-directional BLAST searches of coded protein sequences and phylogenetic analysis using ClustalX 2.1 (Larkin et al. 2007). Attributes of the identified tomato (co-)orthologs, of which gene expression was determined, are listed in Supplementary Table S1. Tomato co-orthologs of a single *Arabidopsis* gene were distinguished by assigning an (*a*) or (*b*) at the end of the gene name.

Data analysis

All data were analyzed by way of Analysis of Variance (ANOVA) using GenStat 15th edition software (VSNI, United Kingdom). In Experiment I, fruits 1 and 2 were analyzed based on an average of the two fruits. In Experiment II, fruit 1 was analyzed independently of fruits 2 and 3 because fruit 1 was used in dry matter estimation while fruits 2 and 3 were further observed at the cell and gene level. Outliers were defined in two steps and excluded from the analysis. The first step involved exclusion of fruits whose fresh weight was at least 30% smaller than that of the other fruit on the same truss with which it would be averaged prior to ANOVA tests. The second step was based on a comparison between average fresh weight of two fruits (fruits 1 and 2 in Experiment I or fruits 2 and 3 in Experiment II) from the same truss and the average of all fruits belonging to the same treatment and harvest stage. This means that truss averages that were more than three times the standard error of observations away from the treatment average were considered as outliers.

To ensure that variations in fruit load, anthesis time and thus onset of treatments for different cuvettes were accounted for, harvest week and number of fruits per truss were used as covariates during ANOVA tests on fruit and cell level data. A completely randomized design was assumed during the analysis of gene level data since sample pooling was conducted prior to real time quantitative PCR. The ANOVA test on gene level data was conducted for separate harvest stages. A Shapiro-Wilk test for normality was also conducted for data at all levels of observation and followed by a $\log_{10}$ transformation of datasets prior to ANOVA tests for parameters where normality could not be proven.

Results

Does a fruit's light environment influence its size and carbohydrate content?

We tested whether the phenotype of fruits would differ between fruits grown under natural light conditions (control) or 12 h white light, and 24 h white light or darkness during two

experiments (I and II) conducted in successive years. Data from the two experiments were separately analyzed. In both experiments, the effect of the presence or absence of light during fruit growth on fruit fresh and dry weight and diameter was not statistically significant when all fruits were considered (Figure 2 and Tables 1 and 2). In Experiment II, only fruit level phenotyping (fresh and dry weight) was conducted on fruit 1 while more levels (cell and gene) were included in defining the phenotype of fruits 2 and 3. An analysis involving only fruits 2 and 3 shows that dark and 24 h fruits had equal fresh weight but both were significantly lower than that of 12 h treatment fruits at breaker stage (Figure 2B). However, when combined with results of fruit 1 to consider the whole truss response, no significant differences between the light treatments were apparent throughout fruit growth (Figure 2). The observed low fruit weight of fruits 2 and 3 at 24 days after anthesis (DAA) compared to 18 DAA in 24 h fruits was possibly caused by poor pollination (Figure 2). The effects of light color and timing of white light application during fruit development were also investigated in Experiment II. Breaker stage observations on fruit size showed no significant response to light color or timing of light application (Tables 3 and 4).

Fruit carbohydrate analyses were conducted on pericarp tissue of fruits harvested at 24 days after anthesis (DAA) and the breaker stage during Experiment I. The concentration of sucrose, fructose, glucose, and starch was not significantly different among all treatments at both harvest stages. Although not statistically significant, the concentration of starch at 24 DAA in dark grown and control fruits was 81% and 44% higher than in fruits exposed to 24 h

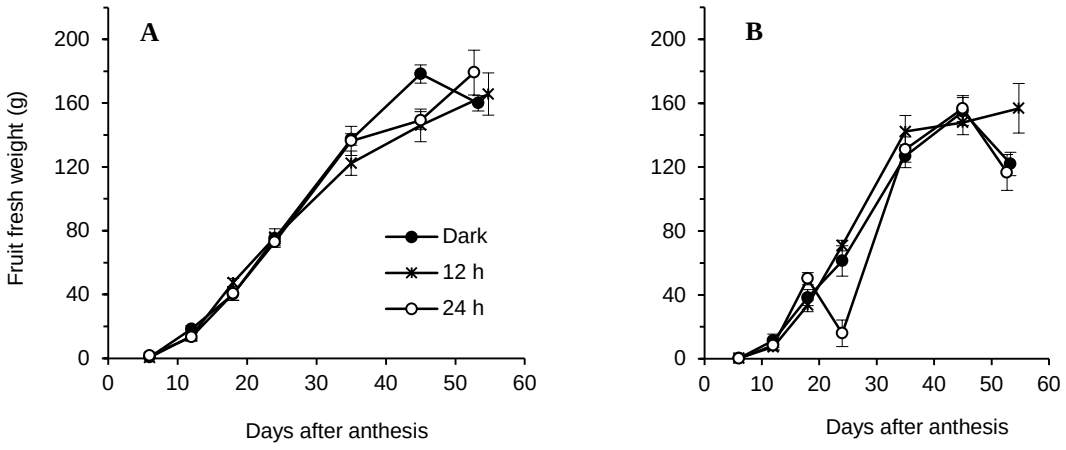

Figure 2: Fresh weights of fruit 1 (A) and 2 and 3 (B, used for gene and cell analysis) grown in the dark, 12 h and 24 h white light conditions from anthesis to the breaker stage during Experiment II. Individual points are averages of fruits from four or five cuvettes. Bars represent standard errors of the means.

Table 1: Phenotype at fruit, tissue and cell level (n = 4 or 5 and each replicate is an average of fruit 1 and 2 on the same truss) for the dark, control and 24 h white light grown fruits harvested at 24 days after anthesis (24 DAA) or breaker stage (Breaker) for Experiment I. Dry weight at breaker stage was estimated for the whole fruit based on the dry matter content determined for one half of fruit 1. Means within a row followed by different letters differ significantly (Fisher's protected LSD test; $P = 0.05$).

	24 DAA				Breaker			
	Dark	**Control**	**24 h**	**P value**	**Dark**	**Control**	**24 h**	**P value**
Growth duration (days)					61	65	61	0.067
Fruit fresh weight (g)	53.17	57.19	58.69	0.320	167.0	191.6	181.1	0.192
Fruit dry weight (g)					7.33	7.56	8.11	0.238
Fruit diameter (mm)	48.73	50.18	51.39	0.080	69.86	74.86	73.61	0.105
Pulp volume (cm^3)	26.7	25.6	29.4	0.514	66.06	86.22	82.88	0.187
Sucrose (µg/mg)	13.35	14.30	12.74	0.621	9.04	5.33	7.13	0.053
Fructose (µg/mg)	199.2	207.3	178.8	0.163	199.5	185.9	196.8	0.359
Glucose (µg/mg)	211.4	215.0	186.9	0.085	217.4	199.8	220.3	0.128
Starch (µg/mg)	51.74	41.13	28.52	0.143	1.51	1.49	0.08	0.201
Exocarp volume (cm^3)	0.34	0.29	0.35	0.200	0.59	0.53	0.58	0.644
Exocarp cell volume ($x10^{-5} mm^3$)	2.6	2.1	2.1	0.202	5.1 **b**	2.9 **a**	3.3 **a**	<0.001
Exocarp cell number ($x10^6$)	13.50	13.51	17.32	0.109	12.11 **a**	18.74 **b**	18.18 **b**	0.025
Mesocarp volume (cm^3)	24.74	23.10	27.92	0.250	83.38	97.83	91.67	0.058
Mesocarp cell volume ($x10^{-5} mm^3$)	1398	1240	1228	0.572	4630 **b**	3790 **ab**	3390 **a**	0.035
Mesocarp cell number ($x10^6$)	1.82	1.90	2.34	0.104	1.89 **a**	2.69 **b**	2.76 **b**	0.021
Mesocarp cell layers	15.5 **a**	18.5 **b**	20.0 **b**	0.004	16.63	18.70	18.20	0.088
Pericarp volume (cm^3)	25.08	23.39	28.27	0.248	83.97	98.36	92.24	0.061
Pericarp cell volume ($x10^{-5} mm^3$)	1378	1225	1213	0.579	4600 **b**	3770 a**b**	3370 **a**	0.035
Pericarp cell number ($x10^6$)	15.32	15.41	19.66	0.088	14.00 **a**	21.43 **b**	20.94 **b**	0.015

Table 2: Phenotype at fruit, tissue and cell level ($n = 4$ or 5 and each replicate is an average of fruit 2 and 3 on the same truss) for the dark, 12 h and 24 h white light grown fruits at breaker stage for Experiment II. Dry weight data were obtained from fruit 1. Means within a row followed by different letters differ significantly (Fisher's protected LSD test; $P = 0.05$).

	Dark	**12 h**	**24 h**	**P value**
Growth duration (days)	55 **b**	55 **b**	50 **a**	0.001
Fruit fresh weight (g)	122.0	156.8	116.6	0.111
Fruit dry weight (g)	7.54	7.57	8.82	0.271
Fruit diameter (mm)	62.62	69.63	61.78	0.086
Pulp volume (cm^3)	38.55	64.43	39.54	0.061
Exocarp volume (cm^3)	0.34	0.46	0.42	0.685
Exocarp cell volume ($x10^{-5} mm^3$)	7.3	4.0	5.2	0.161
Exocarp cell number ($x10^6$)	5.1	11.5	10.7	0.092
Mesocarp volume (cm^3)	62.77	87.11	57.31	0.059
Mesocarp cell volume ($x10^{-5} mm^3$)	6635 **b**	3666 **a**	3246 **a**	0.026
Mesocarp cell number ($x10^6$)	0.86	2.61	2.17	0.082
Mesocarp cell layers	18.09	19.41	21.46	0.078
Pericarp volume (cm^3)	63.10	87.57	57.73	0.061
Pericarp cell volume ($x10^{-5} mm^3$)	6600 **b**	3650 **a**	3220 **a**	0.025
Pericarp cell number ($x10^6$)	5.95	14.14	12.84	0.083

Table 3: Phenotype at fruit, tissue and cell level (n = 3 to 5 and each replicate is an average of fruit 2 and 3 on the same truss) for blue, white and red light grown fruits at breaker stage for Experiment II. Dry weight data were obtained from fruit 1. Means within a row followed by different letters differ significantly (Fisher's protected LSD test; P = 0.05).

	Blue	**White**	**Red**	**P value**
Growth duration (days)	46 **a**	55 **b**	51 **ab**	0.033
Fruit fresh weight (g)	151.9	132.2	139.4	0.750
Fruit dry weight (g)	8.26	8.08	8.66	0.923
Fruit diameter (mm)	69.14	62.54	69.36	0.405
Pulp volume (cm^3)	48.13	36.19	61.52	0.311
Exocarp volume (cm^3)	0.47	0.35	0.41	0.656
Exocarp cell volume ($x10^{-5}\,mm^3$)	6.0	4.2	4.1	0.650
Exocarp cell number ($x10^6$)	13.57	9.27	17.88	0.658
Mesocarp volume (cm^3)	67.50	64.20	76.15	0.640
Mesocarp cell volume ($x10^{-5}\,mm^3$)	3987	3418	3213	0.814
Mesocarp cell number ($x10^6$)	1.85	2.18	3.08	0.668
Mesocarp cell layers	21.80	22.26	20.76	0.845
Pericarp volume (cm^3)	67.97	64.55	76.56	0.643
Pericarp cell volume ($x10^{-5}\,mm^3$)	3960	3400	3190	0.816
Pericarp cell number ($x10^6$)	15.43	11.44	20.96	0.625

Table 4: Phenotype at fruit, tissue and cell level (n = 4 and each replicate is an average of fruit 2 and 3 on the same truss) at breaker stage for Experiment II. Fruits were either grown in the dark for the first 24 days after anthesis (DAA) followed by 24 h white light till breaker stage (DL), or exposed to white light in the initial 24 DAA followed by darkness till breaker stage (LD). Dry weight data were obtained from fruit 1.

	DL	**LD**	**P value**
Growth duration (days)	50	50	0.852
Fruit fresh weight (g)	172.27	181.12	0.666
Fruit dry weight (g)	8.72	7.87	0.266
Fruit diameter (mm)	70.98	73.30	0.582
Pulp volume (cm^3)	59.54	68.50	0.632
Exocarp volume (cm^3)	0.49	0.56	0.602
Exocarp cell volume ($x10^{-5}\,mm^3$)	6.40	3.52	0.125
Exocarp cell number ($x10^6$)	8.81	17.15	0.066
Mesocarp volume (cm^3)	72.82	83.50	0.367
Mesocarp cell volume ($x10^{-5}\,mm^3$)	4226	3332	0.425
Mesocarp cell number ($x10^6$)	1.67	3.07	0.132
Mesocarp cell layers	20.71	19.35	0.452
Pericarp volume (cm^3)	73.31	84.06	0.368
Pericarp cell volume ($x10^{-5}\,mm^3$)	4200	3310	0.427
Pericarp cell number ($x10^6$)	10.49	20.22	0.068

white light (Table 1). The concentration of fructose and glucose at 24 DAA was also similar in control and dark grown fruits but approximately 15% higher when compared to fruits grown under 24 h white light.

Light stimulates cell division at the expense of cell expansion

In Experiment I, samples for histological analysis were collected at 24 days after anthesis (DAA) and breaker stage while in Experiment II only breaker stage samples were considered. It was assumed that cells had attained their maximum size by breaker stage. Histological analyses were conducted on the pericarp: exocarp and mesocarp. No significant effect of light on cell volume within the exocarp was observed at 24 DAA in Experiment I.

However, at breaker stage, the exocarp cell volume was significantly ($P < 0.001$) higher in dark-grown than in control and 24 h white light-grown fruits (Table 1 and Supplementary Table S1). Both control and 24 h white light-grown fruits had a similar exocarp cell volume at the breaker stage. Exocarp tissue and cell volume were found to be at least 140 and 1000 times smaller on average than that of the mesocarp (Tables 1, 2, 3, and 4). This led to the assumption that fruit size increase was influenced more by the mesocarp than exocarp cell growth dynamics. A trend similar to that for exocarp cells was observed in the mesocarp cell volume for the three light treatments at 24 DAA and the breaker stage (Table 1 and Supplementary Table S1). The only exception was that the difference in mesocarp cell volume between the control and dark-grown fruits was not statistically significant at the breaker stage.

Exocarp and mesocarp cell numbers were not different in the treatments of Experiment I at 24 DAA. Dark grown fruits had significantly fewer cells in both the exocarp and mesocarp at the breaker stage compared to control and 24 h white light grown fruits (Table 1). Control and 24 h white light grown fruits had a similar number of exocarp and mesocarp cells. The number of mesocarp cell layers in dark grown fruits was significantly lower than in the control and 24 h white light-grown fruits at 24 DAA. A similar trend was also observed at the breaker stage, although the difference in the number of mesocarp cell layers between dark- and light-grown fruits (control and 24 h white light) was not statistically significant (Table 1).

Observations on exocarp and mesocarp cell volume and number, and mesocarp cell layers at the breaker stage in the dark, 12 h and 24 h white light-treated fruits during Experiment II were in agreement with breaker stage observations in Experiment I (Tables 1 and 2). Compared to fruits grown in the presence of white light (12 h or 24 h), dark-grown fruits generally had a larger cell volume and lower cell number. In Experiment II, however, the large absolute differences in exocarp cell volume and number and mesocarp cell number and layers observed between dark and light (12 h or 24 h) grown fruits were not statistically significant. No differences in anticlinal and periclinal cell diameter of exocarp and pericarp

cells were evident among treatments (Supplementary Table S2). No significant differences between the three light color treatments during Experiment II were observed for measurements on cells at the breaker stage (Table 3 and Supplementary Table S3). In addition, the number and volume of cells did not significantly differ between fruits exposed to 24 h white light during the first 24 DAA followed by darkness thereafter compared to fruits exposed to 24 h darkness during the first 24 DAA followed by white light thereafter (Table 4 and Supplementary Table S4). However, it is worth noting that fruits exposed to darkness in only the first 24 DAA (DL) had 95% fewer exocarp cells than those that received light (LD) in this period ($P = 0.066$).

Light effects on gene expression

The expression of 20 tomato genes involved in the promotion or inhibition of cell division and expansion was studied in pericarp tissue of fruits grown in three treatments; dark, 12 h and 24 h white light. Genes were selected because they had been investigated in earlier studies on either cell division, endoreduplication or cell expansion. The fruits had been harvested at 6, 12, 18, and 35 days after anthesis (DAA) during Experiment II. It was assumed that the first three development stages were representative of the cell division phase while fruits harvested at 35 DAA were in the cell expansion phase of fruit growth. Genes were selected and grouped according to the literature on cell division and expansion in *Arabidopsis thaliana* and tomato.

The first group consisting of genes for Cyclin Dependent Kinases (*CDKA1, CDKA2, CDKB1* and *CDKB2*), Cyclins (*CycA1, CycB2* and *CycD3;3*) and transcription factors *E2Fa-like*, *E2Fb-like* and *E2Fe-like*) encode promoters of the cell cycle (Inzé and De Veylder, 2006; López-Juez et al., 2008). The second group made up of a transcription factor (*E2Fc-like*), Kip Related Protein1 (*KRP1*), a protein kinase (*WEE1*), cell number regulator 1-like (*fw2.2*) and Phytochrome Interacting Factors (*PIF1-like(a), PIF1-like(b)* and *PIF3-like*), which are inhibitors of the cell cycle (Frary et al., 2000; Inzé and De Veylder, 2006; López-Juez et al., 2008; Jang et al., 2010). The third group constituted by *AGPaseB* and *AGPS1* code a small and large subunit respectively of the same enzyme; ADP Glucose Pyrophosphorylase, which promotes starch accumulation (Schaffer and Petreikov, 1997) and consequently fruit growth (Guan and Janes, 1991). We assumed that an increase in expression of *AGPaseB* and *AGPS1* would lead to an increase in cell expansion. The fourth group was

made up of the transcription factor, *E2Ff-like* (Kosugi and Ohashi, 2002) which inhibits cell expansion.

Five distinct gene groups, differing in temporal pattern of expression emerged from this study (Figure 3 and Supplementary Figure S2). The first group, *CDKA2*, *E2Ff-like*, *KRP1*, *PIF1-like(b)* and *PIF3-like* were constitutively expressed between 6 and 35 days after anthesis (DAA). The second group, *AGPaseB*, *CDKB1*, *CDKB2*, *CycA1*, *CycB2*, *CycD3*;3, *E2Fb-like*, *E2Fc-like*, *WEE1*, *fw2.2* and *E2Fe-like*, exhibited a significant decrease in expression as fruits matured. A third group; *CDKA1* and *AGPS1* peaked their expression between 12 and 18 DAA while the fourth group consisting of *E2Fa-like* had its highest expression at 35 DAA. Expression in the fifth group made up of *PIF1-like(a)* decreased between 12 and 18 DAA but again rose at 35 DAA to similar levels as at 6 DAA. No significant effect of light was observed on the expression of *AGPS1*, *CDKA1*, *CDKA2*, *CDKB2*, *CycA1*, *CycB2*, *CycD3*;3, *E2Fa-like*, *E2Fc-like*, *E2Ff-like*, *fw2.2*, *PIF1-like(b)*, *PIF3-like* and *WEE1* from 6 until 35 DAA (Figure 3).

Three genes (*CDKB1*, *E2Fb-like* and *E2Fe-like*) that are associated with promotion of the cell cycle were differentially expressed in fruits from the three light treatments (Figures 3E, 3K and 3M). The expression of *CDKB1* and *E2Fe-like* was significantly lower in 12 h than in dark and 24 h grown fruits at 6 DAA. At 12 DAA, the expression of *CDKB1* was higher in dark grown fruits than that in fruits exposed to 12 h or 24 h light, however, no significant difference among the treatments was observed in older fruits. The expression of *E2Fb-like* was significantly higher in 24 h fruits compared to that in dark and 12 h fruits at 6 DAA (Figure 3K). At 12 DAA, the expression of *E2Fb-like* in 24 h fruits was similar to that in dark and 12 h fruits; however, expression levels were higher in dark compared to 12 h fruits. The expression of two cell cycle inhibitors (*KRP1* and *PIF1-like(a)*) was significantly affected by the light treatments (Figure 3P and 3Q). *KRP1* expression at 6 DAA was highest in 24 h fruits but lower in both dark and 12 h fruits. At 12 DAA, the expression of *KRP1* decreased in the order dark, 24 h and 12 h. *PIF1* expression on the other hand decreased significantly among the treatments at 6 DAA in the order 24 h, 12h and dark. The expression of the cell expansion promoting *AGPaseB* was significantly higher in both 12 h and 24 h fruits compared to dark fruits at 18 DAA but at 35 DAA, expression levels in dark and 24 h fruits was similar but lower than that in 12 h fruits (Figure 3A).

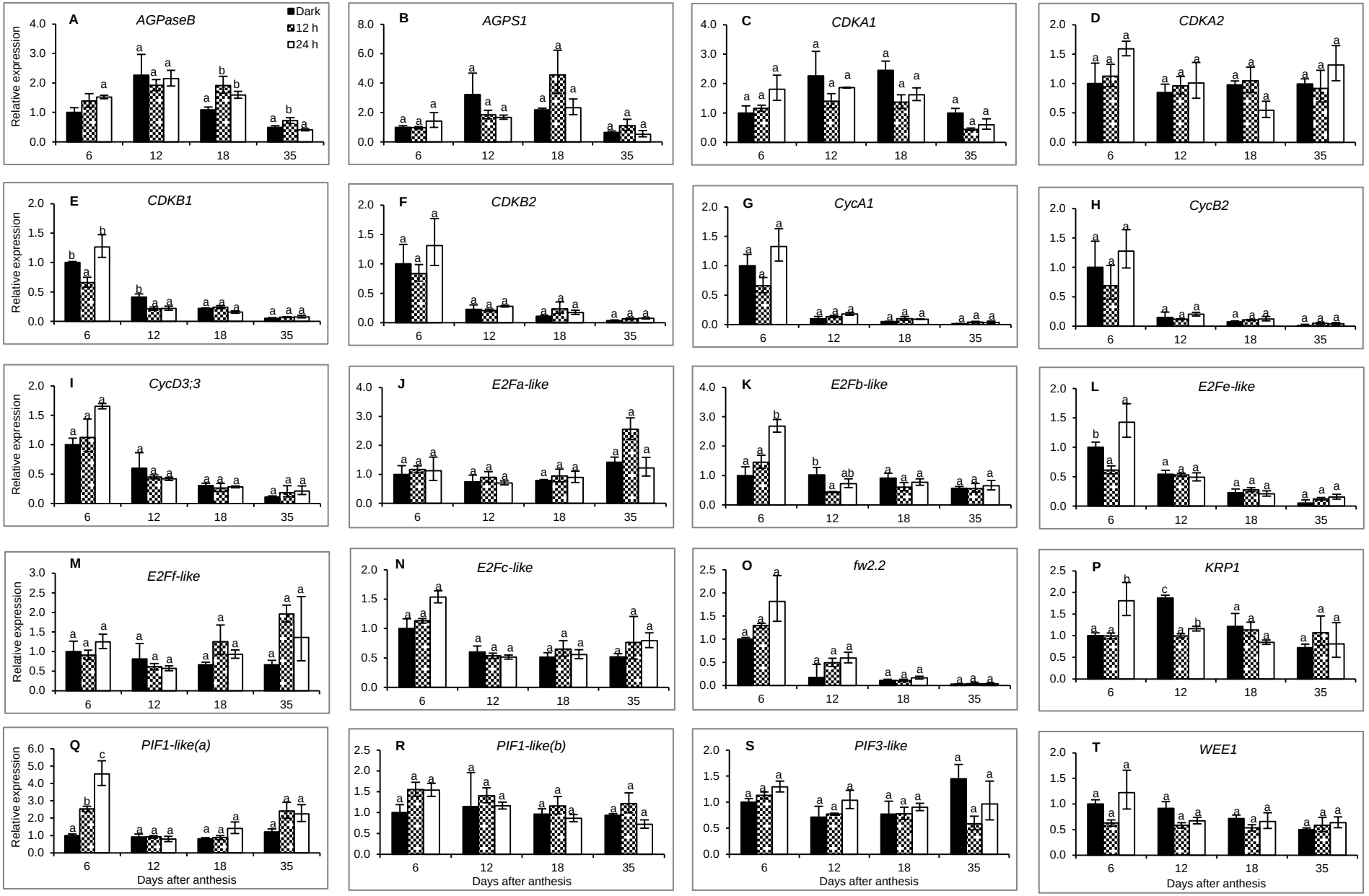
A AGPaseB
B AGPS1
C CDKA1
D CDKA2
E CDKB1
F CDKB2
G CycA1
H CycB2
I CycD3;3
J E2Fa-like
K E2Fb-like
L E2Fe-like
M E2Ff-like
N E2Fc-like
O fw2.2
P KRP1
Q PIF1-like(a)
R PIF1-like(b)
S PIF3-like
T WEE1
Dark
12 h
24 h
Relative expression
Days after anthesis
6
12
18
35

Figure 3: Relative gene (A – B: promoters of cell expansion; C – L: promoters of cell division; M: inhibitor of cell expansion; N – T: inhibitors of cell division) expression in pericarp tissue of dark, 12 h and 24 h white light fruits harvested at 6, 12, 18 and 35 days after anthesis (DAA) during Experiment II. *n* at each harvest point = 3, being biological replicates but a single replicate was a pooled sample of pericarp tissue derived from two fruits. Means are relative to expression in dark fruits at 6 DAA. Means followed by different letters at a given harvest stage differ from each other significantly (P = 0.05). No letters have been indicated at harvest stages where no significant differences were found. Bars represent standard errors.

Discussion

Light effects on fruit size and primary metabolites

Dark-grown fruits were pale white, while light-grown fruits were green until breaker stage. Light triggers chlorophyll formation in tomato fruits. In the dark, amyloplasts and etioplasts are formed instead (Khudairi, 1972; Anstis and Northcote, 1973). Unlike etioplasts, amyloplasts are known to be storage sites for starch (Wise et al., 2006). Chloroplasts (Giuliano et al., 1991), amyloplasts and etioplasts (Khudairi, 1972) accumulate carotenoids responsible for the red color during ripening.

Fruits are capable of photosynthesis, although rates are lower than in leaves and contribution to fruit growth is minor under normal conditions (Tanaka et al., 1974; Steer and Pearson, 1976; Paval and De Jong, 1993; Marcelis and Hofman-Eijer, 1995; Hetherington et al., 1998). The insignificant difference in fruit size and carbohydrate concentration (Figure 2, Tables 1 – 4) under different light conditions suggests that the absence of own photosynthesis in dark grown fruits was compensated by extra assimilate import. Our results do not support the conclusion that light increases fruit sink strength as shown by Guan and Janes (1991) in *in vitro* grown tomato fruits. The authors argued that light activates the rate limiting enzyme during starch biosynthesis; ADP Glucose Pyrophosphorylase (ADPGPP). Consequent up-regulation of starch accumulation increases the sucrose gradient between the fruit and plant and stimulates sucrose import and fruit growth. Observations at 24 DAA in Experiment I indicate the contrary; a tendency towards a higher starch concentration in dark and control fruits (Table 1). More starch accumulation has also been reported in dark compared to light-exposed potato tubers (Ewing and Struik, 1992). Apparently, *in vitro* and *in vivo* fruit growth responses to variations in environmental conditions can be different.

Our findings confirm the conclusion by Lytovchenko et al. (2011) that limitations in tomato fruit photosynthesis under normal growth conditions are compensated by enhanced import of assimilates. The fact that 24 h white light grown fruits were exposed to a higher

light intensity (66 µmol m^{-2} s^{-1}) compared to blue (30 µmol m^{-2} s^{-1}) and red (15 µmol m^{-2} s^{-1}) light did not differ in fruit size provides more support for this school of thought (Table 3), as photosynthetic rates increase with increasing light intensity in these ranges. The effects of light colour and intensity in the current study could not be separated. However, the resulting variations in fruit photosynthesis were likely to be relatively small at the fruit level considering the differences in light level between treatments and that the study was conducted under conditions of non-limiting assimilate supply. Low photosynthetic rates due to low light intensity in the blue or red light treatments compared to the white light treatment must have been compensated by extra assimilate import. Exposure of whole plants to continuous light can lead to severe injury in tomato (for a review see Velez-Ramirez et al., 2011). This damage is visible in leaves as chlorosis and necrosis but has not been reported to occur in tomato fruits as well. One hypothesis that is often fronted as a possible cause for continuous light injury is the down regulation of photosynthesis due to carbon imbalance. Exposing fruits but not whole plants to continuous light as was done in the 24 h light treatments could have led to a continuous supply of assimilates but we expect that this did not lead to a significant carbon imbalance as fruit photosynthesis contributes only a small fraction of the overall fruit assimilate demands.

Light effects on cell division and expansion

Fruit size is a consequence of cell division and expansion (Gillaspy et al., 1993). Our results show that light exposed fruits developed more but smaller cells compared to dark grown fruits, independent of the type of light treatment (Tables 1, 2 and 3). Despite these differences at the cell level, dark and light grown fruits did not differ in fruit fresh and dry weight. This implies that deficiencies in cell division in dark grown fruits were compensated for by increase in cell volume. A compensatory mechanism for cell division by cell expansion has also been reported in cucumber (Marcelis, 1993), parthenocarpic tomato fruits (Bünger-Kibler et al,. 1982; Bohner and Bangerth, 1988), *Arabidopsis* shoot apical meristem (Bemis and Torii, 2007) and leaves (Horiguchi and Tsukaya, 2011), tobacco (Hemerly et al., 1995), *Oryza sativa* (Barrôco et al., 2006) and *Antirrhinum majus* (Delgado-Benarroch et al., 2009). Surprisingly, cell division appeared to occur beyond 24 days after anthesis (DAA; Table 1). Such a late cell division is rare but has also been shown by Bertin et al. (2009). Results from the DL and LD (Table 4) treatments where white light or darkness was continuously provided at different stages of fruit development relative to 24 DAA also support this finding. Although

there was a tendency towards few cells and large cell size in the dark, antagonistic effects due to exposure to light or darkness at different stages of fruit development were still apparently leading to no overall significant differences between DL and LD fruits.

How did light stimulate cell division and why did exposure to darkness stimulate cell expansion? Cell division is largely regulated by cyclin (Cyc) and cyclin dependent kinase (CDK) dimers. Regulation is mediated through hormones and metabolites ensuring that cells divide when environmental conditions are favorable. Auxin, for example, plays a leading role in regulating cell proliferation by inducing the expression of *CycD3;1* and *CDKA;1* (Wang and Ruan, 2013). Expression levels of *CycA3;2*, *CycB1;2 CycD2;*1, and *CycD3;2* have been correlated to glucose levels in cell suspension cultures (Riou-Khamlichi et al., 2000, Hartig and Beck, 2006). Skylar et al. (2011), however, noted that the supply of glucose without auxin to cell suspension cultures could not trigger cell division. This led them to conclude that cell division regulation by auxin and glucose is distinct but also coordinated. Our results suggest that sugars were not correlated with treatment differences in cell number (Tables 1 and 2). A likely mechanism could, therefore, involve light regulation of cell division via a signaling mechanism that involves auxin, abscisic acid and cytokinins (Fosket and Tepfer, 1978; Barlow and Pilet, 1984; Myers et al., 1990; John et al., 1993; Wang and Ruan, 2013).

Cell expansion occurs when cell wall extensibility forces become stronger than cell wall rigidity forces. The larger cell volume exhibited by dark grown fruits in this study corroborates the conclusion by Gendreau et al. (1997) that light increases cell wall rigidity. Seedlings that germinate in the dark also tend to grow rapidly; when exposed to light, stem elongation is significantly reduced (Bandurski et al., 1977). Early studies by Sachs (1882) showed that light inhibits cell elongation. Later, other authors concluded that the reduction in stem elongation by light was correlated with a decrease in cell wall extensibility (Heyn, 1940; Lockhart, 1965). Kutschera (1990) and Hodick and Kutschera (1992) also associated the decrease in cell wall extensibility with cell wall thickening following irradiation of etiolated sunflower seedlings. The observed larger cell volume in dark grown fruits could also be attributed to higher cell ploidy level through increased endoreduplication in the dark as observed in etiolated *Arabidopsis* hypocotyls (Gendreau et al., 1997, 1998). Although the physiological role of endoreduplication is still unclear, there is increasing evidence that cells adjust their cytoplasmic volume to a level that is dependent of the nuclear DNA content (Bourdon et al., 2012). However, other authors have shown that light stimulates cell expansion. Van Volkenburgh and Cleland (1990) concluded that light stimulates cell

enlargement in bean leaves through acidification of epidermal cell walls. Light stimulated cell expansion was also reported in *Arabidopsis* cotyledons (Neff and Van Volkenburgh, 1994). Cell expansion in response to light may therefore depend on the organ or species under consideration. Cell size could also be regulated at fruit level in such a way that environmental conditions constraining cell division set the stage for more cell expansion.

Light effects on gene expression

The expression of genes promoting cell division generally tended to decline as fruits developed, except for *CDKA2* and *E2Fa-like* (Figure 3 and Supplementary Figure S2). Expression of inhibitors of cell division also decreased in the course of fruit development except for *E2Ff-like, KRP1, PIF1-like(a), PIF1-like(b)* and *PIF3-like* (Figure 3). Expression of cell expansion promoters (*AGPaseB* and *AGPS1*) tended to peak between 12 and 18 DAA and then decreased by 35 DAA while that of the cell expansion inhibitor (*E2Ff-like*) appeared to be constitutive. These trends were generally expected because different genes function at different stages of fruit development. Similar expression patterns of Cycs and CDKs were reported by Czerednik (2012). The relatively high expression of genes (*AGPaseB* and *AGPSI*) encoding the two ADPGPP subunits at 12 and 18 DAA followed by a significant decline at 35 DAA also agrees with peaks of starch accumulation typical of the period between 21 and 28 DAA in tomato fruit. Although there was no statistically significant difference in the expression of the gene (*AGPS1*) for the large subunit of ADPGPP between light and dark grown fruit, a tendency towards higher expression of the small subunit gene (*AGPaseB*) in the presence of light was evident at 18 and 35 DAA. This finding does not support the observed larger cell volume in dark compared to light grown fruits. However, it supports the observation by Guan and Janes (1991) that ADPGPP is activated by light. It is likely that this effect is mediated by the small ADPGPP subunit.

There was increased cell division when fruits were exposed to 12 h or 24 h light. It was therefore, expected that the expression of cyclins, cyclin dependent kinases and cell cycle promoting E2F transcription factor genes would be higher while expression of cell cycle inhibitors would be lower in light compared to dark grown fruits. Surprisingly, the expression of one cell cycle inhibitor (*PIF1-like(a)*) was stimulated by light at 6 DAA (Figure 3Q) while the expected higher expression of *KRP1* in dark grown fruits was only evident at 12 DAA. Unlike other studies, cell number observations in the current study were not generally corroborated by expression trends of cell cycle promoters and inhibitors. Increase in the

expression of cyclins in response to light has for example been shown in apple (Dash et al., 2012), where a 4.6-fold decrease in *CycA2;2* and *CycA2;3* expression was reported in shaded fruits. Dash et al. (2012) also studied the expression of KRPs in apple fruits and showed that their expression increased at least four times in the dark. The absence of significant differences in transcript levels despite significant differences at the cell level in the current study could imply that treatment differences were effected through posttranscriptional regulation. It is also likely that treatment effects on transcript levels could not be detected because gene expression analysis was conducted using samples from Experiment II where large variation was also observed in fruit and cell parameters.

Conclusion

The aim of this study was to explore light effects on tomato fruit growth at fruit, cell, and gene level. Our findings revealed a strong effect of light on cell number increase coupled with a decrease in cell size without significant effects on fruit fresh and dry weight. This effect of light on cell number and size was independent of the type of light treatment. Light conditions in the first 24 DAA determined final cell numbers and size. At the gene level, increased cell division and decrease in cell size when fruits were grown in the presence of light was not clearly corroborated by the expression pattern of promoters and inhibitors of cell division and expansion analyzed in this study. The cell cycle inhibitory *PIF1-like(a)* was surprisingly stimulated by light while the expected higher expression of *KRP1* was only evident at 12 DAA. Our results suggest the existence of a complex homeostatic regulatory system for fruit growth where deficiency in cell division is compensated by increased cell expansion possibly through posttranscriptional regulatory mechanisms.

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Supplementary material

Table S1: Phenotype at tissue and cell level (n = 4 to 5 and each replicate is an average of fruit 2 and 3 on the same truss) for dark, control and 24 h white light grown fruits harvested at 24 days after anthesis (24 DAA) and breaker stage (Breaker) for Experiment I. Means within a row followed by different letters differ significantly (Fisher's protected LSD test; P = 0.05).

	24 DAA				Breaker			
	Dark	**Control**	**24 h**	**P value**	**Dark**	**Control**	**24 h**	**P value**
Exocarp thickness (mm)	0.08	0.07	0.08	0.081	0.08 **b**	0.06 **a**	0.07 **a**	0.013
Exocarp anticlinal cell diameter (mm)	0.017	0.015	0.016	0.081	0.016 **b**	0.012 **a**	0.013 **a**	0.013
Exocarp periclinal cell diameter (mm)	0.054	0.053	0.049	0.149	0.077 **b**	0.067 **a**	0.068 **a**	0.021
Mesocarp thickness (mm)	4.81	4.66	5.10	0.249	8.41	8.53	8.25	0.634
Mesocarp anticlinal cell diameter (mm)	0.31**b**	0.25 **a**	0.26 **a**	0.002	0.51	0.46	0.46	0.236
Mesocarp periclinal cell diameter (mm)	0.29	0.30	0.30	0.849	0.42	0.40	0.38	0.301
Pericarp thickness (mm)	4.90	4.73	5.18	0.239	8.49	8.59	8.32	0.642

Table S2: Phenotype at tissue and cell level (n = 4 to 5 and each replicate is an average of fruit 2 and 3 on the same truss) for dark, 12 h and 24 h white light grown fruits at breaker stage during Experiment II. Means within a row followed by the same letter do not significantly (Fisher's protected LSD test; P = 0.05) differ from each other.

	Dark	**12 h**	**24 h**	**P value**
Exocarp thickness (mm)	0.07	0.06	0.08	0.614
Exocarp anticlinal cell diameter (mm)	0.013	0.013	0.016	0.614
Exocarp periclinal cell diameter (mm)	0.10	0.08	0.08	0.065
Mesocarp thickness (mm)	8.67	8.81	8.07	0.191
Mesocarp anticlinal cell diameter (mm)	0.48 **b**	0.46 **b**	0.38 **a**	0.016
Mesocarp periclinal cell diameter (mm)	0.61	0.44	0.42	0.194
Pericarp thickness (mm)	8.73	8.87	8.14	0.201

Table S3: Phenotype at tissue and cell level (n = 3 to 5 and each replicate is an average of fruit 2 and 3 on the same truss for blue, white and red light grown fruits at breaker stage for Experiment II. No statistically significant differences were observed (Fisher's protected LSD test; P = 0.05).). P value for mesocarp periclinal cell diameter is based on $\log_{10}$ transformed data.

	Blue	**White**	**Red**	**P value**
Exocarp thickness (mm)	0.08	0.07	0.06	0.197
Exocarp anticlinal cell diameter (mm)	0.017	0.014	0.012	0.197
Exocarp periclinal cell diameter (mm)	0.076	0.076	0.075	0.997
Mesocarp thickness (mm)	8.27	8.85	8.20	0.615
Mesocarp anticlinal cell diameter (mm)	0.38	0.40	0.40	0.806
Mesocarp periclinal cell diameter (mm)	0.47	0.41	0.39	0.661
Pericarp thickness (mm)	8.35	8.91	8.26	0.616

Table S4: Phenotype at tissue and cell level (n = 4 but each replicate is an average of fruit 2 and 3 on the same truss). at breaker stage for Experiment II. Fruits were either grown in the dark for the first 24 days after anthesis (DAA) followed by 24 h white light till breaker stage (DL), or exposed to white light in the initial 24 DAA followed by darkness till breaker stage (LD). No statistically significant differences were observed(Fisher's protected LSD test; P = 0.05).

	DL	LD	P value
Exocarp thickness (mm)	0.07	0.08	0.442
Exocarp anticlinal cell diameter	0.014	0.015	0.442
Exocarp periclinal cell diameter	0.090	0.066	0.056
Mesocarp thickness (mm)	8.13	8.40	0.654
Mesocarp anticlinal cell diameter	0.39	0.44	0.106
Mesocarp periclinal cell diameter	0.51	0.40	0.361
Pericarp thickness (mm)	8.20	8.48	0.650

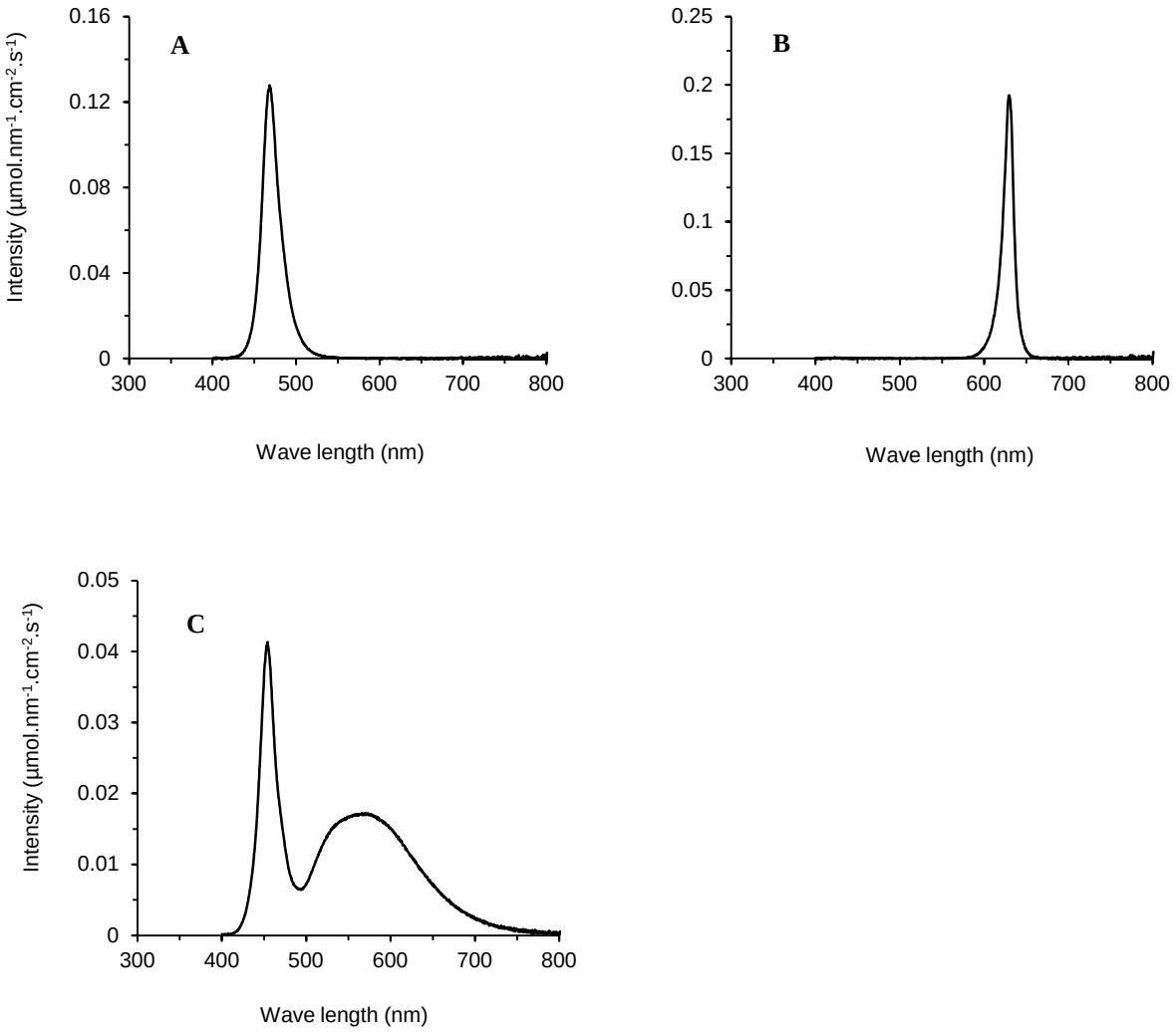

Figure S1: Light spectrum for the blue (A), red (B) and white (C) light emitting diodes (LEDs) attached to cuvettes in respective treatments.

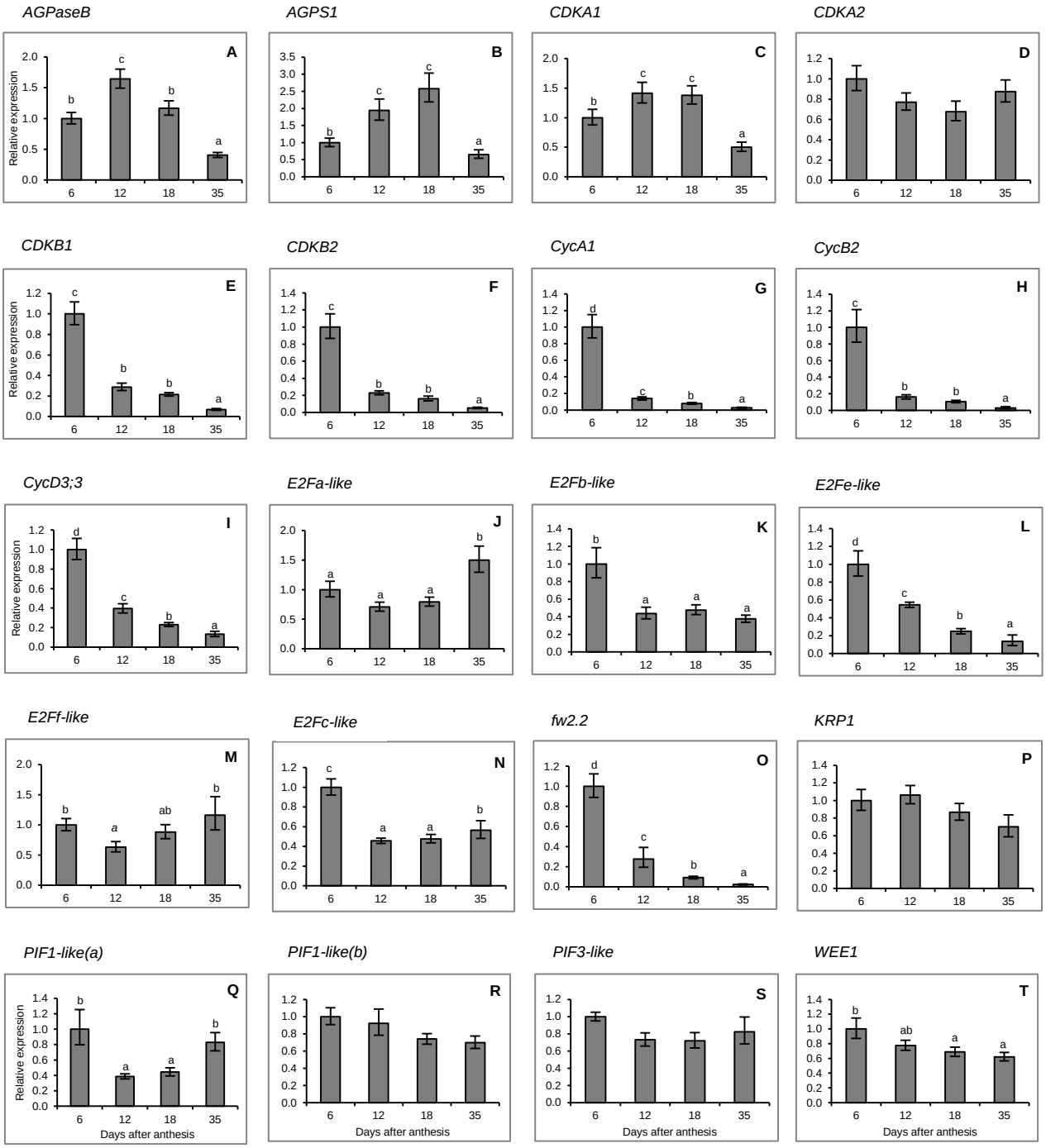

Figure S2: Relative gene (A – B: promoters of cell expansion; C – M: promoters of cell division; N: inhibitor of cell expansion; O – T: inhibitors of cell division) expression in pericarp tissue of fruits harvested at 6, 12, 18 and 35 days after anthesis for Experiment II. Relative expression values were calculated relative to the expression at 6 DAA and they represent averages over all treatments. Bars represent standard errors and different letters indicate significant differences (Fisher's protected LSD test; $P = 0.05$). No letters have been indicated for genes whose expression did not change significantly over time. $N = 9$ at each harvest point and a single replicate was a pooled sample of pericarp tissue derived from two fruits.

Chapter 4

Light mediated regulation of cell division, endoreduplication and cell expansion

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Abstract

Cell division, endoreduplication and cell expansion are key processes for plant growth and development. Light is the main source of energy for plants and as such has a strong effect on plant growth and development. Insight into the role of light in cellular processes is important for our understanding of plant responses to light. Recent advances in artificial plant lighting, cell imaging techniques and molecular biology have provided opportunities to study light responses of plants at the cell and gene level. Regulatory networks for cellular processes have also been unravelled and many transcription factors identified. In this review, we highlight key transcription factors and photoreceptors involved in the regulation of cell division, endoreduplication and cell expansion by light. We suggest that light responses result from either degradation of transcription factors or inhibitory competition between transcription factors for promoter regions of target genes. We also suggest that light stimulates cell division irrespective of the organ under consideration, while endoreduplication and cell expansion responses to light vary from organ to organ.

Keywords: photoreceptors, phytochrome interacting factors, transcription, translation, cell wall

Introduction

Developmental switches in plants are generally controlled by signalling cascades that converge to the level of gene regulation (Kaufmann et al., 2010). In eukaryotes, gene expression is regulated by transcription factors through promoter region binding. This process is intricately regulated to ensure that the right gene expression takes place at the right development stage and when environmental conditions are suitable. In plants, these mechanisms include: 1) inhibitory competition between transcription factors for the same promoter region, 2) transcription factor degradation, 3) binding of transcription factors by inhibitory proteins (Komaki and Sugimoto, 2012), 4) tight binding and coiling of DNA by histone proteins to limit promoter region accessibility (Chua et al., 2001), and 5) feedback post translational regulation by gene products.

In the past decades, environmental factors have also been shown to regulate plant growth and development through modulation of gene expression (Xiong et al., 1999; Molinier et al., 2006; Jiao et al., 2007; Knight and Knight, 2012; Prasch and Sonnewald, 2013; Chapter 2). As sessile organisms, the plants' survival depends on the ability to respond to the environment. Possible strategies for plant response to environmental signals include: 1) reference to short-term past using enzymes or a "cumulative system" like thermal time or the circadian clock, 2) anticipation of future occurrences using evolutionary information obtained through natural selection, i.e., information within the genome is translated into a developmental programme, 3) short term readjustments to unanticipated changes in the environment, and 4) activation of gene expression in response to stress which will help to cope with future stress.

Light perceived by plants varies in duration of the light phase per day, intensity and quality. Compared with other environmental variables, the duration of the light phase is more predictable, following a fixed and cyclic pattern over time depending on latitude. As such, light duration may be an important factor in orchestrating plant growth and development. Plants have, for example, in-built light-dependent (circadian) clocks that have remarkable effects on gene expression regulation (Oh et al., 2012). Besides being predictable in duration, to a considerable extent, light is the main source of energy for plant growth, but light intensity is much more variable and less predictable than the duration of the daily light phase. Studies on thale cress (*Arabidopsis thaliana*) and rice (*Oryza sativa*) seedlings have revealed at least a 20% difference in gene expression during skotomorphogenesis and photomorphogenesis (Tepperman et al., 2001; Ma et al., 2001; Jiao et al., 2005). Light quality (i.e., spectrum) also

varies during the day, the year and within canopies, playing a significant role in neighbour sensing (Pierik and de Wit, 2014), phototrophic responses (Goyal et al., 2013), as well as having a major impact on plant functioning (Sun et al., 2013).

Here, we review transcriptional and post translational regulatory networks associated with photoreceptors and discuss the molecular basis for light responses. Attention is paid to transcription and post translation network components that are linked with fundamental processes for plant organ growth: cell division, cell expansion and endoreduplication (increase in cellular DNA content without cell division).

Light regulation of gene expression is mediated by photoreceptors

Plants perceive the intensity, direction, duration and quality of light using photoreceptors (Nishihama and Kohchi, 2013). The perception of light quality by different photoreceptors is particularly important in signalling responses usually effected through changes in gene expression (Kianianmomeni, 2014). Phytochromes (PHY) were the first photoreceptors to be discovered and to date, five isoforms (PHYA to PHYE) are known in *Arabidopsis* (Sharrock and Quail, 1989). They exist in two interchangeable states; phytochrome red (Pr) and far-red (Pfr) that absorb red and far-red light respectively. UV RESISTANT LOCUS 8 (UVR8) absorbs ultraviolet-B (Tilbrook et al., 2013), while cryptochromes, phototropins, and the family consisting of FLAVIN BINDING, KELCH REPEAT, F-BOX PROTEIN 1 (FKF1), LOV KELCH PROTEIN 2 (LKP2) and ZEITLUPE (ZTL) proteins are receptors for blue light (Quail, 2002; Lin and Shalitin, 2003; Chen et al., 2004). Exposure to light initiates a change in photoreceptor form, for example Pr is converted into the physiologically active Pfr form upon red light absorption. A reversion to Pr occurs when Pfr is exposed to far-red light. Photoreceptors mediate growth and developmental responses to light. Response mechanisms are, however, complex because: 1) more than one photoreceptor may be involved in a given developmental response, 2) responses can be organ and development stage specific, and 3) light signalling networks may be linked to signalling networks of other environmental stimuli (Jiao et al., 2007).

The first response following photon absorption and photoreceptor activation involves a change in photoreceptor localization within the cell. Phytochrome (Pr) is predominantly located in the cytoplasm, while active Pfr translocates to the nucleus. Cryptochrome 1 (CRY1) resides in the cytoplasm following light absorption, but is transported to the nucleus in the dark (Chen et al., 2004). Cryptochrome 2 (CRY2) on the other hand is constitutively

found in the nucleus, while cryptochrome 3 (CRY3) is located in chloroplasts and mitochondria. Phototropins 1 (phot1) and 2 (phot2) are mainly found in the plasma membrane, although a fraction of phototropin 1 translocates to the cytoplasm, when exposed to light (Chen et al., 2004). Light controls the interaction between photoreceptors and genetic information through influences on the localization of photoreceptors or proteolytic degradation of photoreceptors and signalling components (Chen et al., 2004). When inside DNA-containing organelles, photoreceptors interact with chromatin to kick start downstream signalling cascades. These downstream signalling cascades affect plant growth and development through regulation of transcription, post translation modification and degradation of transcription factors of target genes (Bai et al., 2012). Phytochromes, for example, directly inhibit the activity of PHYTOCHROME INTERACTING FACTORS (PIFs) in the presence of light (Oh et al., 2012). Light also affects the expression of target genes directly through modifications that determine nucleosome (DNA bound to histone proteins) accessibility at the promoter region (Guo et al., 2008). For example, Chua et al. (2001) showed that increased acetylation of histones 3 and 4 at the promoter region of a pea (*Pisum sativum*) plastocyanin gene was correlated with its light induced transcription.

Regulation of the cell cycle and endocycle

The canonical cell cycle consists of four distinct phases; gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M). The two gap phases are periods when cells expand in preparation for the next phase or rest when cell division ceases, e.g., under unfavourable environmental conditions or in differentiated cells. Cells growing under unfavourable environmental conditions are typically arrested in G1. Doubling of the cell's genetic material occurs during the S phase before separation into two identical daughter cells during M phase. The cell cycle machinery is largely conserved in different species although regulation varies among species depending on availability of cell cycle components and modulation of the function of regulatory targets (Gutierrez, 2005). Cell cycle phase transitions and progress during a given phase are largely controlled by cyclin dependent kinases (CDKs) grouped into seven categories (A to G). Up to 12 CDKs have been identified in *Arabidopsis* and of these, CDKA has been shown to regulate G1 to S and G2 to M transition, while the plant kingdom specific CDKB is active in the transition from S to M phase (Ramirez-Para et al., 2005). In addition to A and B-type CDKs, D type CDKs play a role in the M phase (Komaki and Sugimoto, 2012). The activity of CDKs is modulated by cyclins and CDK inhibitors like KIP RELATED PROTEINS (KRPs), SIAMESE(SIM)/SIAMESE-RELATED PROTEINS(SMR) and kinases

like WEE1. Cyclins activate CDKs, confer substrate specificity through dimerization and determine subcellular localisation of dimers (Komaki and Sugimoto, 2012).

The duration of cell division following the canonical cell cycle varies among organs and species. In some organs, for example the avocado fruit, cell division proceeds for as long as the fruit is attached to the plant (Schroeder, 1953) while in other organs, like the tomato fruit, it stops at around 14 days after anthesis (DAA) depending on the genotype (Mapelli et al., 1978). Cell division in some tomato genotypes continues beyond 25 DAA (Bertin et al., 2009). Upon cessation of division, many angiosperm cells undergo endoreduplication, a process that involves increase in the genetic material without mitosis. During endoreduplication, cells undergo repeated cycles of the S and an undifferentiated gap (G) phase leading to increase in cellular DNA contents of up to 512 C (where C is the haploid cell DNA content), for example in tomato fruit (Bourdon et al., 2010; Chevalier et al., 2011). This increase in DNA ploidy level has been implicated in genome protection from environmental stresses, e.g., UV damage, salt and water stress and low temperature (Barow, 2006; Ceccarelli et al., 2006; Cookson et al., 2006). Other functions associated with endoreduplication include differentiation (e.g., cell fate determination in *Arabidopsis* trichomes; Bramsiepe et al., 2010) and modulation of transcriptional activity through increase in gene copy number (Bourdon et al., 2012). Positive correlations have also been shown between DNA ploidy level and cell size in a variety of plant tissues and species (Joubés and Chevalier, 2000; Sugimoto-Shirasu and Roberts, 2003; Chevalier et al., 2011; Bourdon et al., 2012). In the tomato fruit, endoreduplication defines a potential that supports a range of cell sizes (Mathieu-Rivet et al., 2010; Nafati et al., 2011); positive correlations between DNA ploidy level and cell size have been reported in some genotypes (Cheniclet et al., 2005). Just like in the canonical cell cycle, cyclins and CDKs drive endocycle entry and progression. Downregulation of the M phase associated CDKB1;1 and degradation of its dimerization partner, CYCA2;3, promote endoreduplication (Boudolf et al., 2004; Boudolf et al., 2009). CDK inhibitors like WEE1, SIM/SMR and KRPs together with inhibitors of the cyclin moiety like the ANAPHASE PROMOTING COMPLEX CYCLOSOME (APC/C) activator; CELL CYCLE SWITCH PROTEIN 52A (CCS52A) (Cebolla et al., 1999) and INCREASED LEVEL OF PLOIDY 1 (ILP1; Berckmans and De Veylder, 2009) induce endoreduplication. Progression of the endocycle then depends on S and G phase specific cyclins and CDKA.

Cell cycle and endocycle regulation by transcription factors is upstream of the cyclins and CDKs. OBP1, E2F and THREE MYB REPEAT (MYB3R) transcription factors are key

regulators of the cell cycle (Skirycz et al., 2008; Komaki and Sugimoto, 2012). E2Fs target genes involved in DNA repair and chromatin dynamics at the transition between G1 to S phase. There are six E2Fs (a to f) in *Arabidopsis*, three of which (E2Fa, E2Fb and E2Fc) are typical, i.e., need a dimerization partner (DPa or DPb) for strong and specific DNA binding, while the others (d to f) are atypical, i.e., bind to DNA as monomers (Berckmans et al., 2011). E2Fa and E2Fb are positive regulators while E2Fc and atypical E2Fs that lack a transcriptional activation domain are negative regulators of cell division. During cell cycle arrest, E2Fs are bound to the RETINOBLASTOMA-RELATED (RBR) protein and rendered inactive. They are activated when CDK-CYCD dimers phosphorylate RBR causing transcriptional activation of DNA replication genes (de Jager et al., 2009; Naouar et al., 2009). On the other hand, OBP1 regulates transcription of E2Fa, CYCD3;3 (Skirycz et al., 2008) and G2 to M phase specific genes while MYB3R target genes are G2 and M phase specific (Komaki and Sugimoto, 2012). Five MYB3R proteins are known in *Arabidopsis* (Haga et al., 2011), from which MYB3R1 and MYB3R4 knockout mutants exhibit deregulation of *CycA1;1*, *CycB1;2*, *CycB1;4*, and *CycB2;1*.

Light interacts with the cell cycle and endocycle through transcription factors

Light effects on the cell cycle

The study of light effects on the cell cycle has been conducted extensively in unicellular green algae (Chlorophytes). These studies have shown that light in the blue region (400 – 500 nm) inhibits cell division in *Protosiphon botryoides* via signals from a yet to be identified blue light photoreceptor (Nishihama and Kohchi, 2013). Red light on the other hand stimulates cell division in *Chlamydomonas reinhardtii* (Beel et al., 2012). Stimulation of cell division in *C. reinhardtii* by light has been reported to be associated with photosynthesis or photoassimilates (Spudich and Sager, 1980). In contrast, cell division in *Ostreococcus* was found to be regulated by the circadian clock independent metabolism (Moulager et al., 2007). Studies on the effect of light in higher plants have revealed comparable results to observations in unicellular algae. For example, Dougher and Bugbee (2004) observed that an increase in the blue light fraction (from <0.1% to 26%) inhibits cell division leading to a decrease in soybean internode length. In field-grown apple fruits, Dash and Malladi (2012) showed that less shading stimulates cell division. Our own work with locally illuminated *in vivo* grown tomato fruits also showed that more cell division takes place within pericarp tissue in the presence of

light than in the dark (Chapter 3). Light in general promotes cell division by stimulating the activity of photoreceptors that suppress cell division inhibitors (Figure 1).

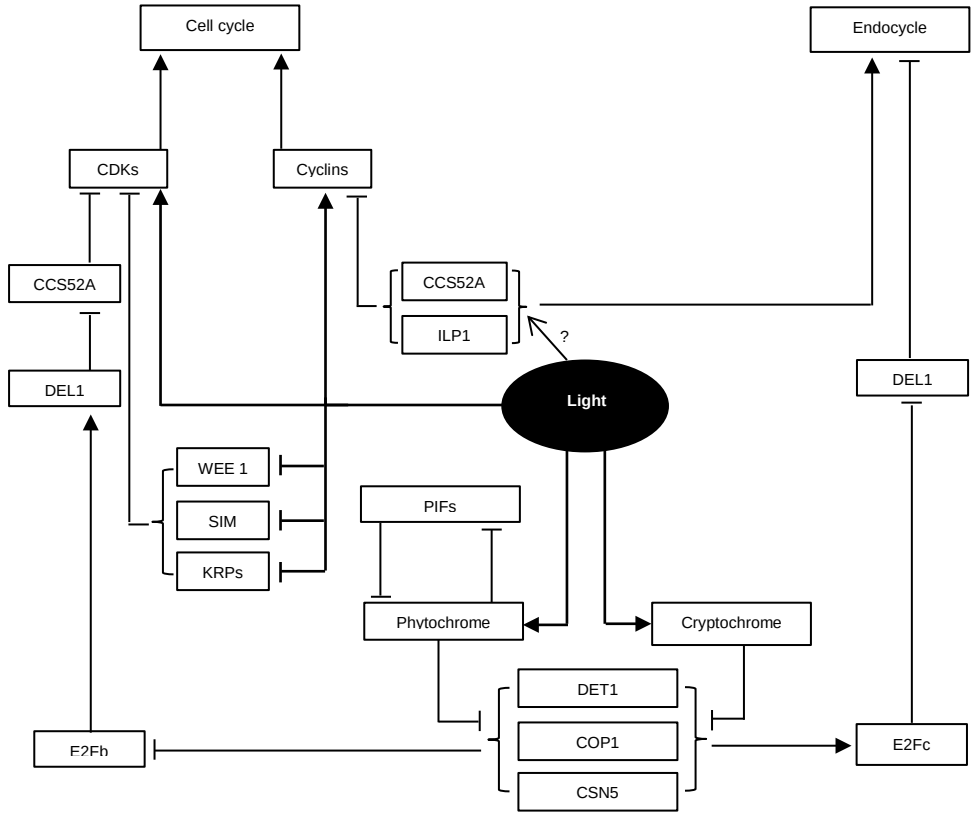

Figure 1: Regulation of cell division and endoreduplication by light (CCS52A, cell cycle switch protein 52A; CDK, cyclin dependent kinase; COP1, constitutive photomorphogenic 1; CSN5, COP9 signalosome complex subunit 5; DEL1, DP-E2F-LIKE1; DET1, de-etiolated 1; ILP1, increased level of polyploidy 1; KRP, KIP related protein; PIFs, phytochrome interacting factors; SIM, Siamese). Question mark indicates a light effect that is yet to be shown through experimentation.

A topical study aimed at elucidating the effect of light on the cell cycle in higher plants was conducted by López-Juez et al., (2008) in *Arabidopsis*. The authors used microarray profiling to investigate the expression of cell cycle related genes in shoot apices and cotyledons of dark- and light-grown (100 µmol. m^{-2}. s^{-1} fluorescent cool-white light) seedlings. In the dark, seedling shoot apex growth is typically arrested and cotyledons remain folded. When exposed to light, shoot apex growth is reactivated leading to development of true leaves while cotyledons unfold and start to expand (Whitelam and Halliday, 2007). López-Juez et al. (2008) showed that shoot apex growth stimulation by light stems from cell division stimulation, while cotyledon growth involves endoreduplication and cell expansion. In the dark, equal proportions of 2C and 4C nuclei were observed in both apical shoots and cotyledons. In the presence of light, a rapid increase in the proportion of 2C relative to 4C

nuclei occurred in shoot apices, while in cotyledons the proportion of 8C nuclei increased at the expense of 4C nuclei. Gene expression data revealed that *KRP1*, a CDK inhibitor that is commonly expressed in endoreduplicating tissues, was highly expressed in cotyledons but downregulated in shoot apices by light. The mechanism for this light stimulation of *KRP1* expression in cotyledons is not yet clear. Increased cell division in the presence of light was associated with activation of cell cycle genes. *CDKB1;1*, *CDKB1;2* and *CYCA2;2*, for example, were activated within one hour of seedling transfer to light. Other D-, A- and B-type cyclins attained maximum expression 6 h after transfer to light. On the contrary, cell cycle inhibitors KRP4, SIM, and CASEIN KINASE I-LIKE 3 (CKL3) were highly expressed in shoot apices of dark-grown seedlings but rapidly repressed by light.

Cell cycle onset begins with dissociation of inhibitory RBR proteins from E2F transcription factors through phosphorylation of RBR by CDK in complex with D type cyclins. Therefore, processes that affect E2F transcription factor stability can have a profound effect on the cell cycle. Using protein extracts from 5-day old dark- and light-grown *Arabidopsis* seedlings, López-Juez et al. (2008) showed that light-grown seedlings could accumulate 2.5 times more E2Fb (activator of cell division) compared with dark-grown seedlings. When seedlings were transferred from darkness to light, both the slow and high mobility form of E2Fc (repressor of cell division) emerged. The high mobility form was specific to light-grown seedlings while the level of the slow mobility E2Fc decreased rapidly in the presence of light. This finding highlights a direct effect of light on cell cycle onset through regulation of E2F transcription factor stability.

The mechanism for light regulation of E2F transcription factors was further studied using mutants with defects in multiple phytochromes, cryptochromes and photomorphogenesis repressors: DE-ETIOLATED 1 (DET1), CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) and the COP9 SIGNALOSOME 5 (CSN5; López-Juez et al., 2008). Phytochrome and cryptochrome defective mutants exhibited delayed activation of leaf primordia growth in light, hence López-Juez et al. (2008) concluded that phytochromes and cryptochromes regulate meristem activity and cell cycle progression. The authors observed that the level of E2F transcription factors was regulated through DET1, COP1, and CSN5. In the dark, activity of DET1, COP1, and CSN5 causes reduced levels of E2Fb and elevated levels of E2Fc. The relationship between DET1 and COP1 and cell cycle regulation was tested using seven-day old knockout mutant seedlings, i.e., *det1-1* and *cop1-4*. Unlike wild type seedlings, the level of E2Fb and E2Fc in *det1-1* and *cop1-4* was not affected by light. To elucidate the role of

CSN5, the authors introduced an RNAi interference construct of CSN5 into dark-grown *Arabidopsis* protoplasts and showed that E2Fb levels could be increased and E2Fc levels decreased in the dark. It is now known that the activity of DET1, COP1, and CSN5 is repressed by the action of UVR8 (Lau and Deng, 2012; Tilbrook et al., 2013), phytochrome, and cryptochrome photoreceptors (López-Juez et al., 2008). Although UVR8 suppresses cell cycle inhibitors (DET1, COP1 and CSN5; Lau and Deng, 2012; Tilbrook et al., 2013), cell cycle arrest still occurs in the presence of UV-B light through direct damage to DNA (Biever et al., 2014).

Light effects on the endocycle

Effects of light on the endocycle have been studied in seedlings mainly during the transition from skotomorphogenesis to photomorphogenesis. Studies generally indicate that light suppresses endocycle promoters by activating photoreceptors (Figure 1). In *Arabidopsis* (Gendreau et al., 1997, 1998), cabbage (*Brassica oleracea*; Kudo and Mii, 2004), and pea (*Pisum sativum*; Van Ostveldt and Van Parijs, 1975) hypocotyls, more endocycles occur in the dark compared with when grown in the presence of light. A study into the molecular mechanism of this response by Berckmans et al. (2011) revealed that the atypical DP-E2F-LIKE1 (DEL1) transcription factor that represses the APC/C activator, CCS52A2, is a mediator for light dependent endoreduplication in *Arabidopsis* hypocotyls. The authors proposed a model in which two transcription factors, E2Fb (activator) and E2Fc (repressor), compete for the same *E2F-2* DNA binding site of *DEL1* (Figure 1). In the dark, E2Fb is degraded and this results in repression of *DEL1* through occupation of its binding site by the repressive E2Fc. Like many light-signalling components, E2Fb is now known to be degraded by the ubiquitin E3 ligase COP1 in the dark (López-Juez et al., 2008).

Etiolated *Arabidopsis* hypocotyls tend to exhibit one more endocycle compared with de-etiolated hypocotyls. Gendreau et al. (1998) reported that photoreceptors mediate this effect of light on endoreduplication in *Arabidopsis* seedlings. They compared the number of endocycles in hypocotyls of wild type, phytochrome (*phyA* and *phyB*) and cryptochrome (*cry1*) knockout mutant seedlings grown in the dark, blue, far-red, red and white light. Their results revealed an extra endocyle in 31% of nuclei of dark-grown wild type and *phyA* and *phyB* seedlings, while growth in white light resulted in an extra endocycle in only 14% of nuclei of *phyB* seedlings. In red light, the number of nuclei with the extra endocycle in wild type and *phyA* seedlings was only half of what was observed in *phyB* seedlings. In far-red light, the extra endocycle was absent in wild type and *phyB* seedlings, however, *phyA*

seedlings were etiolated and exhibited the extra endocycle. They concluded that in red light, PHYB was required but not sufficient to suppress the extra endocycle, while PHYA is necessary for suppression of the extra endocycle in far-red light. In white light on the other hand, neither PHYA nor PHYB was sufficient to completely suppress the extra endocycle implying that other phytochrome isoforms or photoreceptors could play a role in endocycle suppression. To test this, they compared the number of endocycles in wild type and *cry1* seedlings grown in the dark, blue and white light (100 µmol. m^{-2}. s^{-1} fluorescent cool-white). The extra endocycle was evident in both wild type and *cry1* seedlings in the dark and blue light; however, in white light, a small number (1 – 2%) of nuclei had the extra endocycle only in *cry1* seedlings. This led to the conclusion that cryptochromes play a minor role in repression of the third endocycle. To test the role of COP1, Gendreau et al. (1998) studied mutant seedlings deficient in COP1 (*cop1*) in dark and white light conditions. The ploidy level in both treatments was similar to that of wild type seedlings, i.e., no extra endocycle, hence the conclusion that COP1 is necessary for the extra endocycle.

The above findings show that different photoreceptors contribute to the overall suppression of endoreduplication in the presence of light. Hence factors that degrade photoreceptors may also promote the occurrence of more endocycles. For example, PIFs that have now been shown to stimulate COP1 catalyzed ubiquitylation and degradation of PHYB (Jang et al., 2010) could play a role in endocycle promotion. The molecular link between photoreceptors and regulators of the endocycle is still unclear but, possibly, photoreceptors activate endocycle inhibitors, e.g., DEL1. Another possible mechanism could involve transcriptional or post translational suppression of cyclin and CDK inhibitors like WEE1, SIM/SMR, KRPs, CCS52A and ILP1 by photoreceptors. In contrast, UV-B light appears to promote endoreduplication. Radziejwoski et al. (2011) showed a strong downregulation of *DEL1* in *Arabidopsis* when plants were exposed to UV-B. They argued that this could lead to upregulation in the expression of endocycle promoting *CCS52A*.

Cell expansion

In most species, cell expansion takes place after a period dominated by cell division. Cells generally expand through accumulation of macromolecules and organelles (Sablowski and Dornelas, 2014) and water uptake that elicit pressure on the cell wall causing it to extend. The accumulation of macromolecules and organelle growth requires photoassimilates. In sink organs like fruits, cell expansion is directly dependent on the amount of imported

photoassimilates. Fruits like tomato, import sucrose and store it as starch in the first 21 – 28 days after anthesis (Figure 2; Guan and Janes, 1991). In this period, vacuolar acid invertase, ADP GLUCOSE PYROPHOSPHORYLASE (ADPGPP) and starch synthase play important roles in the hydrolysis of sucrose and synthesis of ADP glucose and starch respectively (Yelle et al., 1988; Guan and Janes, 1991; Frommer and Sonnewald, 1995). After approximately 28 days after anthesis, sucrose import declines considerably and accumulated starch is converted by starch phosphorylase into glucose and fructose. Starch accumulation in itself may lead already to some cell expansion, but its break-down into glucose and fructose results in a stronger cell expansion as it causes an increase in fruit osmotic potential leading to increased water uptake and turgor pressure on the cell wall.

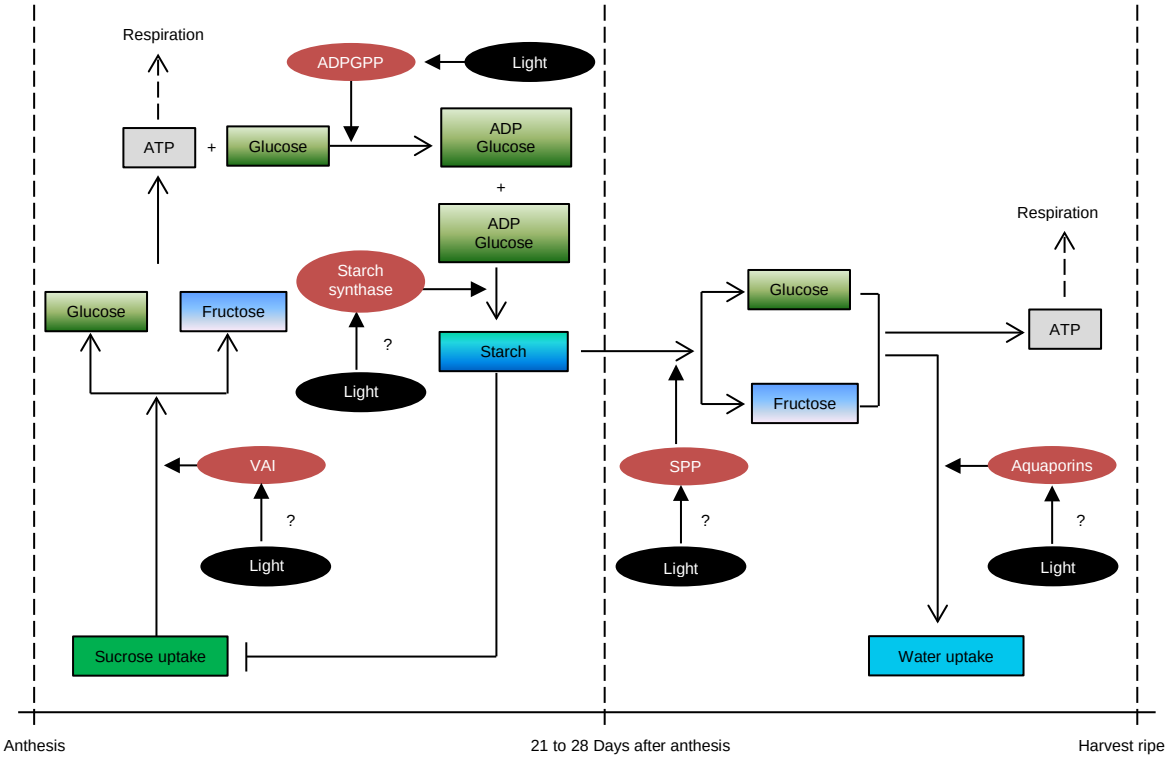

Figure 2: Light effects on carbohydrate metabolism (ADPGPP, ADP Glucose pyrophosphorylase; SP, starch phosphorylase; VAI, vacuolar acid invertase) during tomato fruit growth. Question marks indicate light effects that are yet to be shown through experimentation.

Growing cells have a primary cell wall made up of cellulose microfibrils contained in a matrix of non-cellulosic polysaccharides, pectins and glycans (Figure 3). Of the non-cellulosic polysaccharides, xyloglucans constitute approximately 20 – 25% (Hayashi, 1989) while pectins constitute 35% (Fry, 1988) of plant cell wall dry weight. Glycans, for example

xyloglucan, bind covalently onto cellulose microfibrils and in some cases are trapped inside the microfibrils (Darley et al., 2001; Wolf et al., 2012). A single xyloglucan molecule can bind more than one cellulose microfibril making it a key determinant of cell wall extensibility. By separating individual cellulose microfibrils, xyloglucans also prevent the entanglement and aggregation of cellulose microfibrils leading to increased extensibility of the cell wall. Cellulose microfibrils and non-cellulosic polysaccharides are also intertwined with structural proteins; extensins and arabinogalactans (Darley et al., 2001; Cosgroove, 2005). Extensins are found in small amounts within the cell wall and are known to cause the formation of rigid cell walls towards the end of growth while arabinogalactans appear to be more important as signalling molecules (Gasper et al., 2001).

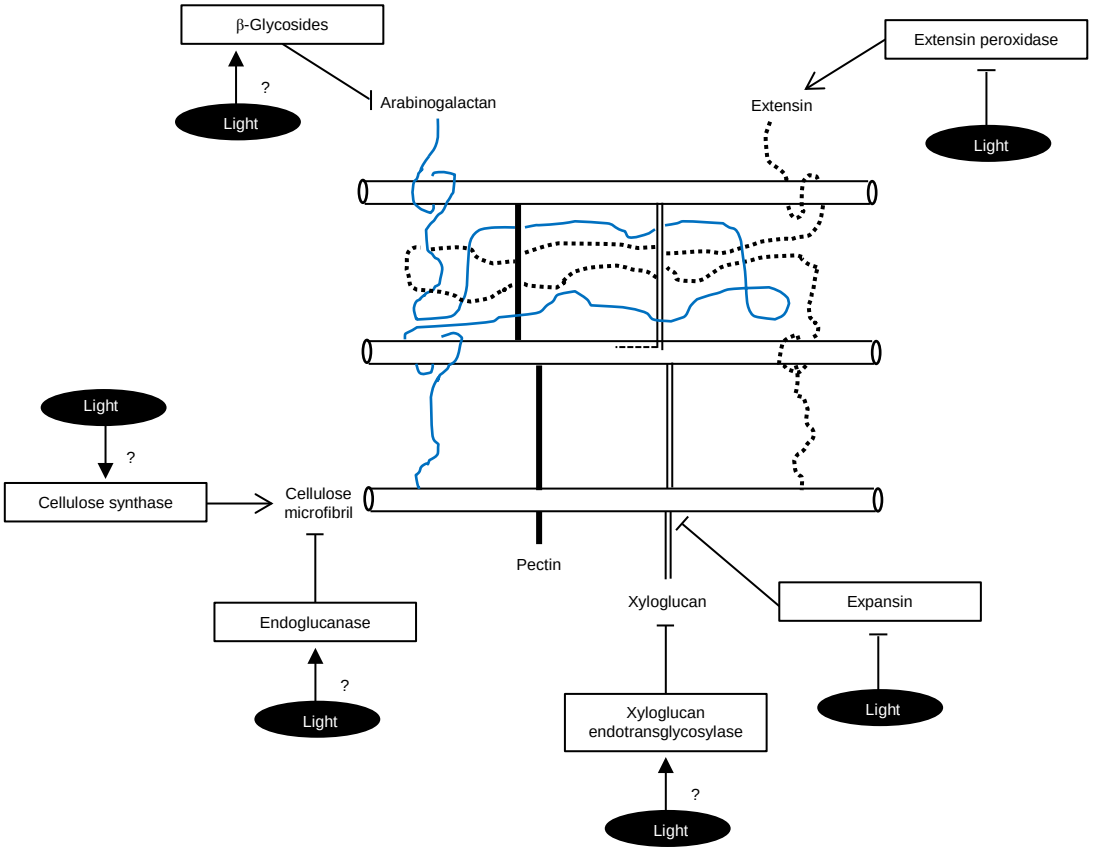

Figure 3: The effect of light on cell wall components. Question marks indicate light effects that are yet to be shown through experimentation.

Cell wall extensibility is a function of new wall deposition, wall composition, bonding between wall components and enzymatic modification of the wall (Darley et al., 2001). Cellulose microfibrils are usually bound to non-cellulosic polysaccharides and proteins

through covalent and non-covalent interactions (Wolf et al., 2012). Recent evidence suggests that covalently bonded pectin and xyloglucan molecules also act as combined units in their interaction with cellulose microfibrils (Dick-Pérez et al., 2011; Popper and Fry, 2008). In general, direct bonding of non-cellulosic polysaccharides with cellulose microfibrils inhibits cell wall extensibility but their presence in between the microfibrils enhances cell wall extensibility by preventing aggregation of the microfibrils. These apparently contrasting roles of non-cellulosic polysaccharides ensure that the cell wall has high tensile strength but is also capable of accommodating cell expansion. Several enzymes (expansins, xyloglucan endotransglycosylases, and endoglucanases) modify the structure of cell walls by acting either on the bonds between cellulose microfibrils and non-cellulosic polysaccharides or on individual polysaccharides.

Expansins for example are thought to act as a molecular grease that disrupts non-covalent bonds between wall polysaccharides (Darley et al., 2001). In addition, expansins have been reported to disrupt the hydrogen bond between xyloglucans and cellulose microfibrils (Cosgroove, 2005; Sablowski and Dornelas, 2014). Repression of expansin activity can have marked effects on cell size. For example, in differentiated *Arabidopsis* root and hypocotyl cells, *E2Ff/DEL3* transcription factors were shown to repress the expression of three expansin genes leading to a decrease in cell size (Ramirez-para et al., 2004). Xyloglucan endotransglycosylases on the other hand allow for the incorporation of new xyloglucan chains into existing chains through cutting and re-joining actions during new cell-wall deposition. Endoglucanases aid in wall loosening by breaking down cellulose microfibrils to release trapped xyloglucans (Cosgroove, 2005).

Effects of light on cell expansion

The effect of light on cell expansion processes results from effects on availability of photoassimilates (Figure 2) and signalling (Figure 3). Guan and Janes (1991) showed that *in vitro* grown tomato fruits accumulate more starch in the presence of light than in the dark. They noted that the increased accumulation of starch was due to activation of ADP Glucose pyrophosphorylase by light. Since accumulated starch is hydrolysed into fructose and glucose leading to an increase in fruit osmotic potential and subsequently water uptake, it is plausible that light stimulates cell expansion in cases where it has a clear effect on carbohydrate availability. For example when apple branches were shaded, fruit cell size was smaller compared with that in fruits from branches that had not been shaded (Dash and Malladi, 2012). However, when carbohydrate availability is abundant, light appears to have a negative

signalling effect on cell expansion. Our own results from studies with tomato fruits grown under non-limiting assimilate supply showed that smaller sized cells were formed in the presence of light compared with when fruits were grown in the dark (Chapter 3). Another example where light reduces cell expansion can be found when etiolated seedlings are exposed to light. Hypocotyl growth is suppressed through cell expansion suppression. However, cotyledon and leaf expansion are stimulated through increased cell expansion upon exposure to light (Van Volkenburgh et al., 1991; Neff et al., 1994; Pfeiffer and Kutschera, 1997; Barkan et al., 2006).

As indicated above, the extent to which a cell expands depends on the accumulation of macromolecules and organelles, and the ability of the cell wall to extend. Lockhart (1965), showed that the extent to which a cell expands, depends on the turgor pressure applied to its cell wall, yield threshold and the cell wall's ability to expand (extensibility coefficient). The yield threshold represents the minimum pressure necessary for the cell wall to extend. This implies that light effects on cell size can be effected through one or more of the above parameters. Following evidence from studies that showed that red light (phytochrome) and blue light (cryptochrome) photoreceptors mediate the inhibition of elongation in etiolated seedlings (Gaba et al., 1983), Kigel and Cosgrove (1991), investigated the effect of blue and red light on cell wall properties in etiolated pea seedlings. They showed that both blue and red light inhibit cell wall extensibility. Red light specifically inhibited seedling elongation by lowering the cell wall extensibility coefficient while blue light increased the yield threshold. In cucumber, Cosgrove (1988) reported that reduced elongation of etiolated hypocotyls in the presence of blue light came as a result of a decrease in the cell wall extensibility coefficient.

How does light lead to reduced or increased cell expansion? Attempts to answer this question have focused on the effects of light on cell wall components since loosening or rigidification of the cell wall has a great impact on cell expansion. Extensins, for example, limit cell expansion by interlacing cell wall polymers. They are deposited in the cell wall by extensin peroxidase. Brownleader et al. (2000) studied the effect of inhibiting extensin peroxidase activity on growth of illuminated and etiolated tomato hypocotyls. They showed that growth inhibition of light-grown hypocotyls could be reduced by 15% when extensin peroxidase activity was inhibited. The inhibitor had no growth enhancing effect on etiolated seedlings, thereby implying that growth inhibition by light occurs through an increase in extensin deposition within cell walls. Promotion of cell expansion in leaves by light on the other hand takes place through cell wall acidification leading to wall loosening (Van

Volkenburgh and Cleland, 1980). These authors noted that light induces proton excretion by leaf cells leading to wall acidification; however, light-induced cell enlargement was inhibited when a neutral buffer was introduced.

In *Arabidopsis*, cell expansion is promoted by HOMOLOG OF BEE2 INTERACTING WITH IBH1 (HBI1) transcription factors. These transcription factors promote the expression of two expansin genes that encode cell wall loosening enzymes (Bai et al., 2012). Temperature, light and plant growth hormones (brassinosteroids and gibberellin) regulate cell expansion by influencing the paclobutrazol-resistant (PRE) family of transcription factors (Bai et al., 2012). PRE transcription factors promote cell expansion by inhibiting ILI1 BINDING BASIC HELIX-LOOP-HELIX 1 (IBH1) transcription factors that are known to be inhibitors of HBI1. Oh et al. (2012) showed that light represses the expression of PRE transcription factors through the inhibitory effect of light on PIFs and the BRASSINAZOLE RESISTANT 1 (BZR1). PIFs are part of the basic helix-loop-helix (bHLH) family of transcription factors. They play an important role in skotomorphogenesis by suppressing the activity of genes that would otherwise initiate photomorphogenesis. Stem elongation observed during skotomorphogenesis is characterized by cell elongation. Light suppresses stem elongation through phosphorylation and degradation of PIFs. Lucas et al. (2008) showed that this effect of light in *Arabidopsis* occurs through destabilization of PIF4 by the light photoreceptor, phytochrome B.

Conclusion and future perspectives

As the main source of energy for plants, light has a significant effect on plant development and growth. The effects of light on cell division, cell expansion and endoreduplication are mediated through various transcription factors. Photoreceptors by default have an important role in regulating these processes. Their interaction with PIFs appears to be central in the regulation of cell division, endoreduplication and cell expansion in plants. The main modes of light regulation are 1) transcription factor degradation and 2) inhibitory competition between transcription factors for promoter regions of target genes. Cell division seems to be stimulated by light irrespective of the plant organ under consideration while the response of cell expansion and endoreduplication varies from organ to organ. This could be because light signalling plays a larger role in cell division while both light signalling and the impact of light on photoassimilate supply are important during cell expansion and endoreduplication. Compared to cell division, the molecular basis of light effects on cell expansion is still

unclear. Studies on the effect of light on genes coding for vacuolar acid invertase, starch synthase, starch phosphorylase, aquaporins, endoglucanases, xyloglucan endotransglycosylases, β-glycosides, and cellulose synthase will bridge the gap in our understanding of cell expansion.

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Chapter 5

What drives fruit growth?

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Abstract

Cell division, endoreduplication (increase in nuclear DNA content without cell division) and cell expansion are important processes for growth. It is debatable whether organ growth is driven by all these three cellular processes. All could alternatively be part of a dominant extra-cellular growth regulatory mechanism. Cell level processes have been studied extensively and a positive correlation between cell number and fruit size is commonly reported while few positive correlations between cell size or ploidy level and fruit size are shown. Here we discuss cell level growth dynamics in fruits and ask, what drives fruit growth and during which development stages? We argue that 1) the widely accepted positive correlation between cell number and fruit size does not imply a causal relationship; 2) fruit growth is regulated by both cell-autonomous and non-cell-autonomous mechanisms as well as a global coordinator, the target-of-rapamycin (TOR); and 3) increase in fruit size follows the neo-cellular theory of growth.

Keywords: growth theory, cell division, endoreduplication, cell expansion, TOR

Introduction

Plant growth involves a permanent increase in size. Although the purpose of growth varies among organs, it ultimately aims at ensuring plant survival and species perpetuation. Roots for instance grow to capture water and nutrients and ensure anchorage with an increasing plant size while stems grow to expose leaves to light, flowers to pollinators and fruits to seed dispersers. Leaves on the other hand grow to maximize light capturing while fruits grow to provide a suitable environment for seed development and attract seed dispersers to ensure colonisation of larger areas. Plant growth can be observed at the plant, organ, tissue or cell level. While plant growth through aggregation of the number and size of its tissues and organs seems straightforward, the cellular basis of organ growth is still unresolved. What drives organ growth is one of the unresolved challenges in plant science.

Three theories of organ growth have so far been fronted, i.e., cellular, organismal and neo-cellular growth (Beemster et al. 2003; John and Qi 2008; Horiguchi and Tsukaya 2011). Proponents of the cellular theory of growth argue that cells are the building blocks of multicellular organisms, hence growth stems from decisions taken at the cell level. The organismal theory on the other hand suggests that cells are compartments of organismal space whose activities are regulated at the organ level; therefore, cell division for example is a consequence but not the cause of growth (Kaplan 2001). The neo-cellular theory of growth combines the cellular and organismal theories of growth and proposes the existence of some level of interaction between cell and whole organ behaviour. Following the organismal or neo-cellular theory of growth, plants can compensate deficiencies in cell division by extra cell expansion. An understanding of growth at the cell level indeed opens up opportunities to manipulate plant growth using current advances in plant breeding and molecular biology.

In this perspective paper, we argue that the importance of cell expansion is often underestimated as a factor driving fruit growth. We also highlight the role of the target-of-rapamycin (TOR) in regulation of both cell division and cell expansion and the importance of DNA content in fruit size determination. We further argue that fruit growth follows the neo-cellular theory of organ growth. Although a few examples are drawn from fruits of other species, emphasis is placed on the tomato fruit because of its long standing status as a model berry fruit (Kimura and Sinha 2008) and the enormous opportunities for genetic manipulation presented by the recent publication of its complete genome sequence (Tomato Genome Consortium 2012).

Fruit development and growth

Generally four phases of fruit development can be discerned. The first phase involves floral initiation, carpel formation and pre-anthesis cell division leading to ovary growth; it ends when the flower is fully formed and open (anthesis). The second phase commences with flower pollination followed by fertilization of ovules, fruit set and re-start of cell division. Cell division is the main process by which fruits increase in size at this early stage. This second wave of cell division continues for approximately two weeks in tomato pericarp tissue (Gillaspy et al. 1993; Tanksley 2004) but in some genotypes it is still significantly evident beyond 25 days after anthesis (DAA; Bertin et al. 2009). The duration of cell division is, however, much longer in the fruit epidermis where it continues until shortly before the fruit attains its full size (Czerednik et al. 2012). The third phase of fruit development is characterized by cell expansion leading to a spectacular increase in cell size and consequently fruit size. This phase involves a great import of water and sucrose and accumulation of starch and hydrolysis of the latter into fructose and glucose. Another phenomenon that is typical of the cell expansion phase of fruit development is endoreduplication, i.e., increase in cellular DNA content without cell division (Chevalier et al. 2011). DNA ploidy levels of up to 512C (where C is the haploid DNA content) have been reported in tomato fruits and there is mounting evidence of a positive correlation between cell DNA content and pericarp cell size (Cheniclet et al. 2005; Bourdon et al. 2012). The last phase of fruit development, ripening, takes place after seed maturation and when the fruit has attained its full size.

Fruit growth is in principle a function of the attributes of its components as defined by processes that independently affect the development of carpels and seeds, and the number, size and DNA ploidy level of cells in fruit tissue. The size of the ovary at anthesis is indicative of the amount of pre-anthesis cell division and number of carpels formed during the first phase of fruit development. Final fruit size is positively correlated with the amount of pre-anthesis cell division (Baldet et al. 2006), post-anthesis cell division (Bertin et al. 2009), carpel number (Houghtaling 1935; Yeager 1937; Lippman and Tanksley 2001), and seed number (Nitsch 1970; Lippman and Tanksley 2001). It has been postulated that seeds are sources and sinks for cytokinin and auxin that stimulate cell division and cell expansion in developing ovaries (Bohner and Bangerth 1988a). In contrast, not many authors have shown a positive correlation between fruit size and cell size or DNA ploidy level (Cheniclet et al. 2005).

Fruit size is a quantitatively inherited trait and as such is controlled by many quantitative trait loci (QTLs) each with varying effects (Paran and van der Knaap 2007). Up to 28 fruit size QTLs have been identified in tomato. The larger fruit size of domesticated species compared to wild species appears to be the result of mutations in a few genes: fw1.1, fw2.2, fw3.1, fw3.2, fw4.1, fasciated and locule-number (Tanksley 2004; van der Knaap et al. 2014). Of these, fw2.2 suppresses cell division (Frary et al. 2000) and accounts for approximately 30% of natural genetic variation in fruit size. Because of its role in cell cycle regulation, fw2.2 is sometimes referred to as Cell Number Regulator (CNR) (Guo and Simmons 2011; van der Knaap et al. 2014). Domesticated tomato has the large fruited allele while wild tomato species have the small fruited allele at the fw2.2 locus. The fasciated and locule-number genes on the other hand affect fruit size via changes in the number of carpels (Tanksley 2004). A clear link is yet to be established between other fruit size QTLs and fruit development and growth processes (Bertin et al. 2009). The correlation between cell size and fruit size QTLs will become clearer when our understanding of the regulation of cell expansion advances to the level of current knowledge of cell cycle regulation.

Cell level dynamics in fruits of contrasting size

Wild tomato species are characterized by small fruit size while cultivated tomato is generally large fruited. Among the cultivated tomato genotypes, fruit size varies enormously from a few grams up to 1,000 g (Tanksley 2004). Fruit load and position on the truss and the environment (temperature, light) during fruit growth have a significant influence on tomato fruit size (Kromdijk et al. 2014). The cellular basis of this variation in fruit size has been a subject of interest in many studies. In the following sections, we provide an overview of findings from experiments in which fruit size differences were noted among fruits growing in different positions and fruit loads on the same truss, in different genotypes or under different environmental conditions and highlight the dynamics of cell division, cell expansion and endoreduplication during fruit growth.

Fruit position and genotypic differences

Previous attempts to unravel the cellular basis of genotypic differences in fruit size have been conducted using genotypes that differ significantly in fruit size or nearly isogenic lines (NILs) harbouring different fruit weight quantitative trait loci (QTLs) or mutants in which specific genes were knocked out or overexpressed (Table 1). For example, in a comparison between a

small (wild-type) and large (mutant) fruited tomato, Bohner and Bangerth (1988a) showed that the mutant had more cells and an extensive and faster cell expansion rate. Although cell division took place over a longer period in the mutant, they concluded that cell number at anthesis was the determining factor for final fruit size in tomato. Similar observations were made by Bertin et al. (2003) in two NILs that differed in fruit weight QTLs (CF12-C, small; CF14-L, large). Proximal and distal fruits of CF12-C had fewer cell layers than CF14-L when ovaries were not pruned. No difference in cell size was noted between the two NILs hence the conclusion that cell division QTLs were responsible for observed differences in fruit size. A comparison between the large fruited proximal and small fruited distal fruits on the same truss in both NILs showed that proximal fruits generally had more cells than distal fruits while cell size did not differ between the two fruit positions. However, when a large fraction of the competing fruits were removed, the difference in fruit size between the two NILs and fruit positions was reduced. This was attributed to a reduction in the difference in cell number between the two lines and promotion of cell expansion in distal fruits. Bertin et al. (2003) also reported a linear relationship between cell number and ovary size at anthesis while that between cell number and fruit size was not significant at the mature green stage. However, Bertin et al. (2009) showed a positive correlation between cell number and fruit fresh weight in fruits of four different NILs. Prudent et al. (2009) further demonstrated a positive correlation between fruit fresh weight and cell number and showed numerous cases of co-localization of QTLs for cell division and fruit fresh weight.

In contrast, the study by Cheniclet et al. (2005) highlights the importance of cell size and ploidy level in the determination of fruit size in genotypes containing a similar number of carpellar locules. Unlike other authors, they found that 1) fruit fresh weight was approximately a cubic function of cell diameter; and 2) mean cell ploidy was positively correlated with both mean cell diameter and fruit fresh weight. A similar effect of cell size and ploidy level on tomato fruit size was also reported by Gonzalez et al. (2007). These authors observed that mutants in which a CDKA inhibitor (WEE1) had been repressed, developed smaller fruits as a result of reduced cell size and ploidy level. In apple, Malladi and Hirst (2010) showed that cell expansion was responsible for fruit size differences between two apple varieties (small, ‘Gala’; large, ‘Grand Gala’). The authors noted that ‘Grand Gala’ had a larger cortex, larger cells and higher ploidy level while cell number was not different in the two varieties. Their finding appears to stem from the fact that unlike other studies, they compared the proportion of large cells in the two varieties rather than the average cell size.

They also reported that a large proportion of nuclei in 'Grand Gala' had an area of 30 – 60 μm^2 while a large proportion of nuclei in 'Gala' had an area of 6 – 30 μm^2.

Studies in other genotypes suggest that fruit size differences can result from changes in both cell number and cell size. In a study by Fanwoua et al. (2012a) involving two ILs of *Solanum chmielewskii* (g36, small; g49, large), it was reported that the large fruited IL (g49) had more and larger mesocarp cells compared to the small fruited g36. Our own results from a study on a large ('Cappricia') and small ('Brioso') fruited tomato cultivar also showed that the larger fruited cultivar had more and larger sized cells (Chapter 2). In addition, Czerednik et al. (2012) demonstrated the same phenomenon using tomato mutants in which the expression of cell cycle promoters CDKB1 and CDKB2 had been altered in fruits. Fruits of CDKB1 and CDKB2 over-expressing mutants were smaller compared to those of the wild-type. Mutant fruits had fewer cell layers mainly in the exocarp and in addition, cell size and ploidy level were reduced.

Temperature induced differences

Temperature is a well-studied environmental factor influencing plant growth. Treatments are often applied at the plant level but some attempts have been made at the fruit level in tomato (Adams et al. 2001; Fanwoua et al. 2012b; Chapter 2). In most cases, heating plants or individual fruit trusses led to reduction in final fruit size. However, Bertin (2005) showed that under non-limiting source conditions, there were no significant differences in fruit size of heated and non-heated tomato plants because of compensation between cell division and cell expansion (Table 1). Bertin (2005) showed that cell expansion was enhanced while cell division was reduced in fruits of heated compared to control plants. This trend was attributed to the relatively shorter duration of cell division and longer period of cell expansion in heated plants. Observations on ploidy level revealed that growth at high temperature was characterized by increased mean ploidy level. Cell size on the other hand seemed to positively correlate with mean ploidy level.

Under limiting source conditions, heating individual fruit trusses caused a reduction in final fruit size and cell volume in tomato. The work by Fanwoua et al. (2012b) showed that tomato fruits that were heated only in the first 7 days after anthesis (DAA) had lower fresh weight and mesocarp cell volume at breaker stage than those that were not heated throughout fruit growth. They also reported that cell number was not significantly affected by local fruit heating. In Chapter 2, heated fruits of a small ('Brioso') and a large fruited ('Cappricia')

tomato variety were heated from anthesis until breaker stage. Heated fruits generally had lower fresh weight and mesocarp cell volume while mesocarp cell number seemed to increase. In cucumber, the response to fruit heating also appeared to depend on source conditions (Marcelis and Baan Hofman-Eijer 1993). Under non-limiting assimilate supply, increase in temperature led to an increase in cucumber fruit size because the decrease in growth duration was more than compensated by an increase in growth rate (Marcelis and Baan Hofman-Eijer 1993). This increase in fruit size was attributed to an increase in cell size while cell number did not respond significantly to fruit heating. Increase in temperature under limiting source conditions, however, led to a decrease in fruit size and cell number without any significant effects on cell size.

Photoassimilate availability induced differences

There is a general tendency towards increase in tomato fruit size with increase in photoassimilate availability (Table 1). How this is put into effect at the cell level has been investigated using defoliation, carbon dioxide enrichment, fruit pruning (Bertin et al. 2002; Baldet et al. 2006) and synchronized pollination (Bohner and Bangerth 1988b) treatments. Synchronization of pollination is one way of manipulating sink activity on the same truss because differences between proximal and distal fruits are associated with differences in access to photoassimilates.

For example, the size of proximal and distal fruits was uniform when pollination was synchronized (Bohner and Bangerth 1988b). Unlike fruits on trusses whose pollination had not been synchronized, cell number was lower in the proximal compared to distal position in the synchronization treatment. Cell size was similar in distal and proximal fruits when pollination was not synchronized, however, synchronization of pollination resulted in smaller cells in distal compared to proximal fruits. The authors concluded that similarity in fruit size of distal and proximal fruits in the synchronized pollination treatment was caused by alteration in cell number. They further observed that defoliation at anthesis did not change the fruit weight relationship between proximal and distal fruits in the synchronized and non-synchronized pollination treatments. In the non-synchronized pollination treatment, defoliation, however, caused a bigger decrease in cell number of distal compared to proximal fruits possibly because proximal flowers had the chance to grow for extra days without limitations in assimilate availability. In the synchronized pollination treatment, the decrease in cell number due to defoliation was similar in proximal and distal fruits.

Table 1: The cellular basis (C: control; +: increase; -: decrease; =: no detectable effect; =/+: depends on genotype; NA: not measured) of the contrast in fruit size in different species as affected by fruit position, genotype (NIL: nearly isogenic lines; IL: inbred line), photoassimilates and light. Comparisons are relative to the control within rows of the same grey level.

Source of variation	Crop	Source condition	Treatment level	Treatment	Fruit size	Cell number	Cell size	Ploidy	Reference
Position on truss	Tomato			Proximal	C	C	C	NA	Bertin et al. (2003)
				Distal	-	-	=	NA	
Genotype	Tomato			Wild-type	C	C	C	NA	Bohner and Bangerth (1988a)
				Mutant	+	+	+	NA	
	Tomato			CF12-C (NIL)	C	C	C	NA	Bertin et al. (2003)
				CF14-L(NIL)	+	+	=	NA	
	Tomato			g36-IL	C	C	C	NA	Fanwoua et al. (2012a)
				g49-IL	+	+	+	NA	
	Tomato			Wild-type	C	C	C	C	Czerednik et al. (2012)
				Mutant	-	-	-	-	
	Tomato			'Cappricia'	C	C	C	NA	Chapter 2
				'Brioso'	-	-	-	NA	
	Tomato			Wild-type	C	NA	C	C	Gonzalez et al. (2007)
				Mutant	-	NA	-	-	
	Apple			'Gala'	C	C	C	C	Malladi and Hirst (2010)
				'Grand Gala'	+	=	+	+	
Temperature	Tomato	Non-limiting	Plant	Heated	=	-	+	+	Bertin (2005)
		Non-limiting		Control	C	C	C	C	
	Tomato	Limiting	Fruit	Heated	-	=	-	NA	Fanwoua et al. (2012b)
		Limiting		Control	C	C	C	NA	
	Tomato	Limiting	Fruit	Heated	-	+	-	NA	Chapter 2
		Limiting		Control	C	C	C	NA	
	Cucumber	Non-limiting	Fruit	Heated	+	=	+	NA	Marcelis and Baan Hofman-Eijer (1993)
		Non-limiting		Control	C	C	C	NA	
	Cucumber	Limiting	Fruit	Heated	-	-	=	NA	Marcelis (1993)
		Limiting		Control	+	+	=	NA	
Photoassimilates	Tomato	Limiting	Leaf	Defoliated	-	-	NA	NA	Bohner and Bangerth (1988b)
		Non-limiting		Control	C	C	NA	NA	
	Tomato	Non-limiting	Flower	Pruned	+	+	NA	NA	Bertin et al. (2002)
		Limiting		Control	C	C	NA	NA	
	Tomato	Non-limiting	Flower	Pruned	+	+	NA	NA	Baldet et al. (2006)
		Limiting		Control	C	C	NA	NA	
	Apple	Non-limiting	Fruit	Pruned	+	+	+	NA	Dash et al. (2013), Dash and Malladi (2012), Wismer et al. (1995)
		Limiting		Control	C	C	C	NA	
	Tomato	Non-limiting	Flower	Pruned	+	+	=/+	NA	Prudent et al. (2010)
		Limiting		Control	C	C	C	NA	
	Apple	Non-limiting	Fruit	Pruned	+	+	=	NA	Goffinet et al. (1995)
		Limiting		Control	C	C	C	NA	
Light	Apple	Limiting	Branch	Shaded	-	-	-	NA	Dash et al. (2012)
		Non-limiting		Control	C	C	C	NA	
	Tomato	Non-limiting	Fruit	Dark	=	-	+	NA	Chapter 3
		Non-limiting		Light	C	C	C	NA	

Bertin et al. (2002) varied source-sink conditions by fruit pruning (2 vs 6 fruits per truss) and CO_2 enrichment. Fruit growth rate was faster and no variation in the number of cells in fruits of successive trusses was reported under non-limiting (2 fruits per truss and CO_2 enrichment) compared to limiting source conditions. Cell number was generally higher in fruits growing under non-limiting compared to limiting source conditions. Proximal fruits had more cells than distal fruits and the gradient in cell number of proximal and distal fruits was steeper in upper trusses under limiting source conditions. This was fronted as evidence that the cell number gradient between proximal and distal fruits was controlled by competition for photoassimilates during floral development. They concluded that cell division was the main factor limiting fruit growth under low assimilate supply conditions. Already at anthesis, proximal ovaries had more cells than distal ovaries implying that proximal ovaries dominated distal ovaries through competition for photoassimilates or through hormonal signals. Bertin et al. (2002) acknowledged that cell size was also important although they did not measure it. In agreement with these findings, Baldet et al. (2006) observed that fruit size was larger in plants grown with low fruit load and large fruit size correlated with higher cell number in the pre-anthesis ovary. They also reported that expression levels of cell proliferation promoters: *CycB2;1*, *CDKB2;1* and *CycD3;1* were higher while that of a putative negative regulator of the cell cycle; *fw2.2* was low in developing flower buds under low fruit load. This led them to conclude that the control of fruit growth through variation in source supply occurs through regulation of cell proliferation related genes.

Dash et al. (2013) examined the effect of early fruit pruning on apple fruit growth. They concluded that fruit pruning increased fruit growth by increasing cell production in the cortex but also reported a minor increase in cell area (11%) of fruits from pruned trees. The expression of the cell proliferation promoting transcription factor; *Aintegumenta* together with that of *CycA2;1*, *CycA2;3*, *CycB1;1*, *CycB2;2*, *CDKB1;1*, *CDKB1;1*, *CDKB1;2* and *CDKB2;2* increased by up to 5-fold in fruits of pruned trees. The expression of genes associated with cell expansion; *expansins* and *cobra* like genes also increased at maturity. The authors noted that treatments aimed at reducing competition for photoassimilates among apple fruits often led to increased fruit growth by promoting cell proliferation (Dash et al. 2012; Goffinet et al. 1995; Wismer et al. 1995). On the contrary other authors have shown that cell size increase also plays a role in increased fruit size under low fruit load (Dash and Malladi 2012; Wismer et al. 1995) in apple. In tomato, the effect of fruit load on cell size was dependent on the genotype (Fanwoua et al. 2012a; Prudent et al. 2010). Prudent et al. (2010) compared cell

division and cell expansion in a parental line (M; *Solanum lycopersicum*) and an introgression line (C9d) from *Solanum chmielewskii* under high load and low load. Fruits were bigger and contained more cells under low compared to high fruit load. However, cell size only increased in M while no effect of fruit load on cell size was detected in C9d.

Goffinet et al. (1995) studied the effect of fruit thinning in apple. They observed that within thinned trees, fruit size was positively correlated with the number of cells in the cortex but not with cell size or the size of the intercellular space. However, cell size and fruit weight in fruits of un-thinned trees were positively correlated ($R^2 = 0.71$). Thinned trees had larger fruits and with more cells compared to un-thinned trees. Fruit size, cortex volume and cell number decreased with increase in the time after bloom when thinning was completed. There were no significant differences in cortex intercellular space and cell size among treatments. A limit to the contribution of cell number increase to fruit size increase was also noted because at the time when fruit size and weight tended towards an asymptote, cell number was still increasing. They concluded that factors other than cell number were necessary to increase fruit size at this stage. In addition, Marcelis (1993) concluded that cell number was not an important determinant of fruit size in cucumber. Using irradiation treatments to vary source supply at different stages of fruit development, he showed that early cucumber fruit development was not important in setting fruit growth potential. Fruit size, cell number and cell size were all low when fruits were grown under continuous low source supply. However, low cell number due to low source conditions in the early stages of fruit development was compensated by increase in cell expansion when assimilate supply was increased at later stages of fruit development.

Light induced differences

Dash and Malladi (2012) investigated the effect of shading during early fruit growth on cell division and expansion in apple. They showed that the growth of fruits on branches that had been shaded approximately 15 to 18 days after full bloom started to decrease by 3 days after treatments had been applied. Reduced fruit growth was linked with a decrease in both cell division and expansion. The expression of cell cycle promoters; *CycA2;2* and *CycA2;3* decreased while that of cell cycle inhibitors; *KRP4* and *KRP5* increased in shaded fruits. Expression of two genes; *COB1* and *EXP10* associated with cell expansion decreased in shaded fruits. Our own results (Chapter 3) from tomato fruits grown either in the dark or in the presence of white light from anthesis until breaker stage indicate that light has no

detectable effect on fruit size. However, we observed a compensatory effect at the cell level. Fruits grown in the dark had fewer but larger cells compared with those grown in the presence of white light.

Drought induced differences

Fruit growth is dependent on photoassimilate import, water influx and carbon metabolism within the fruit (Ripoll et al. 2014). It is therefore beyond doubt that limited water supply limits fruit expansion. In tomato, for example, reduced water availability through high osmotic potential in the root environment has been shown to lead to a decrease in fresh weight, but an increase in dry matter content of harvested fruit (Ehret and Ho 1986; Fanasca et al. 2007). Mingo et al. (2003) also showed that dry conditions in the root environment lead to a decrease in tomato fruit diameter growth rate. To the best of our knowledge, no studies have reported this fruit level response at the cell level in tomato in sufficient detail. It is, however, expected that intense drought conditions indirectly lead to source limitation and consequently reduced cell division in tomato fruit tissue (Bertin 2005; Prudent et al. 2010). In grape (*Vitis vinifera* L.), low berry size under water deficit conditions between anthesis and veraison (onset of ripening) was associated with a decrease in pericarp cell size but no significant effect on cell division (Ojeda et al. 2001). Similar results were also reported by Marsal et al. (2000) in pear fruit. Endoreduplication has been postulated to be one of the mechanisms by which plants adapt to water deficit (Cookson et al. 2006). How the level of endoreduplication in fruits responds to drought conditions is yet to be shown through experimentation.

General trends

Genotypic differences in fruit size stem from variations in either cell division, endoreduplication, cell expansion or a combination of the three. Differences in the size of proximal and distal fruits are largely because of differences in cell number but cell size increase under increased source conditions can significantly reduce the magnitude of this difference between fruits in different positions on the same truss. There is also a general trend towards a decrease in fruit size due to cell size reduction when fruits are heated under limiting source conditions. When fruits are grown under non-limiting source conditions, heating leads to increase in cell size and consequently increased fruit size in some cases. When only source conditions are varied, increase in photoassimilate availability causes an increase in fruit size through cell number increase in tomato while in apple cell size increase is also important.

Exposure of fruits to light can lead to increases in fruit size through increases in both cell number and size in apple but in tomato, no differences in the size of dark and light grown fruits are observed because compensation of low cell number by increase in cell size occurs in the dark. Drought conditions on the other hand limit fruit expansion through a decrease in cell expansion without any significant change in cell number. While cell number increase is important for fruit growth, it is clear that cell size differences can also explain differences in fruit size. In cucumber, cell number is not an important determinant of fruit size.

Cell division, cell expansion and endoreduplication all contribute to fruit size increase

Numerous studies on cell division and expansion in tomato have been conducted in the past decades with the aim of unravelling the cellular process responsible for differences in fruit size. Attention has also been paid to endoreduplication and its role in cell size determination highlighted and linked to fruit growth (Cheniclet et al. 2005; Chevalier et al. 2013; Gonzalez et al. 2007). Treatments ranging from variation in environmental conditions during fruit growth to direct genotypic manipulation aimed at generating overexpression or loss of function mutants suggest a positive correlation between cell number and fruit size in tomato (Bohner and Bangerth 1988a; Bertin et al. 2003; Bertin 2005; Baldet et al. 2006; Bertin et al. 2009). In contrast, only a few studies attribute differences in fruit size to cell size or cell DNA ploidy level (Cheniclet et al. 2005; Gonzalez et al. 2007; Czerednik et al. 2012). This has led to the conclusion that cell division is the primary force that drives organ growth (Komaki and Sugimoto 2012). Therefore, should breeders interested in developing fruits with increased size focus only on cell cycle genes? Although this question could generate a potentially useful answer, it perpetuates the false notion that fruit size is determined by a single attribute of its constituent tissues. We argue below that whilst the above trend in correlations is mathematically correct, there appears to be an overestimation of the importance of cell division and underestimation of the role of cell expansion and endoreduplication in driving fruit growth in literature.

To gain insight into the discrepancy in the frequency of positive correlations between fruit size and cell number, DNA ploidy level or cell size reported in literature, it is important that we ask ourselves whether the correlation between any of the above cellular processes and fruit size is inherently biased. A test of the significance of the correlation between cell number and fruit size seems similar to questioning whether all components of a given entity significantly determine the weight of the entity in question. Indeed, the relationship between cell number and fruit size is mathematically favoured because a count of the total number of cells

represents the contribution of all components of the fruit to fruit size. During cell number estimation, a count is often made within a fruit section and extrapolated to the whole fruit. It is, therefore, not surprising that many studies show a positive correlation between cell number and fruit size. It should, however, be noted that a large number of cells can still occur in a small fruit if growth conditions are not suitable after the cell division phase of fruit development.

In contrast, cell size reported in literature is an estimate of the average size of all cells in the fruit tissue under observation. Cells within a fruit vary in size, therefore, the coefficient of determination for the correlation between average cell size and fruit size is dependent on the extent of variation in cell size within the tissue under study. Cells in the mesocarp of mature fruits are > 30,000 times larger than cells at anthesis (Cheniclet et al. 2005) implying that cell size increases in concert with fruit size increase. It is, therefore, surprising that there are not many reports of positive correlations between cell size and fruit size in literature. The few cases where the role of cell expansion in determining fruit size differences is acknowledged could be those where differences in cell size are extreme, e.g., between cultivars with hugely contrasting fruit size or extreme environmental treatments that lead to extreme differences in cell size (Table 1). A new correlation method, for example the use of proportion of large cells instead of average cell size in a given tissue could reveal the positive correlation between cell size and fruit size more frequently. Such a correlation takes into consideration the fact that large cells have a bigger contribution to fruit size than the small cells.

The phenomenon of compensation observed in determinate organs like leaves (Haber 1962; Tsukaya 2008; Kawade et al. 2010) and tomato fruits (Bertin 2005; Chapter 3) also highlights the importance of cell size in organ size determination. Compensation occurs when cell enlargement is stimulated by a significant decrease in cell division (Tsukaya 2008). Bertin (2005) showed that the decrease in cell division at high temperature was compensated by increase in cell size such that there was no significant difference in fruit size of heated and non-heated plants. In Chapter 3 we also observed that tomato fruits grown in the dark or in the presence of light did not differ in fruit size, however, light grown fruits had more but smaller cells. This compensatory behaviour suggests some level of interaction between cell division and cell expansion hence fruit growth could be driven or coordinated by an extra-cellular mechanism. This mechanism links defects in cell division with post mitotic cell expansion through cell-autonomous or non-cell autonomous mechanisms (Kawade et al. 2010). Cell

autonomous mechanisms are intra-cellularly regulated while non-cell autonomous mechanisms are regulated through proteins that act on more than one cell when produced.

Several physiological functions have been attributed to endoreduplication, for example stimulation of cell expansion, adaptation to stressful conditions (genotoxic stress, low temperature and salt stress), stimulation of cell differentiation, and modulation of transcriptional activity through increase in gene copy number. Bourdon et al. (2012) have shown that rRNA and mRNA transcription is strongly related with endoreduplication. The link with cell size and fruit size is of particular interest in tomato given the fact that high ploidy levels (up to 512C) occur in the pericarp. How endoreduplication triggers increase in cell size is currently speculative. A potential mechanism is one involving the physical increase in nuclear volume when chromatid number increases. This in turn stimulates an increase in cytoplasmic volume because of the inherent balance between nuclear and cytoplasmic volume as stipulated in the karyoplasmic ratio theory of Thoedor Boveri (Wilson 1925). Increase in nuclear DNA content stimulates cytoplasmic volume growth through an increase in ribosome biogenesis (Sugimoto-Shirasu and Roberts 2003). Evidence of the karyoplasmic ratio theory in tomato fruit was recently provided by Bourdon et al. (2012). These authors used fluorescent *in situ* hybridization (FISH) and showed that variation in cell size and nuclear size within pericarp tissue was associated with variation in ploidy level. The small nuclei and cells characteristic of the outer epidermis had low ploidy levels (2C and 4C) while the large nuclei and cells of the mesocarp had ploidy levels in the range of 4C to 256C. Cytoplasmic volume was also found to increase in concert with increase in ploidy level. In contrast to other studies, the use of the FISH technique in the study by Bourdon et al. (2012) enabled the establishment of a ploidy map that was related with cell size hence eliminating the use of average cell size and average ploidy level in establishing the relationship between endoreduplication and cell expansion.

Fruit growth drivers are development stage specific and coordinated by an extra-cellular mechanism

The first phase of tomato fruit growth that ends with a fully formed and open flower (anthesis) sets the initial potential for fruit growth. During this phase, cells within the ovary divide and differences among genotypes can already be apparent. Ovary size at this stage is determined largely by cell division activity. Cells present in the ovary make a significant contribution to later fruit growth because they determine the number of cells that are involved in post anthesis cell division. Post anthesis cell division accounts for most of the fruit size

increase during the first approximately 2 weeks after anthesis. A significant increase in pericarp cell size begins at about 10 days after anthesis (DAA; Tanksley 2004; Bertin et al. 2009). This onset of cell expansion together with endoreduplication appear to be developmentally determined and could be another level at which potential fruit size is defined. Evidence of endoreduplicating cells has been reported in some tomato fruit cells as early as the cell division phase of fruit development (Bertin 2005) but the largest increase in DNA ploidy level occurs after cessation of cell division. The next phase of fruit growth is characterized by adjustment of the earlier set potential to prevailing environmental conditions. Fruits are fairly large at this stage, have high sink strength and exert a considerable pull on the pool of photoassimilates present within the plant. Photoassimilates arrive in fruits in the form of sucrose and are hydrolysed into hexoses (fructose and glucose) and stored as starch. Stored starch is later hydrolysed into fructose and glucose. The accumulation of fructose and glucose leads to a considerable increase in cell size and consequently fruit size through water uptake as a result of the increased fruit osmotic potential.

It is likely that fruits have a central mechanism to coordinate cell division, endoreduplication and cell expansion during growth because all three processes lead to increase in fruit size. Moreover, for an individual cell they are sequential in time, whereas for the fruit as a whole they are overlapping. This coordination could be achieved through global kinases and moderated by photoassimilate import into the fruit (Figure 1).

An obvious candidate for such a global kinase is the target-of-rapamycin (TOR). TOR is a member of the phosphoinositide 3-kinase-related protein kinase family that signal metabolic and genomic stress (Sablowski and Dornelas 2014) and as such is stimulated under high sugar concentration (Figure 1). The amount of photoassimilates imported depends on the source-sink balance, the plasma membrane area developed within the fruit (through cell division or cell expansion with possibilities for feedback) and the availability of carbohydrate metabolism enzymes like vacuolar acid invertase, sucrose synthase and ADP glucose pyrophosphorylase (Sergeeva et al. 2006). TOR plays a dual role with respect to progression of the cell cycle and cell expansion. It activates cell division through direct phosphorylation of E2Fa/b but also inhibits cell division through 1) repression of the cell cycle activator: EBP1, and 2) activation of the cell cycle repressor: S6K (Figure 1). Through repression of EBP1 that has been reported to encourage ribosome biogenesis, TOR is able to repress cell expansion. However, cell expansion is also promoted by TOR through stimulation of cell wall extension, glycolysis, the tricarboxylic acid (TCA) cycle, starch storage and ribosome biogenesis and

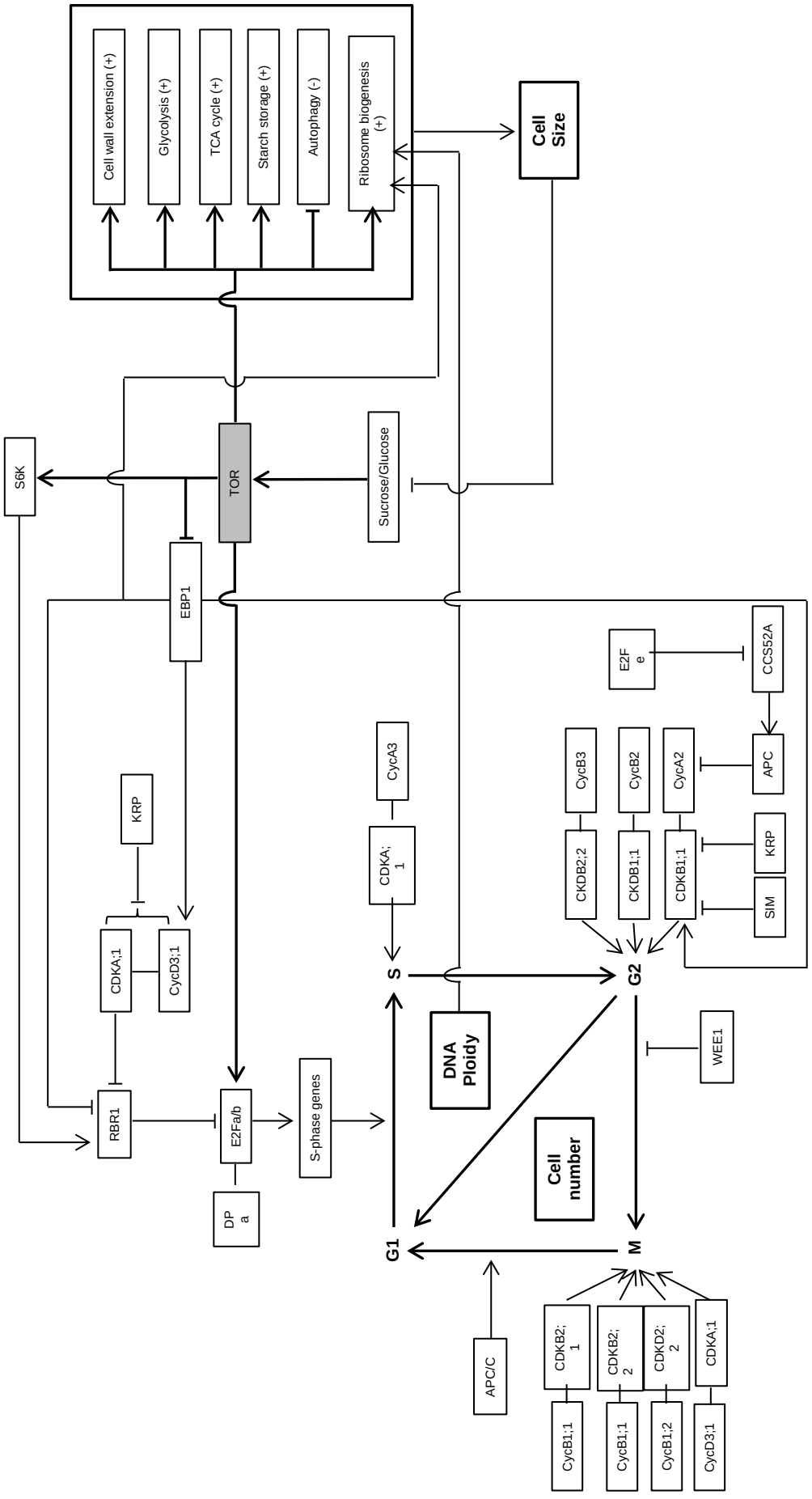

Figure 1: Schematic representation of the regulation of cell division, endoreduplication and cell expansion by the target-of-rapamycin (TOR) when activated by imported photoassimilates. TOR interacts with the cell cycle through stimulation of E2Fa/b transcription factors and inhibits the cell cycle through the eIF4E-binding protein 1 (EBP1) and S6 kinase (S6K). Cell expansion is regulated by the TOR through stimulation of cell wall extension, glycolysis, TCA cycle, starch storage and ribosome biogenesis and inhibition of autophagy. The effect of cell expansion processes on cell size are indicated in brackets (+: stimulates; -: inhibits). Lines ending with an arrow represent a positive feedback while blocked arrows represent a negative feedback.

repression of autophagy (Xiong et al. 2013). Contrasting roles of TOR during cell expansion and division could be separated in time to cater for the different fruit development stages when growth occurs through cell division or cell expansion.

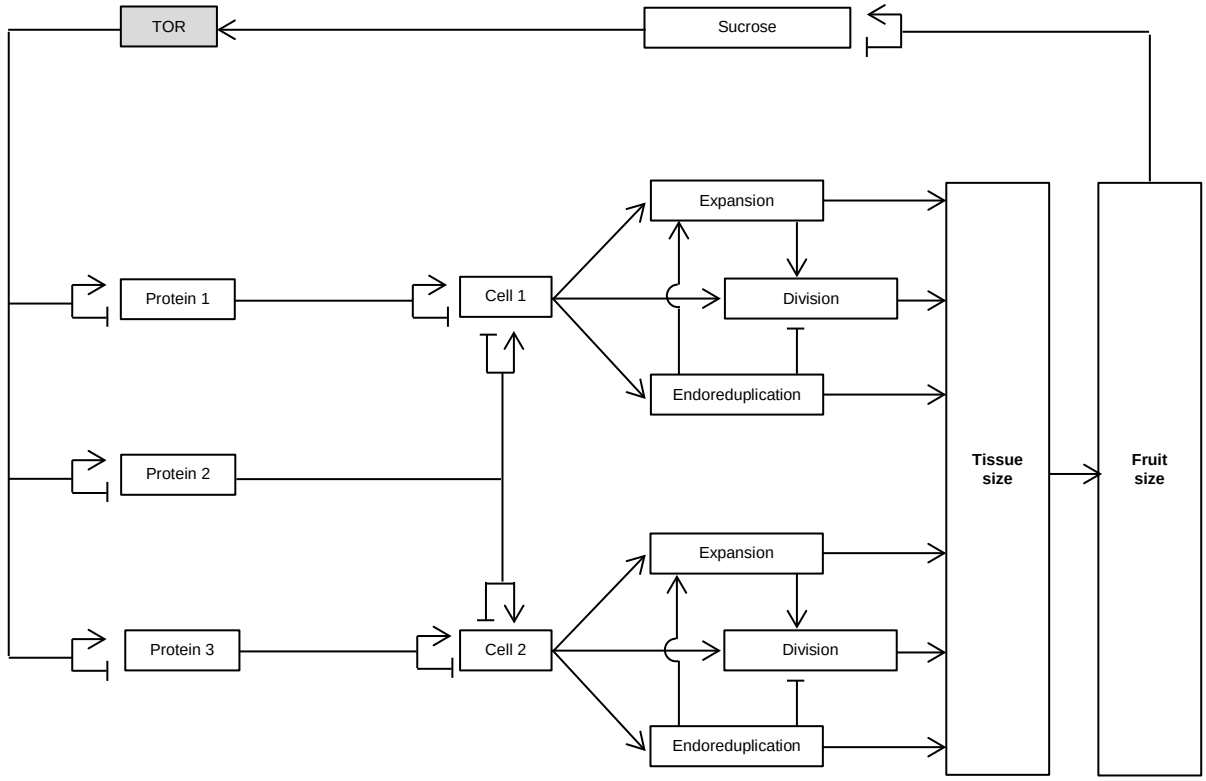

Figure 2: The neo-cellular theory of fruit growth. Lines ending with an arrow represent a positive feedback while blocked arrows represent a negative feedback.

Given that cell expansion is also linked to endoreduplication, it is possible that fruit growth depends on the amount of DNA present as a result of replication during the cell cycle or endocycle. This implies that cell division and endoreduplication occur during fruit growth to increase the fruit's transcriptional capacity through a mechanism that operates at the organ level, within individual cells or within and between cells. The latter, i.e., following the neo-cellular theory of growth, seems the most likely (Figure 2). Compensation of one process by another would then be coordinated through a kinase like TOR. Why fruits have the capacity to compensate cell division by cell expansion is not exactly clear but it is likely that it is a means to achieve a genetically predestined fruit size when environmental conditions are not optimal during the cell division phase of fruit growth. Besides compensation possibly via a global coordinator for cell division and cell expansion like the TOR, evidence of intra- and extra-cellular regulation of growth comes from the cell-autonomous or non-cell autonomous nature

of regulators of the cell cycle. Kawade et al. (2010) for example showed that overexpression of the cell cycle inhibitor: *KRP2* leads to cell autonomous compensation in *Arabidopsis* leaves. On the other hand, Weinl et al. (2005) showed that an inducer of endoreduplication in leaves: *KRP1* could move between cells and hence acts non-cell-autonomously. Since *KRP1* and *KRP2* are also expressed in tomato fruits, it is possible that they have similar modes of action as in leaves.

Conclusion and outlook

Many studies report positive correlations between cell number and fruit size while positive correlations between fruit size and cell size or ploidy level are rare. This correlation based inference has led to underestimation of the importance of cell size in driving fruit growth yet compared to cell division, cell expansion has a larger contribution to fruit size increase (Azzi et al. 2015). The positive correlation between cell number and ovary or fruit size does not imply that fruit growth is mainly driven by cell division. We propose two hypotheses for fruit growth regulation: 1) fruit growth through cell division or cell expansion is driven by photoassimilate import and regulated via the target-of-rapamycin (TOR), and 2) organ size is dependent on the amount of DNA attained through replication during cell division or endoreduplication hence fruits can grow through increase in cell number or size. The amount of DNA determines the size of instructions available for growth while imported photoassimilates represent the building blocks used to execute growth instructions. While it has been shown that increase in ploidy level leads to increase in nuclear volume, number of mitochondria and cell size (Bourdon et al. 2012), a mechanistic link is yet to be made with fruit size. The observation that fruit growth duration in endoreduplicating species is shorter than in non-endoreduplicating species (Chevalier et al. 2011) suggests that endoreduplication increases fruit growth rate to the extent that unusually large fruit sizes can be attained. The above hypotheses can be tested in future studies using loss of function mutants and/or gene over-expression analyses. Such studies will enable breeding programmes aimed at developing large fruited genotypes to specifically optimize either cell division, endoreduplication or cell expansion. In addition, the effect of drought on berry fruit growth at the cell and gene level needs more research attention.

Demonstration of the cell-autonomous action of *KRP2*, non-cell autonomous action of *KRP1* and recent evidence of compensation of cell division by cell expansion are major steps towards unravelling the level at which organ growth is regulated. The likely existence of cell-

and non-cell autonomous regulatory proteins in fruits and a compensatory mechanism suggest that fruit growth follows the neo-cellular theory of growth (Figure 2). This implies that fruits have a mechanism to compensate intra-cellular deficiencies through intercellular movement of regulatory proteins. Future studies should focus on further unravelling the cell-autonomous and non-cell-autonomous regulation of fruit growth.

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Chapter 6

General discussion

The aim of this study was to explain tomato fruit growth through underlying processes; cell division, endoreduplication, and cell expansion. How these processes determine fruit growth was studied in genotypes with contrasting fruit size, at several fruit development stages between anthesis and breaker stage, and under contrasting environmental (light and temperature) fruit conditions. This approach ensured that fruit phenotypes could be analysed over time and at several levels of aggregation (gene, cell, tissue and fruit) as proposed in the plant systems biology approach. Temperature and light effects were varied at the fruit level using climate controlled cuvettes. Unlike other studies, the use of fruit level treatments ensured that fruit and plant level responses could be separated. In this general discussion, strengths and limitations of the approach are discussed and results reviewed. Recommendations are also provided for future research.

Genotypic differences in tomato fruit size

Fruits from different species vary tremendously in size. However, as noted by Darwin (1859), variation also exists within members of the same species. Tomato fruit exhibits enormous variation in size. Cultivated species are usually larger than the wild species because the cultivated tomato species were selected for large fruit size. It is thought that early humans continuously selected fruits from plants in which mutations favouring large fruit size had taken place. This gradually led to accumulation of the large fruit trait in cultivated tomato (Tanksley, 2004). Large-fruited genotypes were preferred because it is less laborious to harvest large-fruited compared to small-fruited genotypes for a given target yield. Nowadays however, a wide variety of fruit sizes are demanded. The largest cultivated tomato (Beafsteak) can weigh up to 1,000 g while the smallest (cherry and cocktail) tomato weighs about 10 g when full grown. How these differences arise within a given species is important for breeding programmes aimed at developing fruits of specific sizes given the ever increasing specificity in consumer preferences.

In this study, small (‘Brioso’; 30 g) and large (‘Cappricia’; 80 g) sized tomato cultivars with similar vegetative growth characteristics were compared at the tissue, cell and gene level and during different stages of fruit development between anthesis and breaker stage. Expression of cell division, endoreduplication and cell expansion related genes, carbohydrate (sucrose, fructose, glucose and starch) content, growth duration and growth rate were also analysed in order to explain from a multilevel perspective why these two cultivars differ in fruit size. Large fruit size in ‘Cappricia’ was attributed to a faster growth rate, longer growth

duration, larger pulp and pericarp volume, larger pericarp cell volume and cell number compared to that observed in 'Brioso'. The two cultivars did not differ in carbohydrate content.

At the gene level, expression of only a few genes encoding cell division (*CDKB2*, *CycA1*, *E2Fa-like*, and *fw2.2*) and cell expansion (*AGPaseB*) proteins tallied with the observation that more and larger cells were observed in 'Cappricia' than in 'Brioso'. The expression of *fw2.2*; a gene that has been postulated to account for at least 30% of the variation in fruit size (Frary et al., 2000) was clearly highly expressed in the small fruited 'Brioso'. Although the exact mechanism by which *fw2.2* inhibits cell division is not yet known, its expression level corroborated the fewer cells observed in 'Brioso' compared to 'Cappricia'. Studies on peach (Scorzal, 1991), strawberry (Cheng and Breen, 1992), melon (Higashi et al., 1999), tomato (Bertin et al., 2003) and cherry (Olmstead et al., 2007) attribute differences in fruit size amongst genotypes to differences in cell number. On the contrary, observations in this study as well as in the study of Fanwoua et al. (2012a) suggest that differences in both cell number and cell size can explain genotypic differences in fruit size.

Any given pair of contrasting genotypes can differ at the genome level with respect to a given trait in that the gene encoding the trait of interest is completely deleted or part of it is duplicated or transformed (Zhang, 2003). A deletion would imply that the gene is not expressed while a duplication suggests the occurrence of several copies of the same gene visible as an overexpression in transcriptional studies. Transformation for instance via an insertion on the other hand can lead to formation of a completely different gene sequence that subsequently encodes a different protein. Small sequence differences due to deletions, duplications and transformations in one or a few nucleotides within the promoter or coding region of a gene can also have a significant effect on protein function. For all the genes observed in this study, none appeared to be deleted or transformed in any of the two cultivars. Clear differences in the expression of *fw2.2* between the two cultivars suggest a duplication in 'Brioso'. However, conclusions in the current study are limited because proteins involved in post-transcriptional interaction were not studied.

Higher concentrations of hexoses in 'Cappricia' compared to 'Brioso' were noted in the early stages of fruit growth and this corroborated the differences in cell number between the two cultivars. High assimilate conditions have been reported to lead to increases in the expression of cell cycle genes and hence an increase in cell division activity (Baldet et al., 2006). Higher expression of some cell division promoters (*CDKB2*, *CycA1*, *E2Fa-like*) in the

early stages of fruit growth in ‘Cappricia’ compared to ‘Brioso’ corroborate these observations. However, unlike observations by Fanwoua et al. (2012a), genotypic differences in carbohydrate content were not significant at breaker stage in the current study. Fanwoua et al. (2012a) reported significantly higher levels of both fructose and glucose (hexoses) in the large-fruited g49 compared to the small fruited g36 at breaker stage. Although not significant, there was even a tendency towards higher concentrations of hexoses in the small-fruited ‘Brioso’ compared to ‘Cappricia’. Results in the current study to some extent show that the strong expression of some genes that promote the cell cycle is associated with higher concentrations of hexoses in ‘Cappricia’ compared to ‘Brioso’ during early fruit growth. On the contrary, larger cell size in ‘Cappricia’ compared to ‘Brioso’ could not be corroborated by observations on either the concentration of carbohydrates or the expression level of genes associated with cell expansion in the current study.

Temperature effects on tomato fruit growth

Tomato plants cease to grow at temperatures below 12 °C (Criddle et al., 1997). A further decrease in temperature over prolonged periods can lead to plant death (Brüggeman et al., 1992). Chilling temperatures generally affect photosynthesis, respiration, membrane integrity, water relations and hormone balance in the crop (Graham and Patterson, 1982). The optimum temperature for tomato growth in commercial greenhouses is 19 – 20 °C (Van der Ploeg and Heuvelink, 2005). Growth at suboptimal temperatures but above 12 °C can lead to reduced fruit set due to poor pollen quality (Picken, 1984). When plants are grown at higher temperatures between 14 °C and 26 °C, flower bud opening is hastened and fruit growth duration generally decreases leading to formation of smaller sized fruits (Heuvelink and Marcelis, 1989; De Koning, 1994; Adams et al., 2001). Fruit dry matter content also increases with an increase in temperature (De Koning, 1994). The difference in total fruit yield between fruits grown at suboptimal and above optimal temperatures might not be significant because, an increase in fruit size is counterbalanced by an increase in fruit number at high temperatures. Exposure of tomato plants to temperatures above 27 °C (supra optimal temperatures) leads to heat stress visible as reduced pollen release and viability, and consequently lower fruit set (Peet et al., 1997; Sato et al., 2004). These negative effects on pollen and consequently fruit set have been shown to be a result of disruption of sugar metabolism during pollen formation under heat stress (Sato et al., 2006).

In this study, the effect of fruit temperature on tomato fruit growth was studied in two cultivars with similar vegetative growth but contrasting fruit size (Chapter 2). Temperature treatments were applied from anthesis until breaker stage. The temperature range selected for treatments was above the optimal but below the supra optimal temperature range for tomato (control: 21.4±0.8 °C, heated: 27.1±1.5 °C). The hypothesis tested was that fruit heating accelerates growth processes, enhances carbon import and initially leads to more cell division and cell expansion. However, an increase in temperature within normal ranges intensifies competition for assimilates among cells and together with a decrease in fruit growth duration leads to formation of smaller final cell and fruit size. Fruits were smaller at breaker stage even though growth rate was higher at high temperature. In agreement with findings from other studies (Adams et al., 2001; Bertin, 2005; Fanwoua et al., 2012b), in the current study growth duration decreased when fruits were grown at higher temperature. A similar response to increase in temperature was observed in both cultivars. Van der Ploeg and Heuvelink (2005) also reported that different tomato cultivars may vary in vegetative growth but there are not many instances where significant cultivar by temperature interactions were found for yield.

A significant reduction in pulp volume was observed and, although not significant, a similar trend was evident in pericarp volume when temperature was raised. This shows that whole fruit responses to temperature do not arise from only a single tissue. At the cell level, the reduction in fruit size at high temperature appeared to be caused by a reduction in pericarp cell volume but not pericarp cell number (Figure 1). These results agree with the findings of Fanwoua et al. (2012b) who also highlighted the importance of cell size in determining differences in fruit size at contrasting fruit temperatures.

Gene expression data obtained from fruits grown at contrasting fruit temperatures in the current study are the first to be reported for tomato. Only a few genes out of the 20 analysed in the current study were differentially expressed at the two fruit temperatures (Figure 1). Although the effect on cell number was not significant, growing fruits at a high temperature stimulated the expression of some cell cycle genes but at the same time stimulated the expression of an inhibitor of cell division; *fw2.2*. It is likely that the insignificant effect of increasing fruit temperature on cell division resulted from counter stimulation of both promoters and one inhibitor of cell division. An increase in expression of genes encoding cell cycle promoters has been reported to occur under conditions of high assimilate availability (Baldet et al., 2006). This is corroborated by the higher concentrations of sucrose, fructose and glucose observed in heated fruits in the early stages of fruit growth. These findings

suggest that the increase in expression of cell cycle genes observed in heated fruits was stimulated by increased assimilate import. The expression level of genes encoding proteins associated with cell expansion did not generally tally with cell size observations. In view of the significant effects of fruit heating on cell size, and the tendency towards increased cell number at high temperature but with only a few incidences of significant effects on gene expression, it is likely that including post-transcriptional studies would provide a clearer picture of temperature effects on cell division and cell expansion.

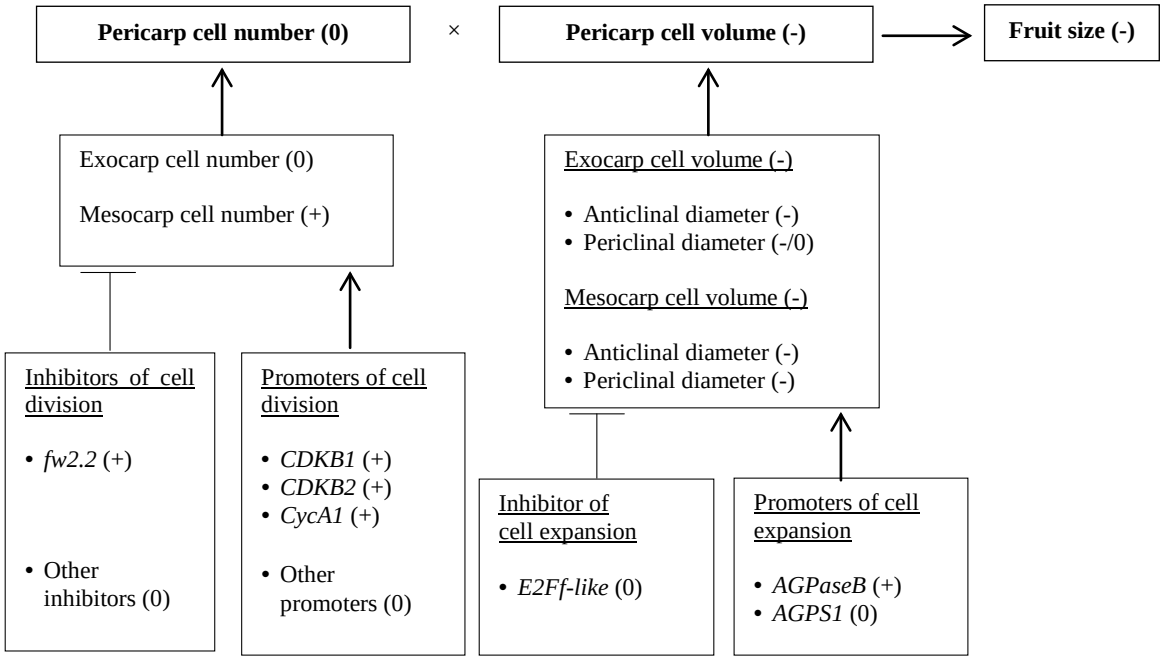

Figure 1: Schematic representation of the effect of temperature on cell characteristics in 'Brioso' and 'Cappricia'. +: stimulates; -: inhibits; 0: no detectable effect; -/0: decreases in 'Brioso' but no detectable effect in 'Cappricia'. A selection of promoters and inhibitors is shown; other promoters of cell division that were not affected include *CDKA1*, *CDKA2*, *CycB2*, *CycD3;3*, *E2Fa-like*, *E2Fb-like* and *E2Fe-like* while other inhibitors of cell division include *E2Fc*, *KRP1*, *PIF1-like(a)*, *PIF1-like(b)*, *PIF3-like* and *WEE1*.

Light effects on tomato fruit growth

Light is the main source of energy for plant growth. It affects plant growth through photoassimilate availability and direct signalling. Large differences in gene expression have been reported in plants grown in the dark and in those grown in the presence of light (Jiao et al., 2005). Studies in *Arabidopsis* show that light stimulates cell division by suppressing the activity of inhibitors of the cell cycle like *KRP1*, *KRP4*, and *E2Fc* (López-Juez et al., 2008). Promoters of cell division like *CDKB1;1*, *CDKB1;2*, *CycA2;2*, and *E2Fb* are stimulated by light. In contrast, growth in the dark promotes the activity of *DET1*, *COP1*, and *CSN5* all of

which are known to promote the expression of the cell cycle inhibiting *E2Fc*. Light has also been reported to promote cell division by promoting the activity of photoreceptors which in turn suppress activity of *DET1*, *COP1*, and *CSN5* (López-Juez et al., 2008).

Endoreduplication is generally suppressed by light. In many species, growth of hypocotyls in the dark takes place through cell expansion that is linked to endoreduplication (Geandreau et al., 1998; Kudo and Mii, 2004). This is achieved via repression of the cell cycle promoting *DEL1* by E2Fc in the dark (Berckmans et al., 2011). In the presence of light, E2Fb occupies the DNA binding site of *DEL1* and prevents the repressive association of E2Fc thereby promoting cell division. These observations in the literature show that factors that inhibit the activity of photoreceptors, for example phytochrome interacting factors (PIFs), suppress cell division and promote endoreduplication. Light effects on cell expansion are mediated through effects on cell wall extensibility and accumulation of photoassimilates. By enhancing the deposition of extensin proteins into the cell wall, light appears to lower cell wall extensibility (Brownleader et al., 2000). Another mechanism by which light inhibits cell expansion is through suppression of expansin genes via PRE transcription factors (Bai et al., 2012).

In this study, two hypotheses were tested in order to elucidate the effect of light on tomato fruit growth, i.e., 1) light-grown fruits are stronger sinks than dark-grown fruits, 2) responses depend on the type of light treatments and fruit development stage (Chapter 3). The size of fruits grown in the presence of light did not significantly differ from those that were grown in the dark. However, there were clear differences at the cell level. Light-grown fruits had more but smaller cells compared to dark-grown fruits. This effect was independent of the combined light colour (blue, red and white) and intensity treatments applied and fruit development stages in which light treatments were varied. These findings highlight the importance of multilevel analyses in revealing compensatory mechanisms that take place at the cell level. Compensation of cell division by cell expansion was also reported by Bertin (2005) using plant level temperature treatments.

In order to identify cell cycle, endoreduplication and cell expansion genes associated with cell level observations in the current study, gene expression analysis was conducted for 20 different genes encoding either promoters or inhibitors of cell division, endoreduplication and cell expansion (Chapter 3). Gene expression data, however, did not corroborate cell level observations. This could be attributed to the fact that there was quite some variation in cell level data from the experiment in which samples for gene expression analysis were obtained. It is also likely that a better understanding of observed gene expression profiles would be

obtained if more detailed observations were considered. For example a consideration of post-transcriptional regulation (proteomics and metabolomics studies), representing a scale between gene and cell level, is likely to provide clues on why significant treatment differences were observed at the cell level but not at the gene level.

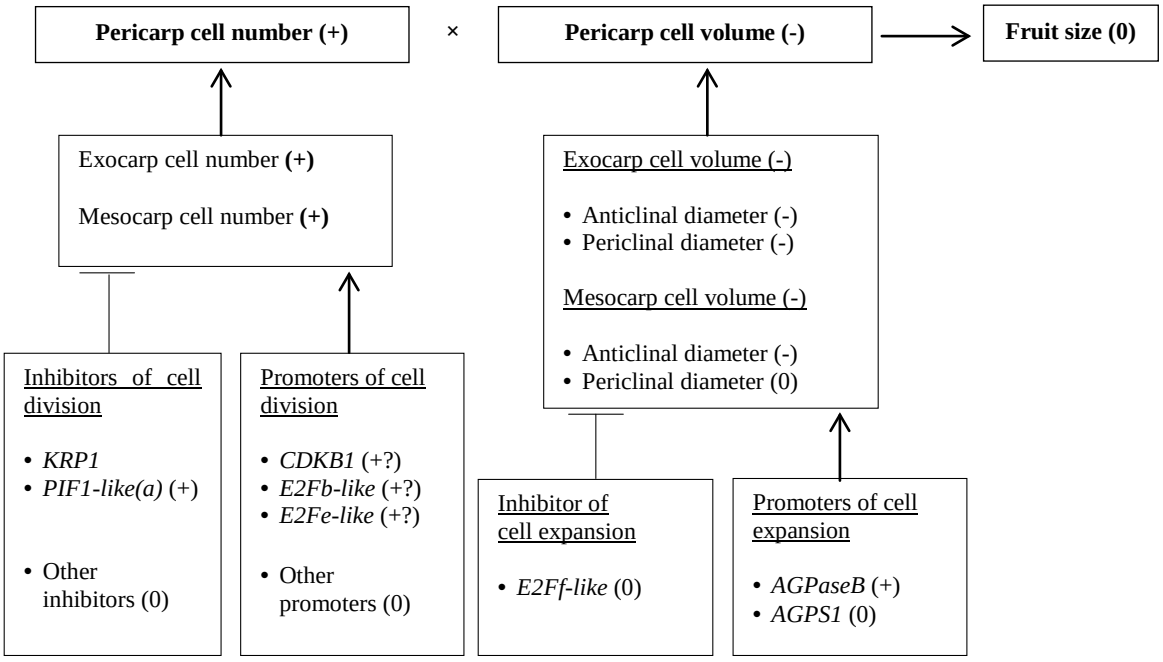

Figure 2: Schematic representation of the effect of light on cell characteristics. +: stimulates; -: inhibits; 0: no detectable effect; +?: stimulated in the dark and 24 h white light at some fruit development stages. A selection of promoters and inhibitors are shown; other promoters of cell division that were not affected include *CDKA1*, *CDKA2*, *CDKB2*, *CycA1*, *CycB2*, *CycD3;3*, *E2Fa-like*, and *E2Fb-like* while other inhibitors of cell division include *E2Fc-like*, *fw2.2*, *PIF1-like(b)*, *PIF3-like* and *WEE1*.

Fruit growth from a multilevel perspective

Unlike other studies, this study investigated tomato fruit growth at several levels of aggregation. Three processes were studied at the cell level (i.e., cell division, endoreduplication and cell expansion). Whilst it was the intention during this study to explain fruit growth based on endoreduplication, ploidy level measurements were not successfully completed. The method that was tried during this study involved chopping fresh pericarp tissue into small pieces in a PARTEC buffer solution followed by filtration and ploidy level measurements using flow cytometry. Clear peaks representing nuclei containing different ploidy levels were visible in pericarp tissue from younger fruits and trials with leaf samples. However, peaks from samples of older pericarp tissue were blurred and higher ploidy level peaks were not distinct. It was assumed that tissue chopping was not suitable for the larger nuclei (higher ploidy levels) that are characteristic of older pericarp tissue. A possible

alternative method could have been fluorescent in situ hybridization described by Bourdon et al. (2012). Nevertheless, the expression of genes encoding promoters of the endocycle were analysed. It was expected that a trend observed at the fruit level as a result of genotypic variation or environmental perturbation would be observed at the cell and gene level. Cell level observations were in agreement with fruit level observations. However, only a few gene level observations tallied with cell and fruit level observations. In this study, genes had been selected for analysis in such a way that promoters and inhibitors of a given process would be studied at the same time point. This approach did not lead to expected results possibly because different genes peaked in expression at different times of the day. Samples for gene expression analysis in this study were collected at similar time points during the day. Therefore, current data on genes whose expression peaked at a different time during the day may not be representative of their effect at the cell and fruit level. The daily pattern of peak gene expression could vary from genotype to genotype. It is likely that the sampling time was suitable for some of the genes in 'Cappricia' and 'Brioso' (Chapter 2) because cell and fruit level observations were corroborated by the expression pattern of a few genes at different temperatures. This corroboration was absent in the experiment with 'Komeett' (Chapter 3). The fact that cell size observations were not corroborated by gene level observations also suggests that the peak expression level for genes encoding different processes may occur at different times of the day. For the case of cell expansion, it is likely that more representative gene expression data would be obtained if samples were collected at the end of the day or at night.

It was also expected that measured carbohydrate concentrations would reflect the fact that fruit growth is driven by photoassimilates present in the fruit. There were, however, only a few instances where carbohydrate concentrations tallied with cell and gene level observations. This could be because the method of analysis used in the current study did not quantify how much photoassimilate was imported or produced and utilized by fruits for growth. Studies in *Arabidopsis* have shown correlations between photoassimilate availability and gene expression and cell phenotype via a growth coordinator; the target-of-rapamycin (TOR; Xiong et al., 2013).

Conclusion and future research perspectives

This study is among many attempts aimed at explaining tomato fruit growth. The approach taken was one that links up several levels of aggregation. Treatments (temperature and light)

during experiments were applied at the fruit level and responses investigated at the gene, cell, tissue and fruit level. In addition carbohydrate concentration measurements were conducted and related with observations at the higher levels of aggregation. Findings from experiments in which two cultivars were grown at two different temperatures showed that,

1) there was a similar response to variations in temperature for the two cultivars,
2) genotypic differences in fruit size arise from differences in both cell number and cell division,
3) fruits were smaller when grown at high temperature because cell size decreased,
4) final fruit carbohydrate content did not differ amongst fruits grown at different fruit temperatures.

Experiments involving light treatments revealed that under non-limiting source conditions, growth of fruits in the dark or in the presence of light does not lead to a significant difference in fruit size. More cells are, however, formed in the presence of light although these cells are smaller in volume compared to those grown in the dark. Synthesis of earlier research on fruit growth showed that cell division, endoreduplication, and cell expansion all contribute to fruit growth. Since all these processes are important for fruit growth, it is likely that plants evolved a mechanism that regulates fruit growth via either cell division, endoreduplication or cell expansion. It is hypothesized that the target-of-rapamycin (TOR) is the protein kinase that coordinates fruit growth through effects on cellular processes. This regulation follows the neo-cellular theory of fruit growth where target proteins of TOR can act within individual cells or among cells. Studies on intra- and extra-cellular activity of cell division, endoreduplication and cell expansion regulators would be very useful in providing experimental evidence of the mechanism by which fruit growth is coordinated.

In general, differences in cell number, cell size, and tissue volume could explain treatment differences, however, gene expression and carbohydrate concentration data were not consistently linked with final cell and fruit level observations. It was only in a few cases during the early stages of fruit growth that carbohydrate concentration and gene expression data appeared to corroborate cell number observations. Carbohydrate measurements in the current study could not account for respiratory losses. A better understanding of fruit growth would be achieved if observations in this study were combined with detailed measurements of fruit respiration, and carbohydrate import by for instance using nuclear magnetic resonance

(NMR) techniques or radioactive labelling of carbon. In addition, whilst genes were selected based on earlier work reported on the regulation of the three cellular processes investigated in this study, a wider spectrum of genes regulating cell expansion could have been selected. Some genes that should be considered for future investigation include those coding for expansins, xyloglucan endotransglycosylase, endoglucanase, cellulose synthase, β-glycosides, and extension peroxidase. The 24 h expression pattern of different genes also needs to be studied in order to establish when different genes peak in expression. This would ensure that samples are collected at appropriate time points.

Fruit growth can be regulated at the transcriptional and post-transcriptional level. This study focused on transcriptional regulation, therefore, progress can be achieved if these observations can be combined with measurement of the concentration of encoded proteins. Important proteins would include enzymes relevant in sugar metabolism such as vacuolar acid invertase, sucrose synthase, hexose carriers as well as the previously mentioned TOR (Sergeevaet al., 2006; Xiong et al., 2013). Identification of the direct targets of *fw2.2* during cell division would also be an important breakthrough in efforts to explain tomato fruit growth. The major QTL *fw2.2* has been associated to only one gene encoding a transmembrane protein of unknown function acting as a negative regulator of cell division during early fruit development (Frary et al., 2000). However, the direct link between FW2.2 and the potential regulation of the cell cycle remains a real enigma.

Recent studies have shown positive links between endoreduplication and cell size, however, there is need for more studies on tomato fruit in which ploidy level measurements are conducted concurrently with cell number and cell size measurements. Whilst cell division is well researched, endoreduplication and cell expansion are still not well understood. The structure of plant cell walls has been studied in detail but how the cell wall of tomato fruit responds to changes in environmental conditions is not clear. Techniques to measure the physical properties, for example, extensibility of cell walls also need to be developed.

Attempts to model tomato fruit growth have highlighted the need to link gene expression data with cell and fruit level data (Fanwoua et al., 2013). This study was an attempt to address this need. Findings from this study provide clues on the basis of variation in fruit size at the gene level for a few genes. Further investigation will aid in bridging the gene-cell-fruit gap but should only be done after establishment of the daily peak expression pattern of genes of interest. A next step would be to conduct studies at the level between genes and the cell in order to quantify the relationship between gene expression and cell and fruit phenotype.

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Summary

There is enormous variation in tomato fruit size. This variation arises from genotypic differences, variations in the environment during fruit growth and their interactions. Fruit growth response to temperature has been studied extensively and there is general agreement that growth at high temperature leads to formation of small fruits. *In vitro* studies show that light stimulates sucrose uptake by fruits leading to an increase in fruit size. However, *in vivo* studies on the effect of localized light on tomato fruit growth have not been conducted before. Furthermore, understanding the fruit's response to light and other environmental conditions (e.g. temperature) requires a multi-level, systems biology approach to aggregate the responses at gene and cell level to fruit level. While attempts have been made to explain genotypic and environmental responses of whole fruits, not many studies have investigated the genetic and cellular basis of tomato fruit growth. Cell division, endoreduplication and cell expansion are hardly investigated simultaneously during tomato fruit growth. What drives fruit growth is also still a subject of debate. The aim of this study was to explain whole fruit growth based on gene related processes at cellular and subcellular level under different environmental (light and temperature) conditions. The specific objectives were to determine 1) the genetic and physiological basis for differences in tomato fruit size, 2) the mechanisms by which fruit growth is driven, and 3) how light and temperature modulate the drivers of fruit growth. Experiments were conducted and observations made at several levels of aggregation (from gene to whole fruit).

After the description of the background, problem statement and objectives in Chapter 1, in Chapter 2, the genetic and physiological basis for fruit size difference between a small ('Brioso') and large ('Cappricia') fruited tomato cultivar was investigated in a greenhouse. In addition the effect of temperature on the growth of fruits of the two tomato cultivars was tested using climate-controlled cuvettes. Two temperature treatments; 21.4±0.8 °C (control) and 27.1±1.5 °C (heated) were applied at the fruit level from anthesis until breaker stage. 'Cappricia' fruits were found to be larger than those of 'Brioso' because of 1) faster growth rate, 2) longer growth duration, 3) formation of more cells, and 4) formation of larger cells. The concentrations of fructose and glucose (hexose) were higher in 'Cappricia' than in 'Brioso' during the early stages of fruit growth. Gene level observations revealed that out of the 20 genes studied, the expression of three promoters (*CDKB2*, *CycA1*, and *E2Fa-like*) of cell division and one promoter of cell expansion (*AGPaseB*) was higher in 'Cappricia' than in

'Brioso'. On the other hand, the expression of the cell division inhibitor *fw2.2* was higher in 'Brioso' than in 'Cappricia'. Both 'Brioso' and 'Cappricia' exhibited a similar response to increase in fruit temperature. Heated fruits had a faster growth rate, shorter growth duration and were smaller in size than control fruits. Cell number did not significantly differ between heated and control fruits. However, cell size was smaller in heated than in control fruits. Hexose concentrations were higher in heated than in control fruits during the early stages of fruit growth but differences were absent at breaker stage. Fruit heating stimulated the expression of three promoters (*CDKB1*, *CDKB2*, and *CycA1*) and one inhibitor (*fw2.2*) of cell division. However, only one promoter (*AGPaseB*) of cell expansion appeared to be promoted by fruit heating. These results show that genotypic differences in fruit size arise from differences in both cell number and cell size while the reduction in fruit size at high temperature is largely due to a decrease in cell size.

In Chapter 3, the effect of light on *in vivo* grown fruits was tested in a large fruited tomato cultivar ('Komeett') under non limiting source conditions. Fruits were either grown in natural light, presence of white (12 h or 24 h), blue (24 h) or red (24 h) light or in the dark using climate-controlled cuvettes from anthesis until breaker stage. Additional treatments involved 1) growing fruits in the dark during the first 24 days after anthesis (DAA) followed by white light (24 h) until breaker stage, and 2) white light (24 h) during the first 24 DAA followed by darkness until breaker stage. Dark-grown fruits were initially white in colour but turned red at breaker stage just like fruits from other treatments. Fruits grown in the presence or absence of light did not differ in size at breaker stage. There were also no differences in fruit size regardless of the light colour or phase during fruit development when light treatments were applied. At the cell level, fruits grown in the presence of light had more but smaller cells than those that were grown in the dark. Cell number and size did not differ among fruits exposed to different light colours. Exposing fruits to light or darkness during different stages of fruit development did not result in significant differences in cell number or size at breaker stage. There were no significant differences in carbohydrate concentration of dark-grown or light-grown fruits. Significant differences between dark-grown and white light-grown fruits in the expression of genes encoding promoters or inhibitors of cell division, endoreduplication or cell expansion were also not noted. These findings highlight the existence of a homeostatic regulatory system that enables compensation of cell division by cell expansion during tomato fruit.

In Chapter 4, a review of the literature on the regulation of cell division,

endoreduplication and cell expansion by light is presented. Different species and organs were considered in order to come up with general response mechanisms but also because there are hardly any studies of light effects on cell division, endoreduplication or cell expansion in tomato fruit. Findings show that light stimulates cell division irrespective of the organ but effects on endoreduplication and cell expansion vary depending on the organ under consideration. It is also shown that light stimulates cell division by inhibiting the inhibitors of the cell cycle. These inhibitors include KIP-RELATED PROTEINS (KRPs), E2Fc transcription factors, and phytochrome interacting factors (PIFs). Growth in the dark appears to activate inhibitors of the cell cycle like DE-ETIOLATED 1 (DET1), CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), COP9 SIGNALOSOME 5 (CSN5). Inhibition of cell division in the dark leads to promotion of endoreduplication in some cases. The stimulation of cell expansion takes place through cell wall acidification while inhibition of cell expansion by light is achieved through deposition of extensin proteins or inhibition of the activity of expansins. Light effects are mediated through promotion of the activity of photoreceptors while these are degraded by PIFs in the dark. These findings generally show that the interaction between phytochromes and PIFs is central in the regulation of cell division, endoreduplication and cell expansion under different light conditions.

Chapter 5 tackles the mechanism by which fruit growth is driven. Literature on fruit growth is explored to identify variations at the cell level in experiments where treatments led to variation in fruit size in order to identify what drives fruit growth. The sources of variation in fruit size in these experiments include genotype, fruit position on a truss, photoassimilate availability, temperature, and light. It is shown that variation in fruit size can arise from changes in cell number, ploidy level or cell size. The need for a global regulator of these cellular processes is highlighted and the target-of-rapamycin (TOR) is proposed as a global regulator for fruit growth. Activity of TOR is shown to be modulated by fruit sugar levels. Fruit growth is further proposed to follow the neo-cellular theory of fruit growth. The findings of this chapter show that the commonly reported positive correlation between cell number and fruit size does not imply a causal relationship.

Chapter 6 discusses the findings of this study, highlights strengths and weaknesses of the findings and proposes future research directions. This work contributes to our understanding of fruit growth by linking gene, cell, tissue and fruit level observations. Although gene-level observations did not always tally with cell-level observations, important clues on for example the response to temperature of a few promoters and one inhibitor of the cell cycle are

provided. Evidence of compensation of cell division by cell expansion is shown. In addition, three fundamental processes of fruit growth (cell division, endoreduplication and cell expansion) are connected and it is shown that cell division does not solely drive fruit growth. A global coordinator and growth theory for fruits are further proposed, however, more studies need to be directed at proving the existence of regulators that act intra- or extra-cellularly.

Samenvatting

Er bestaat een enorme variatie in vruchtgrootte bij tomaat. Deze variatie komt voort uit genotypische verschillen, variaties in het milieu tijdens de vruchtgroei en interacties tussen genotype en milieu. De reactie van vruchtgroei op temperatuur is uitgebreid bestudeerd en er is algemene overeenstemming dat de groei bij hogere temperatuur leidt tot de vorming van kleinere vruchten. *In vitro* studies tonen aan dat licht de sucrose opname door de vrucht stimuleert met een grotere vrucht tot gevolg. *In vivo* studies naar het effect van gelokaliseerd licht op tomatengroei zijn echter nog niet eerder uitgevoerd. Voor het begrijpen van dergelijke reacties van de vrucht op licht, en op andere omgevingsomstandigheden als temperatuur, is een multi-level, systeembiologische aanpak nodig om de mechanismen op gen- en celniveau te aggregeren naar vruchtniveau. Hoewel studies zijn verricht naar de genotype en milieu respons op vruchtniveau, hebben weinig studies de genetische en cellulaire basis van tomaatgroei onderzocht. Celdeling, endoreduplicatie en celexpansie zijn zelden tegelijkertijd onderzocht gedurende de tomatengroei. Vraag hierbij is wat feitelijk de vruchtgroei stuurt. Het doel van deze studie was om de vruchtgroei te verklaren op basis van gen-gerelateerde processen op cellulair en subcellulaire schaal en beïnvloedt door verschillende omgevingsfactoren (licht en temperatuur). De specifieke doelstellingen de bepaling van 1) de genetische en fysiologische basis voor verschillen in vruchtgrootte van tomaat, 2) de mechanismen waardoor de vruchtgroei wordt gestuurd, en 3) hoe licht en temperatuur deze mechanismen van vruchtgroei beïnvloeden. Experimenten werden uitgevoerd en waarnemingen gedaan op verschillende schaalniveaus (van gen tot hele vrucht).

Na de beschrijving van de achtergrond, probleemstelling en de doelstellingen in hoofdstuk 1, wordt in hoofdstuk 2 onderzoek gepresenteerd naar de genetische en fysiologische basis van vruchtgrootte tussen een kleine ('Brioso') en een grote ('Cappricia') vruchten leverend tomatenras middels een kasproef. Hiernaast werd het effect van temperatuur op de groei van vruchten van de twee tomatencultivars getest door plaatsing van de tomatentrossen binnen cuvetten met een geconditioneerd binnenklimaat. Twee temperatuurbehandelingen, i.e. 21.4 ± 0.8 ° C (controle) en 27.1 ± 1.5 ° C (verwarmd) werden toegepast op vruchtniveau vanaf anthesis tot breaker stadium. Vruchten van ras 'Cappricia' bleken groter dan van 'Brioso' vanwege 1) snellere groei, 2) langere groei, 3) de vorming van meer cellen, en 4) de vorming van grotere cellen. De concentraties van fructose en glucose (hexose-) waren hoger in 'Cappricia' dan in 'Brioso' tijdens de vroege stadia van vruchtgroei. Uit de studie naar

expressieniveau van 20 relevante genen bleek dat de expressie van 3 celdelingsgenen (*CDKB2, CycA1* en *E2Fa-achtige*) en van een promotor van de celexpansie (*AGPaseB*) hoger waren in 'Cappricia' dan in 'Brioso' . Anderzijds, de expressie van de celdelingsremmer *fw2.2* was hoger in 'Brioso' dan in 'Cappricia. Zowel 'Brioso' en 'Cappricia' vertoonden een vergelijkbare reactie op verhoging van de vruchttemperatuur. Verwarmde vruchten hadden een snellere groei, lagere groeiduur en waren kleiner in omvang dan de onbehandelde vruchten. Celaantal verschilde niet significant tussen verwarmde en onbehandelde vruchten. Echter, celgrootte was kleiner in verwarmde dan in onbehandelde vruchten. Hexose concentraties waren hoger in verwarmde dan in onbehandelde vruchten tijdens de vroege stadia van vruchtgroei, maar de verschillen waren afwezig bij het breaker stadium. Vruchtverwarming stimuleerde de expressie van de drie promotoren (*CDKB1, CDKB2* en *CycA1*) en een remmer (*fw2.2*) van de celdeling. Slechts één promoter (*AGPaseB*) van celexpansie leek te worden bevorderd door verwarming. Deze resultaten tonen aan dat genotypische verschillen in vruchtgrootte voortvloeien uit verschillen in aantal cellen en celgrootte terwijl de vermindering van vruchtgrootte bij hoge temperatuur grotendeels wordt veroorzaakt door een afname in celgrootte.

In hoofdstuk 3 wordt onderzoek gepresenteerd naar het effect van duur en kleur van belichting van *in vivo* geteelde tomaten. De studie vond plaats aan de cultivar ('Komeett'), een grove tomaat, onder niet-limiterende assimilatenvoorziening. Vruchten werden ofwel geteeld in natuurlijk licht, met wit (12 uur of 24 uur), blauw (24 uur) of rood (24 uur) licht, of in het donker, door plaatsing van de trossen in geconditioneerde cuvetten, in de periode van anthesis tot breaker stadium. Aanvullende behandelingen betroffen 1) donker tijdens de eerste 24 dagen na anthesis (DAA), gevolgd door wit licht (24 h) tot breaker fase, en 2) wit licht (24 h) gedurende de eerste 24 DAA gevolgd door duisternis tot breaker stadium. In donker geteelde tomaten waren aanvankelijk wit van kleur, maar werden rood bij breaker stadium net als vruchten van de andere behandelingen. Tomaten gekweekt in de aanwezigheid of afwezigheid van licht verschilden niet in grootte tijdens breaker stadium. Er werden ook geen verschillen in grootte gevonden bij wijziging van de lichtkleur of fase in vruchtontwikkeling waarin werd belicht. Op celniveau hadden de in licht geteelde vruchten meer, maar kleinere, cellen dan de vruchten die werden geteeld in het donker. Celaantal en -grootte verschilden niet tussen de tomaten blootgesteld aan de verschillende lichtkleuren of aan wel of niet belichting in de eerste of laatste 24 dagen van de vruchtgroei. Er waren geen significante verschillen tijdens breaker stadium in koolhydraat concentratie van in donker of in licht geteelde tomaten. Ook

de expressie van genen voor promotie of remming van celdeling, endoreduplicatie of cel expansie was niet verschillend tussen de behandelingen. Deze bevindingen benadrukken het bestaan van een homeostatisch, regulerend systeem dat de compensatie van celdeling door cel expansie faciliteert tijdens de tomaat groei.

In hoofdstuk 4 wordt een literatuuroverzicht gegeven ten aanzien van de regulering van celdeling, endoreduplicatie en cel expansie door licht. Verschillende plantensoorten en -organen worden onder de loep genomen ten aanzien van een mogelijk algemene reactiemechanisme op licht. Er wordt aandacht besteed aan de lichteffecten op celdeling, endoreduplicatie en/of cel expansie in tomaten. Uit de bevindingen blijkt dat licht de celdeling stimuleert ongeacht het orgaan in kwestie, maar effecten op endoreduplicatie en celexpansie variëren afhankelijk van het type orgaan. Ook is aangetoond dat licht de celdeling stimuleert door remming van de remmers van de celcyclus. Deze remmers behoren tot de KIP-gerelateerde eiwitten (KRPs), de E2Fc transcriptiefactoren, en de fytochroom-interacterende factoren (PIF). De groei in het donker lijkt remmers van de celcyclus te activeren, zoals DE-geëtioleerde 1 (DET1), constitutieve PHOTOMORPHOGENIC1 (COP1), en COP9 SIGNALOSOME 5 (CSN5). In sommige gevallen leidt remming van de celdeling in het donker tot bevordering van endoreduplicatie. De stimulering van de celexpansie vindt plaats door verzuring in de celwand, terwijl een sterkere celwand en remming van de expansie via licht wordt bereikt door afzetting van extensine eiwitten of remming van de activiteit van expansines. Lichteffecten werken uit via het bevorderen van de activiteit van de fotoreceptoren, terwijl deze in het donker worden afgebroken door PIF. Deze bevindingen laten zien dat onder wisselende lichtomstandigheden de interactie tussen fytochromen en PIF centraal staat in de regulatie van celdeling, endoreduplicatie en cel expansie.

Hoofdstuk 5 behandelt het mechanisme dat vermoedelijk ten grondslag ligt aan de vruchtgroei. Literatuur over vruchtgroei is onderzocht op de mogelijke relatie tussen variaties op celniveau en de variatie in vruchtgrootte als gevolg van experimentele behandelingen. De bronnen van variatie in vruchtgrootte in deze experimenten bestonden uit genotype, vruchtpositie op een tros, assimilatenbeschikbaarheid, temperatuur en licht. Er wordt aangetoond dat de variaties in vruchtgrootte voortvloeien uit veranderingen in celaantal, ploïdiegraad en/of celgrootte. Deze cellulaire processen worden zeer waarschijnlijk op hoger aggregatieniveau gereguleerd en gecoördineerd. Een waarschijnlijke kandidaat voor deze coördinerende rol is target-of-rapamycin (TOR). Activiteit van TOR wordt beïnvloed door het suikergehalte in de vrucht. Verder wordt voorgesteld de neo-cellulaire theorie te gebruiken ter

verklaring van vruchtgroei. De in de literatuur gemelde positieve correlaties tussen het aantal cellen en vruchtgrootte betekenen nog niet dat het celaantal de vruchtgroei verklaart.

Hoofdstuk 6 bediscussieert de resultaten van de voorgaande hoofdstukken, geeft een analyse van de sterke en zwakke punten en stelt enkele toekomstig onderzoeksrichtingen voor. Gesteld wordt dat dit werk bijdraagt aan ons begrip van de vruchtgroei door de processen op gen-, cel-, weefsel- en orgaanniveau te koppelen. Hoewel waarnemingen op genniveau niet altijd overeenkomen met die op celniveau zijn er belangrijke aanwijzingen gevonden voor o.a. de temperatuurrespons van enkele promoters en een remmer van de celcyclus. Er wordt aangetoond dat vermindering van celdeling kan worden gecompenseerd door verhoogde celexpansie. Bovendien zijn drie fundamentele processen van vruchtgroei (celdeling, endoreduplicatie en celexpansie) gekoppeld en wordt aangetoond dat celdeling niet in algemene zin als verklaring voor vruchtgrootte kan worden beschouwd. Verder worden een globale coördinator en een bepaalde groeitheorie voorgesteld voor de verklaring van vruchtgroei. Er is echter behoefte aan meer onderzoek voor het aantonen van de rol van zo'n coördinator die zowel intra- als extra-cellulair werkzaam is.

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would make this part of the thesis very long but I trust that you know that I will never forget the details.

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List of publications

Okello R.C.O., Heuvelink E., de Visser P.H.B., Lammers M., de Maagd R.A., Struik P.C., Marcelis L.F.M. (2015). A multi-level analysis of fruit growth of two tomato cultivars in response to fruit temperature. *Physiologia Plantarum* 153: 403–418.

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Robert Cyrus Ongom Okello was born on December 7th 1977 in Walukuba, Jinja, Uganda. He attended primary school education at Mwiri Primary School, Jinja (1984 – 1990), ordinary level secondary school education at Jinja College, Jinja (1991 – 1994) and advanced level secondary school education (Physics, Chemistry, Biology and Subsidiary Mathematics) at Busoga College Mwiri, Jinja (1995 – 1997). He then obtained a scholarship from the Inter University Council for East Africa to study Horticulture at the Sokoine University of Agriculture, Morogoro, Tanzania from September 1997. He graduated with an Upper Second Class Bachelor of Science Degree in Horticulture in 2001 and immediately got employed as an Assistant Agricultural Services Manager at Somuc Agricultural Consultants Ltd in Mbarara, Uganda. In 2003, he joined Wagagai Chrysanthemums Ltd, Entebbe, Uganda (an out grower of Deliflor Chrysanten BV) as a Production Manager and also worked as a Part Time Teaching Assistant on Floriculture at Busoga University, Iganga, Uganda. With sponsorship from the USAID-IDEA Project, he followed the Applied Tropical Floriculture Course (2003) and obtained a Certificate of Excellence. He became a resource person on Chrysanthemum mother plant production at the same course in 2004. Because of his interest in teaching, Okello joined Makerere University, Kampala, Uganda on a temporary basis as a Teaching Assistant and taught Greenhouse Production and Management in 2005. He later joined Mountains of the Moon University, Fort Portal, Uganda on a full time basis to establish the Department of Horticulture and went on to teach Introduction to Genetics and Plant Breeding, Introduction to Plant Biotechnology, Principles of Horticulture, Principles of Vegetable Production, and Nursery Production and Management. While at Mountains of the Moon University, he obtained a scholarship from NUFFIC to pursue the Practical Management Course for Horticulturists at the PTC+, Ede, the Netherlands (2005) and later a Master's Degree in Plant Science with Specialization in Greenhouse Horticulture at Wageningen University (2007 – 2009). He conducted his Master's major thesis research on axillary bud break in roses at the Horticulture and Product Physiology Group, and Centre for Crop Systems Analysis. He worked on scent production in roses for his Master's minor thesis at the Louis Mann Laboratory, University of California at Davis, USA. He was employed in September 2009 through funding from the Technological Top Institute for Green Genetics to conduct a PhD research on tomato fruit growth at the Centre for Crop Systems Analysis,

About the author

Horticulture and Product Physiology Group, and the Business Unit of Greenhouse Horticulture. The results of this research are presented in this thesis.

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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the 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 (6 ECTS)
- Environmental regulation of cell division, endoreduplication and cell expansion

Post-graduate courses (4.2 ECTS)
- The art of modelling; PE&RC (2010)
- Generalized linear models; PE&RC (2013)
- Mixed linear models; PE&RC (2013)

Deficiency, refresh, brush-up courses (3 ECTS)
- Basic statistics (2012)
- Introduction to R for statistical analysis (2012)
- Bioinformatics - a user's approach (2013)

Competence strengthening / skills courses (4.5 ECTS)
- How to write a world class paper (2010)
- PhD Competence assessment; WGS (2011)
- Project and time management; PE&RC (2011)
- Techniques for writing and presenting a scientific paper; WGS (2011)
- Ethics and philosophy in life sciences; WGS (2012)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)
- PE&RC Day: on the origin of communication (2009)
- PERC Weekend (2010)
- PE&RC Day: selling science: why and how scientists sell science? (2010)
- PERC Day: innovation for sustainability: what are the neighbours doing? (2011)
- PERC Day: extreme life: exploring life in the extremes and the extremes in life (2012)

Discussion groups / local seminars / other scientific meetings (6 ECTS)
- Frontier Literature in Plant Physiology (FLOP) (2009-2014)
- How to write a world class paper (2010)

International symposia, workshops and conferences (6.6 ECTS)
- Networking meeting: Technological Top Institute for Green Genetics (2011)
- International Symposium on Light in Horticultural Systems (2012)
- Systems Biology Retreat (2013)
- Systems Biology for Food, Feed and Health (2013)
- Society for Experimental Biology (SEB) (2013)

Lecturing / supervision of practical's / tutorials
- Research methods in crop science (2009, 2011, 2012, 2013)
- Crop ecology (2009, 2013)

Supervision of MSc student
- Effects of temperature on cell division and expansion, sugar metabolism and fruit growth of two tomato cultivars

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