

# The cell size distribution of tomato fruit can be changed by overexpression of *CDKA1*

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## Summary

Tomato is one of the most cultivated vegetables in the world and an important ingredient of the human diet. Tomato breeders and growers face a continuous challenge of combining high quantity (production volume) with high quality (appearance, taste and perception for the consumers, processing quality for the processing industry). To improve the quality of tomato, it is important to understand the regulation of fruit development and of fruit cellular structure, which is in part determined by the sizes and numbers of cells within a tissue. The role of the cell cycle therein is poorly understood. Plant cyclin-dependent kinases (CDKs) are homologues of yeast *cdc2*, an important cell cycle regulator conserved throughout all eukaryotes. *CDKA1* is constitutively expressed during the cell cycle and has dual functions in S- and M-phase progression. We have produced transgenic tomato plants with increased expression of *CDKA1* under the control of the fruit-specific *TPRP* promoter, which despite a reduced number of seeds and diminished amount of jelly, developed fruits with weight and shape comparable to that of wild-type fruits. However, the phenotypic changes with regard to the pericarp thickness and placenta area were remarkable. Fruits of tomato plants with the highest expression of *CDKA1* had larger septa and columella (placenta), compared with wild-type fruits. Our data demonstrate the possibility of manipulating the ratio between cell division and expansion by changing the expression of a key cell cycle regulator and probably its activity with substantial effects on structural traits of the harvested fruit.

**Keywords:** tomato, fruit development, cell cycle, CDKA overexpression.

## Introduction

The fruit of tomato is a complex organ composed of two or more carpels, separated by a radially oriented septa, placenta and a central axis called columella with parenchyma-like cells. The largest part of the tomato fruit is the pericarp (flesh), which consists of multiple layers of large thin-walled cells enclosing many intercellular spaces. Parenchymatous tissue of the placenta grows around the funiculi and encloses the developing seeds. At the end of fruit development, the cells of this parenchyma tissue form a jelly.

Consumer perception of tomato fruits depends on numerous factors, including fruit size, texture, colour and aroma (Chaïb *et al.*, 2007; Mounet *et al.*, 2009; Serrano-Megías and López-Nicolás, 2006; Szczesniak, 2002). These traits are controlled by many genetic loci, influenced by environmental factors, hormonal regulation and cultivation practices (for review see Montforte *et al.*, 2014). Cell number and cell size are the determinants of fruit shape and size, influence the structural dry matter through the amount of cell wall material and also play an important role in qualitative characteristics of the ripe product, such as firmness, mealiness, juiciness and shelf life (Bertin, 2005; Chaïb *et al.*, 2007; Giovannoni, 2001; Higashi *et al.*, 1999; Mizukami, 2001; Nesbitt and Tanksley, 2001; Paran and Van der Knaap, 2007; Prudent *et al.*, 2009; Seymour *et al.*, 2002).

Tomato fruit growth depends on successful pollination, fertilization and on the number of developing seeds and is caused by cell division and cell expansion (Gillaspy *et al.*, 1993). The

development of the fruit is marked by a first increase in size due to cell division activity, leading to an increased cell number. The cell division stage determines to a large extent the final number of cells in the fruit and therefore also the final fruit size (Lemaire-Chamley *et al.*, 2005). This is followed by a cell expansion stage marked by an increase in the average cell volume. During this process, cells enlarge up to 20-fold and become polyploid due to multiple rounds of endoreduplication (Bergervoet *et al.*, 1996; Cheniclet *et al.*, 2005; Chevalier *et al.*, 2011). The ripening stage initiates after growth has ceased and involves rapid biochemical and structural changes that determine fruit aroma, colour, but not fruit size and shape (Giovannoni, 2001).

The control of cell division and expansion through regulation of the cell cycle is a crucial determinant of the final size, shape and texture of the fruit. Cyclin-dependent kinases (CDKs) are key regulators of the cell cycle, which need to interact with Cyclins (Cyc) for their activity. The A-type CDKs form a major class of CDKs encoded by multiple (1–4) distinct genes in plants (Adachi *et al.*, 2009; Dudits *et al.*, 2007; Joubès *et al.*, 2000). In plants, they are constitutively expressed throughout the cell cycle and play roles during S- and M-phase progression (Joubès *et al.*, 2000). CDKAs are able to form active complexes with several cyclins (Boruc *et al.*, 2010; Inzé and De Veylder, 2006; Van Leene *et al.*, 2010). Activated by D-type cyclins, CDKA is involved in the G1-S transition by phosphorylation of RBR, releasing it from the E2F/DP complex and so restoring the transcriptional activity of the E2F/DP dimer (De Veylder *et al.*, 2001; Inzé and De Veylder,

2006; Joubès *et al.*, 2001; Nakagami *et al.*, 1999). In contrast, CDKA-CycA and CDKA-CycB complexes are involved in DNA replication, G2-M transition, and mitosis (Imai *et al.*, 2006; Inzé and De Veylder, 2006).

CDKA1 appears to be involved in the maintenance of cell proliferating competence in differentiating tissues in *Arabidopsis thaliana* (Joubès *et al.*, 2000). In addition, several reports provide evidence that CDKA is essential for endoreduplication (Gonzalez *et al.*, 2007; Hemerly *et al.*, 1993; Joubès *et al.*, 1999; Leiva-Neto *et al.*, 2004; Segers *et al.*, 1996; Verkest *et al.*, 2005a) and contributes to the development of the male and female gametophyte, embryo and endosperm (Iwakawa *et al.*, 2006; Leiva-Neto *et al.*, 2004; Zhao *et al.*, 2012). In tomato, two CDKA genes have been identified: *SICDKA1* and *SICDKA2*. The encoded proteins are highly similar, having 94% amino acid sequence identity. The differences, if any, in their roles in the cell cycle and in plant development are so far not clear (Joubès *et al.*, 1999). Joubès *et al.* (1999) studied the expression of *CDKA1* in tomato fruits. They observed an increase in expression in gel up to the mature green stage, and high expression in pericarp until 20 days after anthesis (DAA), while in the epidermis the expression remained constant throughout development from pollination until the mature green stage.

Several studies on the function of CDKA using ectopic expression lines of *Arabidopsis* or tobacco suggested that it may be possible to manipulate the ratio between cell division and cell expansion during tomato fruit development (Hemerly *et al.*, 1995; Hemerly *et al.*, 2000; Imajuku *et al.*, 2001; Verkest *et al.*, 2005a,b; Iwakawa *et al.*, 2006; Roeder *et al.*, 2010). For example, the overexpression of a dominant-negative mutation of the CDKA gene in tobacco inhibited cell division, which was compensated by an increase in cell size. As a consequence the organ size and shape had not changed (Hemerly *et al.*, 1995). Other examples, confirming this compensation phenomenon, are overexpression of a dominant-negative mutant version of *CDKB1;1* (Boudolf *et al.*, 2004) and overexpression of *CycA1* (Doerner *et al.*, 1996) in *Arabidopsis* or *OsKRP1* overexpression in rice (Inzé and De Veylder, 2006). In these examples, despite changes in the cell division/expansion ratio, the final change in plant morphology was mild or negligible. Several studies in tomato presented the potential for manipulating the cell size/cell number ratio by changes in expression of cell cycle regulators, that was usually connected with remarkable influence on the fruit size and phenotypical properties (Czerednik *et al.*, 2012; Fernandez *et al.*, 2009; Gonzalez *et al.*, 2007; Mathieu-Rivet *et al.*, 2010; Mohammed *et al.*, 2012; Nafati *et al.*, 2011).

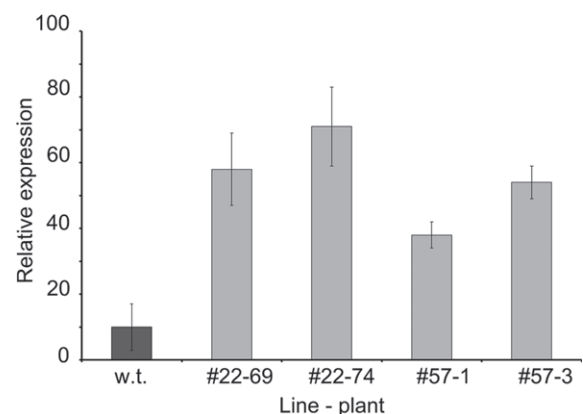
Here, we describe the effect of overexpression of *CDKA1*, under control of a fruit-specific promoter, on cell division and expansion in tomato fruit. In the produced overexpression lines, the placenta and septa were enlarged. Furthermore, we observed an increase in the number of pericarp and placenta cells, which was correlated with a decrease in their size and ploidy level, without affecting the final size and shape of the tomato fruit.

## Results

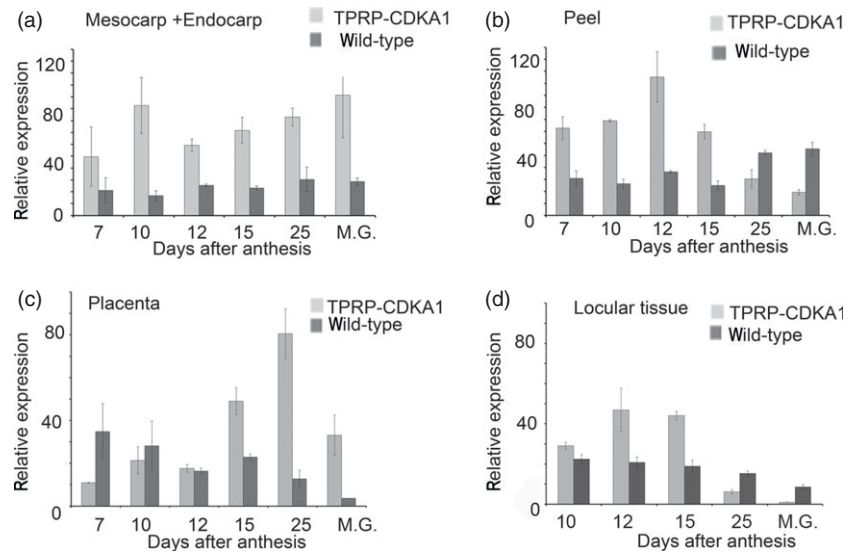
### Fruit-specific overexpression of the *CDKA1* gene resulted in phenotypic changes in fruit tissues without affecting the final fruit size

To study the possibility of regulating cell numbers and sizes in the fruit pericarp by changing *CDKA1* levels, we analysed transgenic

plants of tomato variety M82 ectopically expressing the encoding gene. The upstream regulatory region of the fruit-specific gene *TPRP* (*TM7*) encoding a tomato proline-rich protein was used to drive the expression of *CDKA1* in the fruit only (Czerednik *et al.*, 2012; Fernandez *et al.*, 2009; Salts *et al.*, 1991, 1992). The TPRP promoter is active throughout fruit development in all its tissues with the highest expression during the cell division. From the 34 obtained transgenic tomato lines containing the pTPRP-*CDKA1* construct, three lines were found to have a high level of *CDKA1* overexpression in the pericarp, which was 5- to 10-fold higher than in wild-type control plants. Plants from two lines with high *CDKA1* expression, #22 and #57, were selected for further study. For more reliable analysis of the phenotype and expression levels, progeny (T1) of the primary transformants were examined. Two transgenic sibling progeny each from parental lines #22 and #57, showed up to 7-fold increased expression of *CDKA1* compared with the controls (non-transgenic siblings) at 12–15 days after anthesis (DAA), during the cell expansion phase of growth (Figure 1). We subsequently measured *CDKA1* expression in mesocarp, peel, placenta and locular tissue at different time points of fruit development of line #22 T1 progeny. The results presented in Figure 2 show increased *CDKA1* expression in all tested tissues, although the up-regulation varied in the different tissues and developmental stages, reflecting the differential activity of the *TPRP* promoter during fruit development (Czerednik *et al.*, 2012; Fernandez *et al.*, 2009). In combined mesocarp and endocarp of TPRP-*CDKA1* plants, the expression of *CDKA1* was up to 3-fold higher than in control plants throughout fruit development. In the peel, this expression was higher in the transgenic fruits at the earlier stages, while the overexpression ceased at late stages. In the placenta, significantly increased expression in the transgenic fruits was only observed from 15 DAA onwards reaching the highest value 20–25 DAA, when the expression of *CDKA1* was 10-fold higher in the transgenic fruits compared with wild-type fruits. The expression of *CDKA1* in the locular tissue was analysed from 10 DAA onwards and a 2.5-fold higher expression was observed in transgenic fruits 12 and 15 DAA; however, at later stages, there was almost no detectable expression.



**Figure 1** Relative expression of *CDKA1* in the pericarp of two progeny (T1) plants each of two transgenic parental lines (#22 and #57) determined by quantitative real-time RT-PCR. Values are relative to wild-type pericarp that was set at 10. Data are means of two biological replicates (four fruits from the stages between 12 and 15 DAA, which had the same diameter) and error bars indicate SE.



**Figure 2** Expression of *CDKA1* in different tissues of tomato fruit, progeny (T1) of primary transformant line #22. (a) mesocarp+endocarp, (b) peel (exocarp), (c) placenta, (d) locular tissue. The lowest expression value of *CDKA1* in each analysed tissue of the wild-type pericarp was set to 10 and all other values were plotted relative to the lowest value on a linear Y-axis scale. Data are means of two biological replicates of a pool from the tissue of three fruits of the same developmental stage  $\pm$  SE.

The T1 TPRP-*CDKA1* fruits (Figure 3) had phenotypes, similar to the primary transformants, that included larger septa and placenta, compared with their wild-type sibling fruits. We analysed several characteristics of the transgenic and control fruits, collected at the ripe stage and the results are shown in Figure 4. The placenta size, fruit diameter and pericarp thickness were measured using the Tomato Analyzer software package (Brewer *et al.*, 2006). Fruits of all investigated transgenic lines had diameters and weights not significantly different from those of wild-type fruits (Figure 4a,b). However, the placenta area was significantly increased in transgenic fruits (for T1 #22-69  $P < 0.05$ , in all other plants  $P < 0.005$ , Student's *t*-test) (Figure 4c). We also observed that the pericarp of the transgenic fruits was significantly thicker ( $P < 0.005$ , Student's *t*-test) compared with the control fruits (Figure 4d). Furthermore, the transgenic fruits developed very few seeds (Figure 4e) and failed to develop jelly, but due to the increased area of placenta, septa and pericarp, no empty cavities were observed (Figure 3e, f).

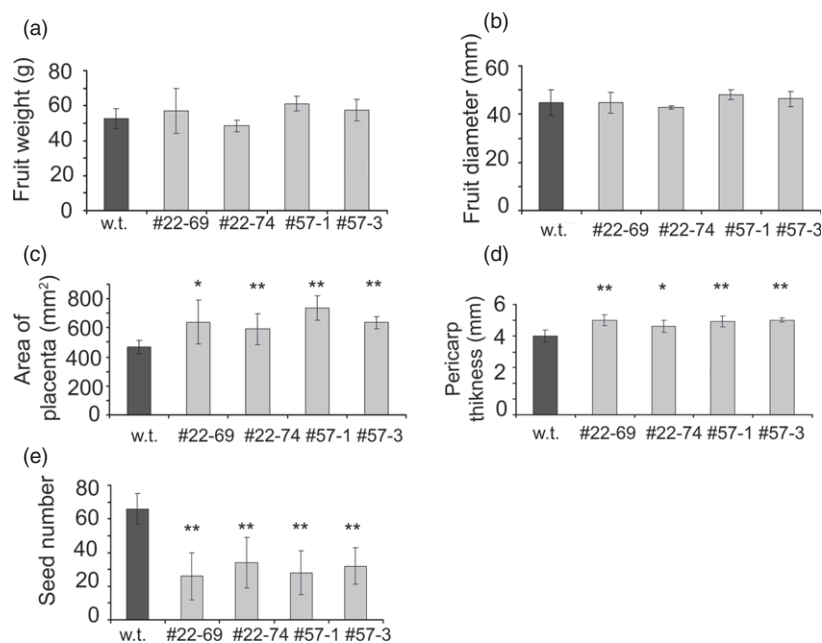
To obtain more seeds from transgenic fruits, we performed cross pollinations of emasculated transgenic flowers with wild-type pollen from cv. M82. Despite the growth of normal sized fruits after cross pollination, there was still a significant reduction in numbers of developed seeds and in jelly development.

#### Overexpression of *CDKA1* in tomato fruits causes increase in cell division

We studied the cell size distribution in fruits and observed changes in pericarp and placenta cell sizes of the transgenic fruits compared with wild-type fruit. In Figure 5, microscopic images of cross sections through the pericarp (Figure 5a–c) and placenta (Figure 5d,e) of wild-type and transgenic fruits are presented. The pericarp cells of the transgenic lines (Figure 5b,c) appear to be smaller than those in the wild type (Figure 5a) and the number of layers with small cells below the epidermis is increased compared with that in a wild-type fruit. We quantified these differences by



**Figure 3** Phenotypes of wild-type and *CDKA1* overexpressing fruits. (a–c) Fruits of non-transgenic sibling progeny of primary transformant line #22. (d–f) Representative fruits from transgenic siblings derived from the same parental. Bar = 10 mm.



**Figure 4** Yield parameters and phenotypic features of T1 wild-type (non-transgenic) and transgenic sibling progeny fruits of primary transformant lines #22 and #57, collected at the ripe stage. The data represents the means  $\pm$  SE of 10–12 ripe fruits per each plant. The *P*-values (Student's *t*-test) are indicated: \*\**P* < 0.005 and \**P* < 0.05. (a) Fruit weight (g). Wild-type and transgenic fruits are not significantly different. (b) Fruit diameter (mm). The differences between wild-type and transgenic fruits are not significant. (c) Area of the placenta analysed with the Tomato Analyser software. The placenta size is significantly enlarged in the transgenic fruits. (d) Pericarp thickness (mm). The pericarp thickness of the transgenic fruits was analysed with the Tomato Analyser software and pericarp of transgenic fruits was significantly thicker. (e) Number of seeds per fruit. The number of seeds in transgenic fruits was significantly reduced.

measuring pericarp cell areas in the microscopic sections and counting the number of cells in defined size classes from small (category 1) to large (category 11) in 2-fold increments of maximum area (Figure 5f, g). The pericarp cell size was significantly decreased in the transgenic fruits (Figure 5f). Significantly increased numbers (*P* < 0.005) were observed for the categories 1 and 2 (cells up to  $0.4 \times 10^{-3} \text{ mm}^2$  and  $0.8 \times 10^{-3} \text{ mm}^2$ , respectively) and significant decreases in the larger cell categories 7, 8 (*P* < 0.05) and 9 (*P* < 0.005; cells up to  $102.4 \times 10^{-3} \text{ mm}^2$ ).

As the overall pericarp thickness of the transgenic fruits had significantly increased (Figure 4d) despite the cells being smaller, we determined the number of cells per area unit and the number of cell layers in the pericarp wall. The former measurement confirmed the observations shown in Figure 4d, that is, transgenic fruits had more (and thus smaller cells) per pericarp section area unit. On the other hand, the overexpression plants had significantly more cell layers in the pericarp than the wild-type fruits (Table 1), suggesting that this increase more than compensated for the decrease in overall cell size. The cell size distribution in the placenta was also shifted towards smaller cells in the transgenic fruit (Figure 5e) compared with the control (Figure 5d, g; Table 1). Transgenic fruits had significantly more cells in the two smallest categories (1 and 2) and significantly fewer cells in all subsequently larger cell categories. A remarkably big difference (2-fold) was observed in the cell sizes in the category 2 (cells up to  $0.8 \times 10^{-3} \text{ mm}^2$ ). Consequently, the cell number per area unit of placenta was also significantly increased (*P* < 0.005) in transgenic fruits when compared to wild-type fruits (Table 1).

#### Ploidy level of cells from different fruit tissues are correlated with their volume

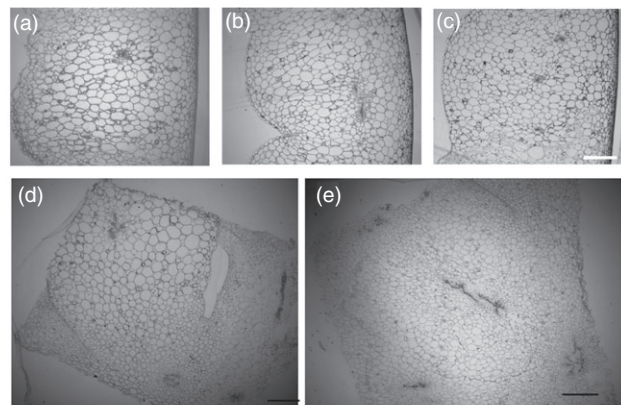
Cell expansion in tomato fruit coincides with endoreduplication, the continuation of DNA synthesis without mitosis (cell division), leading to increased ploidy levels (Bergervoet *et al.*, 1996). Because we observed lower cell sizes in transgenic fruit tissues, we analysed ploidy levels to determine whether changes in endoreduplication are correlated with, and possibly causal to the observed differences.

Fruits were harvested at the breaker stage, and samples of nuclei from cells of mesocarp, placenta and jelly were analysed by flow cytometry for determining the DNA content of the nuclei, after which the endoreduplication index (EI), that is, the mean number of endoreduplication cycles per nucleus, was calculated (Figure 6). Transgenic fruit mesocarp had substantially more 4C nuclei and substantially less 64C or higher nuclei than the controls (Figure 6a), resulting in the EI of mesocarp nuclei in all analysed overexpressing fruits being significantly reduced, by 30% for plant #22-69, 15% for plant #22-74, 20% for fruits of plant #57-1, and 30% for plant #57-3 (Student's *t*-test, *P* < 0.005 in case of #22-69 and *P* < 0.05 for other fruits) (Figure 6b). The differences were more dramatic for placenta nuclei, were many more nuclei of transgenic fruits had 2C and 4C nuclei and substantially less had 8C or higher nuclei (Figure 6c). Thus, the EI of placenta was reduced even more than that of mesocarp: 61% for plant #22-69, 58% for plant #22-74, 61% for plant #57-1, and 62% for plant #57-3 (Student's *T*-test, *P* < 0.001) (Figure 6d). Similar observations were made for nuclei from jelly (Figure 6e) where EI was significantly reduced (*t*-test, *P* < 0.005): 59% for plant #22-69, 39% for plant #22-74, 41% for plant #57-1 and 37% for plant #57-3 (Figure 6f). According to several studies, endoreduplication in tomato is correlated with cell volume and contributes as a major determinant to the final fruit size (Bertin *et al.*, 2001; Cheniclet *et al.*, 2005; Chevalier *et al.*, 2011). In our study, this correlation between reduction in cell sizes and of ploidy levels was confirmed.

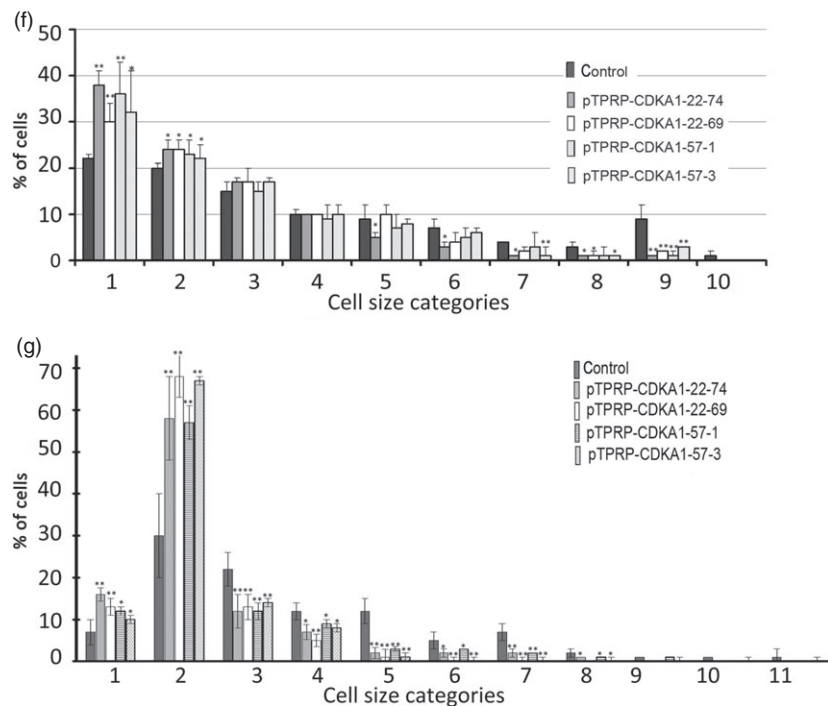
#### Discussion

Plant breeders search for ways to improve the quality of tomato fruits and make them more attractive to consumers. Important structural features of the ripe fruit, such as cell number are determined during the first developmental stages after fruit set, and perturbations during this early development may have a crucial influence on the quality characteristics (Chaïb *et al.*, 2007; Seymour *et al.*, 2002). The size and character of the pericarp and placenta are such important quality characteristics of ripe tomato





**Figure 5** Phenotypic changes in pericarp and placenta due to overexpression of *CDKA1*. (a–c) Microscopic cross section through a pericarp of a control fruit (a) through a pericarp of a *CDKA1* overexpressing progeny (T1) fruit from primary transformant #22 (b) and one from progeny of primary transformant #57 (c). (d, e) Microscopic cross section through a placenta of control fruit and of *CDKA1* overexpressing progeny of line #22 respectively. Bar = 0.2 mm. (f, g) Percentage of cells from pericarp (f) and placenta (g) grouped into 11 different size categories. Cells in category 1 have an average size of up to  $0.4 \times 10^{-3} \text{ mm}^2$  and the maximum cell size in each subsequent category is twice that of the preceding category. Standard errors are indicated for a minimum of 4 measurements from three different fruits for each bar. The *P*-values (Student's *T*-test) are indicated: \*\**P* < 0.005 and \**P* < 0.05.



fruit (Chaïb *et al.*, 2007). There is demand for so-called 'plump' and 'beefsteak' tomatoes, which are characterized by an increased ratio of flesh to jelly and increased volume of the placenta, which is often accompanied by a reduced amount of seeds (Heuvelink, 2005).

By overexpressing tomato *CDKA1*, we obtained transgenic plants with fruits that did not significantly differ in overall size or weight from wild-type fruits. However, the number of seeds in these fruits was significantly reduced ( $P < 0.005$ , Student's *t*-test) and the development of jelly failed. The transgenic fruits had a thicker pericarp and larger placenta size compared with wild-type fruits. Microscopic analysis showed that cell size in pericarp and placenta was reduced, but that the number of cells significantly increased in the transgenic fruits compared with wild-type fruits. Thus, increased cell numbers in the pericarp and placenta compensated for the reduction in cell size, resulting in no apparent change in final fruit size.

#### Overexpression of *CDKA1* in tomato fruits causes an increase in cell division

Interestingly, in other plant systems, increased cell divisions may be accompanied by a decrease in cell expansion, resulting in

organs with no apparent size changes. This phenomenon, known as the 'compensation effect', suggests that the final shape and size of an organ is predetermined and relatively independent of cell size or cell number (Beemster *et al.*, 2003; Bertin, 2005; Mizukami, 2001; Tsukaya, 2003). Also, endoreduplication rate can be uncoupled from final fruit size. For example, a decrease in number of endoreduplication rounds had no effect on tomato fruit morphology when overexpressing *SIKRP1*, encoding an inhibitor of CDK activity during fruit development (Nafati *et al.*, 2011). In our previous study, the suppression of *CDKA1* expression in tomato fruit had the reverse effect, reducing cell numbers and resulting in a reduction of the pericarp width, but the size and weight of fruits were also reduced (Czerednik *et al.*, 2012).

Cell division depends on the mitotic activity of CDK–Cyclin complexes. In the cell cycle of plants CDKs from groups A and B are active, forming an actively phosphorylating complex with Cyclins. *CDKA1* is involved in both the G1-S as well as in the G2-M transition, with different interacting cyclins. Depending on the cell cycle phase, *CDKA1* is able to form active complexes with CycD3, CycA2 or CycB1/B2 (Dewitte *et al.*, 2003; Inzé and De Veylder, 2006; Menges *et al.*, 2005; Verkest *et al.*, 2005a; Weingartner *et al.*, 2004). *CDKA1* activity is controlled by

**Table 1** Quantification of the number of cells per surface unit area in pericarp and placenta and number of cell layers in the pericarp of mature green wild-type and transgenic fruits

Line	Cells/mm <sup>2</sup> pericarp	Cells/mm <sup>2</sup> placenta	Number of cell layers in pericarp
Wild type	15 ± 2.4	33 ± 1.3	27 ± 2.0
Line 22	23 ± 2.2 ( <i>P</i> < 0.005)	56 ± 6.7 ( <i>P</i> < 0.005)	35 ± 2.0 ( <i>P</i> < 0.05)
Line 57	25 ± 1.9 ( <i>P</i> < 0.005)	46 ± 3.3 ( <i>P</i> < 0.005)	33 ± 1.0 ( <i>P</i> < 0.05)

The data represent the means ± SE of five to six measurements from each of three different fruits. For all measurements, the differences between wild-type and transgenic lines were tested for statistical significance. The *P*-values (Student's *t*-test) are indicated.

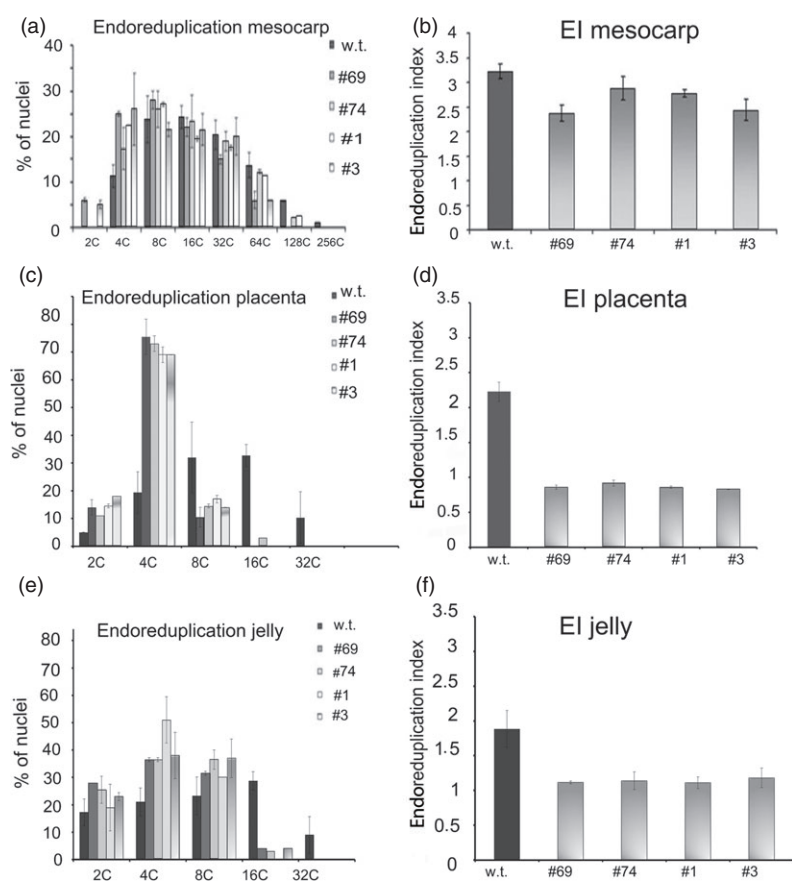
WEE1 protein, which was shown to be a critical target of the DNA replication and DNA damage checkpoint (De Schutter *et al.*, 2007). WEE1 operates in the G2 phase by arresting the cell cycle in response to DNA damage. In tomato fruit, WEE1 is involved in the control of cell size during tomato fruit development through the negative regulation of CDKA activity, which then positively regulates endoreduplication. Down-regulation of *WEE1* in tomato results in smaller pericarp cells coinciding with increased activity of CDKA1 but, in contrast to our CDKA1 overexpression fruits, also in a reduction of pericarp cell layers, pericarp thickness and overall fruit size (Gonzalez *et al.*, 2007). This loss of CDKA1

inhibition resulted in a short G2 phase and a premature entry into mitosis (Chevalier *et al.*, 2014). The central role of CDKA1 in controlling cell number has also been demonstrated using transgenic tobacco plants with reduced A-type CDK activity due to ectopic expression of a dominant-negative form of *CDKA;1* in *Arabidopsis* (Hemerly *et al.*, 1995).

We suggest that overexpressing *CDKA1* in fruits prolongs mitotic activity of pericarp and placenta cells, resulting in an increase in cell numbers. Thus, both our results and those of Gonzalez *et al.* (2007) indicate that CDKA1 activity is a limiting factor for cell division activity in the developing tomato pericarp and placenta. Different from Gonzalez *et al.* (2007), the increased cell division activity of CDKA1 overexpressing fruits more than compensated for the smaller cell sizes.

### Increased *CDKA1* expression affects fertilization

The number of seeds in our *CDKA1* overexpressing fruits was significantly reduced (Fig. 4e). A few studies have shown that *CDKA1* has essential functions in gametogenesis and embryogenesis and that its misexpression or deregulation resulted in defects in male and female gametogenesis and reduced seeds development in *Arabidopsis* and tomato (Gonzalez *et al.*, 2007; Iwakawa *et al.*, 2006; Zhao *et al.*, 2012). Misexpression of *CDKA1* resulted in the production of bicellular pollen grains that failed in double fertilization (Iwakawa *et al.*, 2006). CDKA1 is involved in control of the first and second mitotic division cycle during embryo sac formation in *Arabidopsis* and reduction of CDKA1 function resulted in disruption of seed development (Zhao *et al.*, 2012). In tomato fruits, down-regulation of the *WEE1* gene caused increased activity of *CDKA1* in developing



**Figure 6** Ploidy distribution analyses in nuclei from cells of different fruit tissues. (a) Ploidy distribution analysis of tomato mesocarp cells. (b) Endoreduplication index (EI) mesocarp cycles per nucleus. (c) Ploidy distribution analysis of placenta cells. (d) EI placenta. (e) Ploidy distribution analysis of jelly. (f) EI jelly. Samples were collected at breaker stage, EI is the mean number of endoreduplication. Values are means ± SD (*n* = 3). EI was significantly reduced in mesocarp of transgenic progeny #22-69 and #22-74 and #57-1, and #57-3 compared to non-transgenic siblings (*P* < 0.005 in case of #69, and *P* < 0.05 in case of the other plants). In case of placenta and jelly, EI was reduced significantly (*P* < 0.005) compared with wild type.

embryos and production of fewer seeds (Gonzalez *et al.*, 2007). The TPRP promoter is not active in anthers (Czerednik *et al.*, 2012) and thus *CDKA1* should not be overexpressed in pollen of our transgenic lines. Carmi *et al.* (2003) used the TPRP promoter to drive *rolB* expression in ovules and fruits, resulting in parthenocarpy. This further supports the activity of this promoter in ovules and/or young embryos. As cross pollination of transgenic lines with viable non-transgenic pollen also yielded normal sized fruits with reduced seeds numbers, it is thus more likely that female gametophyte or embryo formation was impaired by *CDKA1* overexpression in our plants. Seeds that did form showed no obvious defects in germination, although smaller differences in germination frequency may have gone unnoticed.

According to several studies, fruit growth and fruit cell size are dependent on embryo and seed development, and the number of seeds in the fruit is a key determinant of its final size, because seeds produce hormones such as cytokinins and auxins. These hormones induce rapid growth of the developing ovary by increasing cell division and cell expansion (Bohner and Bangerth, 1988; Gillaspay *et al.*, 1993; Gonzalez *et al.*, 2007; Matsuo *et al.*, 2012; Pattisona *et al.*, 2014). Parthenocarpic fruits or fruits with decreased number of seeds usually are smaller than normally fertilized fruits, lack jelly and have decreased cell division and cell expansion (Balbi and Lomax, 2003; De Jong *et al.*, 2009; Nitsch, 1970). Tomato fruits with reduced expression of *WEE1* also produced less seeds (Gonzalez *et al.*, 2007).

The reduced amount of jelly surrounding the seeds, which we observed in TPRP-*CDKA1* plants is also observed in varieties with low numbers of seeds, often correlated with a large placenta and less jelly (Heuvelink, 2005). In the early stages of fruit development, the fruit locules are filled by an outward growth of parenchymatous placental cells surrounding the seeds. While firm and compact at first, the intruding parenchymatous tissue becomes gelatinous with fruit maturation (Barrett *et al.*, 1998).

The expression pattern of *CDKA1* in jelly of transgenic fruit was somewhat surprising, because it was 2-fold higher in the jelly of transgenic fruit between 11 and 15 DAA compared with the control, but afterwards the expression dropped to a level lower than in the wild-type fruit. This phenomenon is difficult to explain. On one hand, the activity of the TPRP promoter is significantly lower in jelly compared to mesocarp and placenta, possibly explaining the rapid decrease of expression in 25 DAA and mature green stage (Czerednik *et al.*, 2012), but transgene-mediated silencing of the endogenous gene is also a possibility.

#### Ploidy level of tomato fruit cells correlated with their volume

Tomato fruit development has become a model for study of the karyoplasmic ratio theory, which states that the cytoplasmic volume of a cell is determined by its DNA content (for review see Chevalier *et al.*, 2014). According to several studies, cell volume positively correlates with and for a large part determines the final tomato fruit size (Bertin *et al.*, 2001; Cheniclet *et al.*, 2005; Chevalier *et al.*, 2011, 2014; Nafati *et al.*, 2011). In the course of fruit development, the highest levels of endopolyploidy occur within the mesocarp and in the jelly, in which the seeds are embedded (Bergervoet *et al.*, 1996; Joubès *et al.*, 1999; Cheniclet *et al.*, 2005; Bertin *et al.*, 2007). The correlation between reduction in cell sizes and reduced ploidy levels in our study was obvious, but we did not see a correlation between the final fruit size and mean ploidy level. Therefore, endoreduplication is a major determinant for the final size of the cell, but the cell division and cell

expansion rates can be modulated by changes in activity of *CDKA1*, which in wild-type fruits is strongly inhibited post-translationally at the onset of endoreduplication (Joubès *et al.*, 1999; Gonzalez *et al.*, 2007; De Veylder *et al.*, 2011). During tomato fruit development, the large and hypervacuolarized cells constituting the locular jelly tissue undergo multiple rounds of endoreduplication. In this particular tissue according Joubès *et al.* (1999), *CDKA1* expression is high up to the mature green stage. In the case of transgenic fruits containing the TPRP-*CDKA1* construct, development of jelly was reduced, which was correlated with lower final ploidy level in this tissue.

#### Conclusion

In the present study, we have shown that overexpression of *CDKA1* under the control of the fruit-specific TPRP promoter has effects on different aspects of fruit development, giving fruits of normal size and shape, with thicker fleshy tissues and less jelly. The overexpression leads to fruits with decreased cell sizes, while cell numbers in pericarp and placenta were significantly increased. *CDKA1* expression appears to be a limiting factor for cell division activity and duration and its overexpression might prolong the cell division phase of fruit development, at the cost of the expansion phase. *CDKA1* overexpression thus increased the number of mitotic cycles at the cost of the number of endocycles, confirming the correlation between endoreduplication and final cell size. The other effect of overexpression of *CDKA1* was the reduced number of seeds in developing fruits, probably due to interference with proper gametophytic development. This may affect pericarp cell expansion normally driven by seed-produced phytohormones. Our results demonstrate that fruit internal morphology characteristics and with these, texture aspects of fruit quality, can be modified without negatively affecting yield.

#### Experimental procedures

##### Plant material

Tomato, *Solanum lycopersicum*, cv. M82 and transgenic lines were grown in a greenhouse under a 16 h of light and 8 h of dark regime. Supplementary lights (600 Watt high pressure sodium lights) turned on below 200 W/m<sup>2</sup> and turned off above 300 W/m<sup>2</sup> solar irradiation. Temperature was kept above 20 °C during the light period and at 17 °C during the dark period controlled by a PRIVA Integro version 724 system. Plants were watered daily. The extra tomato fertilizer Osmocote was added in the soil to improve the calcium supply and prevent blossom-end rot in fruits.

##### Binary vectors for transformation

To generate the fruit-specific *CDKA1* overexpression lines, the coding region of *SICDKA1* (accession number Y17225) was amplified using the primers F-5'-CACCATGGACCAGTATGAA AAAGTTGAGAAG-3' and R-5'UTR-GTGGTCACGGCACATACC CAATATCCTTG-3' and cloned into the pENTR/D-TOPO entry vector (Invitrogen). Obtained clones were recombined with the overexpression vector, pARC983, containing the TPRP promoter driving the expression of a Gateway cassette, in which a gene or ORF of choice can be recombined *in vitro* using LR clonase (Czerednik *et al.*, 2012). The correct orientation of the TPRP promoter and *CDKA1* ORF was confirmed by PCR using the pair of primers: F 5'- CTGACCCTTCCTAAATCCC-3' and R 5'- GTA TGTGCCGTGATTGTCTG-3'.

## Transformation of tomato

Transgenic tomato plants were generated by *Agrobacterium tumefaciens*-cocultivation of seedling cotyledon sections of tomato cv. M82, as described in De Jong *et al.* (2009). Plants were selected on kanamycin-containing medium (kanamycin concentration 100 mg/L) and transgene insertion confirmed by PCR with primers specific for the TPRP-CDKA1 construct. Subsequently, lines were tested for ploidy, as only diploid lines were used for further analysis.

## RNA isolation, cDNA-synthesis and q-RT-PCR data analysis

To avoid the differences in source-sink balance affected by fruit number and position (Bertin *et al.*, 2001) we left five fruits on each truss, and the additional pollinated flowers were removed. The first developing truss from each plant was removed as well.

For mRNA isolation, we collected the second and third fruit from the second truss on the plant. Total RNA was extracted with TRIzol Reagent using the standard protocol from Invitrogen (Chomczynski and Mackey, 1995; www.invitrogen.com) and was treated with RQ1 RNase-free DNase (Invitrogen). Photometric RNA measurements were performed to equilibrate the RNA concentrations of different samples and concentrations were corrected by comparing the amount of total RNA on a 1.5% agarose gel. RNA (0.5 µg) was reverse transcribed in a total volume of 10 µL using a cDNA synthesis kit (iScript™, Bio-Rad Laboratories, Hercules, CA) following the manufacturer's protocol. For real-time quantitative PCR, 5 µL of 25-fold diluted cDNA was used in a 25 µL PCR, containing 100 nM of each primer and 12.5 µL iQ-SYBR Green Supermix (Bio-Rad Laboratories). The PCR reactions were performed in a 96-well thermocycler Bio-Rad iCycler (Bio-Rad Laboratories). Technical and biological replications were always performed. Real-time quantitative RT-PCR (qRT-PCR) primers were designed using Beacon Designer Software (Premier Biosoft International, Palo Alto, CA). The absence of genomic DNA in RNA samples was checked by PCR using specific primers that amplify an intron fragment from the tomato actin gene *SITOM 51* (SGN-U60481) using primers F 5'-GCTGTGCTTCTTGTATGC-3' and R 5'-TCAC ACCATCACCAGAGTCC-3'.

Specific amplification for *SICDKA1* cDNA was obtained with the primers: F 5'-AACCCCTGAATAGAACCAAATG-3' and R 5'-G TATGTGCCGTGATTGTCTG-3'.

As reference genes were used *Sl-Actin 2/7* (SGN-U107674) and *Sl-18S* (SGN-U107674). The primer sequences for *Sl-Actin2/7*: F 5'-GGACTCTGGTGATGGTGTAG-3' and R 5'-CCGTTCCAGCAG TAGTGGTG-3'. The primer sequences for *Sl-18S*: F 5'-AGACGA ACAACTGCGAAAGC-3' and R 5'-AGCCTTGCGACCACTACTCC 3'. Relative mRNA levels were calculated following the Bio-Rad outlined methodology based on Vandesompele *et al.* (2002) and corrected for PCR efficiencies. The average of two biological replicates was determined along with the standard error (SE).

## Histological analysis

Fruits were analysed at the breaker stage. Fruits were cut along the equator to remove seeds and pulp. The parameters weight, diameter and weight after removal of seed and pulp were recorded. For microscopy, the procedure as described by Czerednik *et al.* (2012) was used.

## Measurements of fruit characteristics

Fruit sizes and pericarp thickness were determined with the Tomato Analyzer software on images (Brewer *et al.*, 2006).

## Ploidy analysis and EI

Ploidy level was analysed in mesocarp, jelly and placenta of fruits in the breaker stage. Nuclei were isolated according to De Laet *et al.* (1987). Plant material was chopped with scalpel and stained with 'CyStain UV Precise P' kit from Partec. The suspension was filtered through a 30-µm filter and the remaining sample was re-extracted with the same solution. The combined filtrates were analysed with a CyFlow Space cell analyzer (Partec). The EI was calculated from the number of nuclei at each ploidy level multiplied by the number of endoreduplication cycles necessary to reach the corresponding ploidy level  $EI = (1*4C + 2*8C + 3*16C + 4*32C + 5*64C + 6*128C + 7*256C)/100$  (Boudolf *et al.*, 2009).

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