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RESEARCH PAPER

Increase in fruit size of a spontaneous mutant of 'Gala' apple (*Malus*×*domestica* Borkh.) is facilitated by altered cell production and enhanced cell size

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Abstract

Fruit size regulation was studied in the apple cultivar 'Gala' and a large fruit size spontaneous mutant of 'Gala', 'Grand Gala' (GG). GG fruits were 15% larger in diameter and 38% heavier than 'Gala' fruits, largely due to an increase in size of the fruit cortex. The mutation in GG altered growth prior to fruit set and during fruit development. Prior to fruit set, the carpel/floral-tube size was enhanced in GG and was associated with higher cell number, larger cell size, and increased ploidy through endoreduplication, an altered form of the cell cycle normally absent in apple. The data suggest that the mutation in GG promotes either cell production or endoreduplication in the carpel/floral-tube cells depending on their competence for division. Ploidy was not altered in GG leaves. During fruit growth, GG fruit cells exited cell production earlier, and with a DNA content of 4C suggesting G2 arrest. Cell size was higher in GG fruits during exit from cell production and at later stages of fruit growth. Final cell diameter in GG fruit cortex cells was 15% higher than that in 'Gala' indicating that enhanced fruit size in GG was facilitated by increased cell size. The normal progression of cell expansion in cells arrested in G2 may account for the increase in cell size. Quantitative RT-PCR analysis indicated higher *MdCDKA1* expression and reduced *MdCYCA2* expression during early fruit development in GG fruits. Together, the data indicate an important role for cell expansion in regulating apple fruit size.

Key words: Cell cycle, cell division, cell expansion, endoreduplication, fruit growth, gene expression.

Introduction

Fruit size is an important commercial trait in crops such as apple (*Malus*×*domestica* Borkh.). Final fruit size is determined by co-ordinated progression of cell production and cell expansion during fruit growth and development. Early fruit growth in apple is facilitated by cell proliferation where cell number is greatly amplified, within 3-4 weeks after pollination and fertilization (Bain and Robertson, 1951; Denne, 1960; Harada *et al.*, 2005). Later stages of fruit growth are largely associated with cell expansion. Genes that regulate cell production and/or cell expansion may therefore control final fruit size. Several quantitative trait loci (QTL) affecting fruit size have been identified in tomato and apple and two such genes, *FW2.2* and *FASCIATED*, have been isolated from tomato (Liebhard *et al.*, 2003; Tanksley, 2004). *FW2.2* negatively regulates fruit size through the control of cell proliferation during early fruit development (Frary *et al.*, 2000; Cong *et al.*, 2002). *FASCIATED* encodes a YABBY-like transcription factor that affects fruit size by regulating carpel number in tomato (Cong *et al.*, 2008). In addition, several genes that regulate plant organ growth and size through their effects on cell proliferation and/or cell expansion have been identified (Anastasiou and Lenhard, 2007; Bogre *et al.*, 2008; Krizek, 2009). *AINTEGUMENTA* (*ANT*), a transcription factor with an *APETALA2* (*AP2*) like domain, is a key regulator of organ size in *Arabidopsis* and affects the timing

of exit from cell proliferation (Krizek, 1999; Mizukami and Fischer, 2000). *ARGOS*, an auxin-inducible gene, regulates lateral organ size in *Arabidopsis*, at least in part through the alteration of *ANT* expression (Hu *et al.*, 2003). *EBP1*, a plant homologue of the human epidermal growth factor binding protein, regulates organ size through its effect on cell proliferation and cell expansion (Horvath *et al.*, 2006). In addition, genes such as *KLUH*, a cytochrome P450 gene; *STRUWWELPETER*, a putative component of the RNA polymerase complex; *JAGGED*, a C_2H_2-type zinc finger transcription factor; and *BIG BROTHER*, an E3 ubiquitin ligase, control organ size by regulating cell proliferation (Autran *et al.*, 2002; Ohno *et al.*, 2004; Disch *et al.*, 2006; Anastasiou *et al.*, 2007).

In addition to the above genes, plant cell proliferation is also regulated by the cell cycle which, in *Arabidopsis*, is controlled by a core group of over 80 genes consisting of cyclins, cyclin dependent kinases (CDKs), CDK inhibitors (ICK/KRPs), CDK activating kinases, retinoblastoma-related genes, and E2F transcription factors (Inzé and De Veylder, 2006). Overexpression of dominant negative forms of A- and B-type CDKs decreases cell number in *Arabidopsis* (Hemerly *et al.*, 1995; Boudolf *et al.*, 2004). Misexpression of the CDK inhibitor, *KRP2*, reduces leaf size and alters leaf shape in *Arabidopsis* through a reduction in the rate of cell production (De Veylder *et al.*, 2001). Also, overexpression of B-type and D-type cyclins promotes cell production leading to enhanced rate of growth in *Arabidopsis* (Doerner *et al.*, 1996; Dewitte *et al.*, 2003; Kono *et al.*, 2007; Qi and John, 2007).

In addition to the proliferative cell cycle, many plants engage in a modified form of the cell cycle termed endoreduplication, a process involving DNA duplication without mitosis. Cell expansion during organ growth is often associated with endoreduplication (Melaragno *et al.*, 1993; Joubes *et al.*, 1999; Joubes and Chevalier, 2000; Kondorosi *et al.*, 2000; Sugimoto-Shirasu and Roberts, 2003). Final cell size within the fruit pericarp was correlated with the level of endoreduplication in tomato (Cheniclet *et al.*, 2005). Increase in cell size in the hypocotyl and the cotyledon is associated with an increase in ploidy in *Arabidopsis* mutants (Yoshizumi *et al.*, 2006). In addition, suppression of *WEE1* kinase in tomato leads to reduced endoreduplication, decreased cell size, and reduced fruit size (Gonzalez *et al.*, 2007). However, endoreduplication does not appear to be integral to normal fruit development in apple (Harada *et al.*, 2005).

Molecular components involved in regular progression of the mitotic cell cycle also play important roles in endoreduplication. CDKB1 inhibits endoreduplication and induces mitosis in *Arabidopsis* (Porceddu *et al.*, 2001; Boudolf *et al.*, 2004). Increased polyploidy is associated with reduced expression of A2-type cyclins and loss of *CYCA2;3* function in *Arabidopsis* (Imai *et al.*, 2006; Yoshizumi *et al.*, 2006). Overexpression of *Cyclin D3* induces mitotic cell divisions and reduces endoreduplication (Schnittger *et al.*, 2002; Dewitte *et al.*, 2003), while loss of D3-cyclins results in enhanced or ectopic endoreduplication in *Arabidopsis* (Dewitte *et al.*, 2007). KRPs are dose-dependent regulators of endoreduplication as moderate overexpression of *KRP*s in *Arabidopsis* induces endoreduplication while strong overexpression results in exit from the cell cycle (Verkest *et al.*, 2005; Weinl *et al.*, 2005). Overexpression of *E2Fa* and *DPa* in *Arabidopsis* enhances cell proliferation or endoreduplication depending on the competence of cells for division (De Veylder *et al.*, 2002). Competence for cell division is determined, at least in part, by the presence of a mitosis inducing factor (MIF) such as CDKB1 which may prevent entry into endoreduplication cycles through its association with CYCA2;3 (Boudolf *et al.*, 2004, 2009). These studies demonstrate the role of cell cycle genes in regulating endoreduplication and thereby cell and organ size.

Molecular mechanisms involved in regulating fruit growth and final size in apple are not well understood. Recently, a spontaneous mutant of 'Gala' apple with increased fruit size was identified in commercial orchards and is currently marketed as 'Grand Gala' (GG). In this study, the characterization of GG which produces fruits 38% heavier than those of 'Gala' is reported. Evidence is presented for a cell size-mediated increase in fruit size in GG.

Materials and methods

Fruit growth, maturity characteristics, and sink activity of 'Gala' and 'Grand Gala'

'Gala' (n=4) and 'Grand Gala' (GG; n=3) trees growing on M.7 rootstocks, were used for analysis of fruit growth and development in 2004. Flower clusters were thinned to one flower at bloom. Fruit diameter was measured on 10 fruits per tree across the widest part of the fruit using digital calipers at multiple stages during fruit development. For determining fruit maturity characteristics, 10 fruits per tree from 'Gala' (n=5) and GG (n=2) were harvested at maturity. Fruit weight (g), length (mm), and diameter (mm) were measured. To determine if the increase in fruit size of GG was within the core or the cortex, 25 fruits per tree were cut along the radial plane and core diameter was measured. Cortex size was calculated from fruit and core diameter measurements and is presented here as the sum of cortex width on either side of the core. Fruit firmness was measured using a penetrometer equipped with an 11 mm head (Effegi, Italy). Soluble solids content was measured using a digital refractometer (Atago Co., Japan). Starch content was estimated by a starch-iodine test and rated on a scale of 1–10 (1, highest starch pattern; 10, lowest starch pattern). Sink activity was measured using ^{14}C labelled sorbitol according to Archbold (1992). Briefly, fruit cortex discs, 5 mm in diameter and 2 mm in thickness, were obtained from 'Gala' and GG fruits at 60 d after full bloom (n=5). The discs were pre-incubated for 30 min in ice-cold buffer solution [1 mM calcium chloride, 10 mM 2-[*N*-morpholino] ethanesulphonic acid (MES)], transferred to the buffer solution supplemented with ^{14}C labelled sorbitol, and incubated at room temperature for 2 h. The discs were subsequently washed with the buffer. Total sugars were extracted by boiling in 80% ethanol and the amount of sorbitol absorbed was measured using a Beckman scintillation counter.

Microscopy

'Gala' (n=4) and GG (n=3) fruits were collected at regular intervals during fruit development and fixed in CRAF III fixative (Berlyn and Miksche, 1976; ⩾3 fruits per tree). One time point

each in 'Gala' and GG had two replicates due to technical issues with sectioning. Samples were sectioned using a vibratome and digital images were captured using an Olympus microscope. The number of cell layers and the distance between the petal vascular trace and the peel were determined. Average radial cell diameter was determined using cell layers and distance data. At full bloom (FB), FB+28 d and FB+127 d, cell diameter along the long (*A*) and short (*B*) axes of 25–30 floral-tube/fruit cortex cells (n=5) was measured using Image J (NIH Image) and cell area was calculated as: Area=$\pi AB/4$.

For measurement of nucleus size, flowers were collected from 'Gala' and GG at full bloom (n=5) and sectioned at the floral-tube region using a vibratome. Sections were incubated for 30 min in a buffer solution (15 mM TRIS, 20 mM sodium chloride, 80 mM potassium chloride, 2 mM EDTA, 1% β-mercaptoethanol, and 2.5 μg ml^{-1} DAPI: diamidino-phenylindole). DAPI stained sections were observed under a Nikon fluorescent microscope and images were captured using SPOT software and processed in Adobe Photoshop. Projected nucleus area of 30 randomly selected nuclei per flower was measured using Image J software (NIH Image).

Flow cytometry (FCM)

Nuclei were released by chopping fresh tissue in ice-cold Dolezel's LB01 buffer (15 mM TRIS, 2 mM EDTA, 20 mM NaCl, 80 mM KCl, 0.5 mM spermine tetrahydrochloride, 1% β-mercaptoethanol, 0.5% Triton X-100, 2% PVP-40, and 50 μg ml^{-1} RNase; Dolezel *et al.*, 1989). Isolated nuclei were stained with 50 μg ml^{-1} propidium iodide (PI) and incubated for 30 min at 4 °C in the dark. Stained nuclei were filtered through a 41 μm nylon mesh (Millipore, USA) and analysed using a Beckman-Coulter FC-500 analyser. PI excitation was achieved at 488 nm and emission was analysed at 675 nm. A total of 10000 events were collected during early fruit development and 3000–6000 events were collected at later stages of fruit development. Data processing involved gating to isolate debris and aggregates. Ploidy and DNA content were determined using chicken red blood cells and young petunia leaf tissue as standards. Per cent ploidy presented here is from 2003 data as FCM and ploidy distribution analyses during fruit development were repeated during multiple years with similar results (Malladi, 2005).

RNA isolation, reverse transcription, and quantitative RT-PCR

Fruits were sampled from 'Gala' and GG in 2009 (n=4), frozen in liquid N_2 and stored at –80 °C until further analysis. RNA isolation from fruit tissues was performed according to Dong *et al.* (1997). Frozen tissue was ground along with PVPP. Extraction buffer (150 mM TRIS-borate, 50 mM EDTA, 2% SDS, and 1% β-mercaptoethanol) was added to the ground tissue followed by the addition of 0.1 vols of 5 M potassium acetate and 0.25 vols of ethanol. This mixture was extracted with chloroform: iso-amyl alcohol (24:1 v/v), followed by phenol:chloroform: iso-amyl alcohol (25:24:1 by vol.) and chloroform:iso-amyl alcohol (24:1 v/v). The aqueous supernatant was precipitated with iso-propanol (1:1 v/v) at room temperature for 15 min and overnight in 3 M lithium chloride (4 °C). After centrifugation, the RNA was washed in 70% ethanol, dissolved in di-ethyl pyro-carbonate (DEPC)-treated water, and precipitated in 0.1 vols sodium acetate (3 M) and 2.5 vols of ethanol for 2 h. RNA was subsequently washed with 70% ethanol, dried, and dissolved in DEPC-treated water. RNA was treated with DNase (Promega) to remove genomic DNA contamination, according to the manufacturer's instructions. Reverse transcription was performed on DNase-treated RNA (1 μg) using oligo dT (Promega) and ImPromII reverse transcriptase (Promega) according to the manufacturer's instructions. The cDNA was diluted with 7 vols of water and stored at –20 °C until further analysis.

All genes used in this study, except *MdCYCB2* and *MdCYCD3*, were identified from the *Malus* EST database (NCBI). *MdCYCB2* and *MdCYCD3* were isolated in our laboratory. Primers used for quantitative RT-PCR (qRT-PCR) analyses are listed in Supplementary Table S1 at *JXB* online. The qRT-PCR analyses were performed on the Stratagene Mx3005P real-time PCR system using 1 μl of cDNA in a 14 μl reaction with 2× SYBR Green Master Mix (Applied Biosystems). Cycling parameters were: 95 °C (10 min); 95 °C (30 s), and 60 °C (1 min) for 40 cycles. Melt-curve analysis was performed to determine specificity of the amplified product. Efficiency of amplification was determined for all genes. Relative levels of expression of cell cycle genes were determined following efficiency correction (Pfaffl, 2001), and normalization with the geometric mean of expression of apple glyceraldehyde 3-phosphate dehydrogenase (*MdGAPDH*) and actin (*MdACTIN*) genes. Expression for a given gene is presented relative to its expression in 'Gala' fruits at 0 d after full bloom. All analyses were performed using four replicates. Statistical analyses of expression data were performed on $\log_2$ transformed data using Minitab 15.

Results

Phenotypic characterization of 'Grand Gala'

'Grand Gala' (GG) trees exhibited reduced apical dominance and a decrease in plant height compared with 'Gala' trees. GG buds were larger than those of 'Gala' by 10% (P <0.01; data not shown). No significant difference in flowering time was observed between GG and 'Gala'. A few GG flowers (~2%) had a higher number of floral organs, and fused sepals and petals.

GG flowers showed an increase in the carpel/floral-tube size prior to fruit set, compared with 'Gala' (Fig. 1A, inset). Throughout fruit development, GG fruits had a greater fruit diameter than those of 'Gala' except at 6 d and 13 d after full bloom (DAFB), indicating that differences in fruit size were apparent during early fruit growth (Fig. 1A). Relative growth rates of 'Gala' and GG fruits were not significantly different (data not shown). At harvest, GG fruits had a 15% increase in fruit diameter (Fig. 1A), and a 38% increase in fruit weight in comparison to 'Gala' fruits (Table 1). These data indicate a major increase in fruit size in GG. To determine if larger fruit size in GG was due to an increase in core or cortex size, the core diameter and cortex width were measured at harvest. Core diameter was not significantly altered in GG fruits (Fig. 1B). The increase in fruit diameter in GG was due to an increase in cortex width by 18%, suggesting that this tissue contributed most to alteration in fruit size (Fig. 1B).

The fruit length/diameter ratio was reduced in GG (P=0.05) suggesting that GG fruits were less 'typey' or more 'squat' than 'Gala' fruits (Table 1). GG fruits had reduced fruit firmness (P=0.01) and higher soluble solids content (P=0.03), but the starch content was not significantly different from that of 'Gala' fruits (Table 1). GG fruits had reduced seed set as indicated by a lower final seed number (P=0.02).

To determine the physiological basis of increase in GG fruit size, sink activity and source capacity were determined. Fruit cortex cells of GG exhibited an increase in the rate of ^{14}C-labelled sorbitol absorption by 65% in comparison to 'Gala' suggesting enhanced sink activity (Table 1). Specific

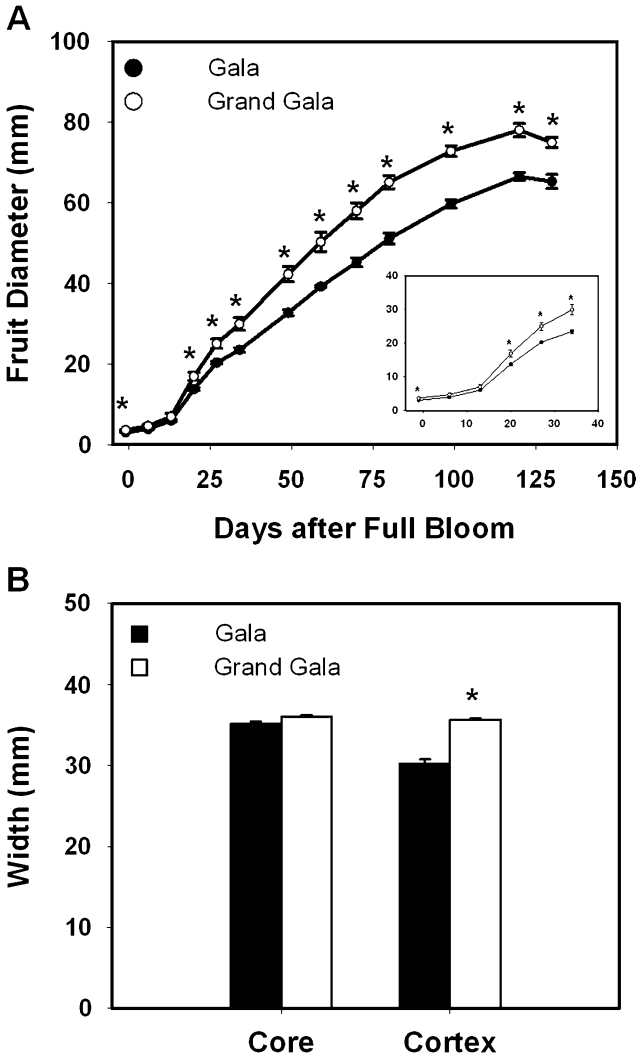

Fig. 1. Comparison of fruit growth between 'Gala' and 'Grand Gala.' (A) Fruit diameter (mm) in 'Gala' (n=4) and 'Grand Gala' (n=3) was measured from before bloom until harvest. Inset shows a magnified view of fruit diameter data during early fruit growth. (B) Breakdown of fruit size according to core and cortex size. Core size is represented as the core width (mm). Cortex size is represented as the sum of cortex width (mm) on either side of the fruit core. Error bars represent standard error of the means. Asterisk indicates significant difference between means ($P \leqslant 0.05$).

leaf weight (SLW) was increased by 24% and 20% in GG spur and bourse leaves, respectively, suggesting an overall increase in net photosynthetic potential (data not shown). In addition, GG pedicels had consistently greater diameter than those of 'Gala' during early fruit growth suggesting enhanced potential for solute and photosynthate movement (data not shown).

Cell production in 'Gala' and 'Grand Gala' fruits

Cell number (cell layers) in the floral-tube tissue was not significantly different between 'Gala' and GG at 6 d before bloom but was higher by five cell layers at bloom and at 4 DAFB (Fig. 2A, inset). The absolute and relative cell production rates were significantly higher in GG at bloom ($P \leqslant 0.05$). In apple flowers, pollination and fertilization occur several days after bloom resulting in the stimulation of cell production and fruit growth. Such an increase in cell production was observed in 'Gala' and GG fruits between 4–9 DAFB. Cell number in the GG fruit cortex was higher than that in 'Gala' during early fruit growth (9-28 DAFB; Fig. 2A). However, cell number was not significantly different between 'Gala' and GG during the later stages of fruit growth (43 DAFB–harvest; Fig. 2A). The absolute and relative cell production rates were not significantly different between 'Gala' and GG during fruit growth. The majority of cell production in 'Gala' fruits occurred between 4–24 DAFB after which cell production occurred at a lower rate (Fig. 2A, inset). In GG fruits, cell production was initiated at the same time as in 'Gala' but continued only until 20 DAFB after which cell production occurred at a lower rate (Fig. 2A, inset). These data indicate that GG fruit cortex cells exited the cell production phase earlier than those in 'Gala'.

Table 1. Fruit maturity characteristics and sink activity in 'Gala' and 'Grand Gala'. 'Gala' and 'Grand Gala' fruits were harvested and maturity characteristics were determined.

Fruit Characteristics	'Gala'	'Grand Gala'
Weight (g)	123.50±7.69	170.90±7.65
Firmness (kg cm^{-2})	9.12±0.36	7.84±0.58
SSC (% Brix)	12.72±0.81	14.41±0.07
Seed number	9.40±0.78	7.50±0.00
Starch index (1–10)	8.22±0.44	8.65±0.49
Length/diameter ratio	0.53±0.011	0.50±0.014
Sorbitol absorption rate (μmol g^{-1} h^{-1})	0.09±0.009	0.16±0.03

Sorbitol absorption rate of fruit cortex discs was determined at 60 d after full bloom to estimate sink activity. Data presented are means ±SD.

Cell expansion in 'Gala' and 'Grand Gala' fruits

Cells in the floral-tube tissue of GG had larger radial cell diameter prior to bloom, at bloom, and at 4 DAFB (Fig. 2B, inset). Between 4–9 DAFB, cells in GG fruits had a lower relative rate of cell expansion (P=0.04). Radial cell diameter in GG fruits was not significantly different from that of 'Gala' from 9–16 DAFB (Fig. 2B). Between 16–20 DAFB, GG fruit cells had a higher relative cell expansion rate (P=0.02) resulting in 24% higher cell diameter at 20 DAFB (Fig. 2B, inset). Thereafter, cell diameter in GG continued to be significantly higher than that in 'Gala'. At harvest, radial cell diameter in GG fruits was 15% greater than that in 'Gala' (Fig. 2B), in spite of a lower relative cell expansion rate between 71 DAFB and harvest (P=0.001). Change in cell size was further characterized through an analysis of the distribution of cell area during fruit growth. In comparison to 'Gala,' a higher proportion of cells in GG had a larger cell area at full bloom (FB), 28 DAFB, and 127 DAFB (see Supplementary Fig. S1 at *JXB* online), confirming an increase in cell size prior to fruit set and during fruit

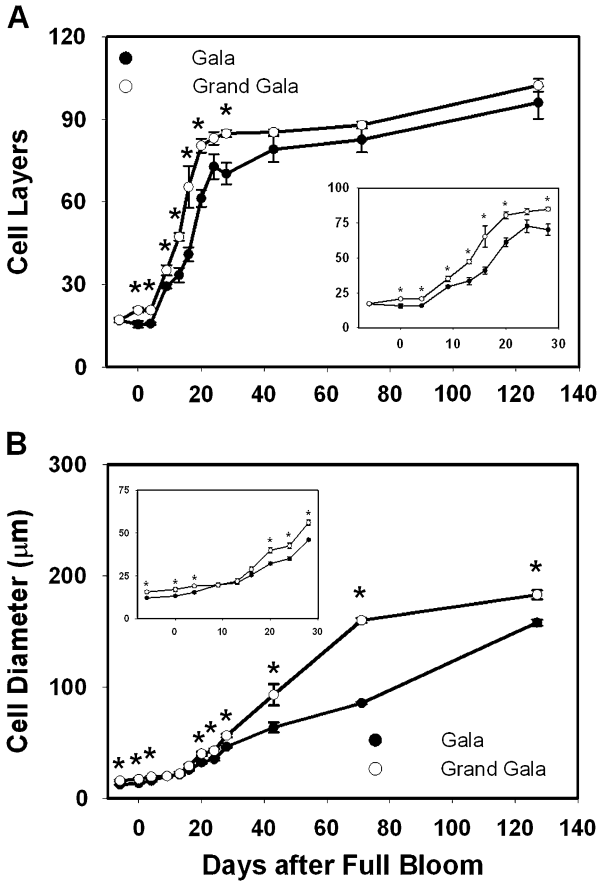

Fig. 2. Cell production and cell expansion in 'Gala' and 'Grand Gala' fruits. (A) Cell number in 'Gala' (n=4) and 'Grand Gala' (n=3) fruit cortex was measured during fruit development. Cell number was determined as the number of cell layers between the petal vascular trace and the peel. Inset presents a magnified view of cell number during early fruit growth. (B) Radial cell diameter in 'Gala' (n=4) and 'Grand Gala' (n=3) fruit cortex was determined during fruit development. Inset presents a magnified view of cell diameter during early fruit growth. Error bars represent standard error of the means. Asterisk indicates significant difference between means ($P \leq 0.05$).

growth in GG. These data indicate that an increase in final fruit size of GG was largely due to the increased cell size.

Nucleus size in floral-tube cells of 'Gala' and 'Grand Gala'

During microscopical analysis, an increase in the size of the nucleus was observed in many cells in the floral-tube tissue of GG. Further analysis of DAPI-stained nuclei at full bloom indicated that the distribution of the projected nucleus area in GG was significantly different from that in 'Gala' (Fig. 3; Mann-Whitney test: P <0.0001). The majority of floral-tube cells in 'Gala' had a projected nucleus area ranging from 6–30 μm^2. While GG had a significant proportion of floral-tube cells with a nucleus area in the above range, a large proportion of cells had a projected nucleus area ranging from 30–60 μm^2 and

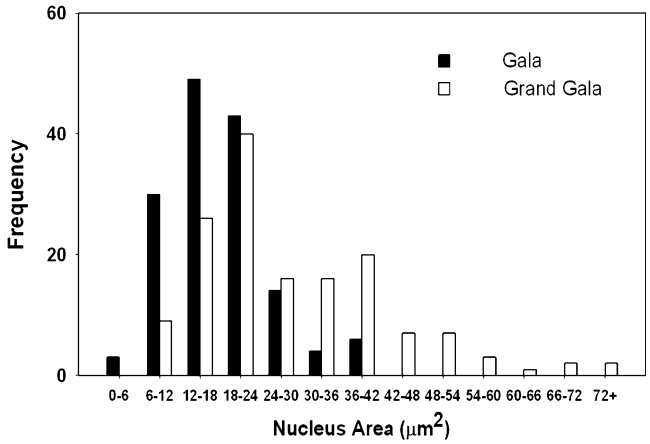

Fig. 3. Distribution of nucleus area in 'Gala' and 'Grand Gala' floral-tube cells. Projected area (μm^2) of DAPI stained nuclei of 30 floral-tube cells was measured in 'Gala' and 'Grand Gala' at full bloom using Image J (n=5). Projected nucleus area distributions were significantly different between 'Gala' and GG (Mann-Whitney test; P <0.0001).

higher. These data indicate an increase in the size of nuclei prior to fruit set in GG.

DNA content of fruit cells in 'Gala' and 'Grand Gala'

An increase in nucleus size is often associated with an increase in DNA content. Using flow cytometry (FCM), it was investigated whether the increase in nucleus size of GG cells was associated with changes in DNA content or ploidy (Fig. 4). Prior to, and around bloom, most cells in 'Gala' had a DNA content of 2C (88–90%) while the remaining proportion of cells had a DNA content of 4C (Fig. 4A, C). The 4C proportion increased during early fruit growth in 'Gala' reaching a maximum of about 13%, indicating cell production during this period (Fig. 4E). At later stages of fruit development, the 4C proportion declined, while the 2C proportion constituted the majority (>96%) indicating the absence of endoreduplication during fruit development in 'Gala' (Fig. 4G, I, K, M, O). Cells with a DNA content of 8C or higher were not detected in 'Gala' fruits.

Around bloom and during early fruit growth, GG had a lower proportion of cells with a DNA content of 2C (31–41%) than 'Gala', while many cells (53–59%) had nuclei with a DNA content of 4C (Fig. 4B, D, F). A small population of cells with a DNA content of 8C was detected around bloom and during early fruit growth (5–8%; Fig. 4B, D, F). These data indicate endoreduplication during bloom and early fruit growth in GG. During the later stages of fruit growth, the 4C proportion increased up to around 77% and was accompanied by a decline in the 2C proportion to around 22% (Fig. 4H, J, L, N, P). These data suggest that some fruit cells in GG exhibited G2 arrest and exited the cell production phase with a DNA content of 4C. The 8C proportion declined to around 1% during the later stages of fruit development. An exponential increase in cell number during early fruit growth may account for the

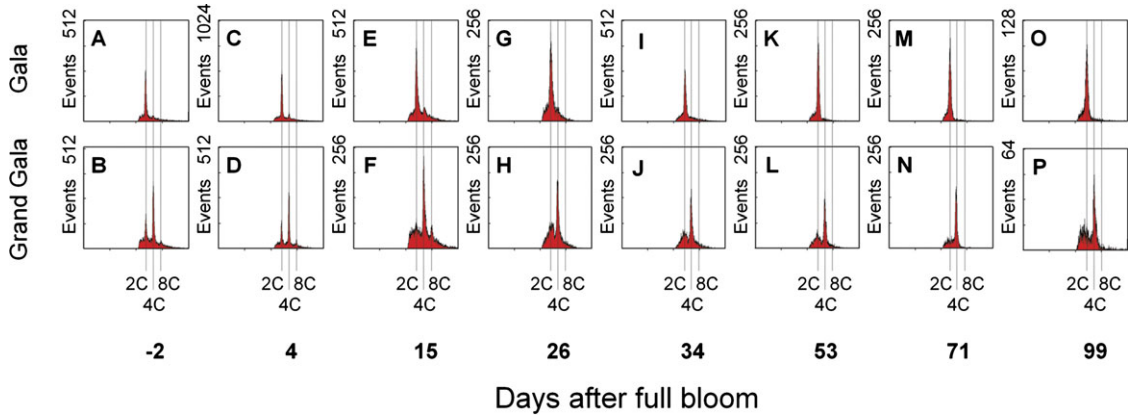

Fig. 4. Flow cytometry (FCM) analyses during fruit development. FCM analyses were performed on 'Gala' (A, C, E, G, I, K, M, O) and 'Grand Gala' (B, D, F, H, J, L, N, P) at different stages during fruit development. (A, B) 2 d before full bloom; (C, D) 4 d after full bloom (DAFB); (E, F) 15 DAFB; (G, H) 26 DAFB; (I, J) 34 DAFB; (K, L) 53 DAFB; (M, N) 71 DAFB; (O, P) 99 DAFB. Dotted lines indicate DNA content/ploidy (2C, 4C, and 8C).

apparent decrease in the 8C proportion. The above data indicate endoreduplication during bloom and G2 arrest during fruit development in GG.

DNA content in mature leaves of 'Gala' and 'Grand Gala'

Previously, some large fruit size spontaneous mutants of apple have been described as polyploids, or cyto-chimeras which exhibit increased ploidy levels in specific histological layers (Tilney-Basset, 1986). To analyse whether GG was a polyploid or a cyto-chimera, the DNA content profiles of mature leaves of 'Gala' and GG were analysed, as apple leaves and fruits are derived from common histological layers (Blaser and Einset, 1948; Dermen, 1951; Tilney-Basset, 1986). FCM analysis did not indicate differences in the DNA content profiles of 'Gala' and GG leaves (Fig. 5). A majority of cells in mature leaves of GG had a DNA content of 2C similar to that observed in 'Gala' leaves (Fig. 5). Occasionally, young leaves of GG had a marginally higher proportion of cells with a DNA content of 4C, suggesting increased cell division. Similar results were obtained from an analysis of sepal tissues of 'Gala' and GG (data not shown). These data demonstrate that GG does not exhibit an overall alteration in the ploidy level and is not a cyto-chimera.

Expression of cell cycle genes during early fruit development in 'Gala' and 'Grand Gala'

It was investigated whether altered cell production and cell expansion in GG fruits were associated with changes in the expression of cell cycle genes. Although cell cycle gene expression was studied in a different season, it is probable that the expression profiles reported here are typical of 'Gala' and GG as fruit growth patterns in these genotypes were similar from year to year, over several years of study. *MdCDKA1* expression was higher in GG fruits by 22% at 8 DAFB but was similar to that in 'Gala' at all other stages analysed (Fig. 6A). *MdCDKB1* and *MdCDKB2* expression

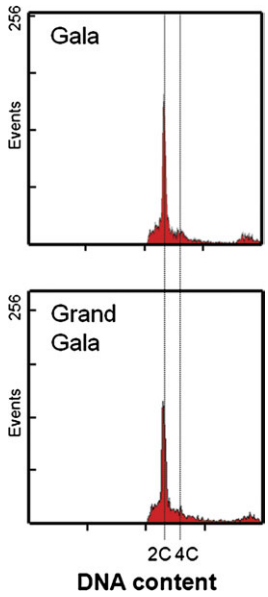

Fig. 5. Flow cytometry (FCM) analysis of 'Gala' and 'Grand Gala' leaves. FCM analysis was performed on mature leaves of 'Gala' and 'Grand Gala' (n=5). Dotted lines indicate DNA content/ploidy (2C and 4C).

were not significantly different between 'Gala' and GG during early fruit development (Fig. 6B, C). Expression of *MdCYCA2* (an A2-type cyclin) in GG fruits was similar to that in 'Gala' during early fruit development but was 25% lower in GG fruits at 28 DAFB (Fig. 6D). Expression of *MdCYCB2*, a B2-type cyclin, and *MdCYCD3*, a D3-type cyclin, was unaltered in GG fruits (Fig. 6E, F). Similarly, expression of *MdKRP* and *MdWEE* in GG fruits was not significantly different from that in 'Gala' at any stage of fruit development (Fig. 6G, H).

Discussion

The mutation in GG resulted in multiple effects on growth and development including reduced apical dominance,

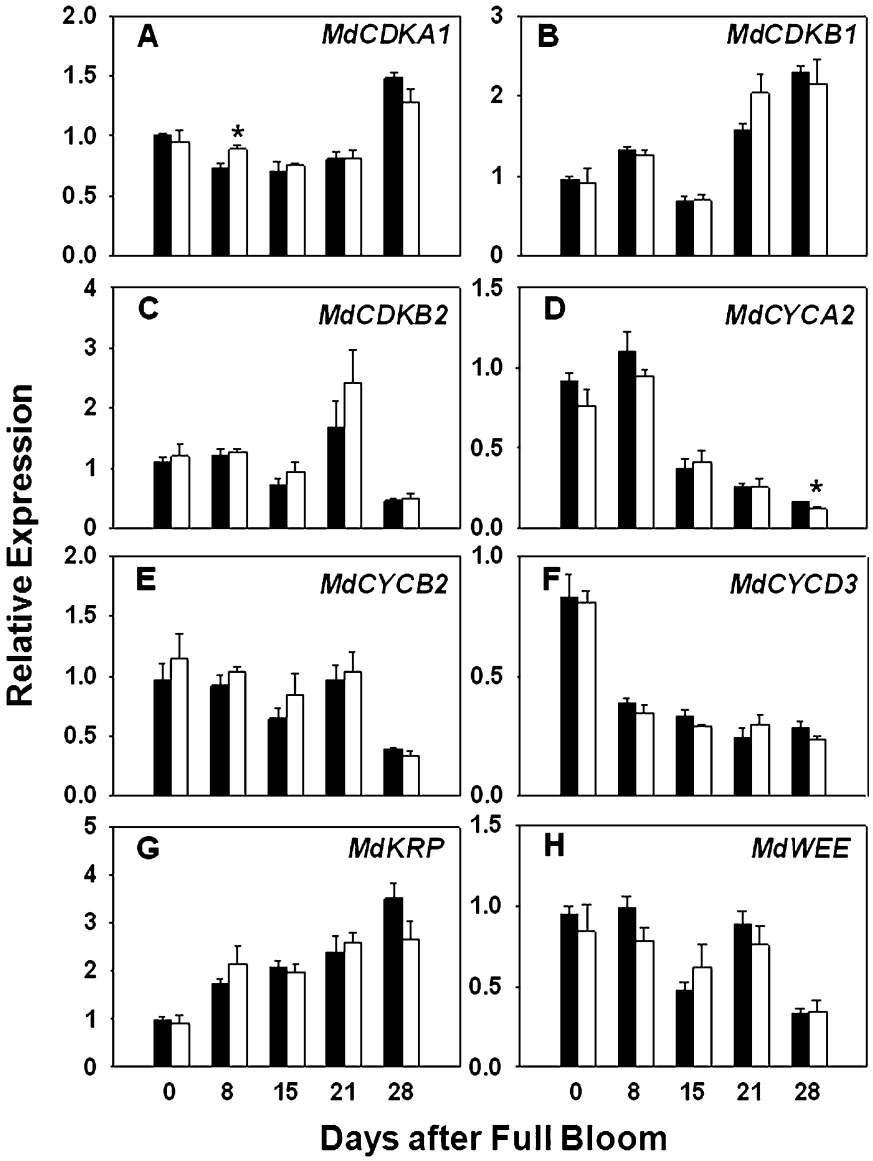

Fig. 6. Quantitative RT-PCR analysis of cell cycle gene expression during early fruit development in 'Gala' and 'Grand Gala'. Expression of eight cell cycle genes was determined during early fruit development in 'Gala' (black bars) and 'Grand Gala' (white bars). (A) *MdCDKA1*; (B) *MdCDKB1*; (C) *MdCDKB2*; (D) *MdCYCA2*; (E) *MdCYCB2*; (F) *MdCYCD3*; (G) *MdKRP*; (H) *MdWEE*. *MdGAPDH* and *MdACTIN* were used for normalization of gene expression data. Data show expression of a gene relative to its expression at 0 d after full bloom in 'Gala' fruits. Error bars indicate standard error of the means (n=4). Asterisk indicates significant difference between the means ($P \leqslant 0.05$).

increased floral organ number, and large fruit size. Reduction in apical dominance in GG trees may be due to the production of heavier fruits which cause excessive shoot bending. Shoot bending in apple reduces polar auxin transport and decreases apical dominance (Sanyal and Bangerth, 1998). Alternatively, the mutation in GG may directly alter transport and/or signalling of auxins and cytokinins thereby reducing apical dominance. An increase in floral organ number in *Arabidopsis* and rice is often associated with an increase in the size of the floral meristem (Clark *et al.*, 1993; Chu *et al.*, 2006). Similar enhancement of meristem size in GG may facilitate an increase in floral organ number. Identification of the molecular nature of the mutation in GG would enable a clear understanding of its multiple effects on growth and development.

The major focus of the current study was to understand the effect of the mutation in GG on fruit size, and on mechanisms such as cell production and cell expansion. Prior to fruit set, an increase in carpel/floral-tube size was observed in GG, along with an increase in cell number and size (Figs 1A, 2; see Supplementary Fig. S1 at *JXB* online). These data indicate that the mutation in GG affects flower growth prior to fruit set. Flower development following bud-break in apple is associated with rapid growth but may be followed by a period of reduced cell production and growth around bloom (see 'Gala', –6 to 4 DAFB in

Fig. 2A), similar to that observed in tomato (Chevalier, 2007). This indicates a cessation of cell production prior to fruit set. However, at least a proportion of cells in the carpel/floral-tube retain competence for cell division during this period as cell production resumes after pollination and fertilization, resulting in a stimulation of fruit growth. Such a mixed population of cells, some of which retain competence for cell division while the rest exit the mitotic cell cycle, is similarly observed during anthesis in tomato (Joubes *et al.*, 1999). The mutation in GG may promote the progression of cells through the G1/S phase of the cell cycle during this period. Cells in the floral-tube tissue, which retain competence for division, may divide in response to G1/S phase promotion resulting in an increase in cell number around bloom in GG. A significant increase in the cell production rate and a higher cell number around bloom in GG support this conclusion (Fig. 2A). Cells that are not competent for division may enter endoreduplication cycles in response to the G1/S phase promotion by the mutation in GG. A large proportion of cells in the carpel/floral-tube tissue of GG exhibited a DNA content of 4C around bloom. While some of the 4C proportion is constituted by cells undergoing division (G2/M phase), the remaining proportion is probably constituted by cells that have undergone one cycle of endoreduplication. In addition, a proportion of cells had a DNA content of 8C during this period, clearly representing cells with two cycles of endoreduplication. These data are further supported by the increase in projected area of the nucleus at bloom in GG. The above data indicate a promotion of cell production and the occurrence of endoreduplication in the GG carpel/floral-tube tissue prior to fruit set. Similarly, an increase in cell production or endoreduplication depending on the competence of cells for division is observed as a result of overexpression of *E2Fa* and *DPa*, and altered expression of the DNA replication licensing components, *CDC6* and *CDT1*, in *Arabidopsis* (De Veylder *et al.*, 2002; Castellano *et al.*, 2004).

Endoreduplication increases DNA content and ploidy in plants and is often correlated with an increase in cell size (Joubes and Chevalier, 2000; Larkins *et al.*, 2001). Cell expansion during tomato fruit development, maize endosperm growth, and nodulation in alfalfa is concomitant with increase in DNA content through endoreduplication (Kowles and Phillips, 1985; Bergervoet *et al.*, 1996; Cebolla *et al.*, 1999; Joubes *et al.*, 1999). Reduction in endoreduplication levels due to altered expression of *KRP1* was associated with reduced cell size in *Arabidopsis* trichomes (Schnittger *et al.*, 2003). In addition, fruit weight was correlated with endoreduplication levels and final cell size in tomato (Cheniclet *et al.*, 2005; Gonzalez *et al.*, 2007). Hence, it is likely that the increase in cell size around bloom in GG is associated with endoreduplication. However, a direct effect of the mutation in GG on enhancing cell expansion during bloom, independently of its effect on endoreduplication, cannot be excluded. Further analysis of a correlation between cell size and ploidy level would help in distinguishing between the above hypotheses.

Early fruit growth in apple is associated with several cycles of cell production triggered by fruit set (pollination and fertilization). This phase of cell production was initiated between 4–9 DAFB in 'Gala' and GG fruits. The higher cell number in GG fruits during early fruit growth (9–20 DAFB) was probably facilitated by a higher cell number prior to pollination and the fertilization-dependent stimulation of cell production (Fig. 2A, inset). Similar relative cell production rates in 'Gala' and GG fruits between 4–20 DAFB support this conclusion. GG fruit cortex cells exited the cell production phase at least 4 d earlier than in 'Gala' as a result of which final cell number was not significantly different between 'Gala' and GG (Fig. 2A). These data indicate that cortex cells in GG progressed through fewer cycles of cell production during fruit growth. An increase in the proportion of cells with a DNA content of 4C and a concurrent decrease in the 2C proportion were observed during the period of exit from cell production in GG, which suggests G2 arrest in GG fruit cells (Fig. 4). One explanation for this observation is that initiation of a final cycle of cell production in GG fruits occurs due to progression through the G1/S phase of the cell cycle. However, mechanisms involved in maintaining final cell number in apple prevent additional cell production in GG by limiting progression through the G2/M phase, resulting in G2 arrest. These data suggest tight regulation of final cell number in apple fruits. The molecular nature of such mechanisms involved in regulating final cell number and initiating G2 arrest in GG fruits warrants further investigation.

Increase in cell size in GG fruits was observed during the period of exit from cell production (between 20–43 DAFB; Fig. 2B; see Supplementary Fig. S1 at *JXB* online) and was concurrent with an increase in the proportion of cells with a DNA content of 4C (Fig. 4), which suggests that the enhanced cell size of the fruit cortex cells in GG is associated with G2 arrest. It is likely that fruit cortex cells in GG expanded normally during this period but prevention of cell division may have resulted in the observed increase in cell size. At later stages of fruit growth, the normal progression of cell expansion in fruit cortex cells arrested in G2 may be sufficient to account for the apparent increase in cell size in GG. Similar relative cell expansion rates in 'Gala' and GG during the later stages of fruit development (20–71 DAFB) supports this conclusion. A lower relative cell expansion rate was observed in GG between 71 DAFB and harvest which suggests a maximum potential limit for final cell size in apple, possibly determined through limitations in source capacity. Notwithstanding, final cell diameter at harvest was 15% higher in the GG fruit cortex, which can potentially increase cell volume by around 50% and may account for the 38% increase in final fruit weight. Analysis of cell area distribution at harvest further supports this conclusion (see Supplementary Fig. S1 at *JXB* online). Together, the above data suggest that enhancement of cell size around bloom and during fruit development contributed greatly to the increase in fruit size of GG. These data provide evidence for a cell expansion-mediated

enhancement of fruit size and demonstrate that cell size is an important regulator of final organ size in apple.

As the relative cell production rate was not significantly altered in GG during early fruit growth, it may not be surprising that expression of several cell cycle genes was not significantly different in GG. Of the eight cell cycle genes studied, only *MdCDKA1* and *MdCYCA2* exhibited significantly altered transcript accumulation in GG. *MdCDKA1* expression was increased in GG fruits by 22% at 8 DAFB. CDKA is considered to be a key factor that regulates the progression of the mitotic cell cycle as well as endoreduplication (Inzé and De Veylder, 2006). However, the significance of change in *MdCDKA1* expression in GG is currently not clear as it does not coincide with the observed changes in cell production, endoreduplication or G2 arrest. *MdCYCA2* expression was reduced by 25% in GG fruits at 28 DAFB which overlaps with the period of exit from cell production, G2 arrest, and an increase in cell size. Increased polyploidy in *Arabidopsis* was associated with reduced transcript levels of A2-type cyclins and loss of function of *CYCA2;3* (Imai *et al.*, 2006; Yoshizumi *et al.*, 2006). Also, *CYCA2;3* may regulate the mitotic to endocycle transition in *Arabidopsis* in association with CDKB1 (Boudolf *et al.*, 2009). It may be speculated that reduction in *MdCYCA2* expression contributes to G2 arrest in GG fruit cells. However, as the reduction in *MdCYCA2* expression is relatively small, it is possible that additional factors are involved in facilitating G2 arrest in GG fruits. Analysis of expression of the family of cell cycle genes during fruit growth may help in identifying such factors. Alternatively, novel cell cycle and/or organ size regulators may facilitate the phenotypic changes observed in GG. Identifying such regulators may aid in developing tools to increase apple fruit size.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. List of primers used in quantitative RT-PCR analyses (5′-3′). F: forward primer; R: reverse primer.

Supplementary Fig. S1. Distribution of floral-tube/fruit cortex cell area in 'Gala' and 'Grand Gala'.

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