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RESEARCH PAPER

Regulation of tomato fruit pericarp development by an interplay between *CDKB* and *CDKA1* cell cycle genes

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Abstract

Growth of tomato fruits is determined by cell division and cell expansion, which are tightly controlled by factors that drive the core cell cycle. The cyclin-dependent kinases (CDKs) and their interacting partners, the cyclins, play a key role in the progression of the cell cycle. In this study the role of *CDKA1*, *CDKB1*, and *CDKB2* in fruit development was characterized by fruit-specific overexpression and down-regulation. *CDKA1* is expressed in the pericarp throughout development, but is strongly up-regulated in the outer pericarp cell layers at the end of the growth period, when *CDKB* gene expression has ceased. Overexpression of the *CDKB* genes at later stages of development and the down-regulation of *CDKA1* result in a very similar fruit phenotype, showing a reduction in the number of cell layers in the pericarp and alterations in the desiccation of the fruits. Expression studies revealed that *CDKA1* is down-regulated by the expression of *CDKB1/2* in *CDKB1* and *CDKB2* overexpression mutants, suggesting opposite roles for these types of CDK proteins in tomato pericarp development.

Key words: Cell cycle genes, fruit development, mutants, tomato.

Introduction

Tomato fruit development starts with the reinitiation of ovary growth, induced by pollination and fertilization events (Picken, 1984; Gilaspy *et al.*, 1998). During fruit development several phases can be recognized: initially the fruit diameter increases due to cell division activities, which rapidly amplify the number of cell layers in the pericarp, followed by a growth phase caused by cell expansion. The expansion phase is accompanied by endoreduplication; that is, a multiplication of the genome without mitosis, leading to an increase in DNA content per cell, which can reach up to 256C at the end of fruit growth (Bergervoet *et al.*, 1996). Fruit growth stops at the mature green stage, when the fruit obtains its final size, which is both genetically and environmentally determined (Chevalier, 2007).

The tomato fruit is composed of different tissues: the pericarp (flesh), which is subdivided into the exocarp, mesocarp, and endocarp, the placenta, septum, and the locules filled with jelly and seeds (pulp) (Bertin, 2005; Mintz-Oron *et al.*, 2008). The mesocarp, being the largest part of the pericarp, encompasses layers of large, highly vacuolated parenchymatous cells and contains vascular bundles. The outer layer of the pericarp, the exocarp, possesses several layers of collenchymatous cells that include mitotically active cells and enlarging cells, and a single layer of epidermal cells which are covered or in some cases encased in a waxy cuticle (Joubès *et al.*, 2000; Lemaire-Chamley *et al.*, 2005; Mintz-Oron *et al.*, 2008).

Growth of the tomato fruit, like any other growing organ of the plant, is intimately associated with the cell cycle. The cell cycle is regulated with strong checkpoints at the Gap 1 (G_1) to Synthesis (S) transition and at the Gap2 (G_2) to Mitosis (M) transition (for a review, see Inzé and De

Veylder, 2006; Francis, 2007). These checkpoints ensure that conditions are appropriate for cells to engage in another round of duplication of DNA in the S phase or for cells to enter the M phase. Both cell division and cell enlargement, the latter being tightly correlated with endoreduplication, are processes controlled by the cell cycle. Protein phosphorylation is a major mechanism for the control of cell cycle progression, and in particular the family of cyclin-dependent protein kinases (CDKs) plays a crucial role in cell division control (Inzé and De Veylder, 2006). They act as serine-threonine kinases in complexes together with the regulatory cyclin subunit (Van Leene *et al.*, 2010). Five families of CDKs (A–F) are known in plants, of which *CDKA* and *CDKB* are the most prominent and numerous classes (Joubès *et al.*, 2000; Dudits *et al.*, 2007). *CDKA* genes are widely present in different organisms and form the largest class of *CDK* genes (Dudits *et al.*, 2007). These proteins are characterized by the presence of the PSTAIRE motif, which is essential for cyclin binding (Joubès *et al.*, 1999). *CDKA* genes control the progression from the G_1 to the S phase and from the G_2 to the M phase (Mironov *et al.*, 1999). In contrast to the *CDKA* class proteins, *CDKB* proteins form a unique class of kinases in eukaryotes and are present in plant cells and budding yeast only (Čížková *et al.*, 2008). The *CDKB* proteins can be further subdivided into two subgroups with different cyclin-binding motifs: PPTALRE for *CDKB1* and PPTTLRE for *CDKB2* (Joubès *et al.*, 2000). While in *Arabidopsis*, tobacco, and alfalfa four members (*CDKB1;1*, *CDKB1;2*, *CDKB2;1*, and *CDKB2;2*) have been reported (Vandepoele *et al.*, 2002; Fountain and Beck, 2003; Dudits *et al.*, 2007), in tomato, only *CDKB1;1* and *CDKB2;1* were identified (Joubès *et al.*, 2001; Chevalier, 2007). In tomato fruits, both *CDKB* genes are highly expressed up to 15 days after anthesis (DAA) and afterwards the expression ceases, suggesting that they play an important role in the cell cycle progression during the division phase (Joubès *et al.*, 2001). The tomato *CDKA1* gene is also expressed at later stages of development and transcripts remain present in the epidermis until the mature green stage (Joubès *et al.*, 1999).

Only a few mutants with altered *CDKB* expression have been described. These studies were primarily performed in *Arabidopsis*, rice, and *Chenopodium rubrum* (Fauntain and Beck, 2003; Lee *et al.*, 2003; Boudolf *et al.*, 2004*a*, *b*; Corellou *et al.*, 2005; Andersen *et al.*, 2008). Changes in the activity of the *CDKB* proteins have led to several meristematic defects (Porceddu *et al.*, 2001; Andersen *et al.*, 2008). *Arabidopsis* plants overexpressing a dominant negative *CDKB1;1* version have cells with a higher 4C/2C ratio in various tissues due to a premature exit from the mitotic cycle and entry into the endoreduplication cycle (Boudolf *et al.*, 2004*b*).

Analysis of *Arabidopsis cdka;1* null mutants showed that *CDKA;1* is required for both the sporophytic and the male gametophytic generations. As sporophytes, homozygous *cdka;1* mutants were not viable and died as young embryos. During male gametophyte (pollen) development, the lack of *CDKA*;1 function caused a cell cycle arrest in the G_2 phase prior to the last mitotic division (Iwakawa *et al.*, 2006).

No information is available about the role of the tomato *CDKA* and *CDKB* genes in cell division and growth during tomato fruit development. Therefore, the expression of the *CDKB* genes was manipulated by overexpressing *CDKB1* and *CDKB2* under the control of a fruit-specific promoter. Furthermore, *CDKA1* was down-regulated in a fruit-specific manner. Both types of transgenic fruits displayed changes in cell numbers and cell sizes in the pericarp and, surprisingly, severe defects in fruit cuticle development were observed. These analyses provide novel information about the role of these genes in fruit development and they suggest an antagonistic mechanism of control between the tomato *CDKA1* and *CDKB* genes.

Materials and methods

Plant material

Tomato plants *Solanum lycopersicum* L., cv. M82, cv. Ida Gold, and the obtained transgenic lines were grown in a greenhouse under 16 h of light and 8 h of darkness. Supplementary lights (600 W high pressure sodium lights) turned on below 200 W m^{-2} and turned off above 300 W m^{-2}. The temperature was kept above 20 °C during the light period and 17 °C during the dark period with the PRIVA Integro versie 724 system. Plants were watered daily and given fertilizer weekly.

Construction of binary vectors for transformation

To generate the fruit-specific *CDKB1* and *CDKB2* overexpression lines, the coding sequences of *LeCDKB1*;1 (accession no. AJ297916) and *Le*CDKB2;1 (accession no. AJ297917) were cloned into the pENTRtm/D-TOPO entry vector (Invitrogen). Both clones were recombined with a binary vector, pARC983, containing the *TPRP* promoter driving the expression of a Gateway cassette, in which a gene or open reading frame (ORF) of choice can be simply recombined *in vitro*.

To knock-down the expression of *CDKA1*, an artificial microRNA (amiRNA) approach was followed (Schwab *et al.*, 2006). Precursor amiRNA molecules were designed that should produce mature amiRNAs, which are able to suppress the expression or activity of the target gene. The amiRNA vector (Schwab *et al.*, 2006) was modified for down-regulation of target genes in the fruit by the pTPRP fruit-specific promoter.

Transformation of tomato

Transgenic tomato plants were generated by *Agrobacterium tumefaciens*-mediated transformation, as described in de Jong *et al.* (2009).

Harvesting plant material

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

For mRNA isolation, the second and third fruit from the second truss on the plant were collected. For each stage, fruits from two plants were collected.

RNA isolation, cDNA synthesis, and qRT-PCR data analysis

RNA was isolated with TRIzol Reagent (Invitrogen), using a standard protocol from Invitrogen (Chomczynski and Mackey,

1995; www.invitrogen.com). Photometric RNA measurements were done to equilibrate the RNA concentrations of different samples. Equal amounts of RNA were DNase treated (Invitrogen) and the absence of genomic DNA was checked by PCR using specific primers that amplify an intron fragment from the tomato actin gene *TOM 51* U60481 only. RNA (0.4 μg) was reverse transcribed in a total volume of 10 μl using a cDNA synthesis kit (iScript™, Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's protocol. Real-time quantitative RT-PCR (qRT-PCR) primers were designed using a computer program (Beacon Designer Software, Premier Biosoft International, CA, USA). Primer pairs are depicted in Supplementary Table S2 at *JXB* online. qRT-PCRs were done using SYBR green mix (iQ-SYBR Green Supermix, Bio-Rad Laboratories). PCRs were performed in a 96-well thermocycler (Bio-Rad iCycler). A 5 μl aliquot of 20-fold diluted cDNA was used per sample. Technical and biological replicates were performed. As control genes *Actin* 2/7 and *Le18S* were used.

Relative mRNA levels were calculated following the methodology outlined by Bio-Rad based on Vandesompele *et al.* (2002) and corrected for PCR efficiencies. The average of two biological repeats and two technical repeats is depicted together with the standard error (SE).

Sudan IV assay

Sudan IV (MP Biomedicals, http://mpbio.com) stock solution [0.1% (w/v) in isopropyl alcohol] was diluted 3:2 with distilled water, mixed well, and filtered through a syringe filter to remove precipitates. The stain was added to thin sections of fruit pericarp for 10 min, rinsed first with 50% isopropyl alcohol, then with distilled water. Slides were mounted in distilled water with a cover slip and observed immediately under a ×63 oil immersion objective HCXPL apo CS ×63/1.30 with a confocal laser scanning microscope (CLSM). Photographs were made with a Leica digital camera, and the software package ImageJ (Rasband, 1997–2008; http://rsb.info.nih.gov/ij/) was used to analyse the photographs and collect size parameters of a coloured cuticle. For the quantitative analyses, 10–15 different images per sample were used.

Scanning electron microscopy (SEM)

Slides of ripe fruit pericarp were fixed on a holder with a layer of carbon-rich conductive glue and frozen with boiling liquid nitrogen, freeze-dried, transferred in the high vacuum cryo-unit, thin sputtered with a thin layer of gold–palladium, and further inserted into the observation chamber with a rod. Microscopic observations of the tissue break-line were made with a JEOL 6335 scanning electron microscope using an acceleration voltage of 3 kV.

Histological analysis

Fruits were analysed at the breaker stage. Fruits were cut along the equator to remove seed and pulp. The parameters weight, diameter (height and width), and weight after removal of seed and pulp were recorded. For microscopy, a triangular wedge (base ~5 mm, height ~5 mm) was cut from the equatorial section, from each fruit in duplicate. Collected tissues were bleached in 0.4% hypochlorite for 90 min to clear cellular content and then were washed with distilled water. Tissues were fixed in fixative containing 10% formaldehyde, 5% acetic acid, and 52% ethanol, vacuum infiltrated for 15 min twice, and left overnight. Pericarp tissues were placed in 70% ethanol and stored for further processing. For the cell distribution studies pericarp samples were embedded in Technovit and thin sections (7–10 μm) were made and stained with toluidine blue. Digital images of sections were analysed using ImageJ (Rasband, 1997–2008, http://rsb.info.nih.gov/ij/). Areas <12.5 μm^2 or >250 μm^2 were excluded from the data, since they most probably represent intercellular spaces and cells that were disrupted. Mean cell size was calculated for each picture. Per plant line four pictures of different pericarp areas were averaged and are depicted with the SE.

Water loss measurements

Three fruits of each line were collected at the ripe stage, and were stored at room temperature for 50 d. Fruit weight was recorded every week, and water loss was calculated as a percentage of weight loss.

Measurements of fruit characteristics

Measurements of fruit firmness in Shore degrees was performed on ripe fruits (measurements in duplicate) using a fruit pressure tester (T.R. Companyhas, catalogue number 53210 Fruit pressure tester http://www.trsnc.com/). Fruit sizes and pericarp thickness were determined with the Tomato Analyzer software on images (Dujmovič *et al.*, 2005; Brewer *et al.*, 2006; Gonzalo *et al.*, 2009).

Ploidy level analysis

Nuclei were prepared from the pericarp of ripe fruits. Two types of tissue were analysed: the bsubepidermal layer and mesocarp. Nuclei were isolated according to De Laat *et al.* (1987) but stained with a 'high resolution DNA kit' (Partec). The suspension was filtered through a 100 mm nylon mesh and the remaining sample was re-extracted with the same solution. The combined filtrates were analysed with a CA-II cell analyzer (Partec).

Results

Expression of CDKB1 and CDKB2 genes

The level of *CDKB1* and *CDKB2* gene expression was analysed in the fruit pericarp of tomato variety M82 during various stages of fruit development from anthesis to the mature green stage. qRT-PCR expression analysis is shown in Fig. 1. *CDKB1* and *CDKB2* are both predominantly expressed in the pericarp during the early developmental stages, when mainly cell division takes place. Both genes have a very similar expression pattern: the expression peaks ~3 DAA, decreases gradually during later stages, and is completely abolished at the mature green stage. These results obtained with fruit pericarp tissues are in agreement with the data reported by Joubès *et al.* (2001) for *CDKB1* and *CDKB2* expression in whole tomato fruits using semi-quantitative PCR.

Fruit-specific overexpression of CDKB genes

To examine if and how *CDKB1* and *CDKB2* may affect the cell cycle and growth of the fruit during development, transgenic plants overexpressing these genes were analysed. Because the aim was to produce modifications in the fruit only, the upstream regulatory region of the fruit-specific gene *TPRP* (*TM7*) (Salts *et al.*, 1991, 1992; Carmi *et al.*, 2003; Fernandez *et al.*, 2009) was used to drive the expression of *CDKB1* and *CDKB2* (see Supplementary Fig. S1 at *JXB* online). From 43 generated primary transgenics containing the pTPRP-*CDKB1* overexpression construct and 20 plantlets with the pTPRP-*CDKB2* vector, lines with the most up-regulated expression of *CDKB1* and *CDKB2* were selected. Four lines with *CDKB1* overexpression and

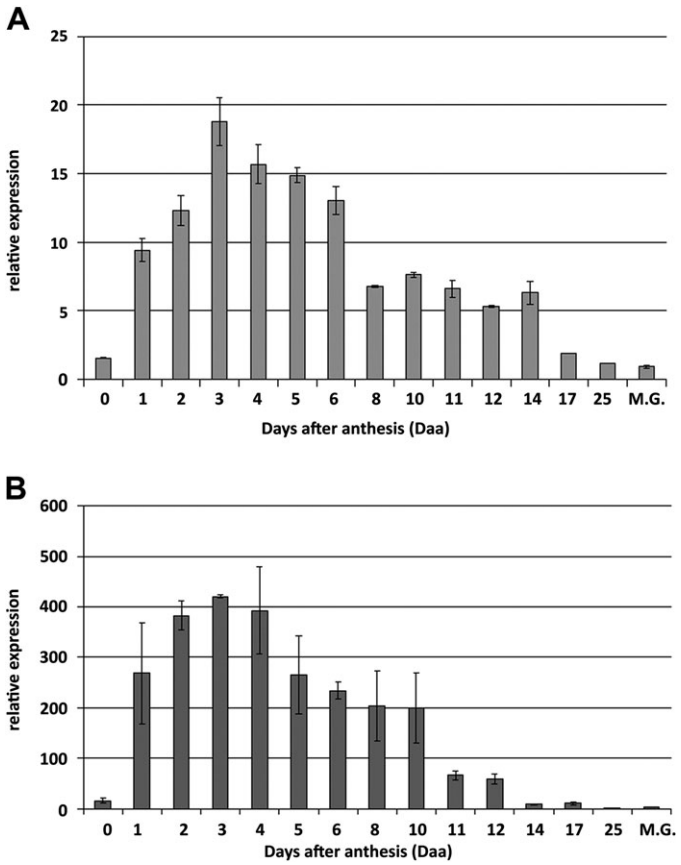


Fig. 1. Expression levels of *CDKB1* and *CDKB2* determined in the pericarp of wild-type tomato fruits. (A) Relative *CDKB1* expression and (B) *CDKB2* expression was determined by qRT-PCR. Samples were collected at various developmental stages from anthesis to mature green. Data are averages of two biological and two technical replications, and the standard error (SE) is indicated. M.G. = mature green stage

10 lines with *CDKB2* overexpression were identified showing a substantial increase in expression, in particular during later stages when the endogenous expression levels drop and the pTPRP promoter is still active. For more reliable analysis of the phenotype and expression levels, offspring plants (T_1) of the primary transformants were examined. Siblings from lines #5 and #57 with *CDKB1* overexpression and from lines #3 and #4 with *CDKB2* overexpression were taken for further investigation and were compared with non-transgenic (segregating) siblings. The (over)expression was determined in the pericarp during fruit development. At later stages of development, the overexpression driven by the pTPRP promoter was >10-fold higher than in wild-type fruits, while during the early stages the transgene expression levels of the *CDKB* genes were hardly elevated (Fig. 2). Therefore, phenotypic alterations in the transgenic fruits at later developmental stages could be expected.

Phenotypes of CDKB1/B2-overexpressing fruits

No alterations were observed in the vegetative growth of the *CDKB*-overexpressing plants. The transgenic fruits developed and ripened very similarly to wild-type fruits, and produced normal amounts of viable seeds. However, the transgenic fruits overexpressing either *CDKB1* or *CDKB2* were smaller than control fruits and had an irregular form (Fig. 2). Several parameters of the fruits were analysed, such as weight, diameter, pericarp thickness, and fruit firmness, and the results of these quantitative analyses are depicted in Supplementary Fig. S2 at *JXB* online. Both the weight and the pericarp thickness were significantly reduced in the transgenic fruits compared with non-transgenic control fruits. Furthermore, the fruit firmness was determined at the red ripe stage and a reduction of firmness was noticed of the fruits overexpressing *CDKB2* and, to a lesser extent, but still significant, fruits overexpressing *CDKB1*.

CDKB overexpression affects the cellular structure of the fruit

To analyse the possible changes in cell division rate and cell growth in the pericarp of the *CDKB1*/*CDKB2*-overexpressing fruits, microscopic sections from fruits at the breaker stage were analysed. These analyses revealed that in both types of transgenics there are significant differences in cell sizes between transgenic overexpressors and non-transgenic plants, as is depicted in Fig. 3. The size of the cells in fruits overexpressing *CDKB2* was more reduced than in fruits overexpressing *CDKB1*. Because mainly the mesocarp was used for the measurements, these data indicate that overexpression of *CDKB1*/*B2*, which is mainly manifested at later developmental stages (Fig. 2), leads to a reduction in cell expansion in the mesocarp.

Next, the number of cell layers was determined in the pericarp of the transgenic fruits, which marks the cell division activity in these fruits. In all four analysed *CDKB1*/*CDKB2* overexpression lines, fewer cell layers were observed than in the pericarp of control fruits (Fig. 3), indicating a reduction in cell division rate. The reduction in cell layers was particularly apparent in the outer layers of the pericarp (exocarp), where mitotic activity is maintained till very late in the developing fruit. These results suggest that overexpression of *CDKB1*/*B2* at later developmental stages mainly affects the cell division rate in the outer epidermal cell layers.

Because the pTPRP promoter already shows activity just before anthesis (see Supplementary Fig. S1 at *JXB* online) the number of cell layers in non-pollinated ovaries of transgenic and wild-type plants was checked. This analysis revealed that the observed reduction in cell layer numbers in the transgenic plants is clearly an effect that takes place after pollination (Fig. 3).

CDKB overexpression changed DNA content in pericarp of the fruit

Since cell size is usually related to the level of endoreduplication in the cells (Joubés and Chevalier, 2000; Cheniclet *et al.*, 2005; Vlieghe *et al.*, 2005), it is expected that fruits with overexpression of *CDKB* and reduced cell sizes also show a reduction in ploidy levels. Therefore, the DNA content in subepidermal and parenchymal regions of the

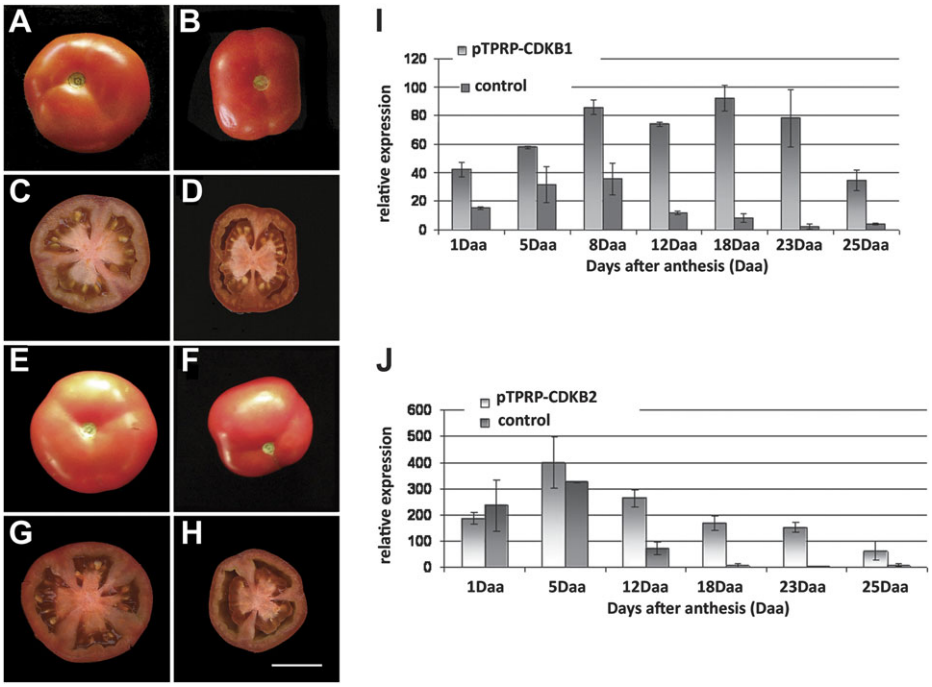


Fig. 2. Phenotypes of control and *CDKB1*- and *CDKB2*-overexpressing fruits. (A and C) A fruit of a non-transgenic sibling derived from a pTPRP-*CDKB1* plant. (B and D) A fruit of a transgenic pTPRP-*CDKB1* plant (line 5). (E and G) A fruit of a non-transgenic sibling derived from a pTPRP-*CDKB2* plant. (F and H) A fruit of a transgenic pTPRP-*CDKB2* plant (line 3). Bar=20 mm. (I) Relative expression of *CDKB1* in the pericarp of wild-type and transgenic pTPRP-*CDKB1* plants. (J) Relative expression of *CDKB2* in the pericarp of wild-type and transgenic pTPRP-*CDKB2* plants. Relative expression was determined by real-time qRT-PCR. Data are averages of two biological pools of samples from three independent fruits per plant at different days after anthesis (Daa) and the standard deviation (SD) is indicated.

pericarp was analysed and the results obtained are depicted in Fig. 4. In both the subepidermal and parenchymal part of the pericarp a reduction of endoreduplication was observed in the *CDKB*-overexpressing fruits compared with the wild type, with a more pronounced effect in the *CDKB2* overexpressors.

CDKB1/B2-overexpressing fruits desiccate faster

Because a difference in fruit firmness was noticed (Supplementary Fig. S2 at *JXB* online), the post-harvest performance of the transgenic fruits was of interest. The desiccation of fruits of *CDKB1*- (line #5) and *CDKB2*- (line #4) overexpressing lines, which were harvested at the breaker stage and stored at room temperature for 50 days, was analysed. Fifty days after harvest the peel of control wild-type plants was still firm, while the peel of the transgenic fruits was shrivelled as shown in Fig. 5. The water loss was determined by measuring the weight of the fruits during the experiment. Fig. 5. shows that the desiccation rate in transgenic fruits is approximately twice that in control fruits.

To examine if such a dramatic water loss was the result of alterations in cuticle structure or thickness, the cuticle microstructure was analysed by SEM and staining by Sudan IV followed by light microscopy (Buda *et al.*, 2009; Isaacson *et al.*, 2009). The results of these analyses are shown in Fig. 6 and reveal that the cuticle thickness of the transgenic mature fruits decreases ~3-fold compared with that in control fruits. This difference may well account for the water loss in the transgenic fruits, which was observed after harvesting.

Relationship between CDKB1/B2 and CDKA1 expression

Overexpression of *CDKB1* or *CDKB2* driven by the pTPRP promoter was most apparent at later stages of fruit development (Fig. 2), which explains why the *CDKB1*/*B2*-overexpressing fruits are mainly affected in expansion of the mesocarp cells and cell division in the exocarp, which continues in the exocarp until maturity. At this point it is not clear whether overexpression of a *CDKB* gene leads directly to these aberrations or that *CDKB* affects the expression of other *CDK* genes in the fruit. Although a relationship of the *CDKB* genes to other *CDK* genes has never been reported, the specific expression of *CDKA1* in the exocarp at later stages of development is striking (Fig. 7).

CDKA1 is expressed throughout fruit development, but is strongly up-regulated at the end of the growth phase (Fig. 7A). This high expression is predominantly caused by a very high expression in the exocarp, as was determined in plants expressing the β-glucoronidase (GUS) gene driven by a *CDKA1* promoter fragment (Fig. 7B). All transgenic lines expressing the reporter show high GUS expression in the exocarp at later stages of development, while the expression in the rest of the pericarp was more diffuse. This pattern of *CDKA1* promoter activity was confirmed with qRT-PCR expression analysis of dissected exocarp and mesocarp/

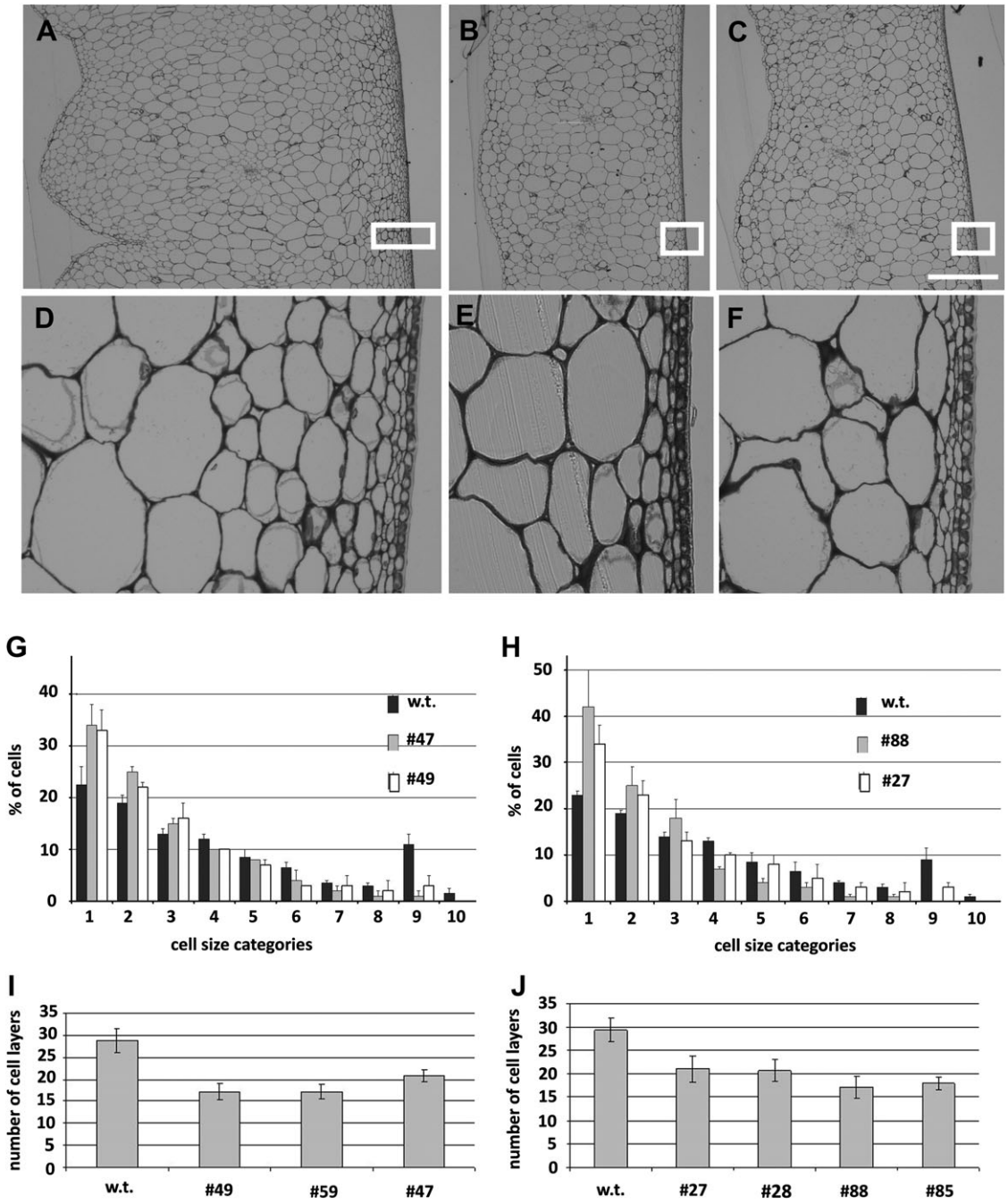


Fig. 3. Phenotypical changes in pericarp due to overexpression of *CDKB1* and *CDKB2*. (A–C) Microscopic cross-section through a pericarp of a control fruit (A), through a pericarp of a *CDKB1*-overexpressing fruit (B), and through a pericarp of a *CDKB2*-overexpressing fruit (C). (D–F) Details of selected areas in A–C, respectively. Note that only 2–3 layers with small cells are present in the exocarp of *CDKB1/2*-overexpressing fruits directly followed by layers with large cells (E and F), while in a control fruit (D) cells in at least 10 layers gradually enlarge. Bar=1 mm in A–C; 0.2 mm in D–F. (H and I) Percentage of cells grouped into 10 different size categories. Cells in category 1 have an average size of up to 0.0004 mm^2 and the maximum cell size in each subsequent category has doubled. The mesocarp region of control fruits (black bars) and two *CDKB1*-overexpressing fruits (T_1 plant #47 from line 57 and T_1 plant #49 from line 5) were analysed (a). The same analysis was done for *CDKB2*-overexpressing fruits (T_1 plant #27 from line 4 and T_1 plant #88 from line 3) (H). Standard errors are indicated for a minimum of four measurements from three different fruits for each bar. (I and J) Average number of cell layers in the pericarp of control fruits (w.t.) and *CDKB1*-overexpressing fruits from three different T_1 plants (#49, #59, #47) derived from line 5 (I). The same analysis was done for *CDKB2*-overexpressing fruits from T_1 plants #27 and #88 from line 4 and T_1 plants #88 and #85 from line 3. Standard errors are indicated for 10 measurements from three different fruits for each bar. All fruits were harvested at the breaker stage.

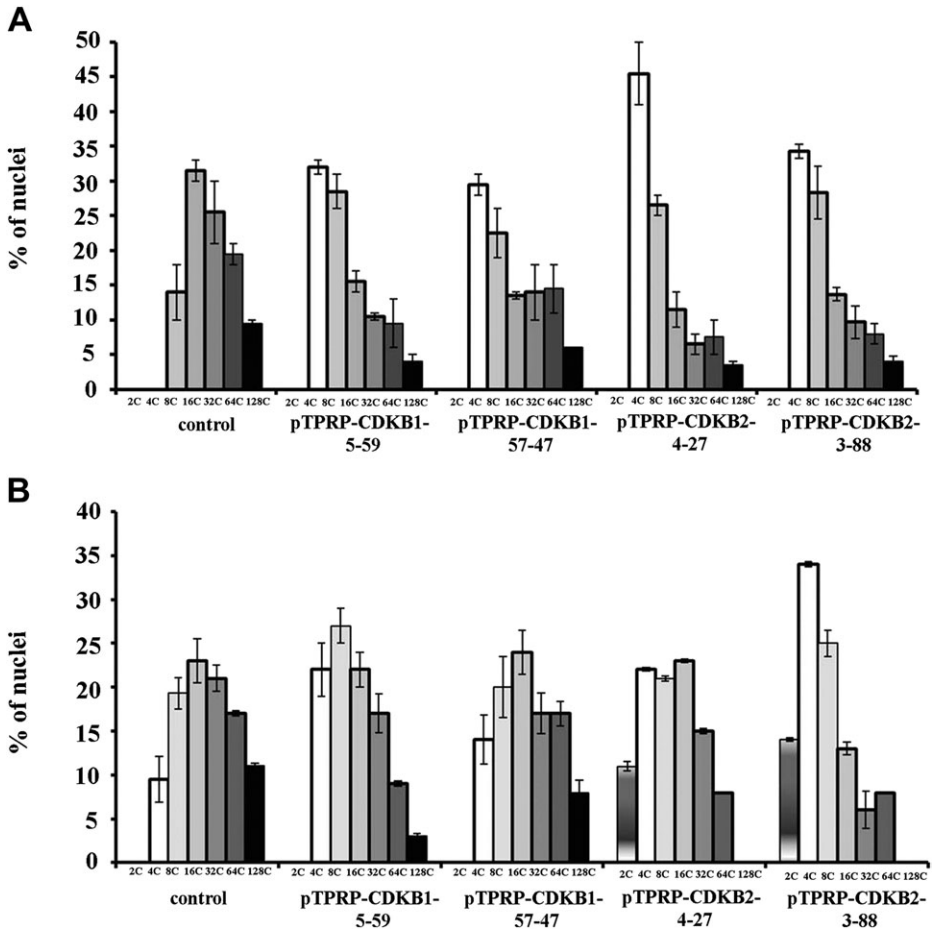


Fig. 4. Nuclear DNA content of pTPRP-*CDKB1* and pTPRP-*CDKB2* overexpressing fruits. The ploidy of transgenic plants was compared with that of the wild-type control in mesocarp (A) and subepidermal layers (B) of the pericarp. The horizontal axis indicates the genome copy number, and the vertical axis shows the percentage of nuclei counted. Error bars represent the SD.

endocarp tissues from different stages during fruit development (see Supplementary Fig. S3 at *JXB* online)

Based on this striking coincidence of the *CDKB1/2* overexpression phenotype and the high expression of *CDKA1* in the exocarp and the complementary expression patterns of *CDKB1/2* and *CDKA1* genes during fruit development (compare Figs 1A, B, and 7A, respectively), it was speculated that *CDKA1* expression could be affected by the overexpression of the *CDKB* genes. To test this hypothesis the *CDKA1* expression in the pericarp of control and *CDKB1/2* overexpression lines was studied using qRT-PCR (Fig. 7C, D). This analysis revealed that *CDKA1* expression is reduced in the *CDKB1/2* overexpression lines compared with levels in control fruits. This reduction occurs only in later stages of fruit development when *CDKA1* expression is up-regulated in wild-type fruits and becomes restricted to exocarp cells. During the early cell division phase, when *CDKB1/2* overexpressor lines do not show a clear elevated expression above endogenous levels (Fig. 2), *CDKA1* expression is not affected.

Down-regulation of CDKA1 phenocopies CDKB1/B2 overexpression

If overexpression of *CDKB* genes leads to a down-regulation of *CDKA1*, one might expect alterations in fruits similar to those in *CDKB* overexpression when the *CDKA1* gene is down-regulated. To confirm this hypothesis, *CDKA1* knock-down plants were generated using expression of an amiRNA (Schwab *et al.*, 2006) and driven by the fruit-specific *TPRP* promoter. Out of eight transgenic lines tested, three lines show a significant down-regulation of *CDKA1* to ~20% of the wild-type transcript level in line #33 (see Supplementary Fig. S4 at *JXB* online). This reduction in *CDKA1* expression was inherited in the T_1 progeny of lines #33 and #39, and the observed phenotype is linked with the presence of the transgene in the segregating T_1 progeny.

No aberrations were observed in other parts of the ami*CDKA1* knock-down plants besides the fruit, demonstrating again the fruit specificity of the *TPRP* promoter. Fruits of these lines develop normal gel and seeds, but are smaller than wild-type control fruits (Fig. 8, see Supplementary Fig. S5). Also the thickness of the pericarp is reduced in these lines, similar to what was observed for the *CDKB1/2* overexpression lines (see Supplementary Fig. S5).

To analyse possible changes in cell size or number in the pericarp of ami*CDKA1* fruits, thin sections from fruits at the breaker stage were analysed. The pericarp of the ami*CDKA1* fruits form fewer cell layers, while the cell sizes in the mesocarp had not significantly changed (Fig. 8). Detailed analysis

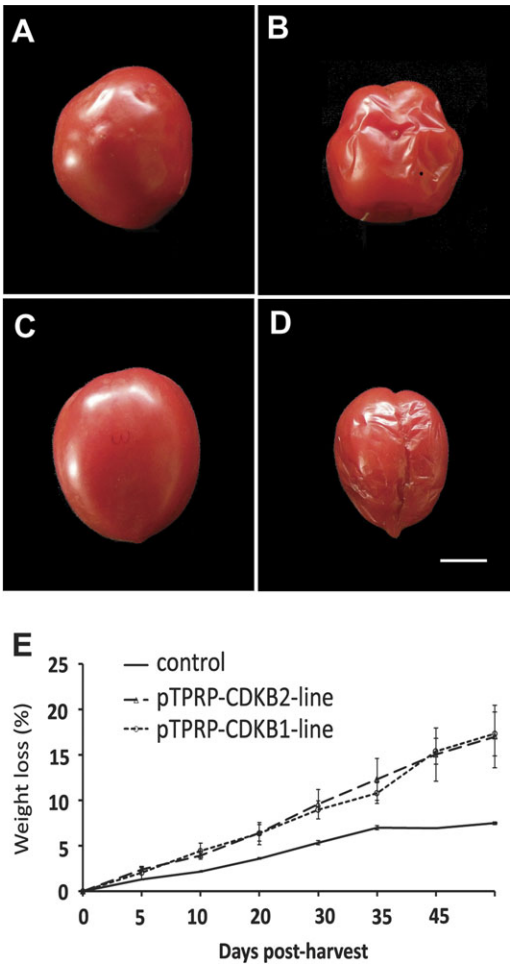


Fig. 5. Post-harvest phenotype and characteristics of *CDKB*-overexpressing fruits. (A–D) Phenotypes of fruits stored for 50 d post-harvest. Control fruits from non-transgenic siblings of *CDKB1*-overexpressing line 57 (A) and *CDKB2*-overexpressing line 4 (C). A transgenic fruit of a *CDKB1*-overexpressing line 57 is depicted in B and a *CDKB2* transgenic fruit of line 4 in D. Bar=10 mm. (E) Percentage weight loss expressed as a percentage of original weight. Every 5 d post-harvest, the fruit weight was measured. Data are means of four fruits ±SD.

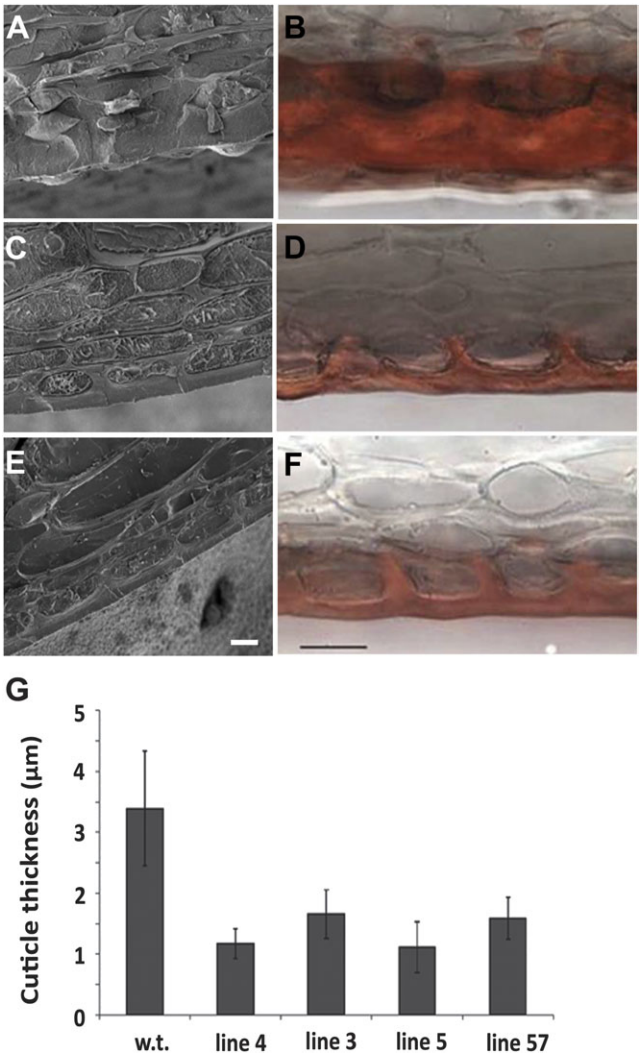


Fig. 6. Characterization of the cuticle layer. The cuticle layer of ripe control (A and B) and transgenic fruits from a pTPRP-*CDKB1* plant (C and D) and a pTPRP-*CDKB2* plant (E and F) shown by cryo-scanning electron microscopy (cryo-SEM) (A, C, E) and Sudan IV cuticle staining of thin sections (B, D, F). The cuticle layer indicated by arrows in the SEM images is visible as a thick layer covering the surface of the epithelial cells. The cuticle layer is stained red with Sudan IV. Bar for cryo-SEMs (A, C, E)=10 µm. Bar in (B, D, F)=50 µm. (G) Cuticle thickness, measured with PlugIns based on images. Fruits from *CDKB1*-overexpressing lines 5 and 57 and from *CDKB2*-overexpressing lines 3 and 4 were taken. Data are means of 10–15 sections ±SD. The cuticle layer is significantly thinner in the transgenic fruits when compared with control fruits (w.t.) (Student *t*-test, $P < 0.0001$ for lines 4 and 3; and $P < 0.0003$ for lines 5 and 57).

of the exocarp of these fruits, as shown in Fig. 8E and F, reveals that only 2–4 layers with small cells are present in the exocarp of ami*CDKA1* fruits directly followed by layers with large mesocarp-like cells, while in the ~10 outer layers of the pericarp from a control fruit the cells gradually enlarge from the outer to the inner layers. This suggests that primarily the exocarp is affected, leading to a small reduction in the number of cell layers in the whole pericarp, although it cannot totally explain the more drastic reduction in pericarp thickness (see Supplementary Fig. S5 at *JXB* online).

In a similar way as was determined for *CDKB*-overexpressing fruits, the ploidy levels of subepidermal and parenchymal cells from ami*CDKA1* fruits were measured. However, in this case a significant difference in ploidy level between control and transgenic fruits could not be detected.

Next, the cuticle thickness was analysed by staining with Sudan IV at the breaker stage, and a decrease of ~2-fold compared with control fruits was observed (Fig. 9). The reduction in cuticle thickness may lead to an increase in desiccation of the fruit after harvesting. Therefore, the phenotype and characteristics of fruits stored for 50 d after harvest were investigated. The fruits from the ami*CDKA1* knock-down lines showed a shrivelled appearance (Fig. 9D) and loss of 30% more weight than the control fruits. The cuticle phenotype and post-harvest

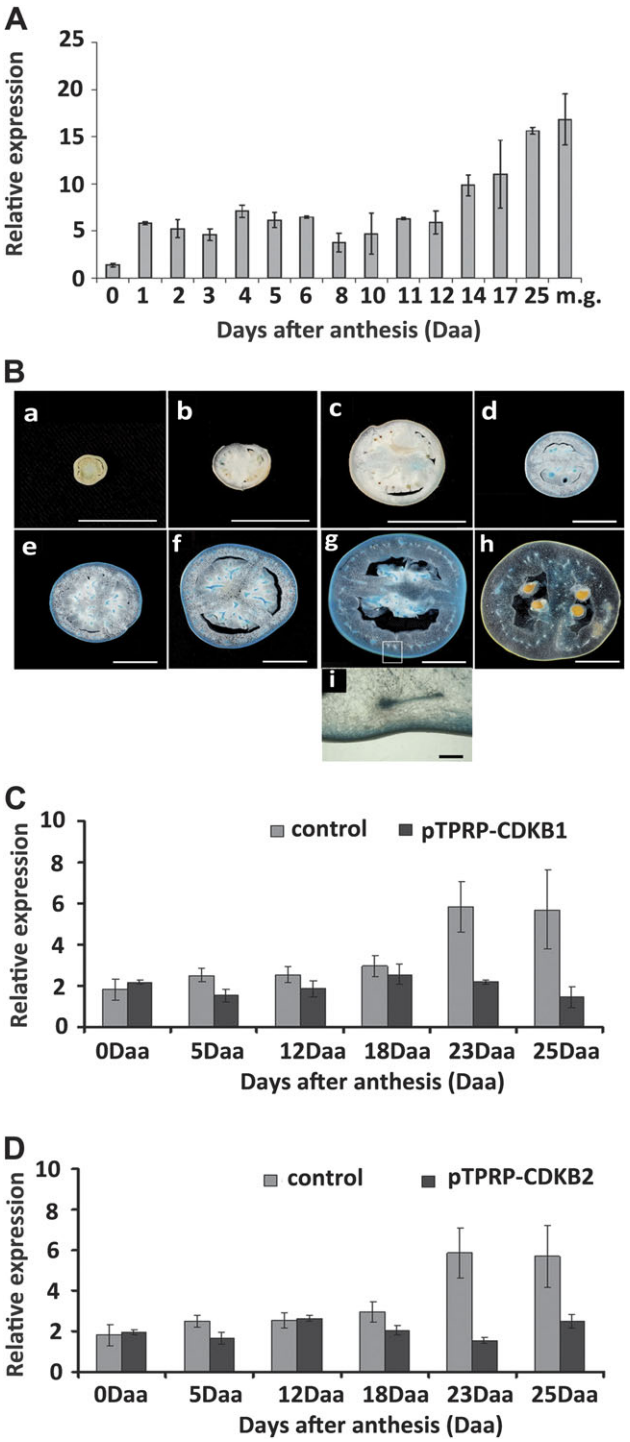


Fig. 7. Expression of *CDKA1* in different genetic backgrounds. (A) Relative *CDKA1* expression was determined in the pericarp of wild-type fruits by qRT-PCR. Samples were collected at various developmental stages from anthesis (class 1) to mature green (class 15). Data are averages of two biological and two technical replications, and the standard deviation (SD) is indicated. m.g. = mature green stage (B) Promoter activity of *CDKA1* in developing fruits (from stages a–h) expressing p*CDKA1*:GUS. Thin sections (~1 mm) of the fruits were stained for GUS activity for 3 h and cleared with ethanol. Bar=1 cm The box in (g) is enlarged in (i). Bar=0.1 mm. (C and D) Relative expression levels of *CDKA1* determined by qRT-PCR in the pericarp samples of transgenic line 5 overexpressing *CDKB1* (C) and line 88 overexpressing *CDKB2* (D). Samples were collected at five different developmental stages, with stage 0 daa corresponding to class 1 and stage 25 daa corresponding to class 14 in A. Data are means of two biological samples containing material from three fruits each.

performance of these ami*CDKA1* knock-down fruits are strongly reminiscent of the characteristics observed for the *CDKB1/2* overexpressors.

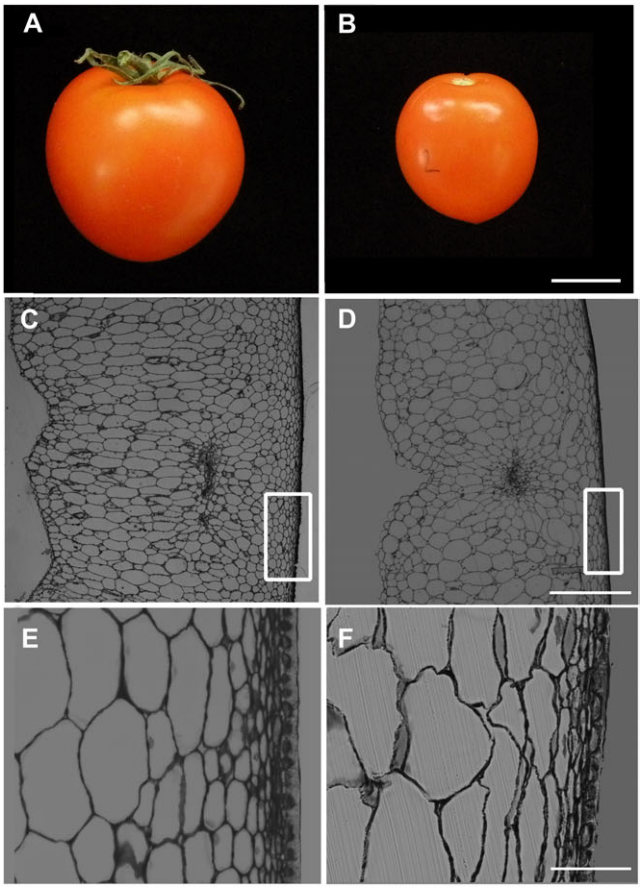
Discussion

Both cell division and cell expansion determine the growth of organs. These processes are precisely controlled by the cell cycle machinery in which the core cell cycle proteins cyclins and CDKs play a central role. A number of different cyclin and CDK types exist, and members of these types form distinct dimer combinations that are active in the different phases of the cell cycle. From tomato (*S. lycopersicum*) two *CDKA* genes (*CDKA1* and *CDKA2*) and two *CDKB* genes (*CDKB1* and *CDKB2*) have been identified (Joubès *et al.*, 1999, 2001). Here the functional characterization of *CDKA1* and the two B-types, *CDKB1* and *CDKB2*, using overexpression and knock-down approaches with a fruit-specific promoter is reported. Both *CDKB1* and *CDKB2* are important for cell cycle transitions during the cell division phase, when they show the highest expression. In all experiments, the two B-type CDKs behaved very similarly and they also showed a comparable expression pattern during fruit development, suggesting that they are interchangeable. *CDKA1* is clearly distinct with respect to both its role in fruit development and its expression pattern.

The *TPRP* promoter that has been used for the manipulation of *CDK* gene expression is active throughout fruit development and in all tissues of the fruit (Salts *et al.*, 1991; Fernandez *et al.*, 2009; Supplementary Fig. S1 at *JXB* online). The highest expression was reported for the earlier stages, and this was confirmed by the *TPRP*–reporter analysis. Surprisingly, overexpression of *CDKB1/2* in the pTPRP-*CDKB* transgenic lines was predominantly observed at the later stages of development when the endogenous *CDKB* expression declines. Apparently, the overexpression of these *CDK* genes is dependent on relatively low endogenous expression levels by unknown mechanisms.

Exocarp of transgenic fruits is predominantly affected

To date only a limited number of mutants with altered CDK functions have been characterized, and these are primarily *Arabidopsis* mutants (Dewitte *et al.*, 2003; Boudolf *et al.*, 2004*a*, *b*; Qi and John, 2007; Imai *et al.*, 2009). In the present study, overexpression of *CDKB* genes resulted in smaller fruits with a reduced number of cell layers in the pericarp, which was particularly apparent in the exocarp. The average size of the cells was also slightly reduced. This phenotype is not easy to explain, particularly when taking into account



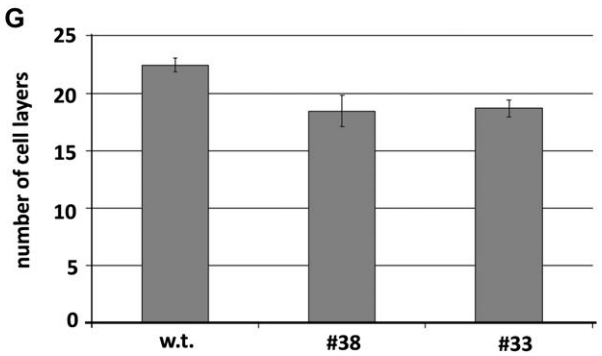


Fig. 8. Analysis of fruits from ami*CDKA1* knock-down lines. (A) Fruit of a control plant. The cultivar is Ida Gold. (B) Representative image of an ami*CDKA1* fruit (from line 33). (C and D) Microscopic cross-sections through a pericarp of a control fruit (C) and through a pericarp of an ami*CDKA1* fruit (line 33) (D). (E and F) Details of the pericarp as shown in C and D, respectively. (G) Reduction of the number of cell layers in the pericarp of ami*CDKA1* fruits (lines 33 and 38) compared with control fruits. Data are means of 3–4 independent sections from three fruits, and the standard deviation is indicated. Bar in A and B=10 mm; bar in C and D=1 mm; bar in E and F=0.2 mm.

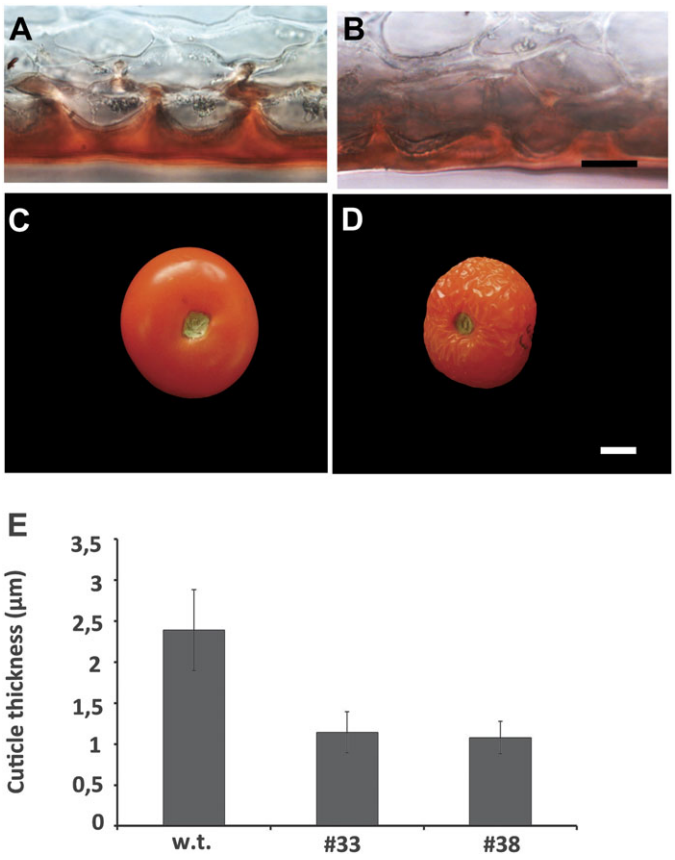


Fig. 9. Cuticle phenotype and characteristics of ami*CDKA1* fruits. (A) Section of a wild-type pericarp stained with Sudan IV. Bar=50 µm. (B) Section of a wild-type pericarp stained with Sudan IV. The cuticle layer covering the surface of the fruit is stained red. Bar=50 µm. (C) Wild-type fruit (Ida Gold) 2 weeks after harvesting. Bar=1 cm. (D) Representative ami*CDKA1* (line 33) fruit 2 weeks after harvesting. Bar=1 cm. (E) Cuticle thickness, measured with PlugIns based on images. Data are means of n=10–15, and the standard deviation is indicated. The cuticle thickness of transgenic fruits is significantly reduced compared with the wild-type (Student t-test, $P < 0.0001$).

that overall *CDKB* levels are only affected at later stages of fruit development. A reduction in cell size could be explained by an extension of the mitotic phase and a delay in entering the endoreduplication phase. This will specifically affect the expansion of the mesocarp cells that undergo a rapid expansion during the later phase of tomato fruit growth. More probably, the overexpression of these B-type *CDK* genes disturbs the delicate balance between the CDK and cyclin proteins, hampering proper cell cycle progression, and hence results in smaller cells and fewer cell layers in the pericarp. Also overexpression of *Arabidopsis CDKB2* in the shoot apical meristem resulted in a reduction in cell division as was also observed for *CDKB2* loss-of-function lines, probably due to similar effects on hormone pathways (Andersen *et al.*, 2008).

CDKA1 is up-regulated in the exocarp at later developmental stages. Down-regulation of *CDKA1* expression was apparent only at the later stages, which explains why aberrations in the ami*CDKA1* lines were only observed in the exocarp. When mesocarp cells enter the cell expansion phase, the exocarp cells maintain mitotic activity and remain dividing up to the mature green stage, generating additional

cell layers for fruit growth (Lemaire-Chamley *et al.*, 2005). In the exocarp of both the *CDKB* overexpression lines as well as in *CDKA1* knock-down lines this mitotic activity seems to be reduced, which resulted in fewer cell layers in the exocarp.

This reduction in cell numbers in the exocarp of mature green fruits most probably affects the cuticle layer. The exocarp of tomato fruits contains the epidermis and additional layers of outer pericarp cells that synthesize and secrete metabolites to the extracellular matrix, forming the waxy cuticle layer (Mintz-Oron *et al.*, 2008; Isaacson *et al.*, 2009). The cuticular layer plays a pivotal role in limiting transpirational water loss of the fruits. Tomato mutants affected in the biosynthesis of the cuticle layer show the same post-harvest phenotype as reported here for the *CDK* transgenic lines (Vogg *et al.*, 2004; Isaacson *et al.*, 2009). Not only the cuticle thickness, but also the wax composition contributes to the permeability properties of the peel (Vogg *et al.*, 2004).

It is not clear yet whether the observed aberrations in the cuticle layer directly result from a reduction in cell division in the exocarp or that the CDK family members are also involved in functions other than the cell cycle (Corellou *et al.*, 2005; Andersen *et al.*, 2008). Overexpression of *CDKB2* in *Arabidopsis* led to an increase in the expression of genes coding for enzymes involved in the jasmonate synthesis pathway. Also the cytokinin signalling pathway was affected in transgenic *Arabidopsis* lines overexpressing or down-regulating *CDKB2* (Andersen *et al.*, 2008). These examples suggest an interaction between hormone synthesis or signalling, and *CDKB2* function.

Interaction between CDKA1 and CDKB genes

Cell cycle progression requires tightly controlled expression and activity of the core cell cycle genes and a continuous cross-talk between the proteins involved. Despite its importance, knowledge of how the activities of *CDK* genes are interconnected and how they control each others' activity is limited (Verkest *et al.*, 2005*a*).

Strikingly, the expression pattern of *CDKA1* is largely complementary to that of *CDKB1*/*CDKB2* during fruit development, being highly up-regulated at the later stages and low at the earlier stages when the expression of *CDKB1*/*2* is high. The analysis of *CDKA1* expression in the *CDKB1*/*CDKB2* overexpression lines revealed that the up-regulation of *CDKA1* at later stages is absent in the transgenic lines, suggesting that these *CDKB* genes negatively regulate *CDKA1* expression. Since these kinase proteins are not known to be transcriptional regulators, this negative regulation should act through an as yet unknown transcriptional intermediate. Possibly, phosphorylation of transcription factors by the CDKs may effect the expression of other *CDK* genes. Candidates for such intermediate transcription factors are E2F and its cognate interaction partner DP, whose activity is controlled via the phosphorylation of the retinoblastoma-related (RBR) protein by CDKs (*CDKA* and *CDKB* proteins) and D- and A-type cyclins during the G_1/S transition (Boniotti and Gutierrez, 2001). Besides the regulation of the E2F/DP transcription factor complex, RBR also interacts with histone-modifying proteins and other transcription factors in animals and most probably in plants as well (for a review, see Gruissem, 2007).

One level of inter-CDK regulation might be at the transcriptional level; a second mode of action could be at the level of CDK–cyclin protein interaction. Different CDK proteins may compete for the same cyclin in the formation of a CDK–cyclin complex (Boruc *et al.*, 2010; Van Leene *et al.*, 2010). Consequently, overexpression of one CDK protein will affect the activity of another competing CDK type, which will disturb the progression in the cell cycle. In tomato, interactions between CDK and cyclins have been studied by yeast two-hybrid assays (Joubès *et al.*, 2001) and they reveal that cyclin A2 interacts with both *CDKA1* and *CDKB2*. Overexpression of *CDKB2* might thus result in a reduction of *CDKA1*–cyclin A2 complexes, causing an arrest in the G_1–S transition.

Another way of interdependency among the CDK proteins is through regulators that inhibit or activate CDK function. Among them are the Kip-related proteins (KRPs), which are related to the class of mammalian Kip/Cip CDK inhibitors and were first identified in *Arabidopsis* (De Veylder *et al.*, 2001; Vandepoele *et al.*, 2002; Verkest *et al.*, 2005*a*, *b*). A model in which *CDKB1;1* controls the level of *CDKA1* activity in proliferating cells through the phosphorylation of KRPs has been proposed (Verkest *et al.*, 2005*a*, *b*; Bisbis *et al.*, 2006). When *CDKB1* levels in the cell are high, it phosphorylates and destoys KRPs, which are the inhibitors of *CDKA1*/cyclin proliferating activity.

Which mode of interdependency is active in the tomato fruit is not resolved, but the present expression studies and functional studies show that the *CDK* B-type genes affect expression and activity of the *CDKA1* gene. The phenotype obtained in the *CDKB1*/*2* overexpression lines mimics the phenotype of the *CDKA1* knock-down lines, which can be explained by a reduction of *CDKA1* in the exocarp due to overexpression of *CDKB1*/*2* at later stages of fruit growth. High activity of *CDKA1* in the exocarp is required to maintain mitotic activity in these outer cell layers throughout fruit growth.

In conclusion, it has been shown that B-type *CDK* genes and *CDKA1* have partly complementary spatial and temporal expression patterns and they might affect each other's activity in a complex antagonistic manner in the tomato fruit pericarp. Manipulating the expression of these *CDK* genes affects cell division and cell expansion, probably by disturbing the delicate balance between these factors that is required for proper cell cycle regulation. It was demonstrated that *CDKA1*, which is highly expressed in the exocarp at the later stages of fruit development, is required to maintain cell division activity in the exocarp. Most probably this cell division is required for the formation of the peel and the cuticle layer that prevents desiccation of the fruit. How the *CDKA1* gene regulates *CDKB* genes and how this delicate balance is controlled remain to be further investigated.

Supplementary data

Supplementary data are available at *JXB* on line.

Figure S1. Expression of the *uidA* (GUS) reporter gene in tomato fruits transformed with the pTPRP:GUS vector.

Figure S2. Post-harvest characteristics of the fruits with *CDKB1* and *CDKB2* overexpression.

Figure S3. Relative expression of *CDKA1* in pericarp tissues of wild-type tomato M82 cultivar in different developmental stages.

Figure S4. Down-regulation of *CDKA1* in pTPRP:ami-*CDKA1* transgenic lines.

Figure S5. Characteristics of pTPRP-ami*CDKA1* lines.

Table S1. Sequences of the primers used for RT-PCR.

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