A multilevel analysis of fruit growth of two tomato cultivars in response to fruit temperature

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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 analyze 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' owing 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-like*) 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.

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

Abbreviations – ADPGPP, ADP glucose pyrophosphorylase; CDK, cyclin-dependent kinase; Cyc, cyclin; DAA, days after anthesis; HPAEC, high performance anion exchange chromatography; QTL, quantitative trait loci.

and fruit maturity decreases with an increase in temperature between 14 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 et al. 1993). In tomato, fruit size increase in the first 2 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, fruit 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 the 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, 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 downregulate 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 multilevel fruit growth modeling 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 performed. 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 carried out 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, De Lier, The Netherlands). 'Brioso' was grafted on 'Maxifort' (Monsanto Vegetable Seeds, Bergschenhoek, The Netherlands) while 'Cappricia' was grafted on 'Stallone' (Rijk Zwaan) rootstock. Graftlings of the two cultivars were planted on Rockwool® slabs in the greenhouse on January 15, 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

$150 \mu mol\, m^{-2}\, s^{-1}$) and carbon dioxide dosing systems. Greenhouse air temperature was $20 \pm 3°C$, while average day time carbon dioxide and relative air humidity were $540 \pm 45\, \mu mol\, mol^{-1}$ and $72 \pm 10\%$, 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, 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 center 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 aluminum foil. Realized average cuvette air temperatures were: $21.4 \pm 0.8°C$ (control) and $27.1 \pm 1.5°C$ (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, SC) with a ventilator attached inside the cuvette as described in the study by 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 and 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 aluminum foil and immediately placed in ice before fresh weight and horizontal plane 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 (Fig. 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 of 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 min 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 (Leica, Rijswijk, The Netherlands) and stained with 1% toluidine blue dye before microscopic analysis as reported in the study by Fanwoua et al. (2012a). An image of one slide per sample was made using

NIS-Elements Software (Nikon Instruments, New York, NY). With the aid of IMAGEJ software (National Institutes of Health, Rockville, MD), pericarp thickness was measured, and the numbers of cell layers and cells (n) in a rectangular section were 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 (Cd; direction parallel to fruit skin), cell volume (Cv), tissue volume (Tv) and number of cells (Cn) in pericarp tissue (mesocarp or exocarp) were then derived according to Eqns 1–4 with the assumption that each cell is an ellipsoid.

$$\text{Periclinal cell diameter } (Cd) = \left(\frac{A}{0.25 \times \pi \times Ch} \right) \quad (1)$$

$$\text{Mean cell volume } (Cv) = \frac{4}{3} \times 0.5 \times Cd \times A \quad (2)$$

$$\text{Tissue volume } (Tv) = \left(\frac{4}{3} \times \pi \right) \times \left(r^3 - (r - Tt)^3 \right) \quad (3)$$

$$\text{Tissue cell number } (Cn) = \frac{Tv}{Cv} \quad (4)$$

where A is the area of rectangular pericarp section in which cells were counted, Ch the mean anticlinal cell diameter (direction perpendicular to fruit skin) in mesocarp or exocarp tissue, r the fruit radius and Tt the 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).

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

where Ev is the exocarp volume, Pv pericarp volume, Ecv the mean exocarp cell volume, Mv the mesocarp volume and Mcv the 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 min. The mixture was then centrifuged (25 000 g for 5 min) and 1 ml of supernatant was dried using a speed vacuum (SpeedVac SPD 2010; Thermo Fisher Scientific, Wilmington, DE) for 105 min. Dried samples were dissolved in 1 ml water using a vortex machine and an ultrasonic water bath and again centrifuged for 5 min. Ten-fold diluted samples were then analyzed 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 × 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 min and again the supernatant discarded. This process was repeated four times to ensure that all the sucrose, glucose and fructose were washed out of the solid material before drying for 20 min 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 min 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 min incubation in a shaking water bath at 60°C. A 1 ml sample of hydrolyzed starch was centrifuged for 5 min, diluted 10 times and its glucose content determined through HPAEC analysis as described above except that the CarboPac1 (250 × 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 (Thermo Fisher Scientific). RNA quality was checked on gel and single stranded complementary DNA (cDNA) was synthesized from 650 ng of total RNA using the TaqMan® Reverse Transcription Reagents kit (Roche Molecular Systems, Branchburg, NJ). 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 or 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-like*) 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.

Statistical analysis

All data collected were analyzed 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 $\pm 3 \times$ 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:

$$Y = A + C \times \exp\left(-\exp\left(-B \times (X - M)\right)\right) \qquad (6)$$

where Y is the fruit fresh weight (g), A the lower asymptote, B the slope of the curve (g d^{-1} or g °C d^{-1}), C the upper asymptote, M the thermal time or day after anthesis (DAA) and X the 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.

$$FGR = \left[C \times \exp\left\{-\exp\left[-B \times (X - M)\right]\right\} \times B \times \exp\left[-B \times (X - M)\right]\right] \qquad (7)$$

where FGR is the fruit fresh weight growth rate (g d^{-1}) and B, C, M and X are as defined in Eqn 6.

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 fruits than in control (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 (Fig. 1). Fruit fresh weight growth rate as a function of the number of DAA was higher in heated fruits during the early stages of growth until approximately 25 DAA in both cultivars (Fig. 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 (Fig. 2B). After this period, fruit fresh weight growth rate steadily decreased regardless of the temperature treatment in both cultivars. In the last one third of the fruit growth period, temperature seemed to have no effect on fruit fresh weight growth rate in 'Brioso' while control fruits of 'Cappricia' exhibited a fresh weight growth rate higher than that for heated fruits (Fig. 2B).

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 (Tables 1 and 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 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).

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 of cultivars or temperature within a row followed by different letters differ significantly ($P < 0.05$). Means within the same row for the main effects of cultivar and temperature were averaged over the two temperature treatments or cultivars respectively.

|	Brioso	Cappricia	P value	Control	Heated	P value	Brioso		Cappricia		
							Control	Heated	Control	Heated	P value
Growth duration (days)	44.78a	49.94b	0.012	52.28b	41.50a	0.007	48.50	40.13	57.00	42.88	0.739
Fresh weight (g)	29.55a	73.15b	<0.001	53.51a	46.21a	0.055	32.22	26.22	80.11	66.19	0.397
Fruit diameter (mm)	40.13a	58.61b	<0.001	50.49b	46.95a	0.049	41.70	38.16	61.48	55.73	0.760
Pulp volume (cm^3)	12.54a	33.52b	<0.001	25.53b	18.90a	0.031	14.41	10.19	39.42	27.61	0.256
Sucrose (µg mg^{-1})	3.39a	5.16a	0.233	4.67a	3.72a	0.482	3.80	2.88	5.76	4.55	0.821
Fructose (µg mg^{-1})	244.9a	231.2a	0.053	233.2a	244.3a	0.175	236.9	254.8	228.6	233.8	0.348
Glucose (µg mg^{-1})	251.9a	234.1a	0.054	240.3a	247.2a	0.806	243.8	262.1	235.9	232.3	0.135
Starch (µg mg^{-1})	4.19a	1.48a	0.288	4.07a	1.62a	0.099	5.30	2.80	2.53	0.44	0.627
Exocarp volume (cm^3)	0.17a	0.31b	<0.001	0.27b	0.19a	0.002	0.20	0.14	0.37	0.25	0.113
Exocarp cell volume (×10^{-5} mm^3)	3.75a	3.87a	0.472	4.37b	3.18a	0.021	4.44	2.89	4.28	3.47	0.103
Exocarp cell number (×10^6)	4.84a	8.63b	<0.001	6.69a	6.56a	0.840	4.63	5.11	9.27	8.00	0.092
Mesocarp volume (cm^3)	16.79a	40.23b	<0.001	29.14a	26.34a	0.168	18.22	15.00	42.78	37.68	0.949
Mesocarp cell volume (×10^{-5} mm^3)	513.8a	667.9b	0.011	679.2b	481.9a	0.005	602.8	402.6	774.8	561.1	0.838
Mesocarp cell number (×10^6)	3.56a	6.29b	<0.001	4.29a	5.46a	0.066	3.25	3.94	5.60	6.98	0.424
Mesocarp cell layers	20.94a	23.48a	0.070	20.69a	23.77b	0.030	20.03	22.08	21.50	25.46	0.723
Pericarp volume (cm^3)	16.96a	40.54b	<0.001	29.42a	26.53a	0.160	18.42	15.14	43.15	37.92	0.959
Pericarp cell volume (×10^{-5} mm^3)	508.6a	662.7b	0.010	672.6b	478.2a	0.005	596.4	398.9	768.0	557.5	0.834
Pericarp cell number (×10^6)	8.40a	14.92b	<0.001	10.99a	12.01a	0.323	7.89	9.04	14.86	14.98	0.369
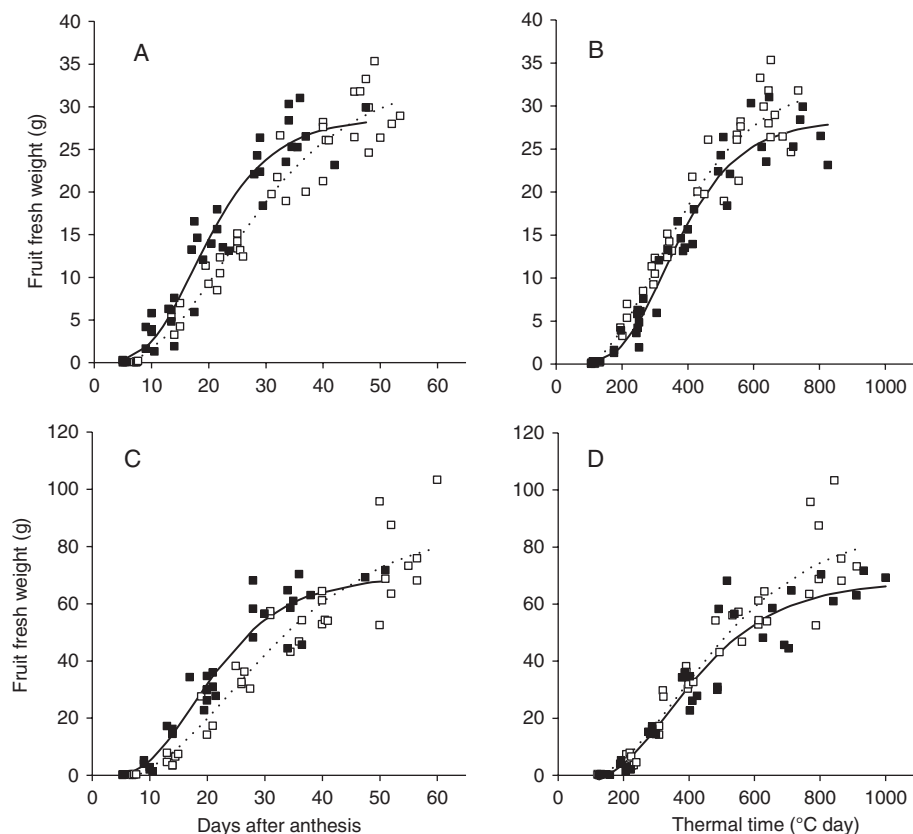
Fig. 1. Effect of fruit heating on a small tomato 'Brioso' (A, B) and intermediate-sized tomato 'Cappricia' (C, D) plotted as a function of the number of days after anthesis (A, C) or thermal time (B, D) in control (□) and heated (■) fruits. Breaker stage was attained at 711 and 907°C day in 'Brioso' 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.

Pulp volume was significantly larger in control compared with heated fruits. A similar 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 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 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 carbohydrate concentration in fruits. The starch concentration peak at about 300°C day matched with the period of maximum growth rate, while sucrose, fructose and glucose concentrations peaked shortly after the period of maximum growth rate in both genotypes and temperature treatments (Fig. 3). Thereafter, sucrose and starch concentrations 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' (Fig. 3A). Between 300 and 550°C day, sucrose concentration was significantly higher in heated than in control fruits (Fig. 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
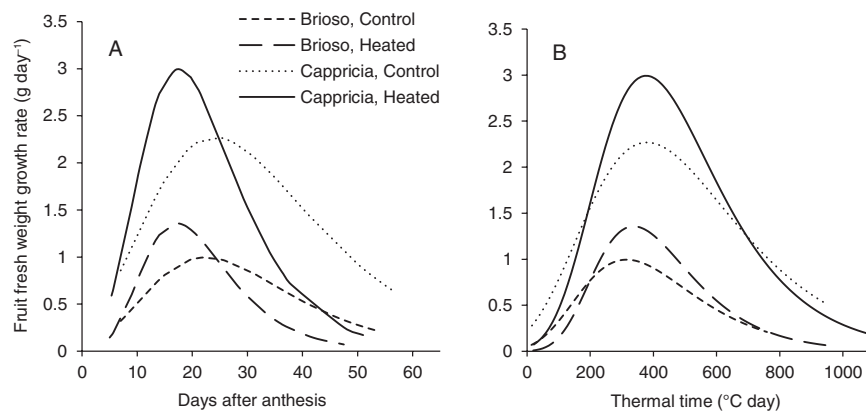
Fig. 2. Fruit fresh weight growth rate for control (21.4 ± 0.8°C) and heated (27.1 ± 1.5°C) small; 'Brioso', and intermediate; 'Cappricia'-sized tomato fruits fitted as a function of number of days after anthesis (A) and thermal time (B). Breaker stage was attained at 711 and 907°C day in 'Brioso' and 'Cappricia', respectively. Curves are derivatives of fitted Gompertz curves for fruit fresh weight in Fig. 1.

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 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; Fig. 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; Fig. 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; Fig. 3D). Pericarp starch concentration seemed to be lower when fruits were heated; however differences were generally not statistically significant (Fig. 3H).

Gene expression analysis

There was a 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 (Figs 4, 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 analyzed (Fig. 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; Fig. 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 with '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; Fig. 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 (Fig. 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; Fig. 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 (Fig. 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 (Fig. 4). Fruit heating stimulated the expression of *CDKB2* (459°C day), *CycA1* (287°C day),
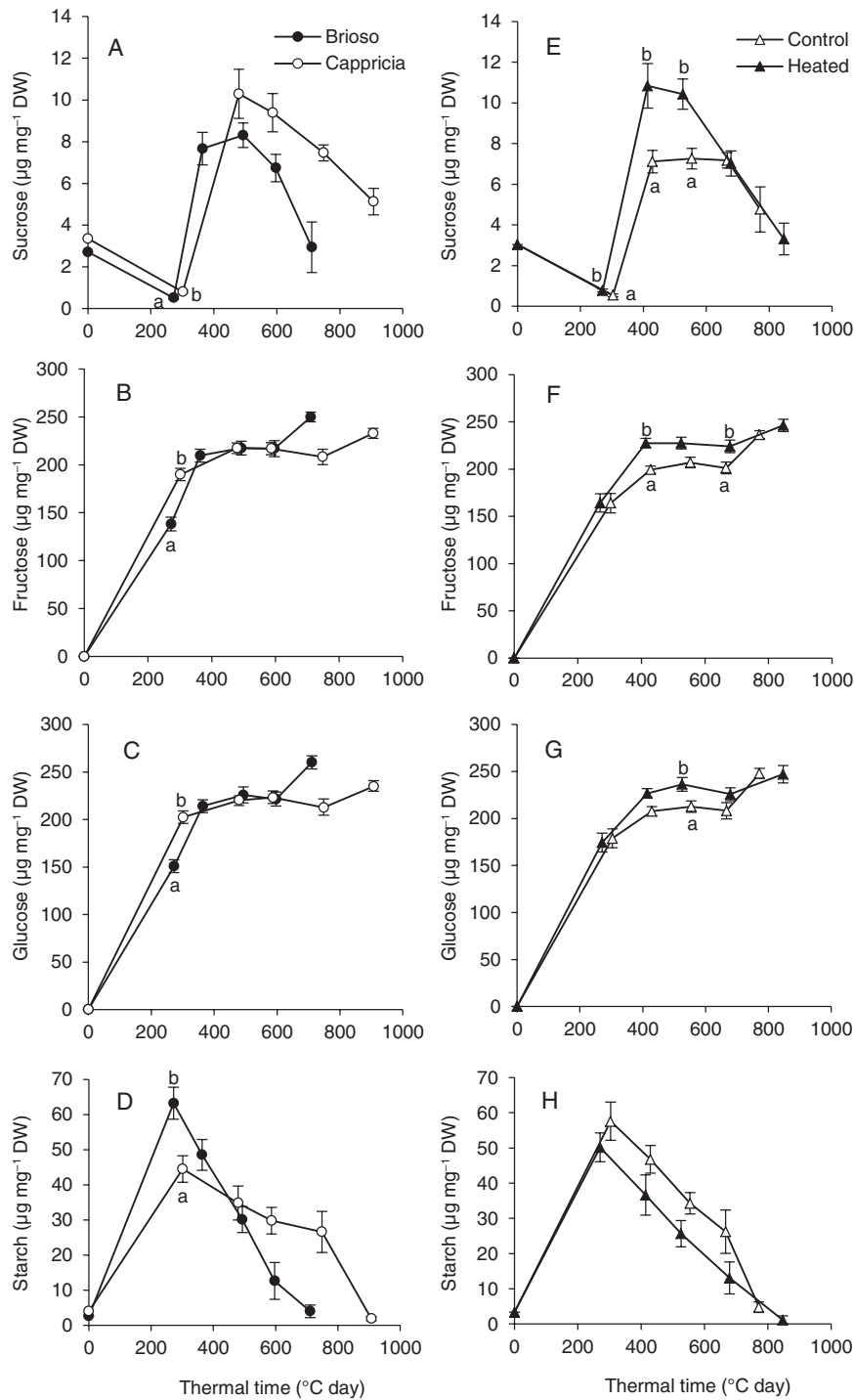
Fig. 3. Sucrose (A,E), fructose (B,F), glucose (C,G), starch (D,H) content in the pericarp of small; 'Brioso' (●), and intermediate; 'Cappricia' (○)-sized, control (△; 21.4 ± 0.8°C) and heated (▲; 27.1 ± 1.5°C) tomato fruits harvested at different stages from anthesis till breaker stage. Breaker stage 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 = 8–12 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.
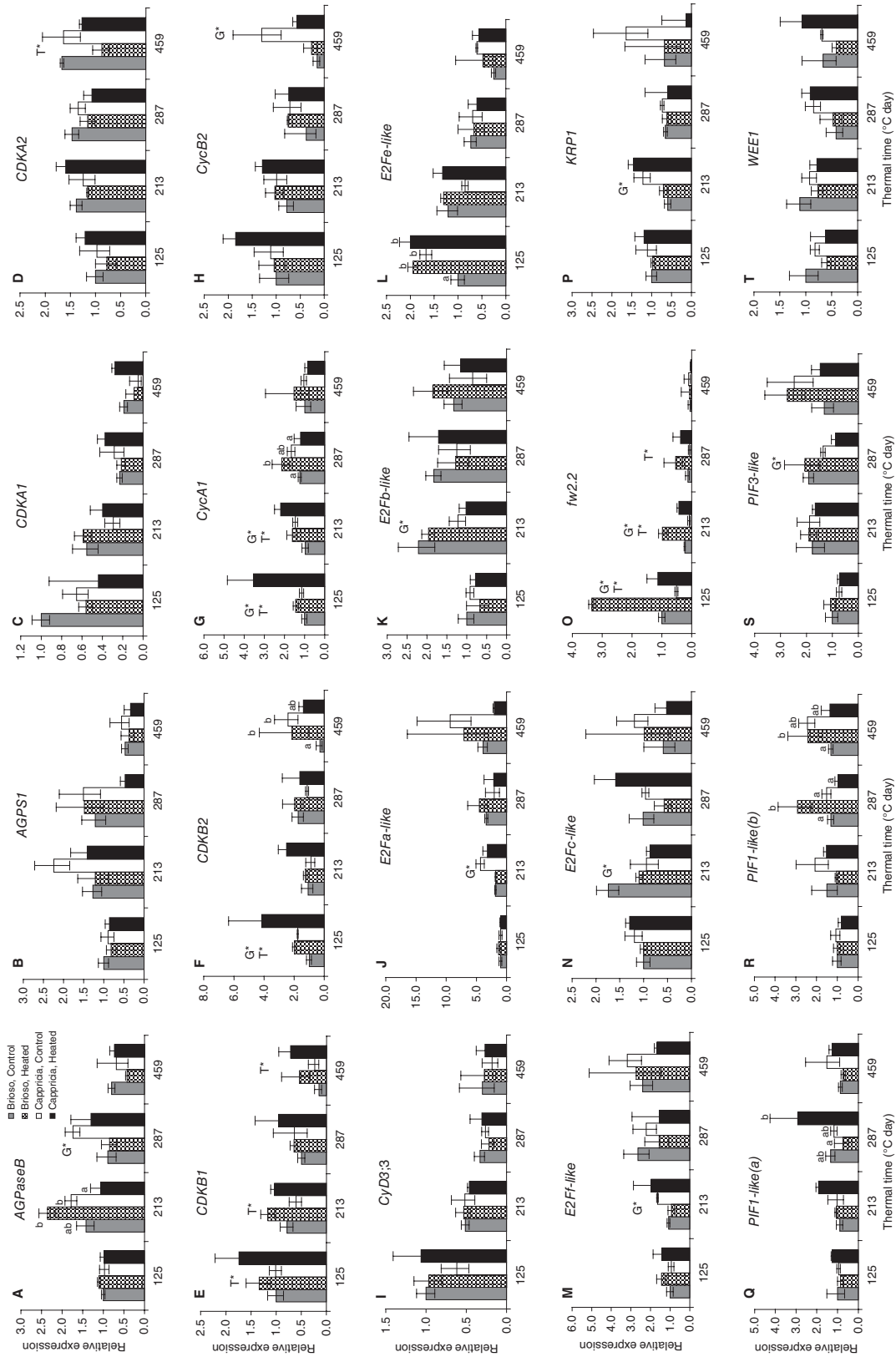
Fig. 4. Relative gene (A–B: promoters of cell expansion; C–L: promoters of cell division; M: inhibitor of cell expansion; N–T: inhibitors of cell division) expression in pericarp tissue of 'Brioso' and 'Cappricia' under control ($21.4 \pm 0.8°C$) and heated ($27.1 \pm 1.5°C$) conditions during the first 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.

and *PIF1-like(b)* (287 and 459°C day) only in 'Brioso'. *PIF1-like(a)* expression at 287°C day was stimulated by fruit heating only in 'Cappricia'. 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 levels. The physiological basis for the fruit size difference 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 variations in fruit size could be investigated simultaneously. Temperature treatments were applied at truss level in order 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 is 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 (Fig. 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' (Fig. 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. 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 (Fig. 1A, C 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 (Fig. 1B, D). This was probably because of the positive effect of high temperature on fruit fresh weight growth rate (Fig. 2A, B) 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 (Figs 1B, D 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 (Fig. 2B). 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 with 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 modeling 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 fruit 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 with heating of fruits from 7 DAA until breaker stage.

The relationship between temperature effects at cell and fruit levels

In agreement with the 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 37% decrease in exocarp cell volume and a 40% decrease in volume and a 29% increase in number of mesocarp cells although the difference in mesocarp cell number was not significant ($P = 0.066$; Table 1). We assumed that fruit size would be determined mainly by mesocarp cell growth dynamics because 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 (Fig. 5). 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 toward 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 diameters when fruits were heated (Fig. 5; 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 carried out 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 with '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; Fig. 3B, C). The tendency toward higher sucrose and hexose concentrations in heated fruits at the beginning of fruit growth (until about 600°C day) also supports this argument as mesocarp cell number and layers tended to be higher in heated than in control fruits (Table 1 and Fig. 3E–G).
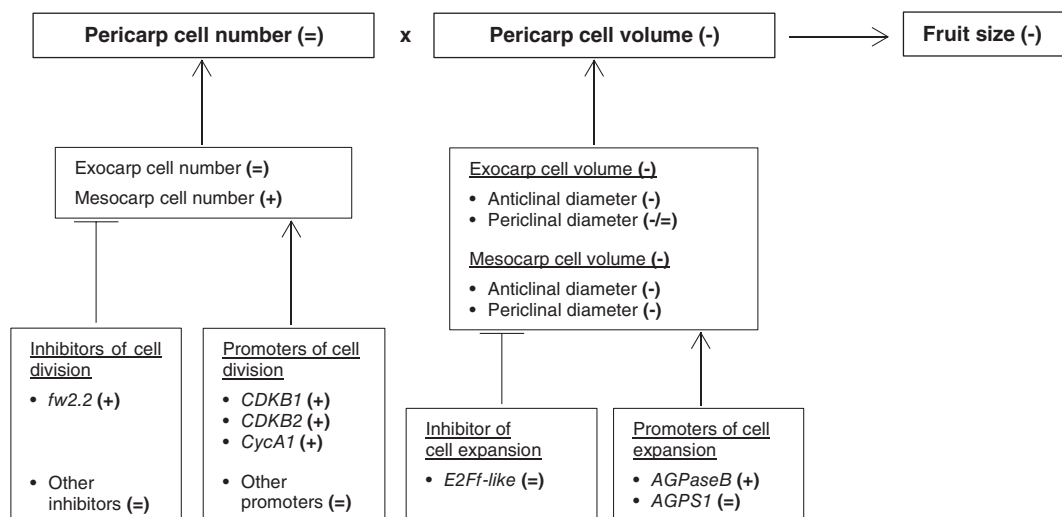

Fig. 5. Schematic representation of the effect of fruit heating (+: stimulates; −: depresses; =: no detectable effect; −/=: decreases in 'Brioso' but no detectable effect in 'Cappricia') on cell characteristics averaged over the two cultivars ('Brioso' and 'Cappricia'). Other promoters of cell division 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.*

Cell volume is a function of both carbohydrate and water content. The accumulation of carbon and water during the cell expansion phase causes a 30 000-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 with 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 analyzed at breaker stage. Gautier et al. (2008) also found no significant effect of increasing fruit temperature (21 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, Czerednik et al. 2012, Fanwoua et al. 2012b). Fanwoua et al. (2012b) studied the expression of cell cycle genes in fruits of inbred lines obtained from a cross between *Solanum lycopersicum* '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 day. On the contrary, the current study showed no significant differences in the expression of *CDKB1* or *CycD3;3* between 'Brioso' and 'Cappricia' (Fig. 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 the 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 the expression of cell division promoters: *CDKB2;1*, *CycB2;1* (Joubès et al. 2000b, Joubès et al. 2000a, 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 toward a higher cell number and layers in the mesocarp of heated fruits, it is not surprising 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 analyzed in this study outweighed the negative effects of *fw2.2* on cell division (Fig. 5). However, high expression of *fw2.2* in heated fruits compared with control and in 'Brioso' fruits compared with 'Cappricia' 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 (NILs) 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 the 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 as the shift in the duration of peak expression was not apparent in the two cultivars (Fig. 4O).

Three genes (*AGPaseB*, *AGPS1* and *E2Ff-like*) 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 with '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-like* was expected to be higher in heated and 'Brioso' fruits as fruits from these two treatments had a lower cell volume compared with control and 'Cappricia' fruits. The expression of *E2Ff-like* 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-like*) 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.

Authors' contributions

R. C. O. O., P. H. B. V., E. H., P. C. S. and L. F. M. M. conceived and designed the study. R. C. O. O. performed the experiments and collected the fruit data. R. C. O. O. and M. L. analyzed the gene expression. R. A. M. conducted the phylogenetic analysis. R. C. O. O., P. H. B. V. and E. H. analyzed the data. R. C. O. O., P. H. B. V., E. H., P. C. S. and L. F. M. M. interpreted the results and wrote the manuscript. All authors edited the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Pericarp contribution to total fruit dry weight expressed as a percentage.

Figure S2 Relative gene expression in pericarp tissue of 'Brioso' averaged over the two temperature treatments during the first four harvest stages when fruits were green.

Figure S3 Relative gene expression in pericarp tissue of 'Cappricia' averaged over the two temperature treatments during the first four harvest stages when fruits were green.

Table S1 Tomato gene names and primer sequences.

Table S2 Fruit phenotype at breaker stage for control and heated 'Brioso' and 'Cappricia'.