Effect of changes in cell cycle gene expression on tomato fruit development

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Doctoral Thesis

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by

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General introduction

Anna Czerednik and Ruud A. de Maagd

This introductory chapter describes the history of tomato and reviews our knowledge about the formation of the fruit. Fruit growth takes place by coordinated cell division and cell growth, both processes controlled by the cell cycle. Because these processes are central to the research described in this thesis, special emphasis will be on the cell cycle and the factors involved.

The history and phylogeny of tomato

Tomato belongs to the genus *Solanum* from the genera Solanaceae, which has approximately 2300 species and is considered to be one of the largest genera of flowering plants (Frodin, 2004). The members of this genera are widely used, particularly for food (potato, tomato, egg-plant, and pepper), stimulants (tobacco), medicines, poisons and drugs. *Petunia*, *Salpiglossis* and *Schizanthus*, as well as *Browallia*, *Cestrum*, *Brugmansia* and *Brunfelsia* are known from their aesthetical importance (Hawkes, 1999).

Wild tomato species are distributed from South, to North America, and they are grown in a variety of habitats, at different altitudes, and adapted to particular microclimate and soil conditions. Different species are found at see level along the arid Pacific coast up to over 3300 m in numerous narrow valleys of the western side of the Western South American Andes from central Ecuador, through Peru, to northern Chile. Charles Darwin found two endemic species from genus *Solanum*, now known as *S. cheesmaniae* and *S. galapagense* during his visit in 1835 on the Galapagos Islands (Rick, 1961; Peralta and Spooner, 2000).

The wild ancestor of the cultivated tomato *Solanum lycopersicum* is the most widespread in comparison to the other members of this genus. According to many historical, linguistic, archeological and ethnobotanical findings the most likely origin of domestication of the wild ancestor *Solanum lycopersicum*, is Mexico, particularly the Vera Cruz-Pueblo area, belonging to the Aztecs in those times. From their Nahual (Aztecan) language come the name “Tomato” - xitomatl (shi-to-ma-tlh) (Rick, 1976; Rick and Holle, 1990).

At the beginning of the 16th century the Spanish conquistador of Mexico, Hernando Cortez, found tomatoes growing in the garden of the Aztecs Emperor Montezuma II and brought seeds to Europe. The plants were grown in gardens as ornamental curiosities, but not eaten, because they were supposed to be poisonous. The “poison” tomato berries were accepted in the Italian cuisine already at the end of 17th century, and the first cook book containing tomato recipes was published in Naples in 1692. In the rest of Europe it became used as a nutritional vegetable only around 1840 (Cox, 2000; Roche, 2010). Tomato is now grown worldwide for the fresh market and processing industry, with thousands of cultivars being selected for different growth conditions as well as for different taste and utility attributes (Rick, 1976; Rick *et. al*., 1990; Cox, 2000).

The status of the wild tomato within the Solanaceae has been controversial since the eighteen century. Tournefort positioned in his “Elements de Botanique” in 1694 the newly discovered species tomato to a distinct genus *Lycopersicum*. In Greece *Lycopersicum* means “wolf peach”, because the fruits of tomato are major food of wild canids in South America. In 1753 Linnaeus placed tomato in the genus Solanum, while in contrary Miller, who followed Tournefort, classified tomatoes in a new genus *Lycopersicum*. The majority of later botanists have followed Miller (Peralta and Spooner, 2000, 2005).

The wild tomato species are morphologically highly diverse (Rick, 1973). Botanists used many criteria for the classification, such as fruit colour and size, pubescence of fruit and leaves, seed size, seed coat cells, locules of fruits, polymorphism in anther characterisctics, floral morphology and mating system (Müller, 1940; Luckwill, 1943; D’Arcy, 1972; Rick , 1977; Child, 1990). Modern systematics includes analysis of isozymes, molecular markers (RAPDs, RFLPs, AFLPs), intron DNA sequences of the single-copy nuclear structural gene for granule-bound starch synthase (Peralta and Spooner, 2000, 2005; Peralta *et al.*, 2005). The latest taxonomic study of tomato species, made by Peralta and Spooner (2005) reveals the phylogeny as is presented in Figure 1.

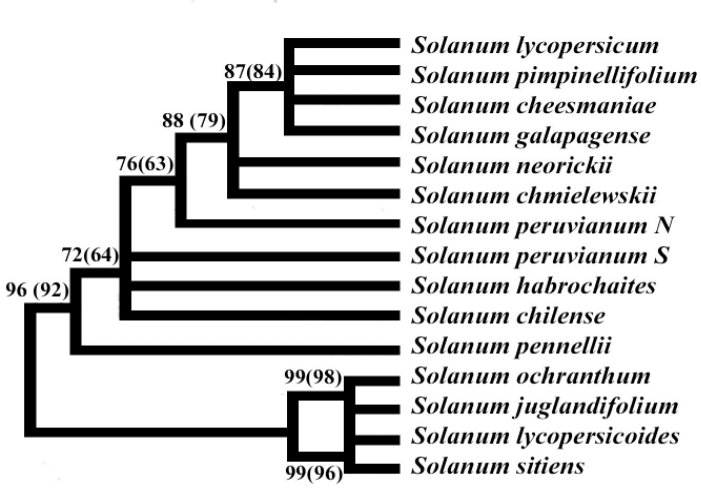

Figure 1. The phylogeny of tomato (from Peralta and Spooner, 2005).

The clade Lycopersicum within the *Solanum* family consists of 15 species. Authors based their classification on crossing relationship, compatibility, similarity in the GBSSI waxy gene, and morphological characteristics, such as the inflorescence and flower structure, flower size and color, plant height, length of internodes, leaf characteristics, fruit color and size, pubescence and trichomes.

Tomato is the model system for studying fleshy fruits because the physiology, biochemistry, genetics, and ripening of this fruit has been studied for decades (Giovannoni, 2001). Also, it is a good model system to investigate the evolution of natural mating systems because of existing variation in compatibility and incompatibility systems (Rick 1976; Rick and Chetelat, 1991). Furthermore, a wide range of genetic resources, intercrossable wild relatives, mutants, and genomic tools, such as BAC libraries and expressed sequence tags (ESTs) are available (Chevalier, 2007). Very recently, the genome sequence of tomato was released, which provides an excellent tool for future genetic, genomic and molecular research (SGN site; http://solgenomics.net/).

Fruit development and ripening

Tomato species possess either an indeterminate or terminal growth. The plant has a sympodial growth architecture: their vegetative and reproductive phases replace each other along the primary and axillary shoots (Lozano *et al*., 2009). The vegetative phase of most tomato species is relatively short, producing 8-12 leaves before the first inflorescence appears. The shoot apical meristem terminates into an inflorescence, while an axillary vegetative meristem continues on the flank of the inflorescence. This vegetative meristem produces usually two-three leaves before it terminates again in an inflorescence. The floral meristems formed from the inflorescence meristem produce mature flowers in about 2-3 weeks, and during this time the identity, number, and shape of all floral organs are determined and the mature ovary is formed (Lozano *et al*., 2009). After anthesis and successful pollination the fertilized ovary proceeds to form the fruit. The formation of the flower, pollination and fruit-set is under control of environmental and endogenous factors (Nitsch, 2009; De Jong *et al.*, 2009a, 2009b).

Fruit development progresses through a number of stages which have been described in detail, among others, by Gillaspy (Gillaspy *et al.*, 1993).

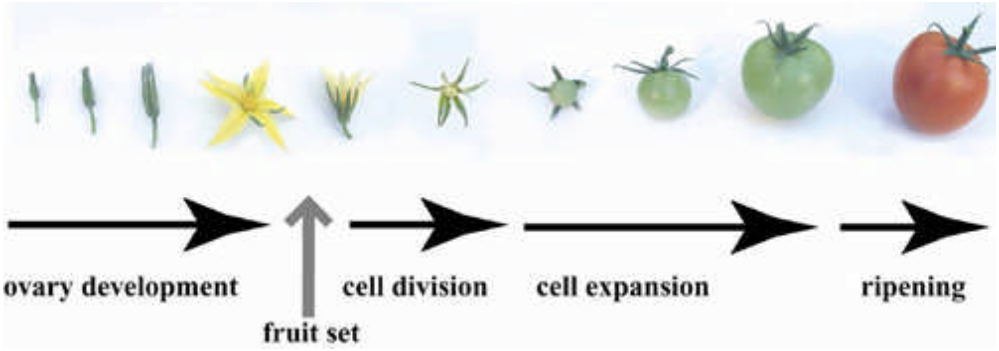

Figure 2. Tomato fruit development divided into five phases.
(Photo kindly provided by Dr. Lisette Nitsch, 2009)

The development of the tomato fruit is marked by a fast increase in size due to extensive cell division activity, leading to an increase in the number of cells. Parenhymatous tissue of the placenta grows around the funiculi and encloses the developing seeds. At the end of fruit development the cells of this parenchyma are thin-walled, giant cells that form a jelly-like homogenous tissue. The phase of cell division is an essential determinant of fruit development, as it fixes the final number of cells inside the fruit and therefore determines to a large extent the final fruit size. The cell division phase is followed by cell expansion leading to 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. The ripening phase initiates after growth has ceased and involves rapid chemical and structural changes that determine fruit aroma, color, and biochemical composition, but not fruit size and shape (Giovannoni, 2001).
In conclusion the formation of the fruit results from the coordinated action of both cell division and cell expansion. The tight control of these two processes is a crucial determinant of the final size, shape and texture of the fruit.

Tomato fruit anatomy

Tomato fruit is classified as a berry fruit because it has a thick pericarp that encloses many seeds (Gillaspy *et al.*, 1993). The fruit is a complex organ composed of two or more carpels, separated by a radially oriented septa and a central parenchymatous axis called columella (Figure 3). The columella develops from placental tissue and its peripheral part develops into gel, or loculair tissue, that surrounds the seeds and fills the locular cavity between the septa. 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 and can be subdivided into exocarp, mesocarp and endocarp (Lemaire-Chamley *et al*., 2005).

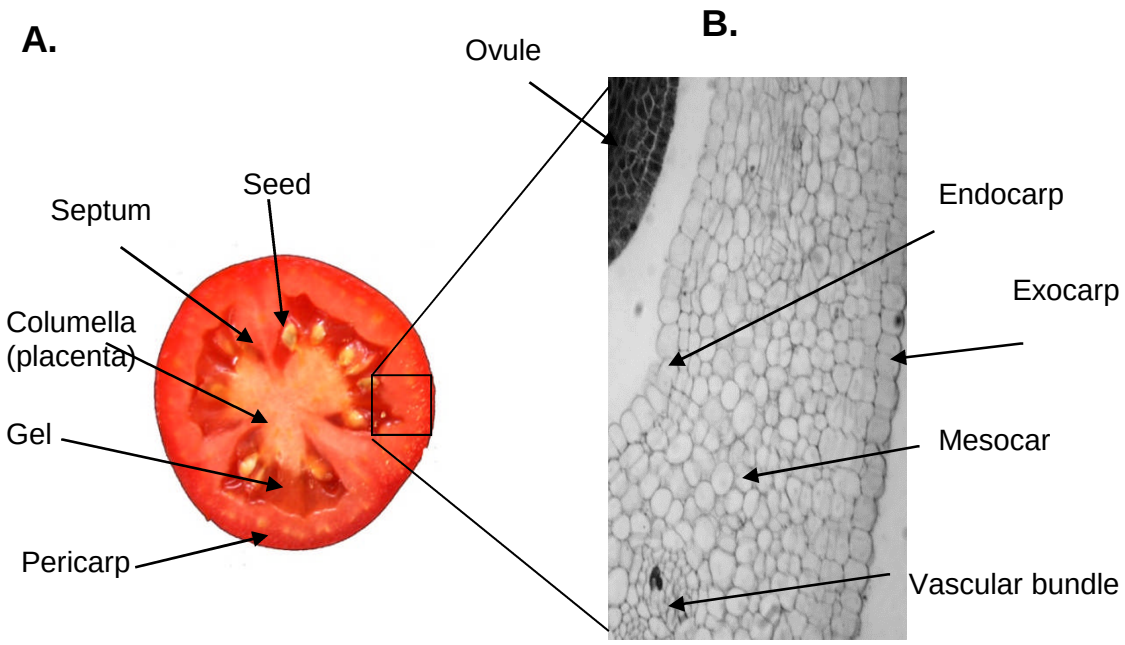

Figure 3. Tomato fruit anatomy

- **A.** Cross section through a ripe tomato
- **B.** Microscopic section through the pericarp, showing the various pericarp layers and cell types. This section was taken from a young fruit in the cell division phase.

The mesocarp, being the major part of the pericarp, in the ripe stage consists of layers of large, highly vacuolated parenchymatous cells and contains vascular bundles. The outer layer of the pericarp, the exocarp, contains several layers of collenchymatous cells, that include mitotically active cells as well as enlarging cells, and a single layer of epidermal cells which are covered or in some cases encased in a waxy cuticle. The pericarp is limited by a single interior cell layer forming the endocarp (Joubés *et al.,* 2000; Lemaire-Chamley *et al*., 2005; Mintz-Oron *et al.,* 2008).

The Plant cell cycle and its regulators

Phases of the cell cycle

Plant development is characterized by indeterminate growth and reiteration of organogenesis and is therefore intimately associated with cell division. For proper development and differentiation of plants it is essential that cell division and cell growth are precisely controlled.

One level at which such control exist is the cell cycle, i.e. entry of cells into the cycle and their progress through mitosis followed by cytokinesis.

The plant cell cycle is typically characterized by two distinguishable phases and two interphases. Figure 4 presents the major points in the cell cycle progression in plants and its important players.

The plant cell chromosomes are duplicated during the S-phase (DNA Synthesis phase), and chromosome associated proteins are synthesized. During the M-phase (Mitosis) the sister chromosomes, synthesized during the S-phase, separate by the mitotic spindle (karyokinesis) and packaged into new daughter nuclei. After that, organelles and cytoplasm are divided during cytokinesis and the mother cell splits itself into two daughter cells. The M and S phases are linked by gap-phases or checkpoints. G1 (gap one) is post-mitotic interphase, which occurs before the S-phase. G2 (gap two), the premitotic or post-synthetic interphase links the S-phase with the M-phase. These checkpoints ensure that conditions are appropriate for cells to engage in another round of DNA duplication in the S (synthesis) phase or for cells to enter the M phase (mitosis). Phosphorylation of proteins is a common biochemical feature of the G1/S and G2/M cell cycle transitions and occurs due to activity of cell-cycle regulators.

CDK and Cyclins are the major cell cycle regulators

Plants have numerous cell-cycle regulators that appear to have overlapping as well as distinct functions. There are at least 80 proteins that regulate the plant cell cycle (Menges *et al*., 2005), but the major checkpoints are controlled by cyclin dependent kinases (CDK), which need for their activation a regulatory cyclin subunit.

The most extended list includes 152 CDKs from 41 plant species (Dudits *et al*., 2007), categorized into eight classes, CDKA to CDKG and the CDK-like kinases (CKLs); which are grouped based on the cyclin-binding motif. Tomato possesses seven known CDKs, which are presented in Table 1.

Table 1. The list of known tomato CDKs

Gene abbreviation	Cyclin-binding motif	Accession	Protein id	References
Lyces;CDKA1;1	PSTAIRE	Y17225	CAA76700	Joubès *et al.*, 2000; Dudits *et al*., 2007
Lyces;CDKA2;1	PSTAIRE	Y17226	CAA76701	Joubès *et al.*, 2000; Dudits *et al.*., 2007
Lyces;CDKB1;1	PPTALRE	AJ297916	CAC15503	Joubès *et al.*, 2001; Dudits *et al.*, 2007
Lyces;CDKB2;1	PPTTLRE	AJ297917	CAC15504	Joubès *et al.*, 2001; Dudits *et al.*, 2007
Lyces;CDKC;1	PITAIRE	AJ294903	CAC51391	Joubès *et al.*, 2001; Dudits *et al.*, 2007
Lyces;CDKC;2	PITAIRE	BT014075		Dudits *et al.*, 2007
Lyces;CDKD;1	NFTALRE	BT013748	BT013748	Dudits *et al.*, 2007

Today, over 160 cyclin genes from 17 plant species have been identified, which can be divided into 7 families. From this collection 8 cyclin genes are known to be present in tomato and they are all involved in the cell cycle progression (Nieuwland *et al.*, 2007).

Table 2. The list of known tomato Cyclins

Gene abbreviation	Accession	Protein id	References
LeCycA1;1	AJ243451	CAB46641	Joubès *et al*., 2000; Nieuwland *et al.,* 2007
LeCycA2	AJ243452	CAB46642	Joubès *et al.,* 2000; Nieuwland *et al.,* 2007
LeCycA3;1	AJ243453	CAB46643	Joubès *et al*., 2000; Nieuwland *et al.,* 2007
LeCycB1;1	AJ243454	CAB46644	Joubès *et al*., 2000; Nieuwland *et al*., 2007
LeCycB2;1	AJ243455	CAB46645	Joubès *et al.,* 2000; Nieuwland *et al*., 2007
LeCycD3;1	AJ002588	CAB60836	Joubés *et al*., 2000; Kvarnheden *et al*., 2000; Nieuwland *et al*., 2007
LeCycD3;2	AJ002589	CAB60837	Kvarnheden *et al*., 2000; Nieuwland *et al*., 2007
LeCycD3;3	AJ002590	CAB60838	Kvarnheden *et al*., 2000; Nieuwland *et al*., 2007

A characteristic mark of most of the cyclins are the cyclin box – a sequence of 100 amino acids, which is required for binding to CDKs and a destruction box, which is a target for ubiquitinilation and rapid protolytic destruction (De Veylder *et al*., 1997).

CDK activation through cyclin binding is accompanied by significant structural changes in the CDK active site. Major changes involve the repositioning of the T-loop that releases the protein substrate-binding site and the reconfiguration of the ATP binding site that corrects the positioning of the ATP phosphates for the transfer to the substrates (Jeffrey *et al.,* 1995; Dissmeyer *et al.,* 2007).

G1-to –S transition and synthesis (Figure 3)

At the G1-to-S transition, an active CDKA/cycD3 complex initiates the phosphorylation of the retinoblastoma-related protein (RBR), a key regulator of the start of DNA replication. The non-phosphorylated form of RBR suppresses the activity of the dimeric E2F-DP transcription factor, which is necessary for the initiation of the S-phase and cell cycle progression. In the G1-to-S cell cycle progression phosphorylation by CDKA/CycD initiates the destruction of the E2F/DP/RBR transcriptional repressor complex by the SCF E3 ubiquitin-protein ligase. As a result of E2F/DP-activation, the expression of S-phase genes is activated (De Veylder *et al.,* 2002).

E2Fs regulate the expression of genes involved in DNA synthesis and replication via binding to E2Fs-sites (TTT(C/G)(C/G)CG(C/G)(C/G)) in their promoters. For example, genes encoding the origin recognition complex (ORC) contain such E2F binding sites in their promoters (Vandepoele *et al.,* 2005). All of the E2Fs need to dimerize with DP proteins in order to bind with high specificity to E2F-binding sites and furthermore the dimerization partner DP is necessary for nuclear localization of E2Fs (Shen, 2002; De Veylder *et al.,* 2003; Ramirez -Parra *et al.*, 2005; Francis, 2007).

A recent study revealed that plants possess a complex set of E2F-DP family members. In Arabidopsis six E2F and two DP genes are present (De Veylder *et al*., 2003; Inzé, 2005; Ramirez-Parra *et al.,* 2007). In tomato, we identified only a single gene for *E2F* and *DP*, whose functions are probably similar to those of the activating E2F-DP complexes of Arabidopsis (data not shown) and are involved in the regulation of G1-to-S transition and S-phase genes activation.

G2-to-M transition and mitosis (Figure 3)

During the G2-M transition CDKs from two classes - A and B, are active. B-type CDKs are unique for plants and their kinase activity peaks in mid-to-late G2 (Francis, 2007). CDKA and CDKB are associated with different cyclins from the classes D, A, and B (Ferreira *et al.,* 1994; Lee *et al.,* 2003; Wang and Yang, 2007; Boudolf *et al*., 2009). Both CDKA/Cyc and CDKB/Cyc complexes are activated by a CAK (CDK activated kinases from the group D associated with CycH), before they can fully drive cells into mitosis by phosphorylation of several G2-specific transcription factors (Umeda *et al*., 2005).

Transcription of CDKB1 is controlled by the G1/S –specific E2F transcription factor and this may provide a mechanism by which the G1-to-S and G2-to-M transitions communicate.

Exit from mitosis requires the proteolytic destruction of the cyclin subunits from the CDKB/Cyc complexes. This destruction is initiated by the activation of the anaphase-promoting complex (APC) through its association with the CCS52 protein (Cebolla *et al.,* 1999).

Cell-cycle progression is strictly regulated by several inhibitors. During the G1-to-S and G2-to-M transitions CDK/Cyc complexes are regulated by binding and inhibiting of CDK by the specific cyclin-dependent kinase inhibitors CKIs or Kip Related Proteins (KRP) (Verkest *et al*., 2005).

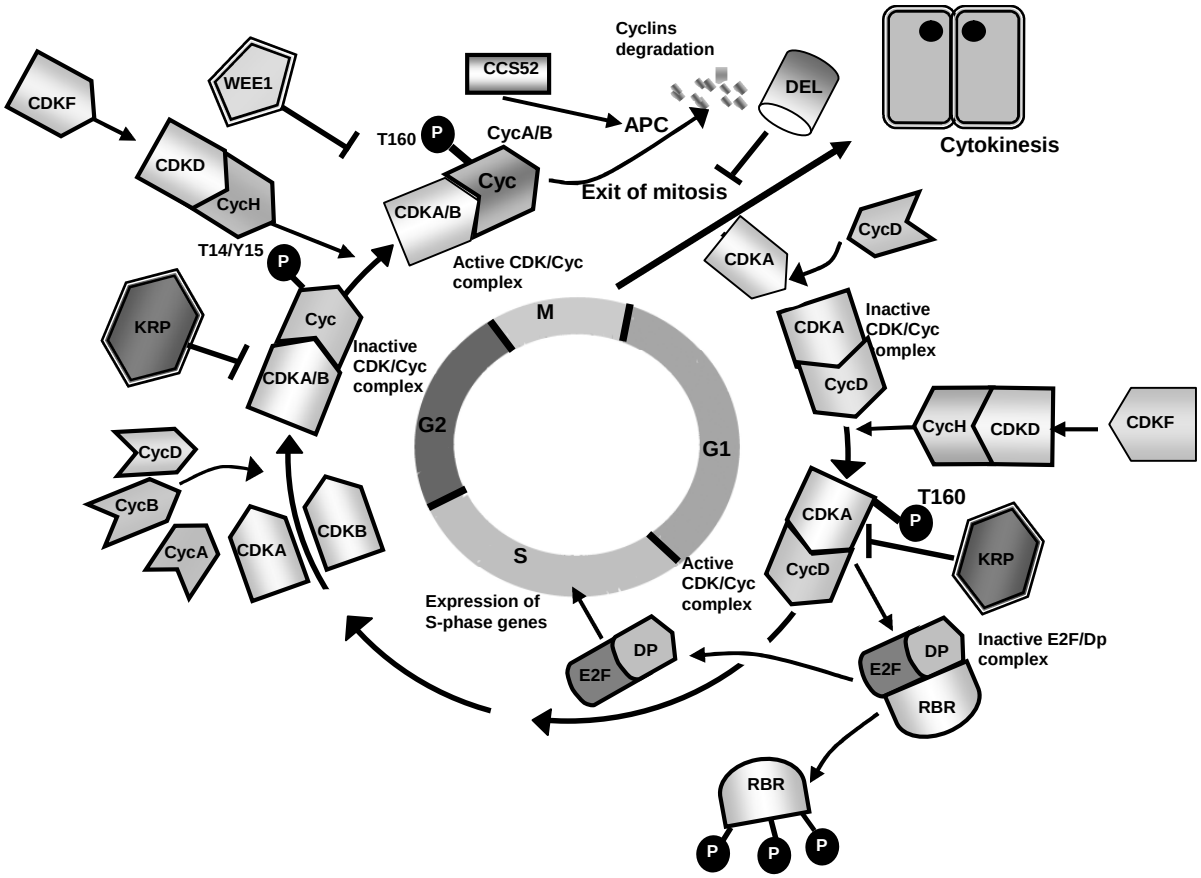

Figure 4. Regulation of the cell cycle. Schematic representation of the regulation of the G1-to-S and G2-to-M transition in plants (adapted from Inzé and De Veylder 2006).

The first check-point of G1-S transition is formed by D-type cyclins (CycD) associated with the A-type CDK. This complex became active through phosphorylation by the CDK-activating kinase pathway - CDKF and CDKD (CAK) associated with an H-type cycline. Active CDK-Cyc complex might be inhibited by KRPs, activated in response to antimitogenic stimuli, such as ABA or cold. CDKA-CycD trigger the G1-to-S transition, by phosphorylation of RBR protein.

During the G2 phase several cyclins – from A, B and D classes associate with A- and B-type CDKs. CDKA/B-Cyc complexes are activated via the CDK-activating kinase pathway, involving CDKF and CDKD-CycH. The activity of CDKs in G2-to-M transition is under control of KRPs, but also WEE1, which is responsible for the inhibition of mitosis. Active CDKA/B-Cyc complexes trigger the G2-to-M transition through the phosphorylation of a plethora of different substrates. At the end of mitosis cyclin is proteolitically destructed by the active anaphase-promoting complex (APC) through the association with the CCS52 protein. The whole mitotic cell cycle finishes by cytokinesis and cell division into two daughter cells. Exit from the mitotical cell cycle is inhibited by the transcription factor DEL (Verkest *et al*., 2005a; Vliege *et al*., 2005; Inzé and De Veylder, 2006).

Endoreduplication

Replication of DNA in the absence of mitosis is a characteristic mark of many plant cells, which become polyploid during development. This modified mitotic cycle is called the endocycle and occurs when cells synthesize DNA in the S-phase and do not reenter into the mitotic phase (De Jager *et al.,* 2005). Endoreduplication is associated with cell expansion, thus may contribute to plant organ growth and plant yield (Chevalier *et al*., 2011). The function of endoreduplication is not fully understood yet. It has been hypothesized that polyploidy supports an increase in metabolic activity needed for differentiation and development of cells (De Jager *et al*., 2005; Guttierez, 2009). Endoreduplication is typical for certain cell types especially for those undergoing differentiation and expansion (Joubés and Chevalier, 2000). This process is well described for trichomes, seed endosperm, leaves and fleshy fruits (Grafi and Larkins, 1995; Gonzalez *et al*., 2007; Chevalier *et al*., 2011). In fleshy fruits most of the cells become polyploid. The number of genome copies in tomato fruit cells can reach 256C or even 512C (where C is the DNA content of the haploid genome) (Joubés *et al.*, 1999; Bisbis *et al.*, 2006). This increase in endoreduplication level in tomato fruit is correlated with cell size and suggests to be an important determinant of organ size (Chevalier *et al.,* 2011).

In the cell cycle several regulators inhibit or promote the endocycle process. Exit from the mitotic cell cycle is negatively regulated by the atypical E2F-like transcription factor DEL, which is a repressor of E2F-regulated genes through competition with E2F-DP for binding sides (De Veylder *et al.,* 2003; Vlieghe *et al.,* 2005).

At the time of exit from the mitotic cell cycle the protein CCS52 is activated and probably targets B-type CDK and associated cyclin A or B for destruction. The decreased activity of the CDKA/CycD-complex is the result of a stabilization of the ICK/KRP proteins, from which two family members were described in tomato: Le*KRP1 (*AJ441249) and Le*KRP2* (AJ441250).

KRP may interact with CycD and inhibits its binding with CDK, which prevent a premature passage into the S-phase (Bisbis *et al.*, 2006).
Another regulator of the cell-cycle and involved in the endoreduplication is the WEE1 kinase, which is responsible for the negative regulation of CDKA/Cyc and CDKB/Cyc by dephosphorylation, resulting in the inhibition of mitosis, but still allows DNA replication to occur. WEE1 kinases are well characterized in maize, Arabidopsis and tomato (Sun *et al.,* 1999; Sorrell *et al*., 2002; Gonzales *et al*., 2007) and are part of the DNA-damage signal transduction pathway operating in the G2 phase by arresting the cell cycle in response to genotoxic stress (De Schutter *et al*., 2007).

Fruit quality characteristics and cell size and number

In The Netherlands, tomato is the most cultivated vegetable and our country exports a quarter of all whole tomatoes produced in the world (GAIN Report, 2005). To keep this position tomato breeder 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).
Consumer perception of tomato fruits depends on numerous factors, including fruit size, texture, color and aroma (Szczesniak, 2002; Serrano-Megias and Lopez-Nicolas, 2006; Chaïb *et al.,* 2007; Mounet *et al.,* 2009). These traits are controlled by many genetic loci, influenced by environmental factors, hormonal regulation and cultivation practices.
Since the fruits of the progenitor species of tomato are small, round berries weighting a few grams and with a diameter less than 1 cm, size has been an important selection criteria in breeding programs. Cultivated *S. lycopersicum* bears fruits with a weight ranging from 3-5 grams (cherry variety) to 1000 grams, exceeding 15 cm in diameter (Frary, *et al*., 2000; Tanksley, 2004). Cell number and cell size are main determinants of the fruit size and also play an important role in qualitative characteristics of the ripe product (Büngler-Kibler and Bangerth, 1982; Bertin *et al*., 2001; Nesbitt and Tanksley, 2001; Mizukami, 2001; Bertin *et al.,* 2003; Bertin 2005; Giovannoni 2004; Paran and Van der Knaap, 2007). The tomato texture has a significant effect on its quality and influences consumer's acceptance by affecting firmness, mealiness and juiciness. In addition, texture plays important roles in shelf-life of the fruit, resistance to pathogens, and transportability (Seymour *et al*., 2002; Chaïb *et al.,* 2008). These texture characteristics are determined by the interaction of many factors such as cell wall chemistry, cell size and shape, cell packing and cell turgor (Bewley *et al.,* 2000; Thompson, 2001; Waldron *et al.,* 2003; Brumel, 2006; Chaïb *et al*., 2007; McAtee *et al.*, 2009). Differences in firmness already occur at the early stages of fruit development and have effects on important qualitative traits.

For example, highly significant positive correlation exists between firmness and color of the ripe fruit, while firmness is negatively correlated with juiciness of the fruit (Chaïb *et al*., 2007). There are positive correlations found between firmness and a heterogeneous cell distribution in the fleshy part of the tomato pericarp (Figure 3), and the pericarp skin toughness is positively correlated with the presence of small cells under the tomato peel (Chaïb *et al.,* 2007; Guillon *et al.,* 2008). Interestingly, mealiness positively correlates with a thick pericarp composed of elongated cells, while the firmness of the pericarp is positively correlated with total elasticity (Chaïb *et al*., 2007).

The cell number is determined during the early stages of development and relates together with cell size to the dry matter content of the ripe fruit because these processes influence the structural dry matter through the amount of cell wall material (Higashi *et al.,* 1999; Bertin, 2005). Cell number is also a determinant factor of fruit sink strength, which affects the sugar content and hence fruit taste (Prudent *et al.,* 2009).

A substantial problem for tomato breeders is fruit cracking, which occurs when there is a rapid net influx of water into the fruit at the same time when ripening or other factors reduce the strength and elasticity of the tomato skin (Valley, 2002; Moctezuma *et al*., 2003). The rapid fruit growth before maturation due to expansion increases the tendency to cracking. But also the cell composition may influence the cracking behavior as it was noticed that cracking of mature tomatoes was reduced in fruits with more resilient outer skin (Prudent *et al.,* 2009).

Although many genetic loci (genes, QTLs) affecting shape, size and quality parameters have been mapped on the genome, relatively little is known about the underlying molecular mechanisms. Moreover, where these mechanisms have been (partially) elucidated, in most cases they concern processes taking place at later stages of fruit development, from the mature green stage to the red ripe fruit. To improve the quality of tomato fruit it is important to understand some of the basic processes that take place from the start of fruit development and how plant organ size is coordinated by the cell cycle.

Plant organ size reflects both cell number and cell size, and it has been shown that certain types of organs from different plant species may differ in their size due to more cells rather than bigger cells. Also for tomato, larger fruit size is often associated with more cells per unit tissue (Frary *et al.,* 2000; Mizukami, 2001). The results of Cheniclet *et al.* (2005) indicate that at the beginning of development, during anthesis, the cell number and size in the pericarp of tomato does not differ, but in the ripe fruits these two parameters vary significantly among cultivated tomatoes with different fruit size. A large variation in fruit size probably depends on quantitative differences in gene expression.

A Quantitative Trait Locus (QTL), fw 2.2 , which is for a large part responsible for the small fruit size in wild tomato relatives, is a good example of how both level and timing of gene expression affect the final fruit size (Frary *et al*., 2000; Nesbitt and Tanksley, 2001). Plants with the "large size" allele enter the cell expansion phase with more cells, because they start cell division earlier in the carpels (Cong *et al*., 2002; Liu *et al*., 2003).

Another possibility to control the number and size of cells is modifying the activity of cell-cycle genes. For example the overexpression of a dominant negative mutation of the *CDKA* gene in tobacco inhibits cell division, which was compensated by an increase in cell size. As a consequence the organ size and shape was not changed (Hemerly *et al*., 1995). Other examples confirming this compensation phenomenon are overexpression of dominant-negative mutant version of CDKB1;1.N161 (Boudolf *et al*., 2004b), overexpression of *Cyc1At* (Doerner *et al*., 1996) in Arabidopsis and *OsKRP1* overexpression in rice (Inzé and De Veylder unpublished, cited in Inzé and De Veylder 2006). In these examples, despite changes in cell division rate, the final change in plant morphology was mild or negligible.

This led to the hypothesis that increased cell division in tomato may coincide with a reduction in cell expansion, resulting in small changes in size, but possibly substantial consequences for fruit texture and other qualitative characteristics.

Scope of the thesis

A fundamental role for cell division in organogenesis and organ-size determination is implied by the fact that larger organs tend to consist of more cells than do smaller organs (Bohner and Bangerth, 1988; Mizukami, 2001; Nesbitt and Tanksley, 2001). On the other hand, the major increase in size occurs during the expansion phase. Interestingly, more cell division can be complemented by a decrease in cell expansion, resulting in an organ with the same size. This concept is known as the ‘compensation effect” and suggests that the final shape and size of an organ is predetermined. Therefore, we propose that a change in the ratio between cell division/expansion even though no effect on total fruit size, may have substantial effects on various quality traits of the full grown ripe tomato.

The aim of this thesis research is to prove this hypothesis using modifications by overexpression or knocking-down of candidate genes, which are involved in the regulation of cell division and cell expansion.

Gene expression studies from fruits during development are not very well documented, because they mainly concentrate on the ripening stage. Furthermore, microarrays used in these expression studies miss many of the interesting cell cycle regulators. Moreover, our knowledge about cell cycle regulators in plants is predominantly from Arabidopsis.

Therefore, we isolated as many as possible tomato homologs of these regulators and studied their expression patterns throughout fruit development. In **Chapter 2** the qRT-PCR analysis of the potential regulators of fruit growth are described. These are the major drivers of plant cell cycle transitions, CDKs, Cyclins, Rb and transcription factors from the E2F, DP and DEL families. These candidate genes were isolated from the pericarp of tomato cv. M82 at various days after anthesis, and their expression patterns during fruit growth are presented. We also analysed the expression of the fruit specific promoter TPRP-F1 in transgenic tomato plants containing a pTPRP::uidA(GUS) construct. This analysis resulted in a better view on the specificity of this promoter, which is being used to specifically down-regulate or overexpress candidate genes in the fruit. Two other promoters, driving the activities of the *CDKA1* and *CDKB2* genes were isolated and characterized in transgenic plants, using reporter constructs. These reporter analysis provided insight into the spatial and temporal regulation of these genes during fruit development.

In **Chapter 3** the analysis of fruits overexpressing *CDKB1* and *CDKB2* are presented. These overexpressing fruits show a phenotype in the pericarp, which could be phenocopied by down-regulation of *CDKA1* in the fruit. This observation suggests a possible interaction of these *CDK* genes and points to a role of the *CDKA1* gene in the regulation of cell division in the exocarp at later developmental stages.

In **Chapter 4** the further analysis on the role of *CDKA1* in fruit development is presented. We characterized in detail the fruits overexpressing pTPRP-CDKA1 and observed an increase in fruit pericarp thickness and size of the septum (placenta), which might be resulted from an increase in the number of cells, despite of the slight decrease in cell size.

CycA2 is known to be involved in the regulation of endoreduplication and its overexpression in Arabidopsis results in organs with smaller cells (Imai *et al*., 2005; Boudolf *et al*., 2009). In **Chapter 5** the action of *CycA2* in tomato fruit development and specifically the duration of cell division in the pericarp were analyzed. We supposed that *CycA2* is involved in the regulation of cell division duration and may also have a role in cell expansion, since the overexpression of *CycA2* resulted in a reduction in cell size and number. However, the involvement of *LeCycA2* in the cell expansion process was not fully understood.

To knock-down the expression of the candidate genes we followed an artificial microRNA (amiRNA) approach. Precursor amiRNA molecules were designed that should produce mature amiRNAs, which are able to suppress the expression or activity of the target genes. All amiRNA constructs described were driven by the TPRP-F1 fruit specific promoter. In Chapter 6 the effect of downregulation of *CDKB2* and *CDKB1* is presented.

In **Chapter 7** the effect of overexpression of *CycD3;3* on cell division and expansion in tomato fruit and also the changes in the length of these phases in fruit development were investigated.

In the case of fruit specific *CycD3;3* overexpression we observed the compensation effect of cell division and cell expansion, because the decreased cell size was correlated with an increasing cell number.

Finally, in **Chapter 8** the results of this study are summarized and discussed. The manipulation of key cell-cycle regulators had clear consequences on proper fruit development, the duration of cell division and cell expansion processes, as well its cellular characteristics. We identified several candidate genes, whose manipulation gives rise to a clear effect on the cell size/cell number ratio. However, the mechanisms how this was achieved in tomato was not fully understood, but the novel transgenic tomato lines generated give an excellent starting point for future research on several developmental processes in the tomato fruit.

Acknowledgements

We are grateful to Mr. Gerard van der Weerden from Radboud University for critical reading of the manuscript and suggestions in describing the history and phylogeny of tomato.

2

Cell dynamics and expression of cell cycle genes during tomato fruit development

Analysis of fruit-specific and cell cycle gene promoters

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Abstract

Expression patterns of cell cycle associated genes give clues about their involvement in the diverse processes that take place during tomato fruit development. Based on the available knowledge about the regulation of the plant cell cycle we made a list of genes potentially involved in cell cycle control and endoreduplication during tomato fruit growth. We found that several genes have expression patterns changing over time in a way that suggests that they are involved in the first stages of fruit development.

During the first 3 to 4 days after pollination pericarp cell sizes increase only slightly, while in consecutive stages mesocarp cells enlarge exponentially. Nevertheless, cell expansion occurs already from the earliest stages onwards, indicating that the phases of cell division and cell expansion are not completely separated in time. The development of tomato pericarp, the expression patterns of core cell cycle genes in fruit pericarp and other tomato tissues, and the activity of some fruit-expressed gene promoters are discussed.

Introduction

The overall control of the cell cycle is largely similar between plants and other eukaryotes, however at several points the process has aspects that are unique in plants. The fruit is a specific part of the plant, which provides a suitable environment for seeds maturation and a mechanism for the dispersal of mature seeds (Gillaspy *et al.,* 1993). This is a complex organ containing different tissues, among which the pericarp forms the biggest part of the fruit. Pericarp develops from the ovary wall after fertilization, which occurs approximately 20 h after pollination (Gillaspy *et al.,* 1993; Picken, 1984). The number of cells in the pericarp is determined by the mitotic activity of the ovary in the developing flower, and later, by the mitotic activity of cells in the developing fruit (Gillaspy *et al*., 1993; Joubès *et al*., 1999).

Directly after anthesis cell divisions occur at the highest rate and new layers of pericarp are formed. The new cell layers arise from periclinal cell divisions in the outer subepidermal cell layers, and to a lesser extent, in the inner layers (Cheniclet *et al*., 2005). Ten to twelve days later cell division is largely replaced by cell expansion (Büngler-Kibler and Bangerth, 1982; Gillaspy *et al*., 1993). This is the longest phase in fruit development and may contribute up to 90% of the increase in fruit weight, depending on the cultivar (Lemaire-Chamley *et al.,* 2005). In addition, cell expansion contributes to the major structural, biochemical, and physiological changes that characterize a fleshy fruit (Gillaspy *et al*., 1993).

The number of cells and the final size of organs and in the end, the whole plant are controlled by the cell cycle. The cell cycle regulates the proper activity and timing of cell division and later, during cell expansion accompanied by endoreduplication, it controls DNA synthesis and the increase of the number of DNA copies.

All eukaryotes including plants have a similar cell cycle with two phases – the synthesis (S-) phase, when DNA is synthesized, and the mitosis (M-) phase when actual cell division into two daughter cells (cytokinesis) takes place. Two interphases, G1 and G2, control the ability of the cell for DNA synthesis in phase S, and for mitosis/cytokinesis in phase M, respectively. In eukaryotes CDKs (Cyclin Dependent Kinases) are the major drivers of cell cycle progression. CDKs require interaction with specific Cyclins (Cyc) for their activity. CDK-Cyclin complexes are involved in direct regulation of the cell cycle.

Activity of CDKs and Cyclins is furthermore controlled by a range of positive and negative regulators such as E2F-DP transcription factors (adenovirus E2 promoter-binding f factor and E2F dimerisation partner (DP), KRP-proteins (kinase inhibitor-related proteins), DELs (E2F-like proteins), and WEE (mitosis inhibitor protein kinase) (De Veylder *et al*., 2001; Mariconti *et al*., 2002; Sorell *et al*., 2002; Kosugi and Ohashi, 2003; Gonzales *et al*., 2007; Verkest *et al.*, 2005b; Vlieghe *et al*., 2005; Bisbis *et al*., 2006; Sozzani *et al*., 2006; Ramirez- Parra *et al*., 2002, 2004).

Expression of cell cycle genes is known to be tissue non-specific, and moreover cell-cycle genes may be involved in a wide range of cellular and developmental processes that are not obviously linked to the cell cycle (Stals *et al*., 1997; Riou-Khamlichi *et al*., 1999; Menges *et al*., 2002; Polko, 2012). Lemaire-Chamley *et al*. (2005) performed a comparative analysis between developing fruit and other plant organs, and showed that most genes active in the fruit are not exclusively expressed there. However, in particular developmental processes in different plant organs, timing and intensity of cell cycle gene activity may differ (Menges *et al*., 2002; Lemaire-Chamley *et al*., 2005).

Cells in developing fruit have evolved a unique gene expression program that reflects their unique functions as compared to cells in other organs (Gillaspy *et al*., 1993).

Because we study the development of tomato fruit and in particular of the pericarp, we analysed how the core cell cycle regulators are expressed in the early developmental stages of the pericarp and compared their expression patterns with those in other tomato plant organs. The study of these genes during fruit development may lead to new views on the role of these genes in the development of fleshy fruits and fruit quality.

Previous reports presented the expression of cell cycle regulatory genes in whole tomato fruits at selected stages of development (Joubès *et al*., 1999, 2000; Lemaire-Chamley *et al*., 2005; Mintz-Oron *et al*., 2008). However, these data provided information about the overall expression level of candidate genes, were obtained by semi-quantitative RT-PCR performed or were obtained with only a limited number of growth stages. In this thesis we aim to study the cell dynamics (cell division, expansion, endoreduplication) in the pericarp, and the expression and function of cell cycle genes.

Furthermore, we made a selection of cell cycle candidate genes, which could play a role in

pericarp development, and their function was studied and described in chapters 3-7 of this thesis. Moreover, we investigated the suitability of the promoter of an early fruit-specifically expressed gene (TPRP/Tfm7) for its use in modification of cell cycle gene expression during tomato fruit development. For this purpose, we analysed the activity of this promoter using transgenic plants expressing the β-glucoronidase (*uidA*) reporter gene under control of the TPRP promoter (*pTPRP-GUS* plants). Additionally, the promoters of the *CDKA1* and *CDKB2* genes were characterized in detail during tomato plant development and their activities presented in this chapter.

Results and Discussion

Cell division and expansion in the growing pericarp

Because we study the development of tomato fruit and in particular of the pericarp, we analysed how the size distribution of cells in the pericarp changes from fertilization to the mature green stage. The diameter of a fruit increases due to active cell division and subsequent cell expansion in all fruit tissues. However, to a large extent this increase depends on the growth of the pericarp, which represents approximately 50% (w/w) of the ripe fresh weight (Mounet *et al*., 2009).

We collected ovaries and fruits and used them for two purposes: for total RNA extraction and analysis of transcript levels, and for microscopic analysis, which enabled us to link these histological data to the gene expression data.

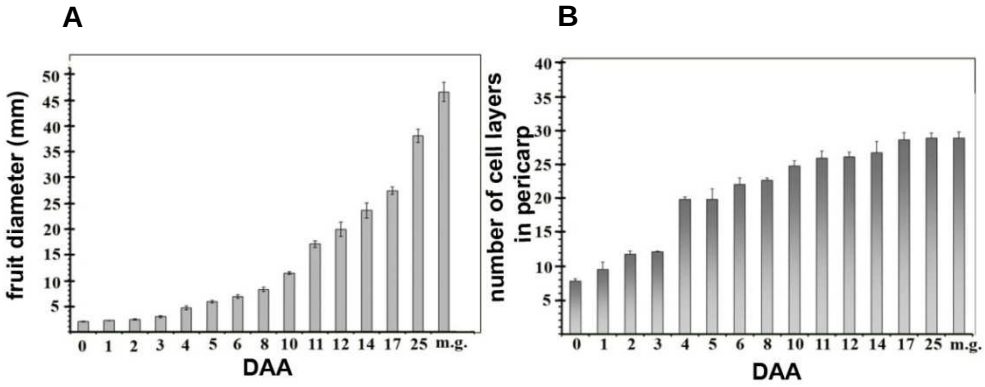

Figure 1. Dynamics of fruit growth and increase of cell layers in the pericarp

(A) Fruit diameter at different times after anthesis. DAA- days after anthesis.
(B) Increase of number of cell layers in growing tomato pericarp at different days after anthesis DAA).

The increase of the fruit diameter and increase of the number of cell layers in developing pericarp are depicted in Figure 1A and B respectively. The detailed microscopic analysis of the pericarp at different developmental stages is presented in Figure 2.

The tomato fruit of cultivar M82 increases in size 20-fold from anthesis up to mature green stage (Figure 1A). At anthesis the ovary diameter was 1,8-2 mm and possessed 8 cell layers, which was similar to the size measured by different tomato cultivars (Cheniclet *et al*., 2005; De Jong, 2010) .

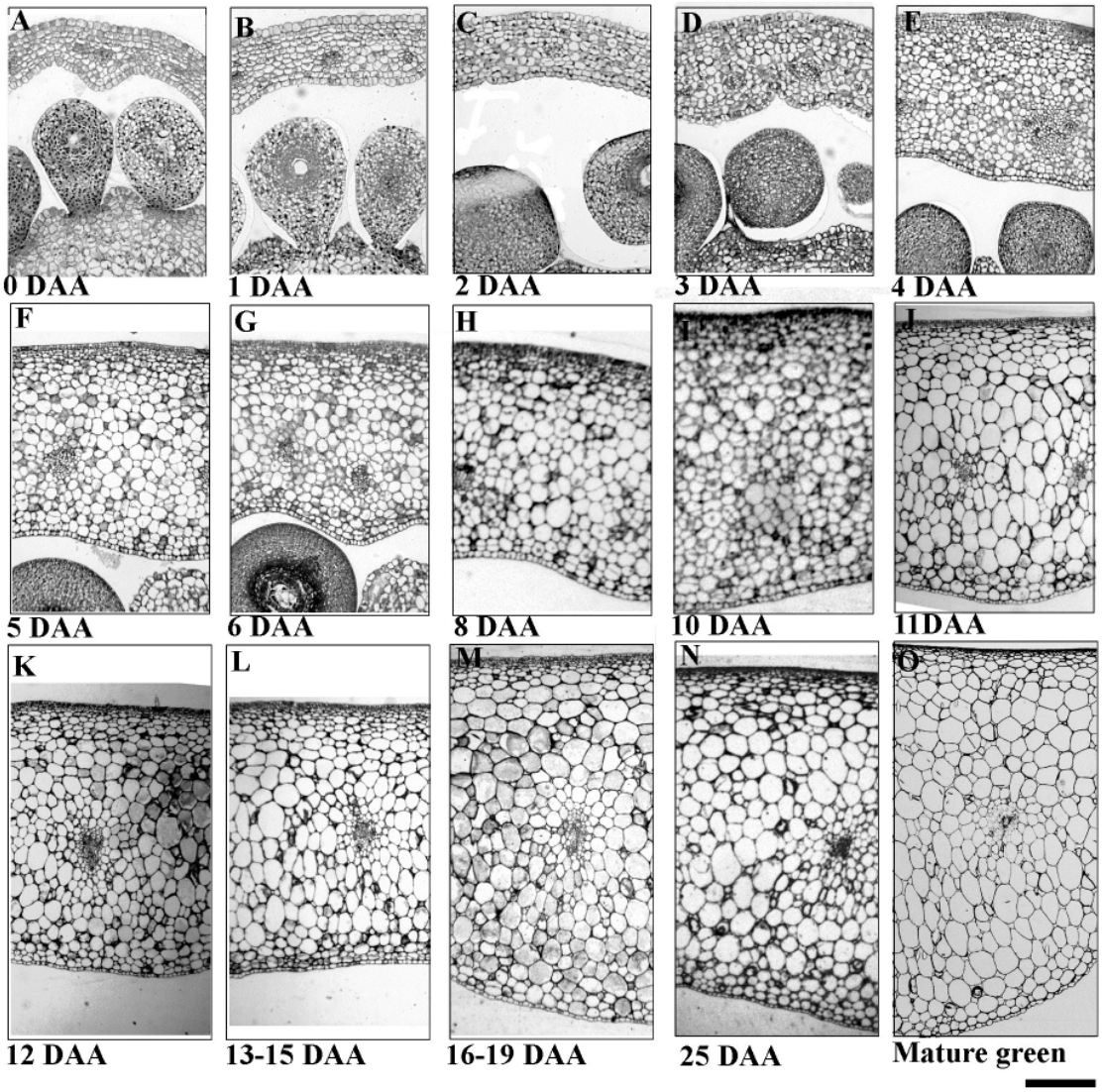

Figure 2. Microscopic analysis of the pericarp during fruit development (cv. M82), Bar=1 mm.

At the early stages from anthesis to 12 DAA, cells in the whole pericarp were actively dividing and created new cell layers. After 12 DAA, new layers of pericarp were mostly created due to division activity of exocarp cells only. A rapid 2-fold increase in number of cell layers took place between 3 and 5 DAA. At this particular stage most cells in all pericarp layers were able to divide. At later stages the increase in cell layers was much slower, from on average of 20 layers at 5 DAA to average of 30 layers at the mature green stage.

In contrast to the results of Cheniclet *et al*. (2005) we observed that the process of cell division continued through the expansion phase up to 25 DAA. The new cells were produced by periclinal cell division in the subepidermal layers of the exocarp until the mature green stage. We analysed the changes in cell size at different days after anthesis. The data are depicted in Figure 3.

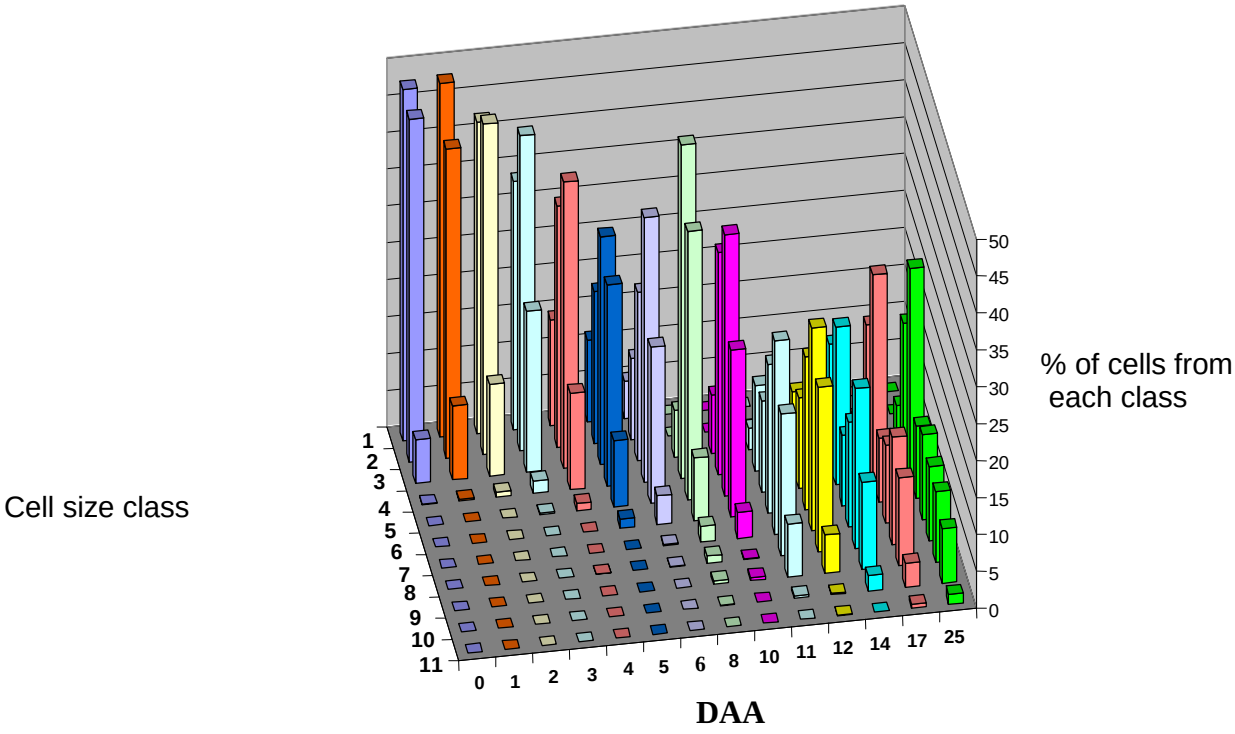

Figure 3. Cell size distribution in developing tomato mesocarp.
Cell numbers were pooled according to size in eleven different size categories, with category 1 having an average size of up to 0.4 x 10^{-3} mm^2 and the maximum cell size in each subsequent category doubling each time.

Most cells in the pericarp expanded substantially during fruit growth and could reach diameters of 200 µm and beyond. During the first 3-4 DAA developmental stages the cell size increases only slightly, while in consecutive stages the mesocarp cells enlarge exponentially.

Nevertheless, cell expansion occurs already from the earliest stages onwards, indicating that the phases of cell division and cell expansion cannot be separated completely. This detailed study of pericarp development was used to link candidate cell cycle gene expression to the cellular processes.

Expression patterns of candidate genes in developing tomato pericarp

Plants contain a number of cell cycle regulators for which overlap or differentiation in function remains largely unclear. Most data referring to the expression of cell cycle regulators were obtained from *Arabidopsis thaliana* and *Nicotiana tabacum.* However, we should take into account that the development of tomato fruit, because of its structure, is clearly different from the development of Arabidopsis siliques or tobacco capsules. We selected genes potentially involved in cell cycle control and endoreduplication (Table 1).

Table 1. Overview of genes used for the experiment

Gene name	Gen Accession number
cycA1;1	AJ243451[a]
cycA2; 1	AJ243452[a]
cycB1;1	AJ243454[a]
cycB2;1	AJ243455[a]
cycD1	SGN-U325387[b]
cycD2	SGN-U330045[b]
cycD3;3	AJ002590[a]
cdkA1	Y17225[a]
cdkA2	Y17226[a]
cdkB1;1	AJ297916[a]
cdkB2;1	AJ297917[a]
DP	SGN-U223495[b]
E2F	SGN-U572319[b]
RBR	SGN-U223495[b]
DEL	SGN-U221655[b]

[a] GenBank accession number;

[b] SOL Genomics Network EST identifier, http://www.sgn.cornell.edu

Our choice was based on the available knowledge about the regulation of the plant cell cycle in other plant species (Doerner *et al*., 1996; Riou-Kamlichi *et al*., 1999; Cockcroft *et al*., 2000; De Veylder *et al*., 2000, 2002; Hemerly *et al*., 1995; 2000; Porceddu *et al*., 2001; Schnittiger *et al*., 2002a, 2002b; Dewitte *et al*., 2003; Boudolf *et al*., 2004a, 2004b; Ebel *et al*., 2004; Leiva-Neto *et al*., 2004; Umeda *et al*., 2000, 2005; Vliege *et al*., 2005).

The temporal expression patterns of these candidate cell cycle genes during fruit growth from anthesis to mature green were investigated using quantitative RT-PCR on the same samples used for the morphological analysis presented in Figure 2. Figure 4 shows the expression results.

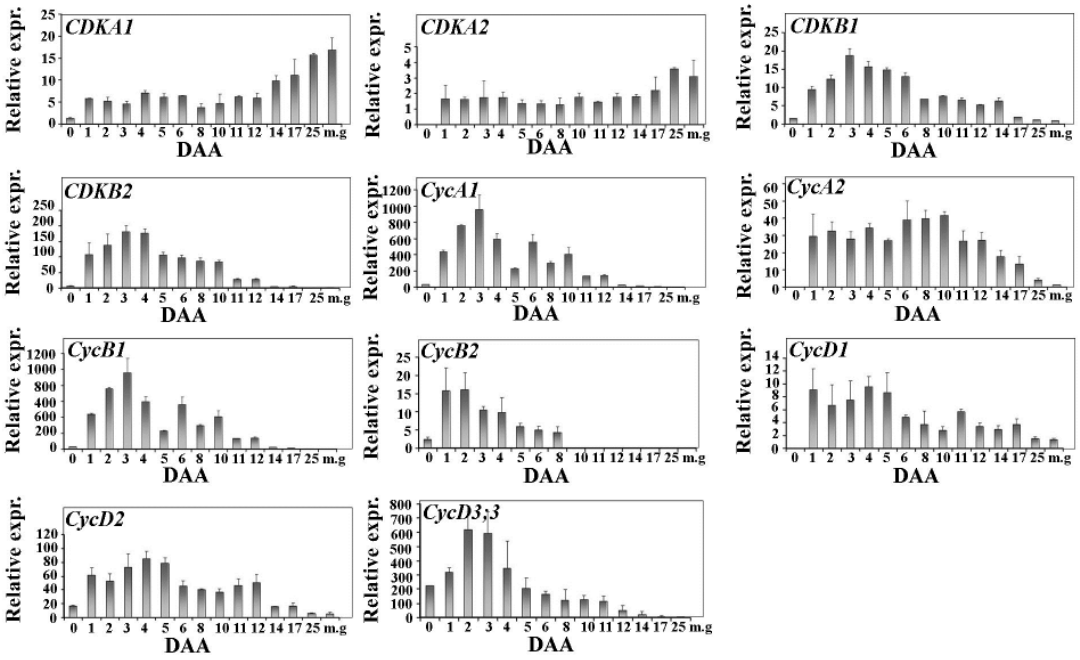

Figure 4. Expression pattern of cell cycle genes during fruit development.

Samples were collected at various days from anthesis (0) to mature green (m.g.). Data are means of two biological and two technical replications ± SE (standard error).

Additionally, we performed qRT-PCR experiments to check the level of gene expression in different plant tissues: 2-weeks-old seedlings, flowers and fruits of tomato plants. Results are shown in Figure 5.

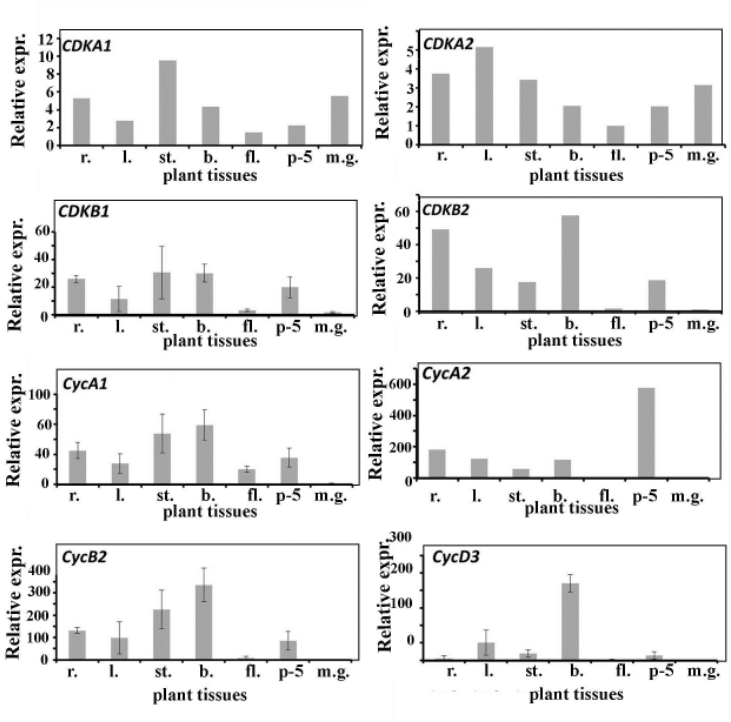

Figure 5. Expression of cell cycle genes in different tissues of tomato.

r. – root **l**.- leaf, **st. -** stem **b.** – flower bud, **fl.** – flower, **p-5** – pericarp of 5 DAA fruit, **m.g.**- pericarp at the mature green stage.
Data are means of two biological replications ± SD standard deviation

Essentially, we obtained three distinct temporal expression patterns for the candidates genes in pericarp. One pattern is in which genes are expressed at higher level between day 1 and day 10 after anthesis (*CDKB1*, *CDKB2*, *CycA1*, *CycB1*, *CycB2*, *CycD1*, *CycD2*, *CycD3;3*); a second group in which expression is highest at very late stages from day 17 till mature green (*CDKA1* and *CDKA2*); and a third class, which expression is more or less uniform throughout pericarp development (*CycA2*).

High expression of genes from the first group pointed to their involvement in cell division and suggest their role in the early developmental stages of tomato tissues in the examined fruits (Figure 4 and Figure 5). Expression of *CDKB1*, *CDKB2*, *CycD1* and *CycD2* was remarkably high during the whole period of cell division with the highest peak at 3 to 5 DAA, when intensive cell division takes place (Figure 2 and Figure 4). Expression of *CDKB2* was reduced two-fold in the period between 6 to 11 days after anthesis, as compared to the expression peak at 3 and 4 DAA. In the cell expansion phase this reduction was more extreme, and finally the expression was only barely detectable after 19 DAA. The results obtained are similar to those reported by Joubès (1999) and De Jong (2010).

In growing tissues of tomato plants the highest level of expression of *CDKB2* was observed in buds and young roots (Figure 5). Comparable results were obtained by De Jong (2010) and Baldet *et al.* (2006), who found that these genes are especially involved in intensive cell division in meristems of plants.

Other genes from the first group – *CycA1*, *CycB1*, *CycB2* and *CycD3;3* seem not to be expressed at all, or at very low levels, during endoreduplication and expansion. Their expression peak was observed between 2 and 5 DAA, and afterwards their expression declined. As we described previously, at this time of pericarp development we observed the increase of pericarp thickness due to division activity of most of the cells in the pericarp (Figure 2). The analysed genes showed also high expression in seedlings. Additionally, these genes showed high expression in flower buds, which is in agreement with data from Dewitte *et al*. (2003), Baldet *et al*., (2006) and De Jong (2010).

The expression of *CycD1* and *CycD2* is reported here for the first time. According to our results CycD1 shares 76% amino acid identity with CycD1;1 of Arabidopsis (ID: 843357); and CycD2 shares 85% identity at protein level with CycD2;1 of *Nicotiana tabacum* (Appendix Figure 1 and Figure 2).

We assayed *CDKA1* and *CDKA2* from the second group of genes, which are expressed at a relatively constant level during the first days after anthesis and increased later around 12 to 14 DAA. Both *CDKA1* and *CDKA2* genes were also highly expressed in young plant tissues (Figure 5). In contrast to *CDKA1, CDKA2* seemed to be relatively stable expressed in all plant tissues. The expression data of both *CDKA*s suggested further analysis of these genes (see chapter 3 and 4 of this thesis) because this pattern hints to a role in cell expansion and endoreduplication.

From the third group of expression patterns we presented here only *CycA2*, which was highly expressed during the cell division phase (1 to 11 DAA) and at the onset of the expansion stage (12 to 14 DAA), after which expression decreased but was still well detectable until the mature green stage. In young tissues of two-week-old plants the expression of *CycA2* was 7-fold lower than in pericarp.

Other candidate genes, which we listed in Table 1 – *DP*, *E2F*, *RBR* and *DEL* were analysed and belong to the third group of expression patterns (data not shown). Our analysis of expression patterns of candidate genes was done in parallel with histological analysis of pericarp development and gave us the directions for further investigations on the effect of modifying the expression of the selected candidate genes on fruit development.

Analysis of fruit specific and cell cycle genes promoters

TPRP-F promoter

Tomato Proline-Rich Proteins (TPRPs) are structural cell wall proteins, which are particularly expressed in growing tomato fruits (Salts *et al*., 1992). The *TPRP-F1* promoter was suggested to be useful for studying early fruit development (Salts *et al*., 1991,1992; Davuluri *et al*., 2005; Fernandes *et al.*, 2009).

We transformed a *pTPRP-GUS* vector (gift of Lisette Nitsch) into cv. M82 to analyse the activity of the *TPRP* promoter. After transformation, 17 regenerants were obtained and 6 of them showed high expression of the *uidA(GUS)* gene in fruit. We selected line #31 with the highest expression and analysed the activity of the *TPRP* promoter at different developmental stages and in different tissues of the fruit, as well as in leaves, stems and roots. We performed an analyses of GUS enzyme activity and gene transcript levels, of which results are shown in Figures 6, 7 and 8. Already after 3 hours incubation in the GUS substrate we observed the indigo color in fruits of different sizes, indicating high expression (Figure 7). Expression was detected at the base of flower buds (receptacle) before ovary fertilization and in fruit placenta, pericarp, gel and ovules all the way to the mature green stage. In ripe fruits, the *TPRP* promoter was still active in the seeds and in some vascular bundles. We did not observe any signal in other parts of the plant (root, stem, leaf), even not after 24 hours of GUS staining (Figure 8).

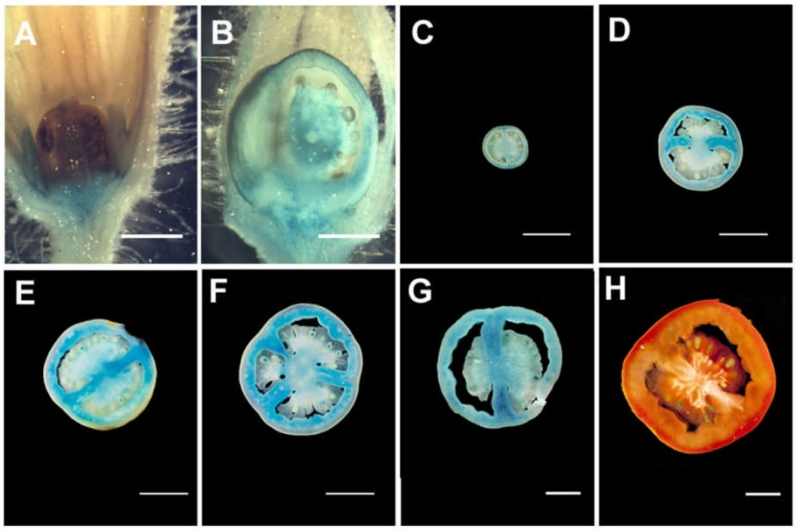

Figure 6. Expression of *uidA(GUS)* gene in tomato fruits transformed with a *pTPRP-GUS* vector.
GUS substrate incubation was performed for 3 hours.
(A) Ovary in flower bud before pollination. Bar = 1 mm
(B) Ovary at the day after pollination. Bar = 1mm
(C), (D), (E), (F), (G), (H) Different developmental stages. Bars = 1 cm

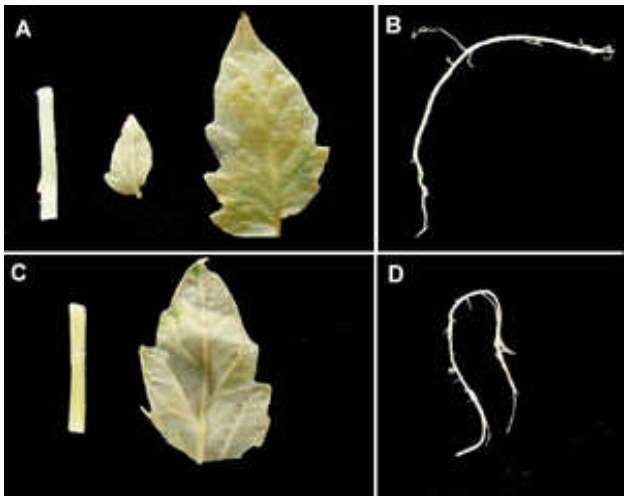

Figure 7. Results of GUS-assay on stems, leaves and roots of transgenic **(A), (B)** and wild-type **(C), (D)** plants. No activity was detected.

We verified these results by performing qRT-PCR analysis of *uidA* expression on the samples from pericarp, placenta and gel, isolated from the fruit at different stages. qRT-PCR analysis was also performed for different plant tissues: young roots, developing leaves and stems. In accordance with the data presented in Figure 8, the *TPRP* promoter was found to be highly active in young fruits and to a lesser extent in fruits in the mature green stage. Although we did not observe any indigo color in GUS assays, the qRT-PCR analysis showed a low level of *uidA* expression in other organs of the plant. In the tomato fruit the highest expression of *uidA* was observed in pericarp and placenta and to a lesser extent in gel (Figure 8B). The highest expression in tomato fruit was observed 10 to 15 days after anthesis, which confirmed the results obtained in the GUS assay.

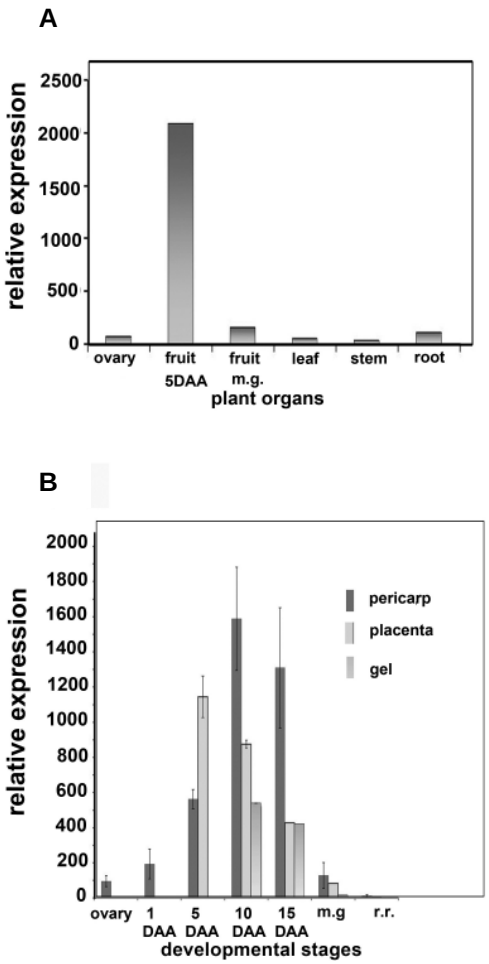

Figure 8. Expression of *uidA* (GUS) gene in transgenic tomato plants.

A. *uidA* expression in different plant organs and fruits in not pollinated ovary, 5 DAA and mature green. Experiment was performed twice and biological replicates showed the same trend but the absolute amounts were different. Therefore, only one biological replica is presented and error bars were omitted in the graph.

B. *uidA* expression in different fruit tissues during development. Data are means of two biological replications ± SD (standard deviation).

m.g.- mature green
r.r.-red ripe
DAA- days after anthesis

These data showed that the use of this promoter aiming to change the expression of cell cycle genes in fruits only, should avoid possible disadvantages associated with using constitutive promoters, which might also affect the vegetative plant tissues. Although qRT-PCR analysis showed some transcript accumulation of *uidA* under control of the *TPRP* promoter in vegetative plant parts, this expression was negligible compared to expression in the fruit, as was also clear in the analysis by GUS activity staining. In our research described in the next chapters we did not observe any aberrations in growth or development of plants tissues other than fruit.

CDKB2 promoter

In order to find markers to follow cell division and cell expansion in tomato fruits *in vivo* we made reporter constructs composed of cell-cycle gene promoters and *uidA*. The ORF of the *CDKB2* gene was identified by BLASTN on a BAC and the *CDKB2* promoter was amplified from this BAC clone. The isolated fragment, containing 2.4 kb preceding the transcriptional start site, was amplified and we assumed that the amplified fragment should possess all regulatory elements needed to drive proper transcription of the reporter gene.

Cotyledons of seedlings from tomato cultivar Ida Gold were transformed by *Agrobacterium tumefaciens*, containing the vector *pCDKB2-GFP-GUS.* Because the ensuing callus is a tissue composed of actively dividing cells we tested it for GUS activity by substrate incubation for 2,5 hours. Interestingly, an intense blue colour observed in this tissue was in agreement with the activity of the *CDKB2* promoter during cell division (Figure 9A).

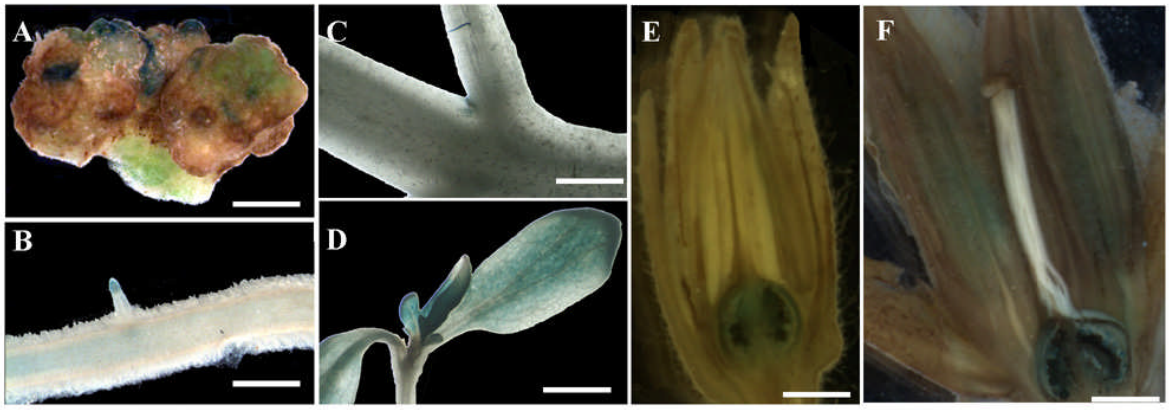

Figure 9. Expression of uidA (GUS) gene in tissues of transgenic tomato transformed with the *pCDKB2-GFP-GUS* vector.

(A) Callus from shoot induction medium during transformation procedure, **(B)** Root from a plantlet on the root induction medium; **(C)** and **(D)** Cotyledon 1 week old, **(E)** Flower 2 days before anthesis. The flower was emasculated, **(F)** Flower 1 day after pollination. Bar = 1 mm.

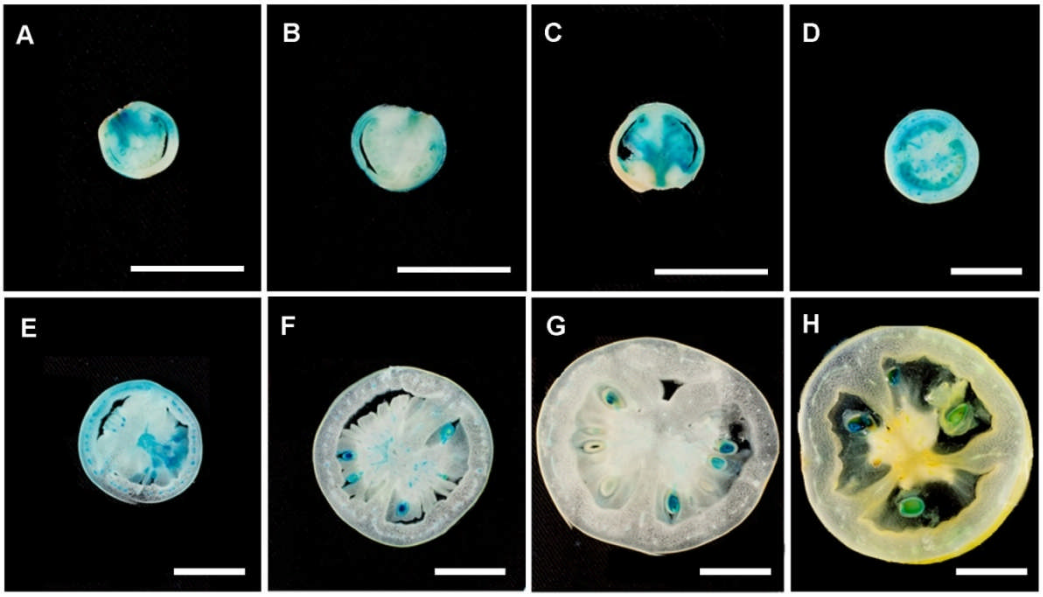

Figure 10. Expression of *uidA (GUS*) gene in tomato fruit transformed with *pCDKB2-GFP-uidA*. Different developmental stages **(A)-(H).** Bars = 1 cm.

In the obtained transgenic tomato plants, we analysed activity of the promoter in ovaries before pollination, in ovaries 1 day after pollination (Figure 9 E and F), in T1 seedlings (Figure 9 B, C and D), and at different developmental stages during fruit growth (Figure 10). Five from seventeen analysed plantlets showed strong expression of *uidA* in fruit tissues during early stages of fruit development. Furthermore, the expression was observed in cells surrounding vascular bundles in fruits at any stage until mature green. Activity of the *CDKB2* promoter was also observed in seeds of ripe fruits.

Our detailed analysis of *CDKB2* promoter activity in *pCDKB2-GFP-uidA(GUS*) transgenic plants confirmed the results of gene expression pattern, obtained by qRT-PCR (Figure 4 and Figure 5). The activity of the *CDKB2* promoter in seeds of the ripe fruit was surprising. This may indicate a particular involvement of *CDKB2* in seed development. The *CDKB2* promoter was also active till ripening in vascular bundles, which is not surprising as division activity continues in this tissue.

CDKA1 promoter

The *CDKA1* promoter was also a candidate for a marker for cell division or expansion *in vivo*. We amplified the promoter using a plasmid containing the end-BAC sequence SL_EcoRI0063D22 as a template, because only these sequence was known at the time we performed investigation. The promoter was amplified using inverse PCR and subsequently sequenced.

The amplified fragment was 4 kb and proceeded the transcriptional start site of *CDKA1* gene. The obtained vector *pCDKA1-GFP-GUS* was transformed by *Agrobacterium tumefaciens* mediated transformation of cotyledons of tomato cultivar Ida Gold. Also here, we observed the activity of the *CDKA1* promoter already in dividing cells of callus. The blue colour after 2 hours of substrate incubation indicated high activity of the promoter (Figure 11 A and B).

We obtained 12 transformants in which the presence of the *pCDKA1-GFP-GUS* construct was detected by PCR analysis. The activity of the *CDKA1* promoter was analysed in ovary and fruit tissues at different stages of development (Figure 11 D, E and Figure 12). Figure 12 shows that the expression of *uidA* under the control of the *CDKA1* promoter in tomato pericarp has a pattern similar to that shown by qRT-PCR analysis of the gene (Figure 4). *uidA* (*GUS*) was highly expressed in tomato pericarp and vascular bundles. The expression was already detected before fertilisation in ovules (Figure 11 **D** and **E**) and highly induced after fertilisation (Figure 12).

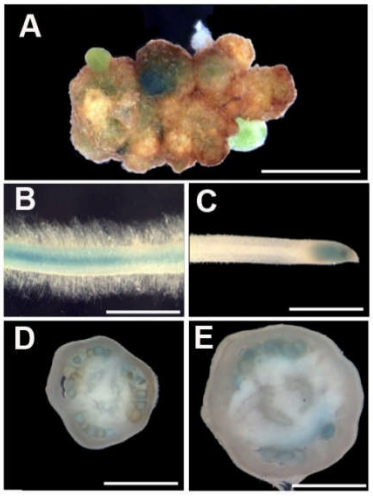

Figure 11. Expression of *uidA*(GUS) in different tissues of transgenic tomato.

(A) Callus on medium
(B) Root on medium
(C) Root tip on medium
(D) Ovary 3 days before pollination (flower was emasculated)
(E) Ovary 1 day after pollination

Bars in all cases = 1 mm

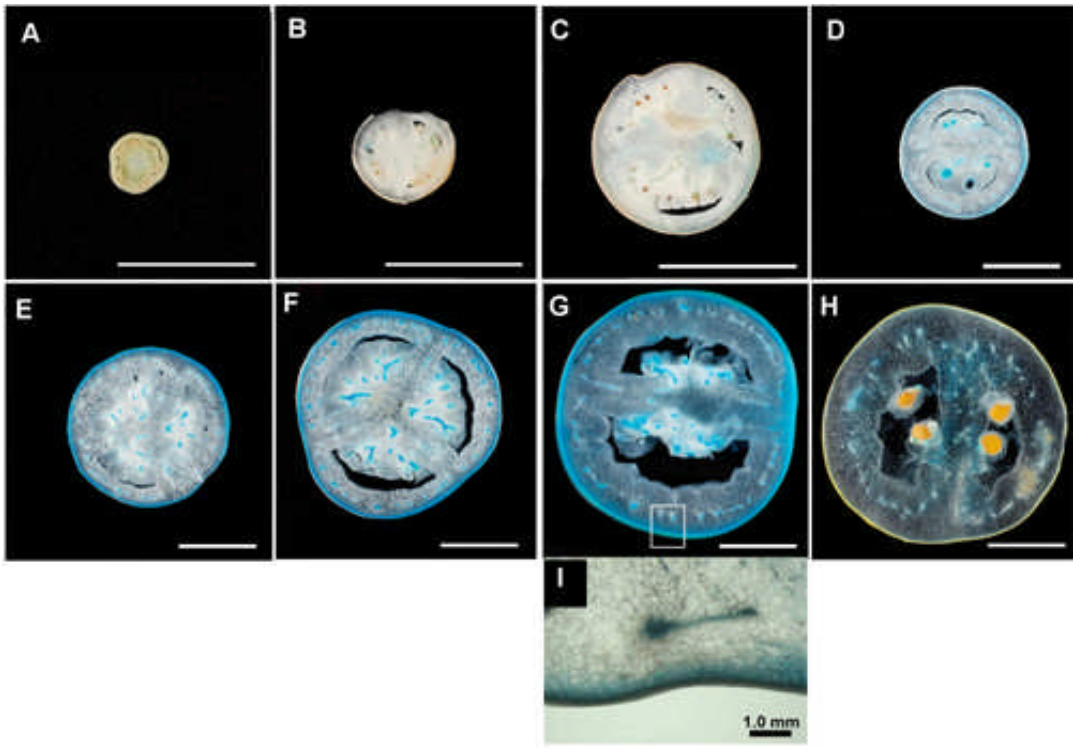

Figure 12. Expression of the *uidA* gene in tomato fruit transformed with the *pCDKA1-GFP-GUS* vector.

Different developmental stages **(A)-(H).(I)** is an enlarged section of pericarp from the picture **G.**
Bars = 1 cm, except for **(I)** bar =1 mm.

The analysed promoters of *CDKA1* and of *CDKB2* seem not to be useful for our approach to monitor (and distinguish) cell division and cell expansion *in vivo* in growing tomato pericarp because they both showed expression throughout the whole course of fruit development.

Nonetheless, the obtained results gave new information about the expression pattern of *CDKB2* and *CDKA1* in tomato tissues during development. These data confirmed our results from the qRT-PCR analysis, and were helpful for our investigation on the changes in fruit development due to overexpression and knock-down of *CDKA1* and *CDKB2* (Chapter3, 4 and 6 of this thesis).

Conclusions

Altogether, the results presented in this chapter show the dynamic changes at cellular level during tomato fruit development and expression of genes that are involved in cell division, expansion and endoreduplication. This knowledge, these genes and their promoters were our toolbox for further detailed studies on the early stages of fruit development.

Material and methods

Plant material and growth conditions

Tomato plants *Solanum lycopersicum*, cv. M82, cv. Ida Gold and obtained transgenic lines were grown on soil in a greenhouse under a 16-h of light and 8-h of dark conditions. Supplementary lights (600 Watt high pressure sodium lights) turned on below 200 W/m^2 and turned off above 300 W/m^2 solar irradiation. Temperature was kept above 20^{0}C during the light period and 17^{0}C during the dark period controlled with the PRIVA Integro version 724 system. Plants were watered daily and given fertilizer weekly. For cultivar M82 additionally was applied Osmocote, to prevent blossom-end rot. To avoid the differences in source-sink balance affected by fruit number and position (Bertin *et al*., 2001) we left at each truss 5 fruits, while the other pollinated flowers were removed. The first developing truss form each plant was also removed.

For *in vitro* culture, seeds were surface sterilized by treatment with 70 % ethanol for 1 min and with 2.5 % hypochlorite solution for 20 min. After rinsing six times with sterile distilled water, seeds were spread on 100 ml of Murashige and Skoog (MS) culture medium, containing 1x Nitsch and Nitsch vitamins mixture, 1.5% w/v sucrose, 0.5 % MES and 0.7% w/v phytoagar, pH 5.7. Transgenic seeds were selected on MS culture medium, containing 100 mg L^{-1} kanamycin. Germination and seedling growth took place in a growth chamber with a 16 h light period (photosynthetic photon flux density 50 µmol m^{-2} sec^{-1}) and 8 h dark period at a constant temperature of 22 ^{0}C.

For mRNA isolation in experiments for measuring expression patterns during the whole course of early fruit development we collected the second and third fruit from the second truss on the plant. For each stage, fruits from two plants were collected. All collected tissues were frozen in liquid nitrogen and stored at -80^{0}C until RNA extraction.

Real-time quantitative PCR

Total RNA was extracted with TRIzol Reagent from the frozen tomato plant tissues 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 done to equilibrate the RNA concentrations of different samples and concentrations were corrected by comparing the amount of total RNA on an 1,5% agarose gel.

The absence of genomic DNA was checked by PCR using specific primers that amplify an intron fragment from tomato actin gene *TOM 51* (SGN-U60481) only.

RNA (0,5 µg) was reverse transcribed (RT) in a total volume of 10 µL using a cDNA synthesis kit (iScript ^{tm}, Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's protocol. For real-time quantitative PCR, 5 µL of 25-fold diluted cDNA were used in a 25 µL PCR reaction, containing 400 nM of each primer and 12,5 µL iQ-SYBR Green Supermix (Bio-Rad Laboratories). The PCR reactions were performed in a 96 well Bio-Rad iCycler (Bio-Rad Laboratories, CA, USA).

Real-time–quantitative RT-PCR (qRT-PCR) primers were designed using Beacon Designer Software (Premier Biosoft International, CA, USA). Primer pairs are listed in Table 3. PCR reactions were done using SYBR green mix (iQ-SYBR Green Supermix, Bio-Rad Laboratories) and performed in a 96-well thermocycler (Bio-Rad iCycler). Five microliters of 20-fold diluted cDNA was used per sample. Technical and biological replications were always performed. As reference genes were used *Le-Actin* 2/7 (SGN-U107674) and *Le18S* (SGN-U107674).

Cloning of the *CDKA1* promoter

The coding sequence (CDS) of *CDKA1* gene (CDKA1 core nucleotide, gi|3123614|emb|CAA76700, SGN-U315006) was used to search by BLASTN**,** the SGN tomato BAC sequences database for matches**.** The BLASTN search results identified several BAC-end sequences, partially matching fragments of the *CDKA1* CDS. Two BACs (SL_EcoRI0063D22 & LE_Hba0238N17), possibly containing the *CDKA1* promoter, were selected for further investigation and ordered at the USDA-ARS (United States Department of Agriculture-Agriculture Research Service), Boyce Thompson Institute, Ithaca, NY .

The plasmids possibly containing the *CDKA1* promoter were isolated from the bacteria. Since the promoter sequence was not known we were unable to design primers to perform a direct isolation of the promoter fragment and used inverse PCR to circumvent this problem. Following normal procedures inverse PCR is used to identify flanking sequences of a known single DNA fragment. Inverse PCR consists out several successive steps. During the first step the target DNA is slightly digested into fragments of a few kilobases.

In the next step these fragments are promoted to self-ligate, producing numerous circular DNA fragments of which only one specimen contains the known DNA fragment. Finally a successive sequencing reaction, using primers designed to anneal to the known sequence, can retrieve the unknown sequence flanking the known fragment.

After digestion a self-ligation reaction was initiated, creating circular DNA fragments. Finally the PCR reaction was performed using a forward primer (BB-CDKA1-inverse-R) located near the 3' end of the CDKA1 CDS sequence overlapping with the BAC-end and a reverse primer (BB-CDKA1-inverse-F) located near the 5' end of the CDKA1 CDS outside the region of the known BAC-end region. The self-ligation products acted as DNA templates in the inverse PCR.

The amplified fragment 4kb containing *CDKA1* promoter was cloned in the pGEM-T-Easy vector and subsequently resulting plasmids were transformed to DH5α cells. The transformation mixture was placed on LB medium containing the antibiotic ampicillin (100 μg/ml) and additives X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) and IPTG (Isopropyl-β-D-Thiogalactopyranoside), facilitating blue/white screening. Colonies were grown overnight at 37^{o}C. Successively a colony PCR reaction was performed on a selection of the positive (white) colonies using the M13-F & R primers. Colonies whose amplified fragment was 4kb were identified as positives. Plasmids were isolated with QIAprep Spin miniprep kit. Samples were prepared for sequencing according to a standard protocol.

Isolation of the *CDKB2* promoter

The tomato *CDKB2* cDNA sequence was taken from the NCBI database (http://www.ncbi.nlm.nih.gov/, *CDKB2* core nucleotide gi|11125684|emb |AJ297917.1, SGN-U323897) and was used to search (BLASTN) the SGN tomato BAC sequences database for matches (http://www.sgn.cornell.edu/index.pl). The BLASTN results indicated that the SL_MboI0059M16 BAC (also identified as C04SLm0059M16) contained the full sequence of the *CDKB2* gene.

The SL_MboI0059M16 BAC was ordered at the USDA-ARS (United States Department of Agriculture-Agriculture Research Service), Boyce Thompson Institute, Ithaca, NY. *E. coli* DH10B cells carried the BAC recombined with the pEC BAC1 fertility plasmid.

Primer set BB-BAC-CDKB2-1F & BB-BAC-CDKB2-3R was specifically designed to include the promoter region close up to the start codon, where translation is started. Primer nucleotide sequences were based on the sequence of the SL_MboI0059M16 BAC. Additionally the forward primer was modified by adding the 4 nucleotides CACC at its 5' end to promote the directional cloning of the fragment in the PENTR/D vector. A PCR was performed by annealing temperature 62^{o}C and extension time 1'30" (with expected fragment size 2397bp), using SL_MboI0059M16 BAC DNA as a template.

The PCR product was cleaned from the agarose-gel using the Promega PCR clean-up system and this cleaned DNA fragment was cloned into a pENTR/D vector using the pENTR/D-TOPO cloning kit from Invitrogen. Finally CDKB2 promoter was cloned into pKGWFS7 vector, which contains both the *eGFP* (enhanced Green Fluorescence Protein and the GUS (*uidA*, β-glucuronidase) reporter genes (Karimi *et al.*, 2002).

Bacteria and plant transformation

To generate transgenic lines containing *pTPRP-uidA*, *pCDKA1-GFP-uidA* and *pCDKB2-GFP-uidA* vectors were transferred into *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw transformation protocol (Chen *et al.*, 1994).

Transgenic tomato plants were generated by *Agrobacterium tumefaciens*-cocultivation of seedling cotyledons, as described in De Jong *et al.* (2009b). Plants were selected on kanamycin-containing medium and then checked by PCR with primers specific partially for TPRP-promoter and gene of interest on genomic DNA. Subsequently, lines were tested for ploidy, as only diploid lines were used for further analysis.

Histochemical analysis of GUS activity

Analysed tomato fruit were sliced into sections of 500 µm or divided into small sections in case of leaves, stem and roots, and submerged in GUS-staining buffer containing 0.1 % Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10mM EDTA, 1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl ß-D-Glucuronide (X-Gluc), 0.1 mg ml^{-1} in 50 mM phosphate buffer, pH 7.5. After incubation at 37 ^{0}C, the tissues were steadily cleared with 70 % ethanol on 30 min, 80 % ethanol on 30 min, 90 % ethanol on 30 min, 96 % ethanol on 30 min, 80% ethanol on 30 min and 70% ethanol on 30 min. The tissues were viewed under a stereomicroscope (Leica MZFL III, Leica Microsystem, http://www.heraeus-kulzer.com). Images were made with a Leica digital camera (model DFC 420C; Leica microsystems).

Quantification methodology of cell area and number of dividing cells

The mean size of pericarp cells was determined in cross sections of parenchymatous (not vascular) parts of the mesocarp. The most outer and inner layers of pericarp as well as vascular bundles were excluded from these measurements.

For the cell distribution studies pericarp samples were embedded in Technovit and thin sections (7-10 µm) were made and stained with toluidine blue. Pictures were taken with 1.0 and 10x time objective. Contrast was manually adjusted in Photoshop 7.0 to enhance cell wall recognition. Digital images of sections were analysed using ImageJ (Rasband, 1997-2008, http://rsb.info.nih.gov/ij/) in combination with two plug-in macros.

With the first macro the image was pre-processed using the "skeletonize" operation to highlight cell walls, which are subsequently overlaid onto the original image. Subsequently missing cell wall parts were manually edited using ImageJ's drawing tools. A second analysis macro identified and selected cells by inversion of the edited overlay image and subsequently used ImageJ's "analyse cells" operation to measure the cell size parameters. Cell areas smaller than 12,5 μm^2 or larger than 250μm^2 were excluded from the data, since they most likely represent intercellular spaces and cells that were disrupted. Mean cell size was calculated for each picture. Per line 4 pictures of different pericarp area were averaged and depicted with standard errors.

Acknowledgements

We are grateful to Dr. Lisette Nitsch from Wageningen University for sequencing of TPRP promoter and permission to use it in our research. We also like to thank Dr. Elisabeth Pierson and Mr. Dick van Aalst from Radboud University for their help with making pictures, Dr. M. de Jong (University of Cambrige, UK) and Mr. R. Feron (Enza Zaden, the Netherlands) for the primers of reference genes.

Appendix

Table 2. Sequences of primers

Gene name	Forward (F) and reverse (R) primer sequences
CDKA1	F 5'-AACCCCTGAATAGAACCAAATG-3' R 5'- GTATGTGCCGTGATTGTCTG-3'
CDKA2	F 5'-AAGAGAATCACTGCCCGAAG-3' R 5'- AACAGATTGGATGTCATTGGAG-3'
CDKB1	F 5'-ATGGAGAAATACGAGAAATTGGAG-3' R 5'- ACAGTGAAATATGAAAGTGACAAG-3'
CDKB2	F 5'-ATGCTGGTAAGAGTGTATCGG-3' R 5'- CGGAGAGTAGTTGGAGGAAC-3'
CycA1	F 5'-GCCAGGGAGATAATGTGAGAAG-3' R 5'- CAAACAAAGATGCTCTGCTAAGG-3'
CycA2	F 5'-ACGACTTGAAGTTCCCTGTG-3' R 5'- GTGATGCTTGAACGAATCTCC-3'
CycB1	F 5'-CGTTACTAGGAGGTCTGCTG-3' R 5'- CCTTTAGTTACAAGAGGCTTCG-3'
CycB2	F 5'-ATTCAATCTTGGAGAGGATTAAAG-3' R 5'- GTAGCCATTTCAGCCCTATC-3'
CycD1	F 5'- CACTTCCTGCTACTTCTTCTTC-3' R 5'- AACGGTATCGGAGTCTTCG-3'
CycD2	F 5'-GGATTTGAATCATAGAAGAGAAGC-3' R 5'- GACAGCAAGGGATAAACAGG-3'
CycD3;3	F 5'-CTTGTTGCTGTTACTTGTCTTTC-3' R 5'- AATGGTGTTACTGGATTCATCTTC-3'
BB-CDKA1-inverse	F 5'- GTTCCTTCCCCAATCTTCTCA-3' R 5'- GTATCTAGTGTTTGAATATCTTGACTT-3'
BB-BAC-CDKB2	F 5'- CACCCTGTCTTCCTTCTTGGTGATTAAGCATTGG-3' R 5'- CTTCACTCTCGCTGTGTTTGTG-3'
Le 18S	F 5'-AGACGAAACAACTGCGAAAGC-3' R 5'-AGCCTTGCGACCATACTCC-3'
Le Actin 2/7	F 5'-GGACTCTGGTGATGGTGTTAG-3' R 5'-CCGTTCAGCAGTAGTGGTG-3'
Tom 51	F 5'- GCTGTGCTTTCCTTGTATGC-3' R 5'- TCACACCATCACCAGAGTCC-3'

Figure 13. Alignments of identified Tomato CycD1 (SGN-U325387) and Arabidopsis CycD1;1 (ID: 843357)

```
LeCycD1      MSVSYSDCFSDLLCGEDSDTVFSNGRGEDLPECSSSDIESQFADIDESIAGLIEDEQNFV   69
AtCycD1,1    MSVSFSNDM-DLFCGEDSG-VFS---GESTVDFSSSEVDSWPGD---SIACFIEDERHFV   63

LeCycD1      PGFDYIEKFQSQSLSAAARDESVAWILKVQRHYAFQPLTAYLAVNYFDRFLYLRSLPQTNG  129
AtCycD1,1    PGHDYLSRFQTRSLDASAREDSVAWILKVQAYYNFQPLTAYLAVNYMDRFLYARRLPETSG  123
```

Figure 14. Alignments of identified Tomato CycD2 (SGN-U330045) and *Nicotiana tabacum* CycD2;1 (CAA09852.1)

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LeCycD2    LIDLLPCLSEESFSVMLEREKVFLPKDDYLKRLRIGDLDLNHRREAVNWIWKAHVHYGFG  61
NtCycD2,1  LIDL-PSLSEECLSFMVQREMEFLPKDDYVERLRSGDLDLSVRKEALDWILKAHMHYGFG  113

LeCycD2    ELSFCLSINYLDRFLSLYELPRGKIWTIQLLAVACLSLAVKMEEINVPLTVDLQVGEPKF  121
NtCycD2,1  ELSFCLSINYLDRFLSLYELPRSKTWTVQLLAVACLSLAAKMEEINVPLTVDLQVGDPKF  173

LeCycD2    LFEGITIQRMELLVLSTLRWRMQAYTPCTFIDYFMRKMNLDEFPSMRLVSRSIQLILSII  181
NtCycD2,1  VFEGKTIQRMELLVLSTLKWRMQAYTPYTFIDYFMRKMNGDQIPSRPLISGSMQLILSII  233

LeCycD2    KGIDFLEFRSSEIAAAVAMSVSWERPQAKDIDKAMSCFSIQVEKDRVMKCFELIQDLTLV  241
NtCycD2,1  RSIDFLEFRSSEIAASVAMSVSGE-IQAKDIDKAMPCFFIHLDKGRVQKCVELIQDLT--  290

LeCycD2    SGTSAATAAAAATSVPQTPNGVLE-AACLSYKSG-EGIVLSC---QNAKRRKLDTN     292
NtCycD2,1  --TATITTAAAASLVPQSPIGVLEAAACLSYKSGDERTVGSCTTSSHTKRRKLDTS     344
```

3

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 (Cyc) play a key role in the progression of the cell cycle. We have characterized the role of *CDKA1*, *CDKB1* and *CDKB2* in fruit development by fruit-specific overexpression and down-regulation. *CDKA1* is expressed in the pericarp throughout development, but is strongly upregulated 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 the *CDKB1* and *CDKB2* overexpression mutants, suggesting opposite roles of these types of CDK proteins in tomato pericarp development.

Introduction

Tomato fruit development starts with the reinitiation of ovary growth, induced by pollination and fertilization events (Picken, 1984; Gilaspy *et al*., 1993). During fruit development several phases can be recognized: initially the fruit diameter increases due to cell division activities, which amplify rapidly the number of cell layers in the pericarp, followed by a growth phase caused by cell expansion. The expansion phase is accompanied by endoreduplication, i.e. a multiplication of the genome without mitosis, leading to an increase of 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 (G1) to Synthesis (S) transition and at the Gap2 (G2) to Mitosis (M) transition (for review, 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 synthesis 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 (Cyc) subunit (Van Leene *et al*., 2010). Five families of CDKs (A-F) are known in plants, of which the *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 *CDKs* (Dudits *et al*., 2007). They 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 G1 to the S phase and from the G2 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* (Fountain and Beck, 2003; Lee *et al*., 2003; Boudolf *et al*., 2004a, 2004b; 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 of the mitotic cycle and entering in the endoreduplication cycle (Boudolf *et al*., 2004b).

Analysis of Arabidopsis *cdka;1* null mutants showed that *CDKA;1* is required for both the sporophytic as well as the male gametophytic generations. As sporophyte, 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 G2 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, we manipulated the expression of the *CDKB* genes 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 it suggests an antagonistic mechanism of control between the tomato *CDKA1* and *CDKB* genes.

Results

Expression of *CDKB1* and *CDKB2* genes

We analysed the level of *CDKB1* and *CDKB2* gene expression in the fruit pericarp of tomato variety M82 during various stages of fruit development from anthesis to the mature green stage. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) expression analysis is shown in Figure 1. *CDKB1* and *CDKB2* are both highly 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 around 4 days after anthesis (DAA) and 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 (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 we analyzed transgenic plants overexpressing these genes. Because we aimed for 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 Figure 6 and 7, Chapter 2) .

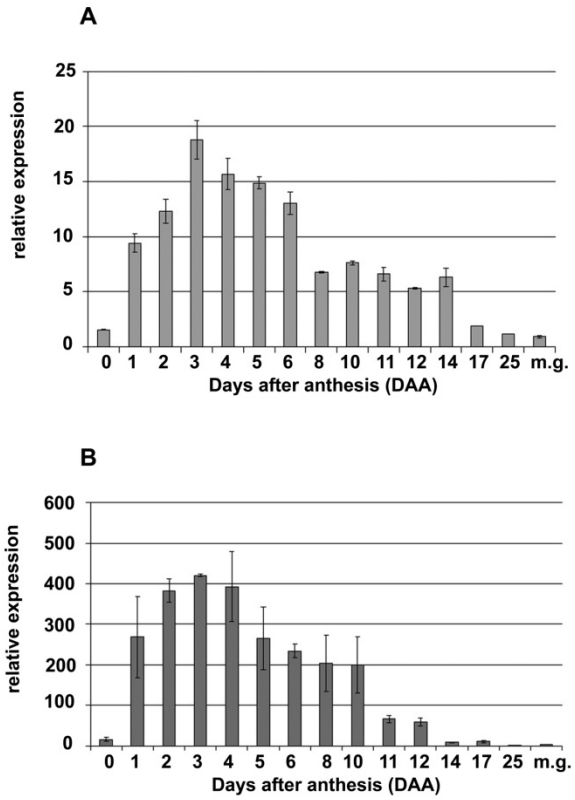


Figure 1. Expression levels of *CDKB1* and *CDKB2* determined in the pericarp of wild-type tomato fruits.

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.

From 43 generated primary transgenics containing the pTPRP-CDKB1 overexpression construct and 20 plantlets with the pTPRP-CDKB2 vector, we selected lines with the most upregulated expression of *CDKB1* and *CDKB2*. Four lines with *CDKB1* overexpression and 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, off-spring plants (T1) 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 to 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 more than 10-fold higher than in wild-type fruits, while during the early stages the transgene expression levels of the *CDKB* genes were hardly elevated (Figure 2). Therefore, we could expect phenotypic alterations in the transgenic fruits at later developmental stages.

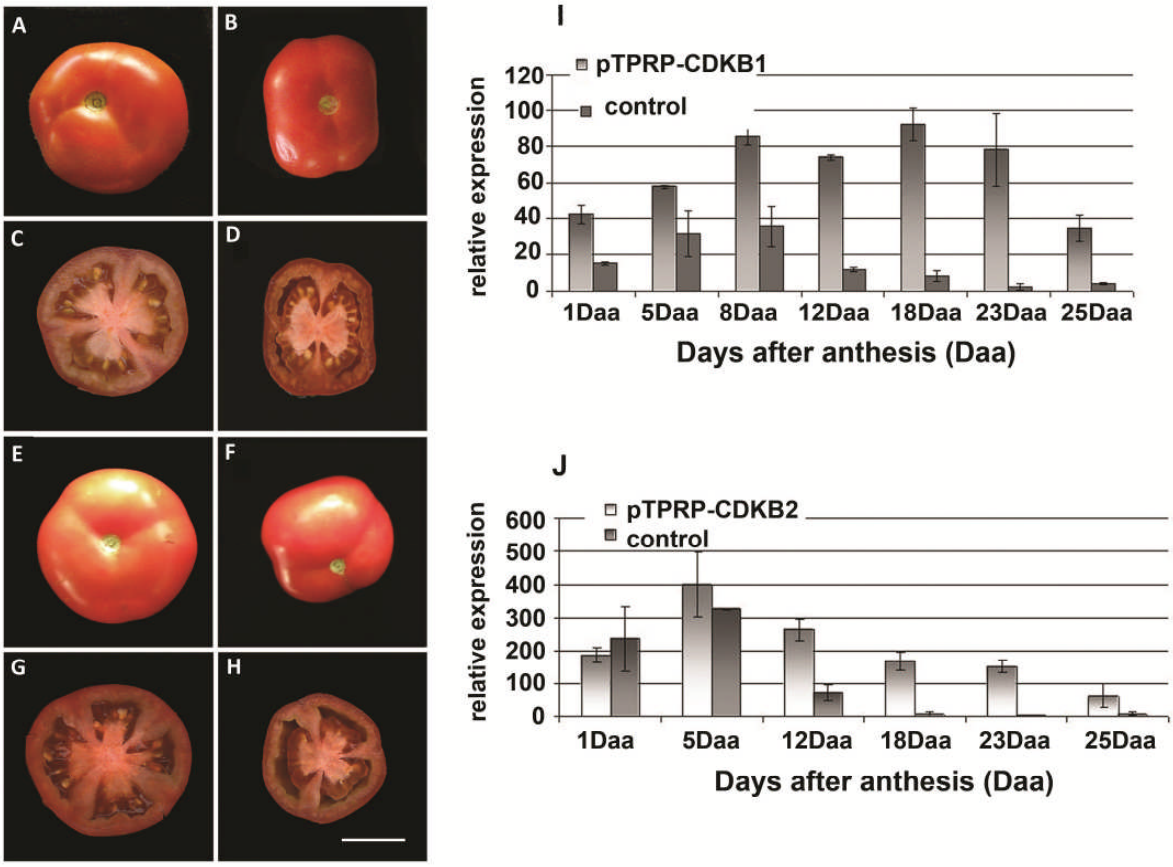


Figure 2. Phenotypes of control and *CDKB1* and *CDKB2* overexpressing fruits.

A and **C**, A fruit of a non-transgenic sibling derived from a *pTPRP-CDKB1* plant; **B** and **D**, A fruit of 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 pericarp of wild-type and transgenic *pTPRP-CDKB2* plants. Relative expression was determined by real-time Q-PCR. Data are averages of two biological pools of samples from 3 independent fruits per plant at different days after anthesis and the standard deviation (SD) is indicated.

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 similar 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 (Figure 2). We analysed several parameters of the fruits, such as weight, diameter, pericarp thickness, fruit firmness and the results of these quantitative analyses are depicted in Supplemental Figure 1. Both the weight and the pericarp thickness were significantly reduced in the transgenic fruits compared to non-transgenic control fruits.

Furthermore, we determined the fruit firmness at the red ripe stage and noticed a reduction of firmness of the fruits overexpressing *CDKB2* and to a lesser extent, but still significant for fruits overexpressing *CDKB1*.

CDKB overexpression affects cellular structure of the fruit

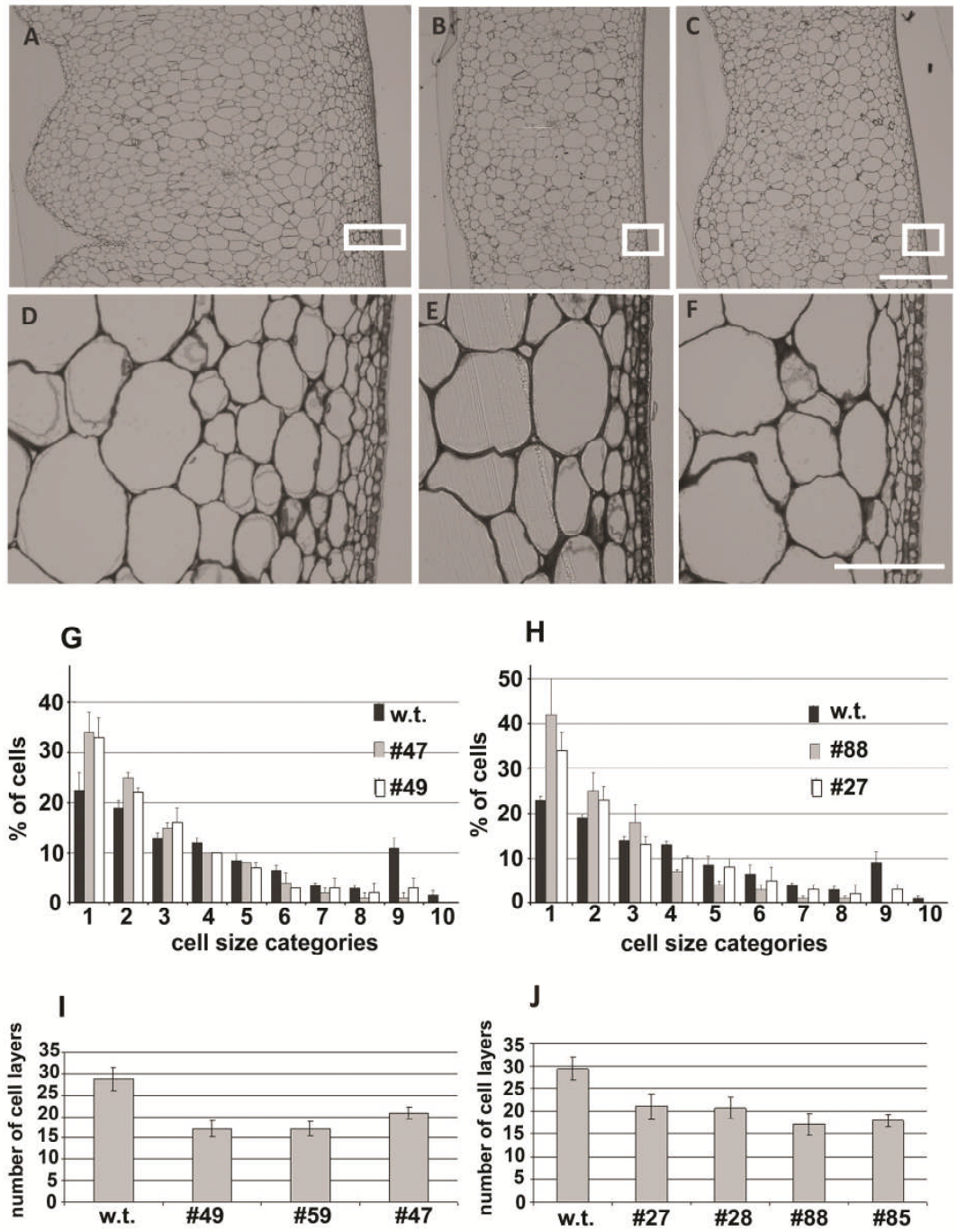
To analyse the possible changes in cell division rate and cell growth in the pericarp of the *CDKB1/CDKB2* overexpressing fruits, we analysed microscopic sections from fruits at the breaker stage. These analyses revealed that in both types of transgenics there are significant differences in cell sizes between transgenic overexpressors and non-transgenic plants as is depicted in Figure 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 (Figure 2) leads to a reduction in cell expansion in the mesocarp.

Next, we determined the number of cell layers in the pericarp of the transgenic fruits, which marks the cell division activity in these fruits. In all 4 analyzed *CDKB1*/*CDKB2* overexpression lines we observed fewer cell layers than in the pericarp of control fruits (Figure 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 shows already activity just before anthesis (see Figure 6 and 7, Chapter 2) we have checked the number of cell layers in non-pollinated ovaries of transgenic and wild-type plants. This analysis revealed that the observed reduction in cell layer numbers in the transgenic plants is clearly an effect that takes place after pollination (Figure 3).

Figure 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**. (**G** and **H**) 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 (T1 plant #47 from line 57 and T1 plant #49 from line 5) were analysed (**G**). The same analysis was done for *CDKB2*-overexpressing fruits (T1 plant #27 from line 4 and T1 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 T1 plants (#49, #59, #47) derived from line 5 (**I**). The same analysis was done for *CDKB2*-overexpressing fruits from T1 plants #27 and #28 from line 4 and T1 plants #88 and #85 from line 3 (**J**). Standard errors are indicated for 10 measurements from three different fruits for each bar. All fruits were harvested at the breaker stage.

CDKB overexpression changed DNA content in pericarp of the fruit

Since cell size is usually related to the level of endoreduplication in the cells (Joubès and Chevalier, 2000; Cheniclet *et al*., 2005; Vlieghe *et al.,* 2005) it is expected that in fruits with overexpression of *CDKB* and reduced cell sizes also show a reduction in ploidy levels. Therefore, we analysed the DNA content in subepidermal and parenchymal regions of the pericarp and the obtained results are depicted in Figure 4. In both the subepidermal and parenchymal part of the pericarp a reduction of the endoreduplication was observed in the *CDKB2* overexpression fruits compared to wild type, with a more pronounced effect in the *CDKB2* overexpressors.

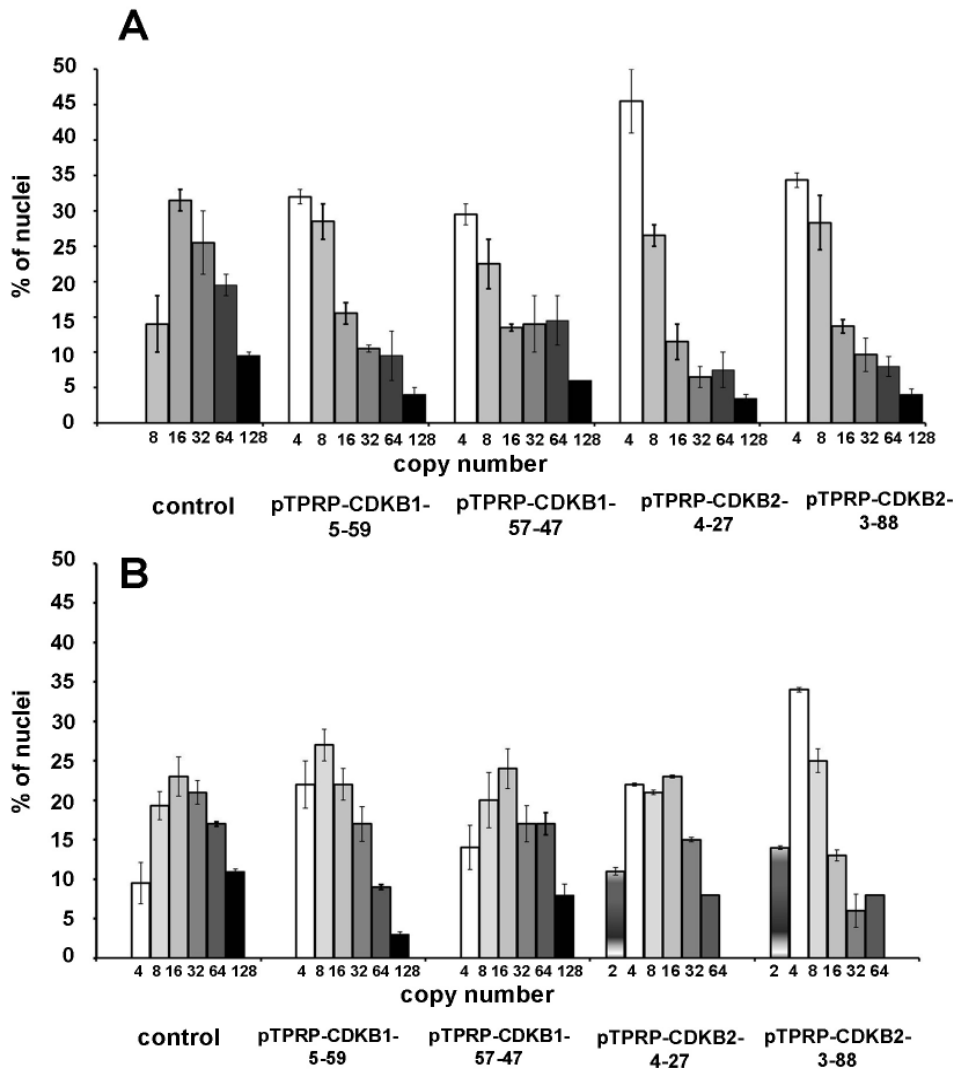


Figure 4. Nuclear DNA Content of pTPRP-CDKB1 and pTPRP-CDKB2.

Ploidy of transgenic plants was compared with wild-type control in mesocarp (**A**) and subepidermal layers (**B**) of pericarp. Ploidy of transgenic fruit pericarp was compared with wild-type control fruit. The horizontal axis indicates the genome copy number, and the vertical axis shows the percentage of nuclei counted. Error bars represent SD.

CDKB1/B2 overexpressing fruits desiccate faster

Because we noticed a difference in fruit firmness (Supplemental Figure 1) we were interested in the post-harvest performance of the transgenic fruits. We analysed 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.

Fifty days after harvest the peel of control wild-type plants were still firm, while the peel of the transgenic fruits were shriveled as shown in Figure 5. The water loss was determined by measuring the weight of the fruits during the experiment. Figure 5 shows that the desiccation rate in transgenic fruits is approximately two times of that in control fruits.

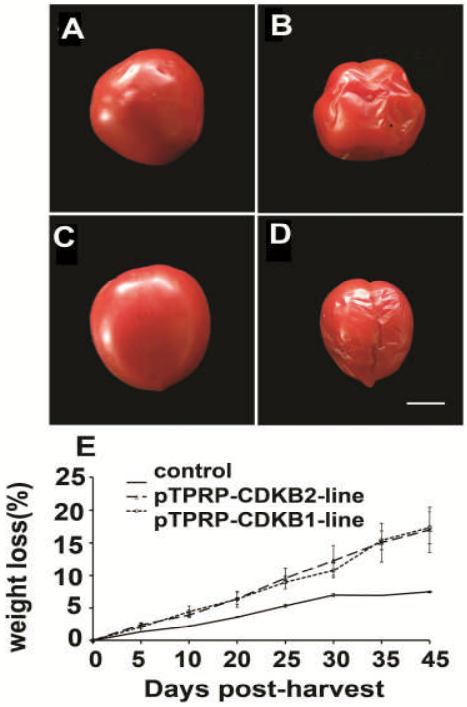


Figure 5. Post-harvest phenotype and characteristics of *CDKB* overexpressing fruits.

A to **D**. Phenotypes of fruits stored for 50 days post-harvest. Control fruit 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 in depicted in (B) and a *CDKB2* transgenic fruit of line 4 in (**D**). Bar=10mm. **E**. Percentage weight loss expressed as percentage of original weight. Every five days post-harvest, the fruit weight was measured. Data are means of 4 fruits ±SD.

To examine if such a dramatic water loss was the result of alterations in cuticle structure or thickness, we analysed the cuticle microstructure by scanning electron microscopy (SEM) and staining by Sudan IV followed by light microscopy (Buda *et al*., 2009; Isaacson *et al*., 2009). Results of these analyses are shown in Figure 6 and reveal that the cuticle thickness of the transgenic mature fruits decreases approximately 3-fold compared to that in control fruits.

This difference may well account for the water loss in the transgenic fruits, which was observed after harvesting.

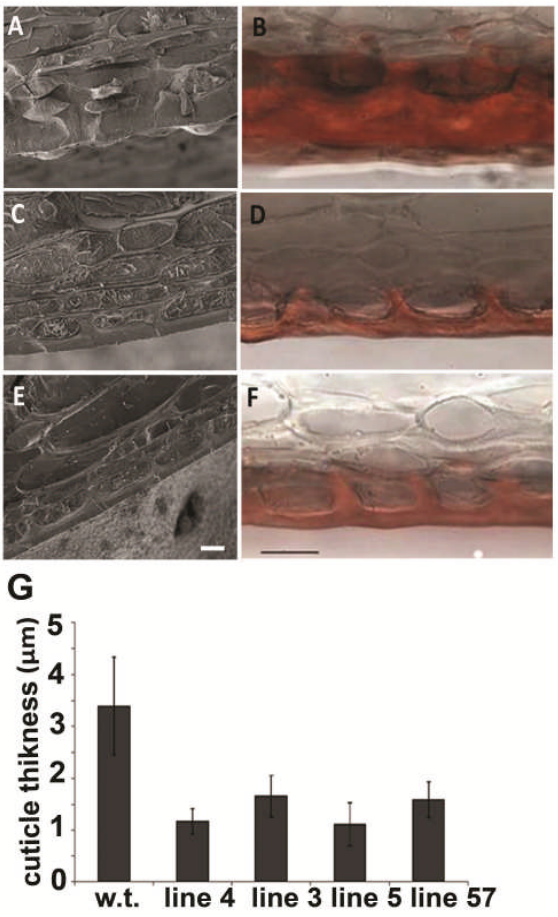


Figure 6. Characterization of the cuticle layer.

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**). Cuticule 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).

Relation 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 (Figure 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 relation of the *CDKB* genes with other *CDKs* has never been reported, the specific expression of *CDKA1* in the exocarp at later stages of development is striking (Figure 7).

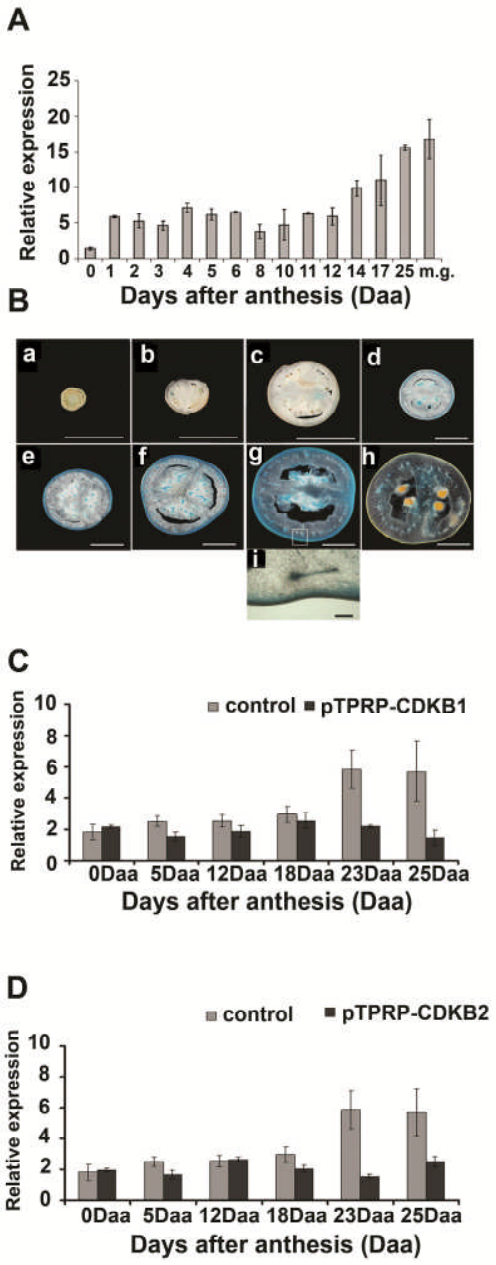


Figure 7. Expression of *CDKA1* in tomato fruit.

(**A**) Relative *CDKA1* expression was determined in the pericarp of wild-type fruits by real-time RT-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 deviation (SD) is indicated.
(**B)** Promoter activity of *CDKA1* in developing fruits (from stages **a-h**) expressing pCDKA1:GUS. Thin sections (~1mm) of the fruits were stained for GUS activity for 3 hours and cleared with ethanol. bar=1cm The box in (**g**) is enlarged in panel (**i**). bar=0,1 mm. (**C,D**) Relative expression levels of *CDKA1* determined by real-time RT-PCR in the pericarp samples of transgenic line 5 overexpressing *CDKB1* (C) and line 88 overexpressing *CDKB2* (D). Samples were collected at 5 different developmental stages with stage 0 Daa and stage 25Daa. Data are means of two biological samples containing material from 3 fruits each.

CDKA1 is expressed throughout fruit development, but is strongly upregulated at the end of the growth phase (Figure 7A). This high expression is predominantly caused by a very high expression in the exocarp as was determined in plants expressing the ß-glucoronidase (GUS) gene driven by a *CDKA1* promoter fragment (Figure 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 quantitative RT-PCR expression analysis of dissected exocarp and mesocarp/endocarp tissues from different stage during fruit development (see Supplemental Figure 3).

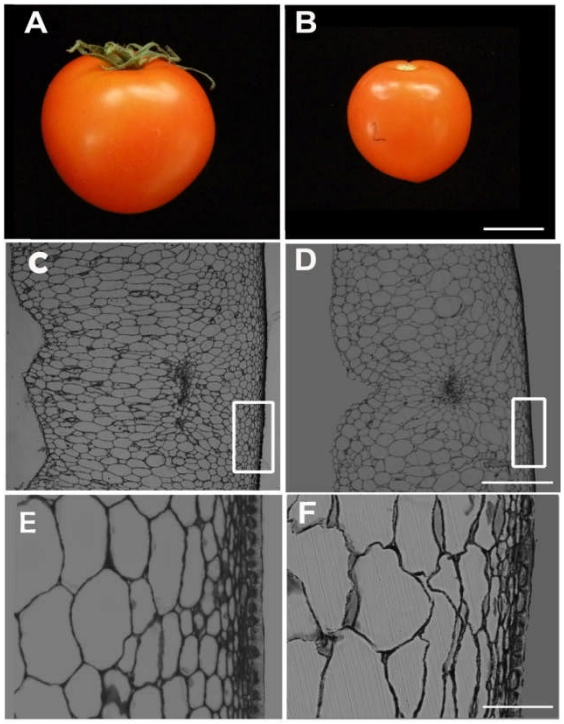
Based on this striking coincidence of the *CDKB1/2* overexpression phenotype and the high expression of *CDKA1* in the exocarp and the complementary expression patterns of *CDKB1/2* and *CDKA1* genes during fruit development (compare Figures 1A,B and 7A, respectively), we speculate that *CDKA1* expression could be affected by the overexpression of the *CDKB* genes. To test this hypothesis we studied the *CDKA1* expression in the pericarp of control and *CDKB1/2* overexpression lines using Q-RT-PCR (Figures 7 C,D). This analysis revealed that *CDKA1* expression is reduced in the CDKB1/2 overexpression lines compared to levels in control fruits. This reduction occurs only in later stages of fruit development when *CDKA1* expression is upregulated 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 (Figure 2), *CDKA1* expression is not affected.

Down-regulation of *CDKA1* phenocopies *CDKB1/B2* overexpression

If overexpression of *CDKB* genes leads to a downregulation of *CDKA1*, one might expect similar alterations in fruits as in CDKB overexpression when the *CDKA1* gene is down-regulated. To confirm this hypothesis, we generated *CDKA1* knock-down plants using expression of an artificial microRNA (amiRNA) (Schwab *et al.,* 2006) and driven by the fruit-specific *TPRP* promoter. Out of 8 transgenic lines tested 3 lines show a significant down-regulation of *CDKA1* to about 20% of the wild-type transcript level in line #33 (see Supplemental Figure 2). This reduction in *CDKA1* expression was inherited to the T1 progeny of lines #33 and #39 and the observed phenotype is linked with the presence of the transgene in the segregating T1 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 (Figure 8, see Supplemental Figure 3). Also the thickness of the pericarp is reduced in these lines similar to what was observed for the *CDKB1/2* overexpression lines (see Supplemental Figure 3).

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



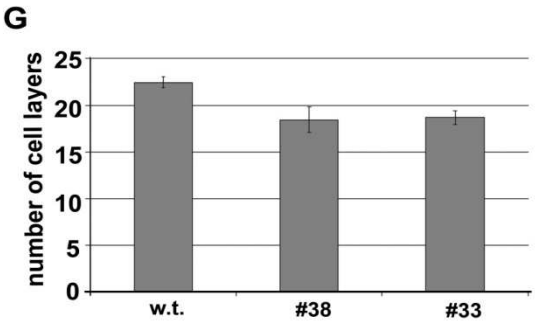


Figure 8. Analysis of fruits from amiCDKA1 knock-down lines.

A. Fruit of a control plant. Cultivar is *Ida Gold***,** B, Representative image of an amiCDKA1 fruit (from line 33); **C** and **D** Microscopic cross-sections through a pericarp of a control fruit (**C**), through a pericarp of an amiCDKA1 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 amiCDKA1 fruits (lines 33 en 38) compared to control fruits. Data are means of 3-4 independent sections from 3 fruits and standard deviation is indicated. Bar in **A** and **B**=10mm; Bar in **C** and **D**=1mm; Bar in **E** and **F**=0,2mm.

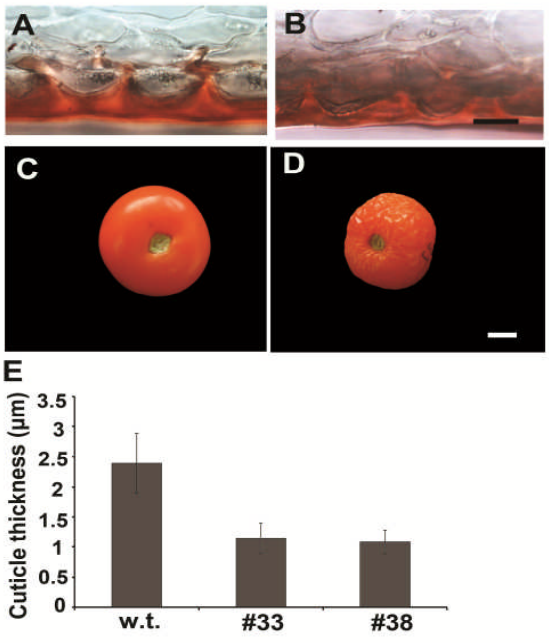


Figure 9. Cuticle phenotype and characteristics of amiCDKA1 fruits.
(A) Section of a wild-type pericarp stained with SUSAN IV. Bar= 50 µm
(B) Section of a wild-type pericarp stained with SUSAN IV. The cuticle layer covering the surface of the fruit is stained red. Bar= 50 µm
(C) Wild-type fruit (Ida Gold) 2 weeks after harvesting. Bar= 1cm
(D) Representative amiCDKA1 (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 standard deviation is indicated. The cuticle thickness of transgenic fruits is significantly reduced comparing to wild-type (Student t-test, $p<0.0001$).

Detailed analysis of the exocarp of these fruits as shown in Figures 8E and 8F, 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 suggest 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 Supplemental Figure 3).
Next, we analysed the cuticle thickness by staining with Sudan IV at the breaker stage and observed a decrease of approximately 2-fold compared to control fruits (Figure 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 days after harvest were investigated. The fruits from the ami*CDKA1* knock-down lines showed a shriveled appearance (Figure 9D) and loss 30% more weight than the control fruits. The cuticle phenotype and post-harvest performance of these ami*CDKA1* knock-down fruits strongly reminisce with 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 Cyclin Dependent Kinases (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 (*Solanum lycopersicum*) two *CDKA* genes (*CDKA1* and *CDKA2*) and two *CDKB* (*CDKB1* and *CDKB2*) have been identified (Joubés et al., 1999, 2001). Here we report the functional characterization of *CDKA1* and the two B-types *CDKB1* and *CDKB2* using overexpression and knock-down approaches with a fruit-specific promoter. 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; Figures 6 and 7, Chapter 2). Highest expression was reported for the earlier stages and this was confirmed by our TPRP-reporter analysis. Surprisingly, overexpression of *CDKB1/2* in the pTPRP-CDKB transgenic lines was only observed at the later stages of development when the endogenous *CDKB* expression declines.

Apparently, the overexpression of these CDK genes is dependent on 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*., 2004a; 2004b; Qi and John, 2007; Imai *et al*., 2009). In our 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 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 likely, the overexpression of these B-type *CDK* genes disturbs the delicate balance between the CDK and CYC proteins, hampering a proper cell cycle progression and hence results in smaller cells and less cell layers in the pericarp. Also overexpression of Arabidopsis *CDKB2* in the shoot apical meristem resulted in a reduction of 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 upregulated 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 *amiCDKA1* lines were only observed in the exocarp. When mesocarp cells enter the cell expansion phase, the exocarp cells maintain mitotic activity and remain dividing till 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 less cell layers in the exocarp.

This reduction in cell numbers in the exocarp of mature green fruits most likely 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 we report 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 other functions 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 signaling 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 signaling, 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, our knowledge on how the activities of *CDK* genes are interconnected and how they control each others' activity is limited (Verkest *et al*., 2005a).
Strikingly, the expression pattern of *CDKA1* is largely complementary to that of *CDKB1/CDKB2* during fruit development, being highly upregulated 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 upregulation 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 a yet unknown transcriptional intermediate. Possibly, phosphorylation of transcription factors by the CDK kinases may effect the expression of other *CDK* genes. Candidates for such intermediate transcription factors are E2F and its cognate interaction partner DP, which 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 G1/S transition (Boniotti and Gutierrez, 2001). Besides the regulation of the E2F/DP transcription factor complex, RBR also interacts with histon modifying proteins and other transcription factors in animals and most likely in plants as well (for 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-CYC protein interaction. Different CDK proteins may compete for the same CYC protein in the formation of a CDK/CYC 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 CYC proteins have been studied by yeast two hybrid assays (Joubès *et al*., 2001) and they reveal that CYCA2 interacts with both CDKA1 and CDKB2. Overexpression of *CDKB2* might thus result in a reduction of CDKA1-CYCA2 complexes, causing an arrest in the G1-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 (KRP), 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*., 2005a, 2005b). 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.,* 2005a, 2005b; Bisbis *et al*., 2006.). When CDKB1 levels in the cell are high, it phosphorylates and destructs KRPs, which are the inhibitors of CDKA1/CYC proliferating activity.

Which mode of interdependency is active in the tomato fruit is not resolved, but our 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 mimic 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, we have shown that B-type *CDKs* 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 affect cell division and cell expansion, probably by disturbing the delicate balance between these factors that is required for proper cell cycle regulation. We 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 likely 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.

Material and methods

Plant material

Tomato plants *Solanum lycopersicum* L., cv. M82, cv. Ida Gold and obtained transgenic lines were grown in a greenhouse under a 16-h of light and 8-h of dark. Supplementary lights (600 Watt high pressure sodium lights) turned on below 200 W/m^2 and turned off above 300

W/m^2. Temperature was kept above 20^{0}C during the light period and 17^{0}C during the dark period with the PRIVA Integro versie 724 system. Plants were watered daily and given fertilizer weekly.

Construction binary vectors for transformation

To generate the fruit specific *CDKB1 and CDKB2* overexpression lines, the coding sequence of *Le*CDKB1;1 (accession number AJ297916) and *Le*CDKB2;1 (accession number 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 ORF of choice can be simply recombined *in vitro*.

To knock-down the expression of CDKA1 we followed an artificial microRNA (amiRNA) approach (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

The transgenic tomato plants were generated by *Agrobacterium tumefaciens*-cocultivation of seedling cotyledons, as described in De Jong *et al.* (2009b).

Harvesting plant material

To avoid the differences in source-sink balance affected by fruit number and position (Bertin et al., 2001) we left 5 fruits at each truss, the additional pollinated flowers were removed. The first developing truss from each plant was removed.

For mRNA isolation we collected the second and third fruit from the second truss on the plant. 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 actine gene *TOM 51* U60481 only. RNA (0,4 µg) was reverse transcribed (RT) in a total volume of 10 µL using a cDNA synthesis kit (iScript ^{tm}, Bio-Rad Laboratories, Hercules, CA, USA) following 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 Supplemental table 2. qRT-PCRs were done using SYBR green mix (iQ-SYBR Green Supermix, Bio-Rad Laboratories). PCR reactions were performed in a 96-well thermocycler (Bio-Rad iCycler). Five microliters 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 Bio-Rad outlined methodology 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 63x oil immersion objective HCXPL apo CS 63x/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 we used 10-15 different images per sample.

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 and a 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 approximately 5 mm, height app. 5 mm) was cut from the equatorial section, from each fruit in duplicate. Collected tissues were bleached in 0,4% hypochlorite for 90 minutes to clear cellular content and washed with distil-water. Tissues were fixated in fixative containing 10% formaldehyde, 5 % Acetic acid and 52% ethanol, vacuum infiltrated for 15 minutes 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 smaller than 12,5 μm^2 or larger than 250μm^2 were excluded from the data, since they most likely represent intercellular spaces and cells that were disrupted. Mean cell size was calculated for each picture. Per plant line 4 pictures of different pericarp areas were averaged and depicted with SE.

Water loss measurements

3 fruits of each line were collected at the ripe stage, and were stored at room temperature for 50 days. 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 (Dujmović *et al.,* 2005; Brewer *et al.,* 2006; Gonzalo *et al*., 2009).

Ploidy analysis

Nuclei were prepared from the pericarp of ripe fruit. Two types of tissue were analysed - subepidermal layer – peel and mesocarp tissues. 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 100mm nylon mesh and the remaining sample was reextracted with the same solution. The combined filtrates were analysed with a CA-II cell analyser (Partec).

Acknowledgments

We are grateful to Dr. Liesbeth Pierson and Mr. Geert-Jan Janssen from Radboud University Nijmegen (The Netherlands) for help with microscopy and scanning micrographs, Gregory Buda from the Rose Lab at Cornell University (USA) for the protocol and suggestions on the SUDAN assay and Gianfranco Diretto from the ENEA Casaccia Research Center in Rome (Italy) for suggestions on the measurements of the tomato firmness. We thank Rijk Zwaan Nederland B.V. for generating transgenic tomato lines TPRP-CDKB1 and TPRP-amiCDKA1.

Supplemental data

Supplemental table 1: Sequences of the primers.

Gene name or number	Primer sequences
TOM 51	5’- GCTGTGCTTTCCTTGTATGC-3’ 5’- TCACACCATCACCAGAGTCC-3’
LeACT 2/7 (actin 2/7)	5’-GGACTCTGGTGATGGTGTTAG-3’ 5’- CCGTTCAGCAGTAGTGGTG-3’
Le S18	5’- AGACGAACAACTGCGAAAGC -3’ 5’- AGCCTTGCGACCATACTCC-3’
Le CDKA1	5’-AACCCCTGAATAGAACCAAATG-3’ 5’-GTATGTGCCGTGATTGTCTG-3’
Le CDKB1	5’-ATGGAGAAATACGAGAAATTGGAG-3’ 5’-ACGATGTAGAGAGAATGAGATAGC-3’
Le CDKB2	5’-ATGCTGGTAAGAGTGTATCGG-3’ 5’-CGGAGAGTAGTTGGAGGAAC-3’
TPRP-CDKB1	5'-TCATTATATTTAACAATCCCACTTGATG-3' 5'-ACAGTGAAATATGAAAGTGACAAG-3'
TPRP-CDKB2	5'-TACATATTACATACCTAACTCAAGCATC-3' 5'-CGGAGAGTAGTTGGAGGAAC-3'

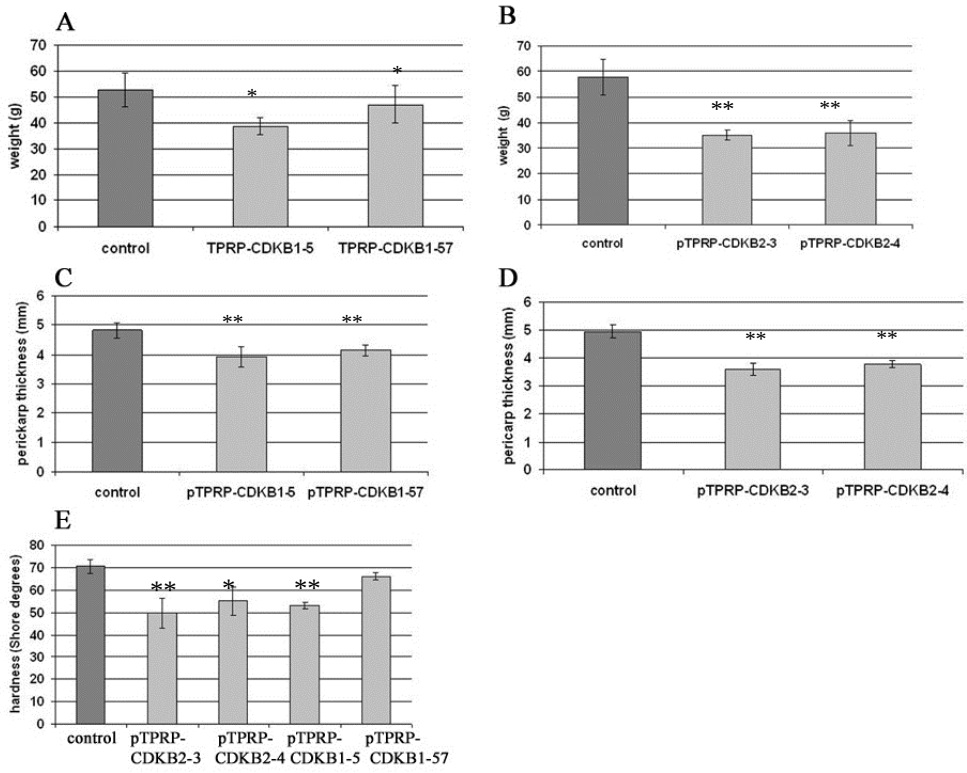


Figure S1. Postharvest characteristics of the fruits with *CDKB1* and *CDKB2* overexpression. **A.** Mean weight of fruits from pTPRP-CDKB1 lines; **B.** Mean weight of fruits from pTPRP-CDKB2 lines; **C.** Pericarp thickness of fruits from pTPRP-CDKB1 lines; **D**. Pericarp thickness of fruits from pTPRP-CDKB2 lines; **E.** Firmness of fruits from pTPRP-CDKB2 and pTPRP-CDKB1 lines. Data are means of 10-12 ripe fruits from each plant ± SD. Significant reduction of the parameter in the transgenic fruit compared to wild-type fruits is indicated with an asterisk **P<0.005, *P<0.05

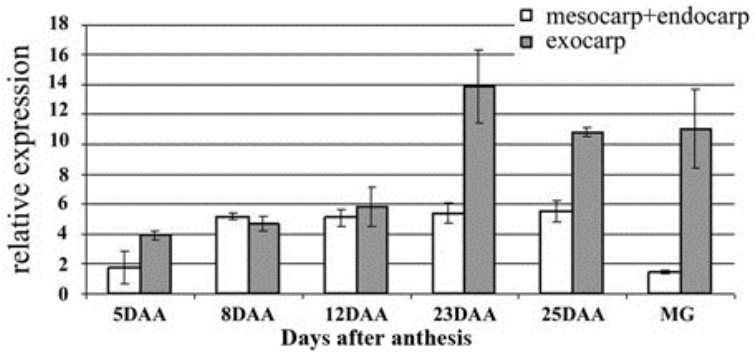


Figure S2. Relative expression of CDKA1 in pericarp tissues of wild-type tomato M82 cultivar in different developmental stages. Bars are means of two biological replicas, each containing 3 different fruits.

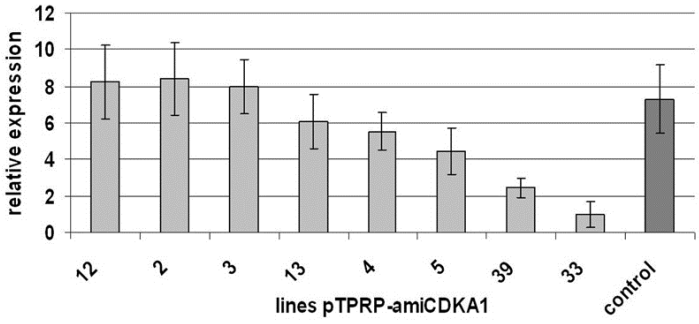


Figure S3. Downregulation of *CDKA1 in pTPRP:ami-CDKA1* transgenic lines.

Fruits of the primary transformants (variety Ida Gold) were harvested at the mature green stage and the expression was determined by Q-RT-PCR analysis. Two biological replicates were taken and the standard deviation is indicated by a thin line. Transgenic lines #5, # 39 and #33 show a significant reduction in expression compared to control wild type fruits

4

The effect of fruit-specific *CDKA1* overexpression on tomato fruit development

Anna Czerednik, Marco Busscher, Ruud A. de Maagd and Gerco C. Angenent

Abstract

CDKA1 is an important cell cycle regulator, which has dual functions during both S and M phase progression. We obtained transgenic 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 phenotypical changes with regard to the pericarp thickness and placenta area were remarkable. Fruits of tomato plants with the highest expression of *CDKA1* had larger septum en placenta, comparing to the wild-type fruits. In this chapter we discuss the possible causes of this phenomenon.

Introduction

Cyclin dependent kinases (CDKs) are key regulators of the cell cycle, which need the interaction with Cyclins (Cyc) for their activity. The A type CDKs form a major class of CDKs present as multiple (1-4) distinct genes in plants (Joubès *et al*., 2000; Dudits *et al*., 2007). At the amino acid sequence level, CDKAs are characterised by the presence of a conserved PSTAIRE cyclin-binding motif (Dicommun *et al*., 1991). In plants, they are constitutively expressed throughout the cell cycle and play roles during both S and M phase progression. They are involved in cell proliferation and in the maintenance of cell division competence in different tissues during plant development. However, their expression has also been detected in non-dividing tissues, suggesting that they are also involved in endoreduplication (Hemerly *et al*., 1993; Segers *et al*., 1996; Joubès *et al*., 1999).

CDKAs are able to form an active complex with several cyclins during the cell cycle (Inzé and De Veylder, 2006; Boruc *et al*., 2010; Van Leene *et al*., 2010). CDKA activated by D-type cyclins is involved in the G1-S transition by phosphorylation of RBR and initiating the degradation of RBR from the E2F/DP/RBR transcriptional repressor complex by the SCF E3-ubiquitin protein ligase. This release of RBR from the E2F/DP complex restores the transcriptional activity of the E2F/DP dimer, which subsequently results in the transcriptional activation of S-phase genes (De Veylder *et al*., 1997; Nakagami *et al*., 1999; Joubès *et al*., 2001; De Veylder *et al*., 2001; Inzé and De Veylder, 2006). 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 seems to be involved in the maintenance of cell proliferating competence in differentiating tissues in Arabidopsis (Joubès *et al*., 2000). In addition, several reports provide evidence that *CDKA* is essential for endoreduplication (Verkest *et al*., 2005(a);

Leiva-Neto *et al*., 2004) and contributes to the development of the male gametophyte, embryo and endosperm (Leiva-Neto *et al*., 2004; Iwakawa *et al*., 2006).
In tomato two genes of the *CDKA* family have been identified: *Lyces; CDKA1* (gi 3123614) and *Lyces;CDKA2* (gi 312316), and the proteins are highly similar sharing 94% amino acid sequence identity. The differences, if any, in their role in the cell cycle and in plant development are not clear yet.
Joubès *et al*. (1999) studied the expression of *CDKA1* in tomato fruits and noticed differences in expression of this gene in jelly and epidermis. They observed an increase in *CDKA1* expression in jelly up to the mature green stage, while in the epidermis the expression remains the same throughout development from pollination until mature green. Our own analysis of *CDKA1* and *CDKA2* expression in the pericarp during development shows unchangeable expression levels up to 14 days after anthesis (DAA) and then the expression is increased up to the mature green stage (Chapter 2, Figure 4).
Here we describe the effect of the overexpression of *CDKA1* under control of a fruit specific promoter on cell division and expansion. In the obtained overexpression lines, the placenta and septa were enlarged. Furthermore, we observed an increase in the number of 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

To obtain information about possible roles of *CDKA1* in the regulation of cell numbers and cell sizes in the pericarp we analysed transgenic plants overexpressing this gene. The promoter region of the fruit-specific gene *TPRP* (*TM7*) was used to drive the expression of *CDKA1* in the fruit only (Salts *et al*., 1991,1992; Carmi *et al*., 2003; Fernandez *et al*., 2009).
We obtained 34 transgenic lines with the p*TPRP-CDKA1* overexpression construct. Three of these lines, showing the highest expression of *CDKA1* were selected for further study (Figure 1A). From each transgenic line two fruits of different stages were analysed. The two fruit stages were: small 4-12 mm roughly, representing the cell division phase and larger fruits (15-25 mm) from the expansion phase. In Figure 1A presented data of *CDKA1* expression in pericarp of fruits in size 4-12 mm. The expression of *CDKA1* in the pericarp of transgenic fruits was compared with expression of this gene in pericarp of wild-type M82 cv, harvested at the same stages as the transgenic fruits. To confirm the obtained results, the Q-PCR analysis on the high expression lines was performed twice.

Results of both analyses indicated 5-fold, 16-fold and 10-fold overexpression of *CDKA1* in lines #22, #57 and #84 respectively, which also showed the most severe phenotypes.

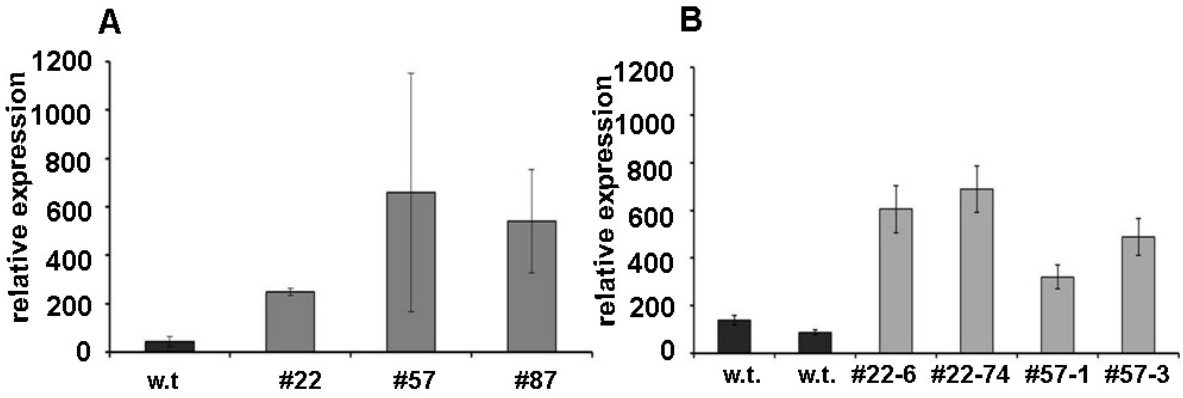


Figure 1. Relative expression of *CDKA1* in the pericarp of several transgenic lines, which showed the most apparent phenotypes and were used for further analysis.
A. Expression of *CDKA1* in pericarp of fruits from the T0 generation. Fruits were 4-12 DAP.
B. Expression of *CDKA1* in the pericarp of fruits from the T1 generation. Fruits were 12-17 DAP.
Relative *CDKA1* expression was determined by real-time RT-PCR in two replications. Data are means of 2 biological replicates and standard deviation is indicated.

For more reliable analysis of the phenotype and expression levels, progeny (T1) of the primary transformants were examined. Siblings from lines #22 and #57, which had the most apparent phenotype and expression of *CDKA1* were compared with (non-transgenic) siblings (Figure 1B). This analysis showed that the overexpression is stably inherited to the next generation.

Phenotypes of *CDKA* overexpressing fruits

Fruits of tomato plants with the highest expression of *CDKA1* had larger septum en placenta, comparing to the wild-type fruits. Furthermore, the transgenic fruits developed very few seeds and failed to develop jelly, but due to an increased area of placenta, septa and pericarp, no empty cavities were observed. The T1 *pTPRP-CDKA1* overexpressing plants showed the same phenotype as the primary transformants (Figure 2 D-F) and further analysis was performed on these T1 overexpressors. We analysed several characteristics of the transgenic and control fruits, collected at the ripe stage and the results are depicted in Figure 3. Fruits of all investigated transgenic lines had shape, size and weight comparable to that of wild-type fruits (Figure 3 A-D). However, these fruits contained significantly less seeds (Figure 2, Figure 3 E) and many pseudoembryos.

Figure 2. Phenotypes of control and *CDKA1* overexpressing fruits.

(A), (B), (C) Fruits of a non-transgenic siblings derived from a pTPRP-CDKA1 plant (line#22). **(D), (E), (F),** Representative fruits from an overexpressing transgenic pTPRP-CDKA1 plant (line# 22). Bar= 10 mm

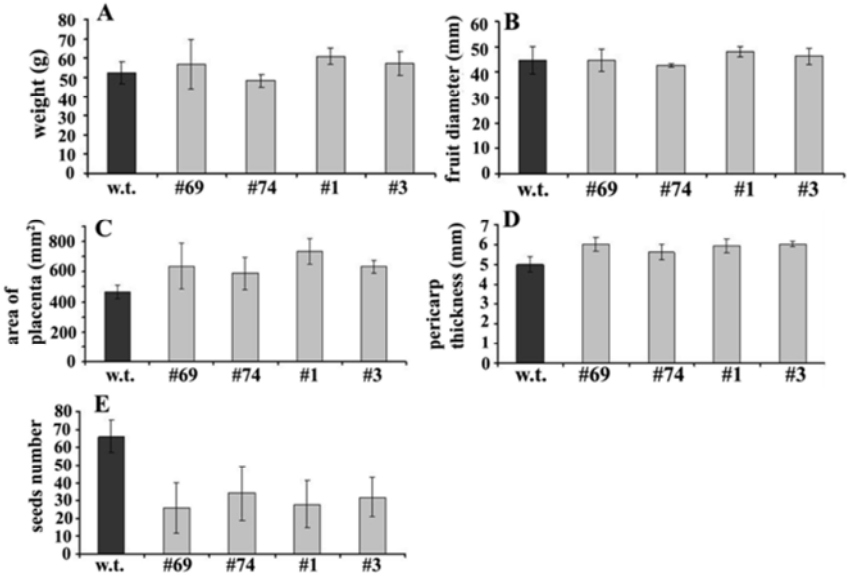


Figure 3. Yield parameters and phenotypic features of wild-type and transgenic fruits, collected at the ripe stage. The data represents the means ± standard error of 10-12 ripe fruits.

The placenta size, fruit diameter and pericarp thickness were analysed using the Tomato Analyzer software (Dujmović *et al*., 2005; Brewer *et al*., 2006; Gonzalo *et al*., 2009). The placenta area was significantly increased up to 2-fold. We also observed that the pericarp of the transgenic fruits was 1-2 mm thicker compared to the control fruits.

Microscopic analysis of transgenic fruits overexpressing *CDKA1*

In order to find the cause of the observed changes in pericarp and placenta of the transgenic fruits, we performed histological analysis of these tissues. In Figure 4, microscopic cross-sections through the pericarp and placenta of wild-type and transgenic fruits are presented. The pericarp cells of the transgenic lines (Figure 4, B and C) appear to be smaller than in the wild-type (Figure 4A). The detailed images of the exocarp region shown in Figures 4 D-F suggest that the number of layers with small cells under the epidermis is increased compared to a wild-type fruit. Therefore, we determined the number of cell layers in the pericarp and confirmed that the overexpression lines have significantly more cell layers in the pericarp than the wild-type fruits (Table 1).

Also the cell sizes in the placenta are affected: there are more small cells in the transgenic fruit (Figure 4 H) compared to the control (Figure 4G). The cell number per area of pericarp and placenta was significantly ($p<0.005$) increased in transgenic fruits when comparing to wild-type. Similarly, the number of cell layers in the pericarp was increased in transgenic fruits (Table 1).

The pericarp cell size was significantly decreased in the transgenic fruits (Figure 4I). Significant differences ($p<0,005$) were observed for the categories 1, 2 (small cells) and the categories 7, 8 ($p<0,05$) and 9 ($p<0,005$) with the largest cells.

A. Fruit weight (g). The differences between wild-type and transgenic fruits are not significant (#69 p=0.33; #74 p=0.62; #1 p= 0.53; #3 p= 0.33; Student's test)

B. Fruit diameter (mm). The differences between wild-type and transgenic fruits are not significant (#69 p=0.99; #74 p=0.36; #1 p= 0.10; #3 p= 0.49)

C. Area of the placenta analysed with the Tomato Analyser software. The placenta size is significantly enlarged in the transgenic fruits (for #69 $p< 0.05$, in all other lines $p<0.005$).

D. Pericarp thickness. The pericarp thickness of the transgenic fruits was analysed with the Tomato Analyser software and pericarp of transgenic fruits was significantly thicker ($p<0.005$).

E. Number of seeds per fruit. The number of seeds in transgenic fruits was significantly reduced ($p<0.005$).

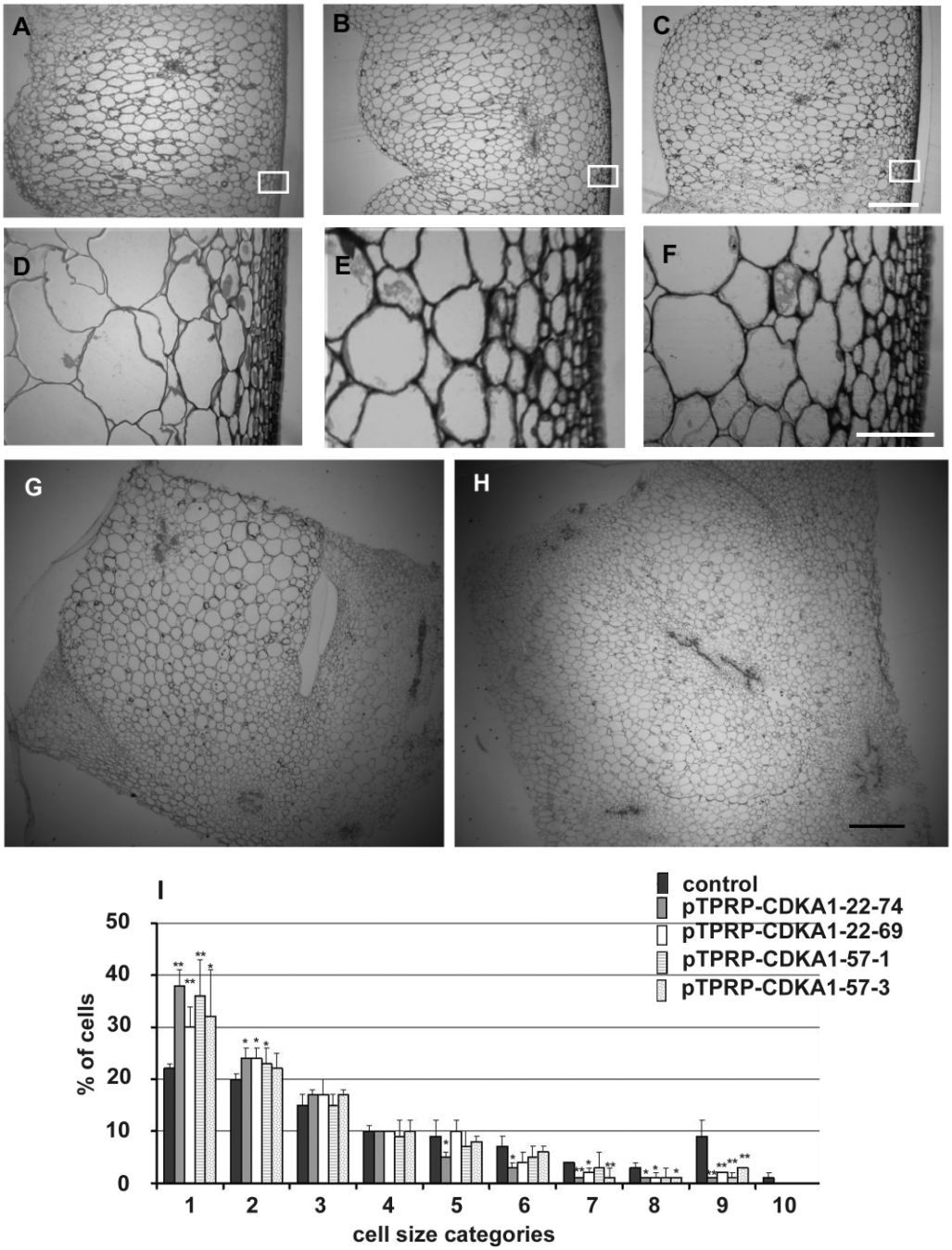


Figure 4. Phenotypic changes in pericarp and placenta due to overexpression of *CDKA1.*

(A)-(C) Microscopic cross-section through a pericarp of a control fruit **(A)**, through a pericarp of a *CDKA1* overexpressing fruit from line #22 **(B)** and one from line #57 **(C).**

(D)-(F) Details of selected areas in **(A)-(C),** respectively. Note that the number of layers with small cells in the exocarp of the transgenic lines is increased compared to a control fruit. Bar=1 mm in **(A)-(C)**; Bar=0.2 mm in **(D)-(F)**

G, H. Microscopic cross-section through a placenta of control fruit and of *CDKA1* overexpressing line#22 respectively. Bar=0.2 mm.

Percentage of cells grouped into 10 different size categories. Cells in category 1 have an average size of up to 0.4 * 10^{-3} mm^2 and the maximum cell size in each subsequent category has doubled. Standard errors are indicated for a minimum of 4 measurements from 3 different fruits for each bar. The p-values (Student's T-test) are indicated: ** $p<0.005$ and * $p<0.05$.

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

The data represent the means ± standard error of five-six measurements from each of 3 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.

line	cells/mm^2 pericarp	cells /mm^2 placenta	Number of cell layers in pericarp
wild-type	15 ± 2.37	33 ±1.3	27 ± 2
Line 22	23 ± 2.20 (p<0.005)	56 ± 6.71 (p<0.005)	35 ± 2 (p<0.05)
Line 57	25.2 ± 1.94 (p<0.005)	46 ± 3.33 (p<0.005)	33 ± 1 (p<0.05)

Analysis of ploidy level in pericarp cells

Cell expansion coincides with the continuation of DNA synthesis without mitosis (cell division), leading to endoreduplication and increase in ploidy level (Bergervoet *et al*., 1996). Because we observed decreased pericarp and placenta cell sizes we determined ploidy levels to find out if changes in endoreduplication could have caused the observed differences.

Fruits were harvested at the breaker stage, and samples of mesocarp were analysed by flow cytometry after which the endoreduplication index (EI), i.e. the mean number of endoreduplication cycles per nucleus, was calculated (Figure 5). The EI of mesocarp cells in all analysed overexpressing fruits was significantly reduced (Student's T-test, p<0.005 in case of #69 and p<0.05 for other fruits), indicating a correlation between reduction of cell size and ploidy level.

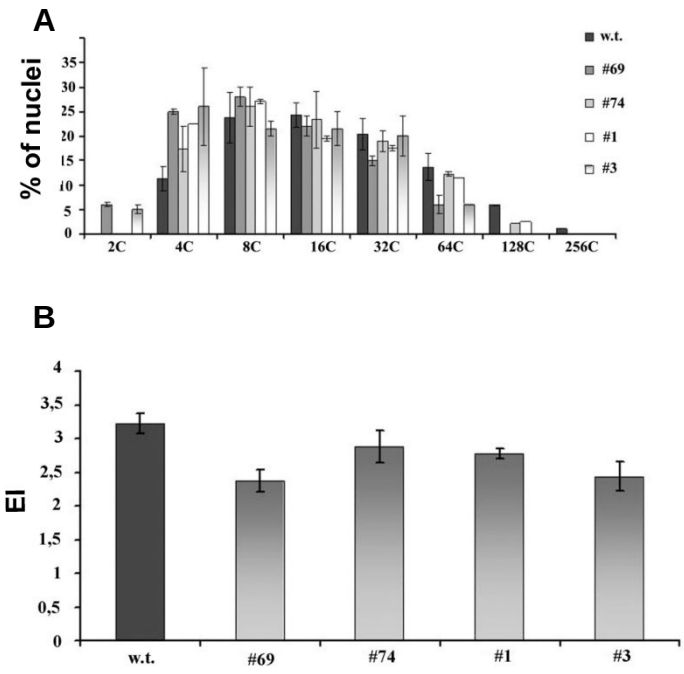


Figure 5. Ploidy distribution analysis in nuclei from mesocarp cells

A. Ploidy distribution analysis of tomato mesocarp cells at the breaker stage.

B. Endoreduplication index (EI), which is the mean number of endoreduplication cycles per nucleus. Values are means ±SD (n=3). EI was significantly reduced in pericarp of transgenic lines (lines #69, 74, 1 and 3) compared to wild-type (w.t.) (p<0.005 in case of #69, and p<0.05 in case of other plants).

Discussion

Tomato fruit growth depends on successful pollination, fertilization and number of developing seeds and is mediated by cell division and cell expansion (Gillaspy *et al*., 1993). The fertilized ovules normally trigger the development of an ovary into a fruit. The absence of fertilization usually results in abortion of flowers or development of fruits with fewer seeds or parthenocarpic fruits. Parthenocarpic fruits or fruits with decreased number of seeds usually are smaller than normally developed fruits, lack gel, and they have decreased cell division and cell expansion (Nitsch, 1970; Balbi and Lomax, 2003; De Jong *et al*., 2009b).

Reduced seed set influence cell size in tomato fruit tissues

A central role for *CDKA1* in controlling cell number has been demonstrated using transgenic tobacco (*Nicotiana tabacum*) plants with reduced A-type CDK activity due to dominant negative overexpression of *CDKA;1* in Arabidopsis (Hemerly *et al*., 1995). Several studies reported that fruit growth is associated with embryo and seed development, and the number of seeds in the fruit is a key determinant of its final size, because seeds produce and act as sinks for hormones such as cytokinins and auxins. These hormones induce rapid growth of the developing ovary by increasing cell division and cell expansion (Varga and Bruinsma 1986; Bohner and Bangerth, 1988). Therefore, seed development correlates with the size of the fruit.

We obtained transgenic plants with fruits that didn't differ in size and weight from wild-type fruits. However, the number of seeds in these *CDKA1* overexpressor fruits was significantly reduced and the development of jelly failed. The transgenic fruits had a significantly thicker pericarp compared to wild-type fruits and significantly larger placenta size. Microscopic analysis showed that cell size in pericarp and placenta was reduced, but the number of cells significantly increased in the transgenic fruits compared to wild-type fruits. Thus, increased cell numbers compensated for the reduction in cell size resulting in no apparent change in final fruit size.

Cell expansion takes place after division and has the largest contribution to the final size of the fruit. During this developmental phase in tomato fruit the volume of cells in the placenta, locular tissues, and mesocarp tissues can increase by more than 10-fold (Chapter 2, Figure 3 this thesis; Gillaspy *et al*., 1993). Cell expansion in tomato tissues depends on auxin signals, which increase the extensibility of cell walls and induce uptake and retention of water and solutes as well as stimulate cell growth (Rayle and Cleland, 1992; Catalá *et al*., 2000; John, 2007). The source of this auxin signal is developing tomato seeds, which contain high amounts of auxin.

According to Gillaspy *et al.* (1993) there is also a possibility that seeds produce a certain threshold level of auxins which is difficult to detect because of rapid consumption during the cell expansion process. Additionally it is supposed that the developing seeds or embryo produces a signal molecule other than auxin that regulates cell expansion and sink activity of the surrounding fruit cells. These regulatory molecule(s) produced by seeds or embryos may direct the sink activity of cells in the pericarp, placenta, and locular tissues and may be involved in cell expansion in tomato tissues (Gilaspy *et al*., 1993).

We hypothesize that the reduction of seed number in our *CDKA1* overexpressing plants may have a direct effect on the amount of auxin or other regulatory molecules and consequently, it influenced expansion negatively, resulting in smaller cells. The reduced amount of jelly locular tissue surrounding the seeds could be explain in two ways. Firstly, seed development impaired also the formation of jelly locular tissue surrounding the seeds, which growth is stimulated by developing seeds. This decrease in the amount of jelly is often observed in fruits with reduced seed numbers or in case of parthenocarpy (Lemaire-Chamley *et al*., 2005; De Jong *et al*., 2009b). Alternatively, the lack of jelly might be linked to the presence of more solid and expanded placenta, which we observed in *CDKA1* overexpressors.

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

Simultaneous with a reduced cell expansion in tomato tissues we observed an increase in the number of cells in the transgenic fruits, both in the number per unit area as well as in the number of cell layers across the pericarp width, which resulted in a significant increase in pericarp thickness and placenta area. 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 the phosphorylatory active complex with Cyclins. CDKA1 is involved in both the G1-S as G2-M transition, while varying the interacting cyclin. Depending on the cell cycle phase CDKA1 is able to form an active complex with CycD3, CycA2 or CycB1/B2 (Weingartner *et al.,* 2003; Dewitte *et al.,* 2003; Verkest *et al*., 2005a; Inzé and De Veylder, 2006; Menges *et al.,* 2005).

We suggest that in fruits overexpressing *CDKA1* the mitotic activity might be prolonged in the cells of the pericarp and placenta, resulting in an increase of cell number. This may indicate that *CDKA1* expression, which in the transgenic lines was increased up to 6-fold, was enough to continue the cell division longer than in wild-type fruits. In Chapter 2 of this thesis we showed that the expression level of *CycD3;1*, *CycA2* and *CycB1,* which are potential partners of CDKA1, is maintained longer in the pericarp during fruit development than CDKA1. These data suggest that *CDKA1* expression is the limiting factor for cell division activity in the developing tomato pericarp in the presence of available Cyclin partners.

Increased *CDKA1* expression affects successful fertilization

It is known that CDKA1 has essential functions in developmental processes such as gametogenesis and embryogenesis. Missexpression of *CDKA1* resulted in defects in male gametogenesis, leading to the production of bicellular pollen grains that fail in double fertilization (Iwakawa *et al.,* 2006). Based on these data from Iwakawa et al. we analysed the activity of the pollen from the *CDKA1* overexpressing plants. In an in vitro pollen tube germination assay, the overexpression of *CDKA1* had no effect on pollen germination (not shown). Furthermore, we did not expect an effect in the anthers because the pTPRP promoter used for the overexpression is not detectably active in the anther.

Therefore, it is likely that the female gametophyte or embryo formation was impaired by *CDKA1* overexpression. To confirm this hypothesis, we performed cross pollinations of transgenic flowers with wild-type pollen from cv. M82. Despite successful pollination and growth of normal sized fruits there was still a significant reduction in numbers of normally developed seeds and a relatively high number of pseudoembryos. We observed an increased number of pseudoembryos, which originate from divisions of cells of the inner integument (Kataoka *et al*., 2003). Possibly, the rapid growth of ovaries may have resulted in the formation of pseudoembryos due to a failure of proper fertilization as was observed in tomato fruits were growth of ovaries was stimulated by gibberellins (Kataoka *et al*., 2003). This all may point to an effect of *CDKA1* overexpression/ectopic expression on either the development of female gametophytes or a prevention of successful fertilization. Further studies are needed to unravel these phenotypes.

In conclusion, we show that overexpression of *CDKA1* leads to fruits with decreased cell sizes, probably indirect caused by abolished seed set, while cell numbers in several fruit tissues were significantly increased. This compensation phenomenon yielded fruits of normal size and shape. How these changes in cellular composition affects the structure and quality characteristics of the fruits need further investigation.

Material and methods

Plant material

Tomato plants *Solanum lycopersicum*, cv. M82, cv. Ida Gold and obtained 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/m2 and turned off above 300 W/m2 solar irradiation. Temperature was kept above 20^0C during the light period and 17^0C during the dark period controlled with the PRIVA Integro version 724 system. Plants were watered daily. The extra tomato fertilizer Osmocote was added in the soil to improved the calcium supply and prevented blossom-end rot in fruits.

Construction binary vectors for transformation

To generate the fruit-specific *CDKA1* overexpression lines, the coding sequence of *Lyces; CDKA1* (accession number Y17225) was cloned into the pENTR/D-TOPO entry vector (Invitrogen) using the primers F - 5'-CACCATGGACCAGTATGAAAAAGTTGAGAAG-3' and R- 5'UTR-GTGGTCACGGCACATACCCAATATCCTTG-3'. 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 was recombined in vitro using LR clonase (Czerednik *et al.,* 2012). The right connection of TPRP promoter and *CDKA1* was confirmed by PCR using the pair of primers: F 5'- CTGACCCTTCCTTAAATCCC-3' and R 5'-GTATGTGCCGTGATTGTCTG-3'

Transformation of tomato

Transgenic tomato plants were generated by *Agrobacterium tumefaciens*-cocultivation of seedling cotyledons, as described in De Jong *et al.* (2009b). Plants were selected on kanamycin-containing medium and then checked by PCR with primers specific partially for TPRP-promoter and gene of interest on genomic DNA. Subsequently, lines were tested for ploidy, as only diploid lines were used for further analysis.

Harvesting plant material

To avoid the differences in source-sink balance affected by fruit number and position (Bertin *et al.,* 2001) we left 5 fruits at each truss, the additional pollinated flowers were removed. The first developing truss from each plant was removed.

For mRNA isolation we collected the second and third fruit from the second truss on the plant.

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

RNA was isolated and reverse transcribed to cDNA following the protocol described in chapters 2 and 3. Also for the real-time quantitative PCR, the same conditions were used as mentioned in chapter 2. Real-time –quantitative RT-PCR (qRT-PCR) primers were designed using a computer program (Beacon Designer Software, Premier Biosoft International, CA, USA).

Relative mRNA levels were calculated following the Bio-Rad outlined methodology based on Vandesomple *et al.* (2002) and corrected for PCR efficiencies. The average of two biological repeats is depicted with the SD.

5

The effect of *CycA2* overexpression on cell cycle regulation in the tomato fruit pericarp

Anna Czerednik, Marco Busscher, Ruud A. de Maagd and Gerco C. Angenent

Abstract

The overexpression of *CycA2* under the control of the fruit specific promoter TPRP has been studied in tomato plants. The obtained fruits with unregulated *CycA2* were more oval in shape and were smaller due to a decrease in size and number of cells in the fruit pericarp. Despite the decreased cell size the number of DNA copies in cells of the transgenic fruits was similar to the control, possibly due to a shortening of the endoreduplication phase, which is associated with a shorter time period for cell expansion in the transgenic fruits.

Introduction

Cyclins are regulatory partners of Cyclin Dependent Kinases (CDKs), and the CDK-Cyc complexes drive crucial transitions of the cell cycle - the entry from G1 into S phase and from G2 into M phase. In plants, more than 100 cyclins from various species have been isolated and they are grouped into nine classes. From them, only three classes, A, B and D are directly involved in cell cycle regulation through the activation of CDKs. The different classes control different transitions and phases of the cell cycle in higher eukaryotes: G1/S (D-type), G2/M (A-type), and mitosis by the B-type cyclins.

All cyclins contain the cyclin core, which is a conserved region of 250 amino acids consisting of two domains, called the N and C domain. Domain N is a 100 amino acids long cyclin box, which is the distinctive domain for all cyclins. In contrast, domain C is less conserved and is not present in all plant cyclins.

The majority of the cyclins, including A- types, possesses a destruction box (D-box) that makes the proteolytic turnover through ubiquitinylation possible (Glotzer *et al.,* 1991). An exception is a very divergent cyclin of pea (Pissa;CycA2;2), which does not contain the prototypic destruction box. The combination of proteolysis and transcriptional regulation controls the abundance of premitotic A and mitotic B cyclins.

The plant A-type cyclins are subdivided into three subclasses, CycA1, CycA2 and CycA3. The general signature for all CycAs is the motif LVEVxEEV (Renaudin *et al*., 1996), although some CYCs have additional functionally conserved amino acid residues. Plant A-type cyclins have a variable N-terminal domain, which is much shorter in cyclins from the CycA3 group than in cyclins from the CycA1 and CycA2 groups. The consensus sequence for the destruction box is different in each CycA group: relatively loose in CycA1 (RxA/PLxNL/IxN), moderate in CycA2 (RAV/ILxDxxN) and highly conserved in all CycA3 proteins (RVVLGEL/IxN). These characteristics may suggest distinct mechanism and/or timing of cyclin degradation for each group (Chaubet-Gigot, 2000).

The CycA family is very complex and depending on the plant species multiple cyclins from a single species can be found in the same subclass. In the Arabidopsis genome two members

of *CycA1*, four A2-type cyclins and four *CycA3* genes are present, while two distinct members of the *CycA1* group have been found in maize and rice, two and four members of *CycA2* in pea and *Arabidopsis*, respectively, and three members of *CycA3* in tobacco (Nieuwland *et al*., 2007).

From tomato, only one single member from each subclass of A-type cyclins (*Lyces;CycA1;1*, *Lyces CycA2;1* and *Lyces CycA3;1*) has been described (Joubés *et al*., 2000). Experiments with synchronized tobacco BY2 cells showed that different A-type cyclins are expressed sequentially at different time points of the cell cycle from late G1/ early S-phase until mid M-phase (Reichheld *et al.*, 1996). *CycA1* and *CycA2* show a peak in expression at G2/M (Menges *et al*., 2005), while *CycA3* is expressed from G1/S to the end of the S-phase.

A-type cyclins interact with CDKB1 and CDKA1, and depending on the combination the phosphorylation activity of such a complex is different. This interaction also depends on the phase of the cell cycle, e.g. A-type cyclins seem to interact exclusively with CDKA1 during the S phase (Van Leene *et al.,* 2010).

A2-type cyclins play an important role in the regulation of endoreduplication in cells of *Arabidopsis thaliana.* This mechanism was described by Boudolf *et al*. (2009), who found that in Arabidopsis cells CycA2;3 is the activating partner of CDKB1;1. The activity of this CDKB1;1/CycA2;3 complex drives the mitotic cell cycle and prevent cells from entering the endocycle program. Co-expression of *CDKB1;1* and *CycA2;3* induces ectopic cell divisions (Boudolf *et al*., 2009), whereas knocking out *CycA2;2* together with overexpression of *CDKB1*;1 results in an increase of DNA ploidy level (Boudolf *et al*., 2004b). When Arabidopsis plants were generated with upregulation of CycA2;3 and CDKA;1, together involved in the endocycle, termination in Arabidopsis trichomes occurred (Imai *et al.,* 2006)

Another function of the CDKB1 and A2-type cyclin complex is maintaining the CDKA1 activity. A model has been proposed in which CDKB1;1 controls the level of CDKA1 activity by its phosphorylation and as a consequence, proteasomal degradation of the CDKA;1 inhibitor ICK2/KRP2 occurs. In such a scenario, the activity of the CDKB1;1-CycA2;3 complex may inhibit the action of ICK2/KRP2 and allows the cells to enter mitosis (Verkest *et al*., 2005b).

Here we describe experiments in which we overexpress *CycA2* from tomato specifically in the fruit. We found that overexpression of *CycA2* affects the cell expansion phase and time to ripening of the fruits. However, we did not find a direct involvement of *CycA2* in the regulation of endoreduplication despite of the reduction in cell sizes in the pericarp.

Results

In Chapter 2 we presented the analysis of the expression of *CycA2* in the pericarp and several plant tissues of wild-type tomato cv. M82 (Chapter 2, Figure 4). It is highly expressed in the first 10 developmental stages, gradually downregulated during the cell expansion and then hardly detectable at the mature green stage.

Overexpression of *CycA2* in developing fruits

To explore the effect of *CycA2* overexpression on tomato fruit development, transgenic tomato lines were generated in which the gene was overexpressed under the control of the fruit specific TPRP promoter (Carimi *et al*., 2003; Fernandez *et al*., 2009). From the 28 generated independent transgenic lines, 6 showed a high overexpression level of *CycA2* in the pericarp as was determined by real-time PCR (Figure 1A). The fruits of these lines were different when compared to wild-type fruits: they were irregular of shape more oval and were clearly smaller (Figure 2). We selected two of these lines (#15 and #26) for further analysis. The relative expression of *CycA2* in the fruit pericarp of these lines was also analysed by qRT-PCR in the transgenic progeny, which confirmed the overexpression observed in the primary transformants, although variation in the level of overexpression was observed (Figure 1, B and C).

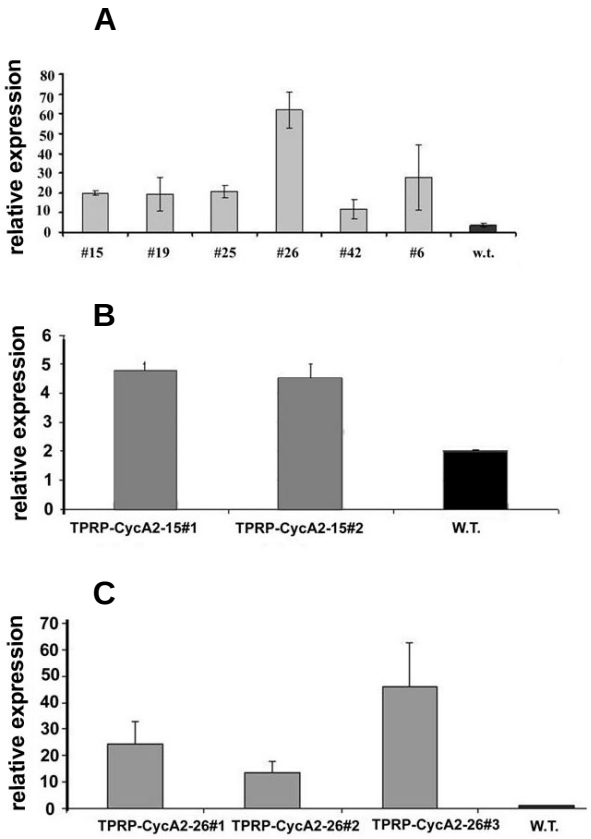


Figure 1. Relative expression of *CycA2* in the pericarp of transgenic lines

A. Relative *CycA2* expression was determined by real-time qRT-PCR in biological replicates 15 days after anthesis. The numbers represent primary transformants. Lines #15 and #26 were selected for further analysis.
B and **C.** Relative expression of *CycA2* in progeny plants from the primary transgenic lines #15 and #26.

Phenotypes of *CycA2* overexpressing fruits

In the second generation (T1) of TPRP-CycA2 plants the phenotype was apparent again. Fruits of the transgenic lines overexpressing *CycA2* have reduced size and weight, thinner pericarp than wild-type (Figure 2) and they reached the breaker stage about 7-10 days earlier compared to the wild-type siblings (Figure 7).

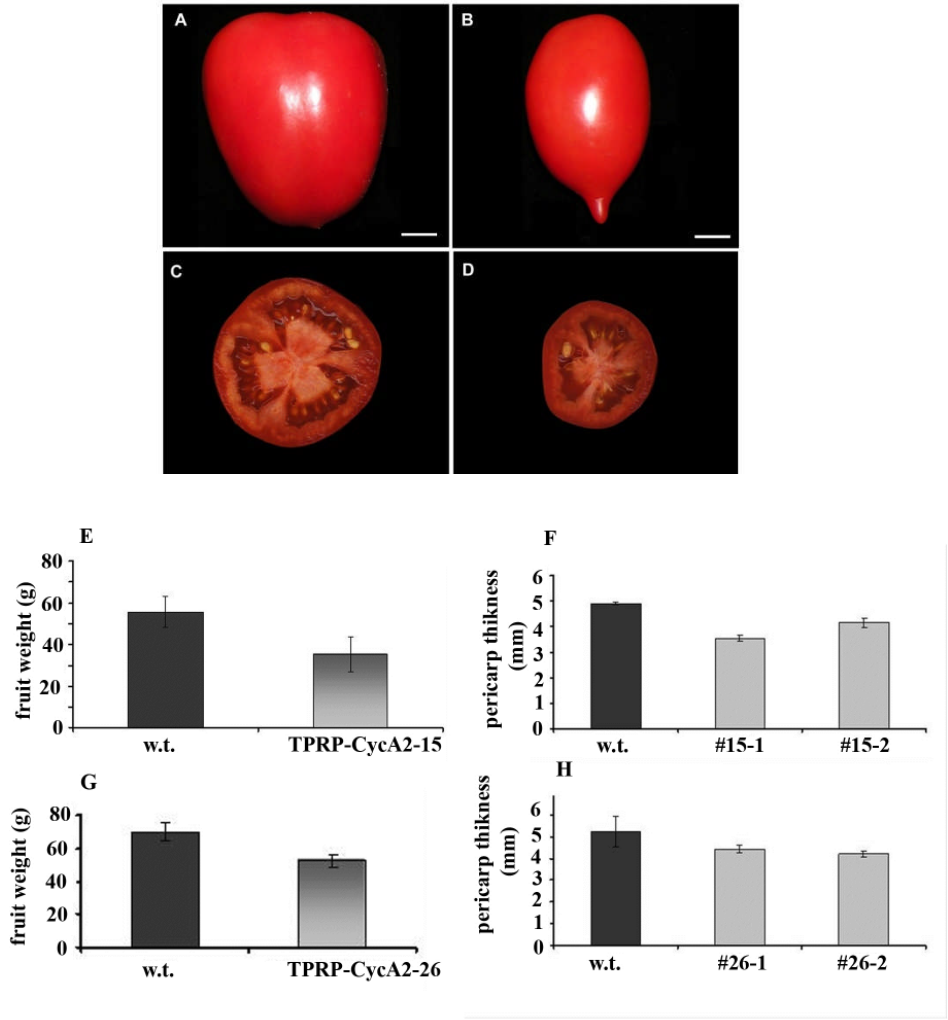


Figure 2. Phenotypes of control and *CycA2* overexpressing fruits.

A, C - Wild-type control
B, D - Transgenic fruit overexpressing *CycA2.* Bar= 10 mm
E, G - Fruit weight of control sibling and *CycA2* overexpressing plants (T1) from line #15 and #26 respectively . Standard deviation is indicated, n=7-10
F, H - Pericarp thickness of control sibling and *CycA2* overexpressing plants (T1). Standard deviation is indicated, n=5.
Differences are significant according to Students t-test, $p < 0.005$

Normal seed set and jelly production were observed, however, seeds obtained from the transgenic fruits had a reduced germination capacity. Only about 15 % of the sowed seeds germinated, while this was over 90% for wild-type seeds. We did not analyse embryo development in the seeds of the transgenic fruits, but taking into account that the TPRP promoter is active in developing seeds, it is possible that embryo development was negatively affected. In Figure 2 the obtained phenotypes are presented.

Histological analysis of transgenic fruits overexpressing *CycA2*

Histological sections of fruits at different developmental stages were studied and cell sizes and cell numbers were determined at the mature green stage. Data are depicted in Figure 3 and in Table 1.

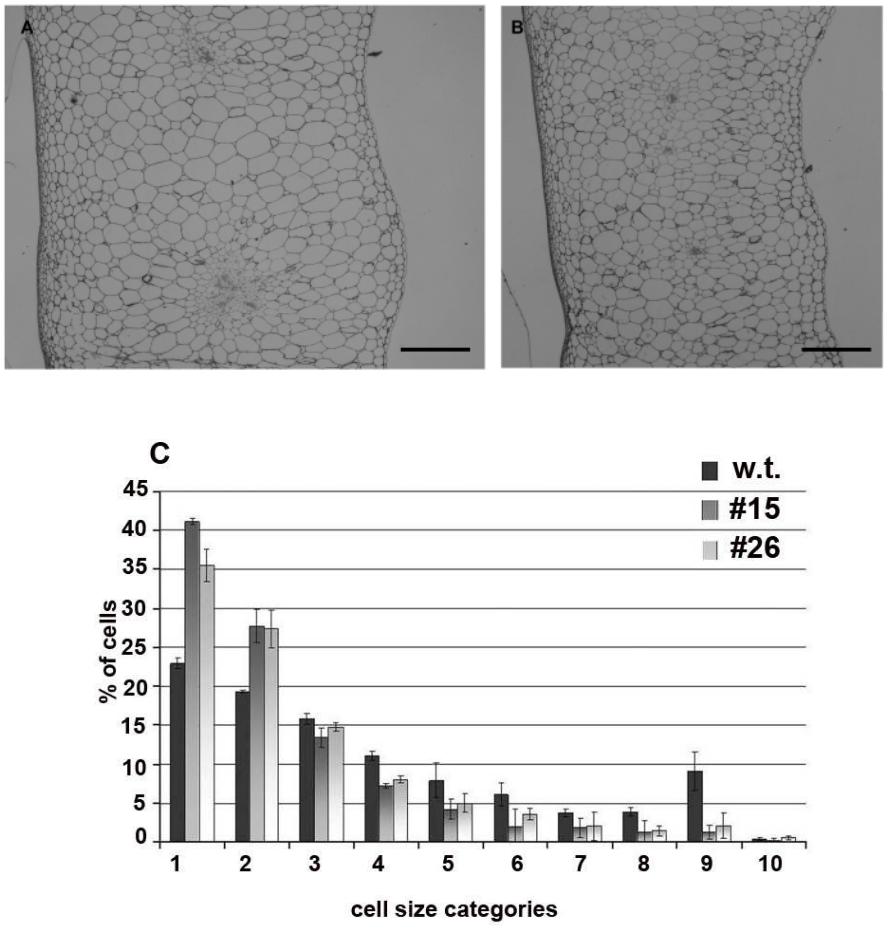


Figure 3. Phenotypic changes in pericarp due to overexpression of *CycA2.*

Microscopic cross-section through a pericarp of a control fruit **(A)** and through a pericarp of a *CycA2* overexpressing fruit **(B)** at the mature green stage. Bar= 1mm
(C) Cell sizes and numbers in pericarp of mature green fruits. Ten different size categories are defined with category 1 having an average size of up to 0.4 x 10^3 mm^2 and the maximum cell size in each subsequent category is doubled. Standard errors are indicated for a minimum of 4 measurements from 3 different fruits for each bar.

At the mature green stage, the pericarp of transgenic fruits is significantly thinner ($p<0.005$) compared to the wild-type (Figure 2, F and H), due to a reduction in size and number of cells (Figure 3, Table 1).

We quantified cell sizes and the number of cell layers in the pericarp of tomato fruits at the mature green stage. The cells were divided into ten cell size categories, the percentage of cells that fell into a certain category are depicted in Figure 3C. A larger percentage of the pericarp cells of *CycA2* overexpressing lines falls into the categories with the smallest cells (category 1 and 2) as compare to wild-type fruits, whereas a considerable amount of cells of wild-type fruits falls into the size categories with the larger cells (category 3-9).

The overall number of cells per surface unit (mm^2) in the mesocarp area is higher in the transgenic fruits, demonstrating that the average cell size is smaller than in wild-type. Also the number of cell layers was significantly reduced in the pericarp of the transgenic lines compared to the control (Table 1).

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

The data represent the means ± standard error of four measurements from 3 different fruits for each line. For all measurements, the differences between wild-type and transgenic lines were tested for statistical significance. The p-values (Students t-test) are indicated.

Line	Cells/mm^2 mesocarp	Cell layers
Wild-type	15.2 ± 2.24	29.78 ± 4.09
#15	25.23 ± 2.78 ($p<0.005$)	21.67 ± 3.33 ($p<0.005$)
#26	22.19 ± 2.17 ($p<0.05$)	25.29 ± 2.29 ($p<0.05$)

Duration of pericarp development is affected in transgenic fruits

To determine whether the differences in fruit size is mainly due to reduced cell division or shortened cell expansion phase we analysed the dynamics of cell size increase and changes in number of cell layers in the pericarp at different developmental stages in transgenic line TPRP-CycA2 #26 and control plants. The obtained results are depicted in Figure 4 and in Table 2.

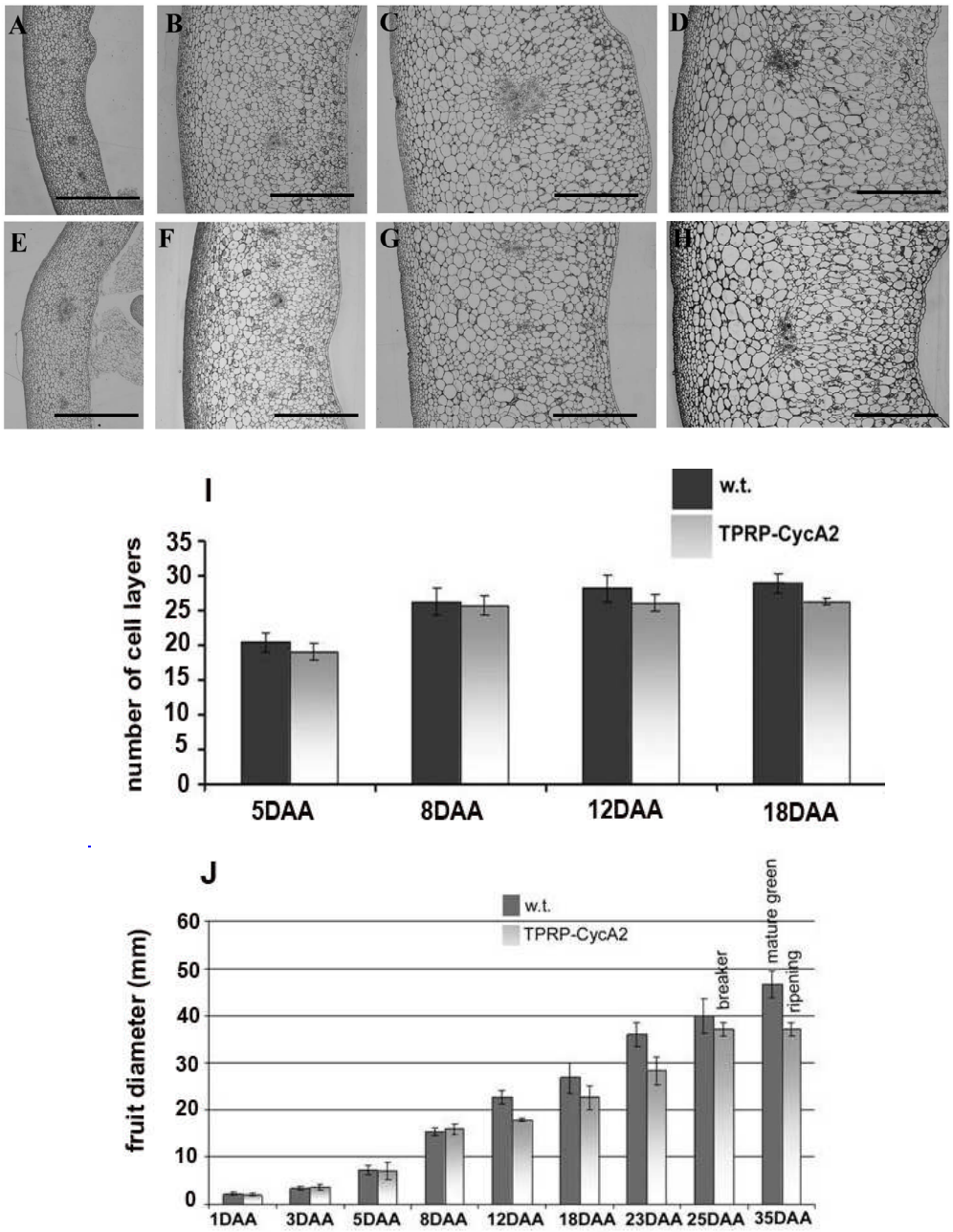


Figure 4. Pericarpcharacteristics duringdevelopment.

A-H Microscopic sections through the pericarp at various developmental stages.
A,B,C,D - pericarp of the wild-type control at stages 5, 8, 12 and 18 DAA, respectively.
E,F,G,H - pericarp of the TPRP-CycA2 overexpression line # 26 at stages 5, 8, 12 and 18 DAA, respectively.Bars=0,5 mm.
I - number of cell layers in fruits from four different developmental stages. Cell layers were counted using the images of the microscopic sections. Bars represent the means of 3-4 analysed imagesfrom three fruits. At 5 and 8 days after anthesis the differences between wild-type and transgenic fruits were not significant. At day 12 and day 18 the differencesarestatistically significant according the Student's t-test - $p<0.039$ and $p<0.013$ respectively.
J- dynamics of fruit diameter growth from one day after anthesis until mature green/breaker stage. At 25DAA, the wild type fruits are still growing up to 35 days after anthesis, while the transgenic fruits are fully grown and start changing color.

Table 2. Quantification of the number of cells per surface unit (cell size) in the pericarp of wild-type and transgenic fruits collected at several developmental stages.

The data represent the means ± standard error of 3-4 measurements from 3 different fruits. For all measurements, the differences between wild-type and transgenic lines were tested for statistical significance (p-values according the Students test).

Line	Mesocarp at 4 different days after anthesis (DAA) Cells/mm^2			
	5 DAA	8 DAA	12 DAA	18 DAA
Wild-type	630± 107	287±33	118±39	68±5
TPRP-CycA2 #26	713± 91(p=0.26)	284± 42 (p=0.92)	146±13 (p=0.13)	89±16 (p<0.05)

The differences in fruit size (diameter) became clearly apparent after 12 DAA (Figure 4, J). Surprisingly, the fruits of the transgenic plants enter the breaker stage already 25-27 days after anthesis, which was about 10 days earlier than wild-type fruits.

Five days after anthesis there were no clear differences between the pericarp thickness and cell size in wild-type and transgenic fruits. The differences in cell size and number of cell layers became visible 12 days after anthesis. Remarkably, the number of cell layers in the pericarp of the transgenic fruits did not increase after 8 days and cells only enlarge, while in the wild-type fruits the number of cell layers were still increasing, although only slightly, indicating that cell division still continues in wild-type fruits.

The number of cells per square mm (=cell size) in the pericarp was comparable between wild-type and transgenic fruits up to 12 DAA. After that stage, the rate of cell expansion seems to be higher in wild-type than in transgenic fruits. A significant difference was observed 18 DAA (Table 2) and in the pericarp of mature fruits (Table 1).

Expression of *CycA2* at the different developmental stages

To show the differences in expression and its changes during fruit development we analysed the expression of *CycA2* in control and transgenic line #26 at different stages from anthesis till mature green. To find possible correlations between changes in pericarp development and expression level of *CycA2* we used for the RNA extraction samples from the same fruits as we collected for microscopic analysis.

Expression analysis by real-time quantitative PCR shown in Figure 5 revealed that in transgenic line #26 the expression level of *CycA2* is 6-7-fold higher than wild-type expression and is longer maintained.

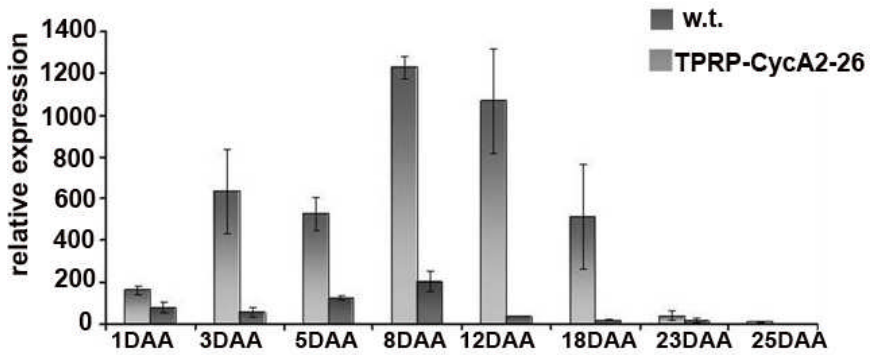


Figure 5. Expression of *CycA2* in the pericarp of transgenic and wild-type plants at different developmental stages. Stages are indicated as days after anthesis (DAA).
Expression determined by qRT-PCR analysis in two biological replicas. Each replica is a pool of three fruits collected at the same stage after anthesis.

Endoreduplication index in transgenic and wild-type fruits

Because several studies (Imai *et al.,* 2006; Yoshizumi *et al*., 2006; Boudolf *et al*., 2009) report a direct connection between the activity of A2-type cyclins and the regulation of the endocycle we analysed the number of DNA-copies in fully developed fruit. After determining the ploidy levels in pericarps of transgenic and control fruits by flow cytometry the endoreduplication index (EI) was calculated. Despite that the size of pericarp cells in transgenic fruits was significantly smaller, the level of endoreduplication and EI index of the mesocarp cells did not differ from control fruits. The results are depicted in Figure 6.

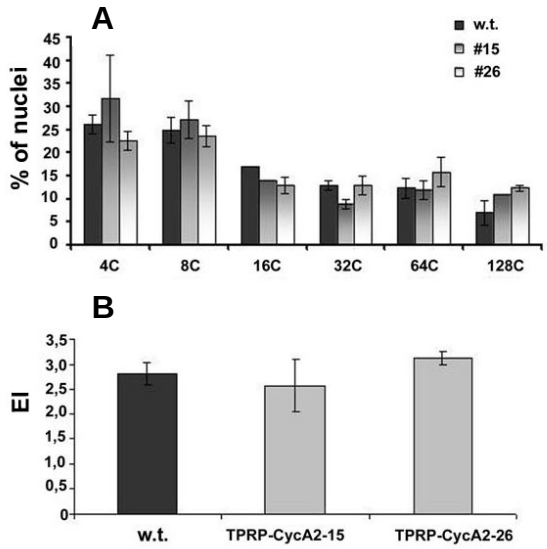


Figure 6. Ploidy distribution analysis.

A. Ploidy distribution analysis of tomato mesocarp at the breaker stage.
B. Endoreduplication index (EI), which is the mean number of endoreduplication cycles per nucleus. Values are means ±SD (n=3). No significant differences in copy number and EI were observed between wild-type and transgenic fruits.

Discussion

A-type cyclins are the largest group of plant cyclins and their functions in the cell cycle and plant development are very divers. We characterize further the role of the tomato *CycA2* in fruit development by overexpressing this gene in tomato.

The tomato *Cyclin A* types were first isolated by Joubés *et al.* (1999; 2001) and their expression patterns were analysed during fruit development and in tomato cell suspension cultured under different hormonal and nutrient regimes. These data, which were obtained by using semi-quantitative PCR on cherry tomato (*Lycopersicon esculentum* Mill. cv. West Virginia 106) are partly according to our own findings by real-time quantitative PCR analysis on tomato pericarp and different plant tissues of the processing tomato M82. We analysed the expression of *LeCycA2* and *LeCycA1* at different developmental stages from anthesis till the mature green stage (Figure 4 in Chapter 2 of these thesis). The expression of *CycA2* is highest during the cell division phase and at the beginning of cell expansion/endoreduplication phase. Previously, it has been shown that A2-type cyclins are active in proliferating tissues and that they operate from the late S through the G2 phase of the cell cycle and they are degraded when cells enter the M-phase (Geley *et al*., 2001; Roudier *et al*., 2003; Imai *et al*., 2006).

The *CycA2* overexpressing tomato fruits show a slight decrease in the number of pericarp cells and a substantial reduction in cell sizes, which resulted in smaller fruits compared to wild-type control fruits. This phenotype is reminiscent to dwarf plants with smaller cells, which was observed when *CycA2* with a modified D-box was overexpressed in Arabidopsis (Umeda *et al.,* 2006; Boudolf *et al*., 2009), so that CycA2 was continuously produced without destruction. This overexpression in Arabidopsis with the modified version of the mitotic CycA2 leads to an abnormal accumulation of CycA2 proteins, which retards the mitotic cell cycle in proliferating tissues instead of promoting cell divisions or, alternatively, it affects the timing of cell divisions. In our experiment the D-box in *CycA2* was not modified, so we can expect the normal destruction of CycA2 during the cell-cycle. Hence, we do not know whether the 6-7 fold increase in mRNA levels in the transgenic fruits compared to wild-type also leads to an increase in stable CycA2 protein.

The *CycA2* overexpressing fruits grow at a similar rate as the wild-type fruits during the initial phase when mainly cell division takes place. At later stages, we observed a slight decrease in the number of cell layers, which is possibly due to a reduction of cell division in the exocarp where cell division retains till the full grown stage. No significant effect on cell size was observed up to 12 days after anthesis, but the cells in the full grown overexpression fruits were significantly smaller and this reduction contributed to a large extent to the total size reduction of the fruit.

These results suggest that the expansion phase was affected, although we could not demonstrate a reduction in endoreduplication, which normally coincides with cell expansion.

The best studied role of A2-type cyclins in plant tissues is their involvement in the regulation of endoreduplication (Imai *et al*., 2006; Boudolf *et al*., 2009). The involvement of *CycA2* in this process was reported by Imai *et al*.(2006) who showed that in Arabidopsis cells *CycA2;3* acts as a negative regulator of the endocycle. CycA2;3 forms a functional complex with CDKA;1 and negatively controls the entry into a new DNA synthesis phase. The functional complex CycA2;3 /CDKA;1 is upregulated at the end of the endocycle when the required number of DNA copies is obtained (Imai *et al*., 2006). Loss of function of CycA2;3 caused an increased level of endoreduplication due to the termination of the mitosis and precocious entering of cells into the endocycle (Imai *et al*., 2006).

In the pericarp of the fruits overexpressing *CycA2* the endoreduplication level was not significantly altered, while we expected a reduction in endoreduplication based on the study by Imai *et al*. (2006). Thus, the endocycle appears to be unaffected, while cell sizes were reduced. This suggests that cell growth is not absolutely coupled to DNA copy number and when the timing or duration of cell division and expansion phases is modified, cell growth and DNA replication seem to be uncoupled. The reduced cell size and the shortened duration of fruit development from pollination till breaker stage support this hypothesis. In both analysed lines the development till full grown fruits was up to 10 days faster than in wild type fruits. A reduction in cell expansion duration combined with reduced levels of cell proliferation was also observed in Arabidopsis plants with an overproduction of CycA2 proteins with a modified D-box (Umeda *et al.*, 2006). These results suggest that *CycA2* has similar functions in Arabidopsis and tomato in the regulation of cell expansion, although the mechanisms are not fully clear and may differ between the two species. The ploidy levels in the tomato tissues are not affected by *CycA2* overexpression, suggesting that the endocycle itself is not affected, which seems to be different from Arabidopsis where upregulation of *CycA2* prevents the entry into the endocycle.

It is generally proposed that in tomato fruits endoreduplication is tightly associated with expansion (Cheniclet *et al.*, 2005; Chevalier, 2007). In his recent review Chevalier *et al*. (2011) proposed that endoreduplication in tomato is more a facilitator of cell growth rather than a driver of fruit growth. The down-–regulation of endoreduplication promoting genes, such as *SIWEE1* and *SICCS52A* resulted in smaller cells and reduced fruit size as a result of decreased DNA-copy number (for review Chevalier *et al*., 2011), while the early onset of endoreduplication due to overexpression of *SIKRP* impaired the cell growth without affecting the final fruit size (Nafati *et al*., 2011). In these particular cases the duration of fruit growth was not altered. We hypothesize that in our experiments with fruit-specific overexpression of *CycA2* the cell expansion phase was affected independently from the endocycle.

Further analysis of the TPRP-CycA2 lines may shed light on the relation between expansion and endoreduplication and how *CycA2* is able to fasten fruit development.

Material and methods

Plant material

Tomato plants *Solanum lycopersicum*, cv. M82, and obtained transgenic lines were grown on soil in a greenhouse under a 16-h of light and 8-h of dark conditions. Supplementary lights (600 Watt high pressure sodium lights) turned on below 200 W/m^2 and turned off above 300 W/m^2 solar irradiation. Temperature was kept above 20^{0}C during the light period and 17^{0}C during the dark period controlled with the PRIVA Integro version 724 system. Plants were watered daily and given fertilizer weekly. The extra tomato fertilizer Osmocote was added in the soil to improved the calcium supply and prevented blossom-end rot in fruits.

Construction binary vectors for transformation

To generate the fruit specific *CycA2* overexpression lines, the coding sequence of *Lyces; CycA2* (accession number Y17225) was cloned into the pENTRtm/D-TOPO entry vector (Invitrogen). Obtained clone was 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 simply recombined *in vitro.*

Transformation of tomato

Transgenic tomato plants were generated by Agrobacterium tumefaciens-cocultivation of seedling cotyledons, as described in De Jong *et al*. (2009b). Plants were selected on kanamycin-containing medium and then checked by PCR with primers specific partially for TPRP-promoter and gene of interest on genomic DNA. Subsequently, lines were tested for ploidy, as only diploid lines were used for further analysis.

Harvesting plant material

To avoid the differences in source-sink balance affected by fruit number and position (Bertin *et al*., 2001) we left 5 fruits at each truss, the additionally pollinated flowers were removed. The first developing truss from each plant was removed.

For mRNA isolation and histological analysis we collected the second and third fruit from a the second or third truss on the plant.

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

RNA was isolated and reverse transcribed to cDNA following the protocol described in chapters 2 and 3. Also for the real-time quantitative PCR, the same conditions were used also mentioned in chapter 2. Real-time –quantitative RT-PCR (qRT-PCR) primers were designed using a computer program (Beacon Designer Software, Premier Biosoft International, CA, USA). Primers for *CycA2* : F 5'-ACGACTTGAAGTTCCCTGTG-3' and R 5'- GTGATGCTTGAACGAATCTCC-3'.

As control genes *Actin 2/7* F- 5'-GGACTCTGGTGATGGTGTTAG-3' and R- 5'- CCGTTCAGCAGTAGTGGTG-3' and *Le18S* F-5'-AGACGAACAACTGCGAAAGC-3' and R-5'-AGCCTTGCGACCATACTCC-3' were used.

Relative mRNA levels were calculated following the Bio-Rad outlined methodology based on Vandesomple *et al*. (2002) and corrected for PCR efficiencies. The average of two biological repeats is depicted with the SD.

Histological analysis

Fruits were analysed at different stages after anthesis. Fruits were cut along the equator to remove seeds and pulp. The parameters weight, diameter (height and width) 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 (Dujmović *et al.,* 2005; Brewer *et al.,* 2006; Gonzalo *et al.,* 2009).

Ploidy analysis and Endoreduplication Index (EI)

Nuclei were prepared from the pericarp of orange ripe fruit. Two types of tissue were analysed - subepidermal layer and mesocarp tissues. Nuclei were isolated according to De Laat *et al.* (1987) and stained with a "high resolution DNA kit" (Partec). The suspension was filtered through a 100mm 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). After flow cytometry the EI was calculated from the number of nuclei of each represented ploidy level multiplied by the number of endoreduplication cycles necessary to reach the corresponding ploidy level EI = (1*4C + 2x8C+ 3*16C+4x32C + 5*64C + 6*128C + 7*256C)/100 (Boudolf *et al*., 2009).

6

Effect of down regulation of *CDKB1* and *CDKB2* expression in tomato fruits

Anna Czerednik, Marco Busscher, Peter de Groot, Gerco C. Angenent and Ruud A. de Maagd

Abstract

Fruit-specific downregulation of *CDKB1* and *CDKB2* in tomato was achieved using an amiRNA approach. The obtained transgenic fruits had reduced weight and decreased cell size in the pericarp. The fruits of the *amiCDKB1* lines were stenospermocarpic, while in the *amiCDKB2* lines seeds developed properly. For both types of transgenic plants a characteristic feature was the lack of abscission of ripe fruits.

Introduction

Plants possess a unique class of Cyclin Dependent Kinases (CDKs), the B-type, for which no evidence has been found in other phyla. These kinases are unable to complement yeast *cdc2/cdc28* mutants as *CDKA1* can (Imajuku *et al.,* 1992; Fobert *et al*., 1996), suggesting that they regulate only plant-specific aspects of the cell cycle. In Arabidopsis, two *CDKB1* genes and two *CDKB2* genes were found, but in tomato only a single gene representing each class is known so far. The unique motifs PPTALRE or PPTTLRE are specific marks of CDKB proteins. The accumulation of *CDKB2* transcripts is strictly specific for G2 and M phases and both B-type CDKs show maximum kinase activity during the M-phase (Porceddu *et al*., 2001). However, the activity of B-type CDKs is linked with the G2-to-M transition phase, although *CDKB1* transcripts accumulate already from late S-phase onwards, which suggests that *CDKB1* is already functional before its observed expression peak at the G2-to-M transition (Segers *et al*., 1996; Porceddu *et al*., 2001; Sorrel *et al*., 2001; Menges *et al*., 2002; Boudolf *et al*., 2009). B-type CDKs generally interact with mitotic cyclins, however, the recent analysis of protein-protein interactions for Arabidopsis cell cycle proteins showed their ability to interact with S-phase cyclins (Van Leene *et al*., 2010; Boruc *et al*., 2010).

Functions of B-class CDKs have been intensively characterized in Arabidopsis plants (Boudolf *et al*., 2004a,b; Andersen *et al.,* 2008; Boudolf *et al*., 2009). CDKBs are key regulators of the mitotic cell cycle and are highly expressed in dividing cells as well as in cells with high competence for cell division (Andersen *et al*., 2008). Moreover, the functional complex CDKB1,1-CycA2;3 regulates endoreduplication levels in Arabidopsis cells (Boudolf *et al*., 2009) and is important for the formation of stomata guard cells (Boudolf *et al*., 2004a). Our results presented in Chapter 3 suggest the possible (regulatory) interaction of CDKB1 and CDKB2 with CDKA1, which may result in modification of the activity of CDKA1, especially in the tomato peel.

During tomato fruit development the phase of cell expansion is tightly connected with endoreduplication, where DNA synthesis occurs in the absence of mitosis. Endoreduplication may contribute to plant organ growth and have an effect on plant yield (for review see Chevalier *et al*., 2011).

The cells of tomato fruit become highly polyploid and it is supposed that the cell size of tomato fruits is correlated with the DNA copy number (Bergervoet *et al*., 1996; Cheniclet *et al*., 2005), while the final size of the fruit is also positively correlated with the ploidy level (Chevalier *et al.,* 2011).

We found that downregulation of *CDKB1* and *CDKB2* affects cell size and number, and ultimately tomato yield characteristics. The fruits with down regulated *CDKB2* were smaller than wild-type fruits due to a decrease in cell size. From the transformation with a TPRP-promoter driven-*amiCDKB1* construct we obtained only one line with reduced expression of *CDKB1*, which failed to produce fully developed seeds. Fruits from both genotypes did not abscise from the truss and desiccated on the plant. We discuss the cause of these phenomena, and hypothesize about the possible reason for defective seed development and altered hormone signaling due to downregulation of *CDKB1/CDKB2* in the transgenic fruits. These transgenic lines may be suitable starting material for studies on improvement of fruit quality through reduced abscission.

Results

To knockdown the expression of *CDKB1* and *CDKB2* we followed an artificial microRNA (amiRNA) approach (Schwab et al., 2006). Precursor *amiRNA* molecules were designed that should produce mature *miRNAs*, which are able to suppress the expression of the target genes post-transcriptionally. Expression of the obtained *pre-amiRNA* constructs was driven by the *TPRP* (*Tfm7*) fruit-specific promoter (Salts *et al*., 1991, 1992, chapter 2).

From the transformation with the p*TPRP-amiCDKB2* construct 12 independent transgenic lines were obtained. Fruits were harvested 5-6 days after anthesis and pericarp tissues were subjected to qRT-PCR to demonstrate the downregulation of *CDKB2*. 6 lines showed clear reduction in *CDKB2* expression, suggesting that the amiRNA strategy was successful (not shown). All transgenic lines with downregulation of *CDKB2* were able to make the transition to flowering and produced seeds with a normal degree of germination. For further studies we selected line #4 and line #6, whose *CDKB2* mRNA levels were reduced 3.5 and 5-fold, respectively. This downregulation was confirmed by qRT-PCR in the T1 generation. From each line two plants with low expression of *CDKB2* in fruit pericarp were selected. The results obtained from qRT-PCR analysis are presented in Figure 1A.

From the 8 plantlets obtained after transformation with the p*TPRP-amiCDKB1* construct only one showed knock-down of *CDKB1* expression in the pericarp. The relative expression level of *CDKB1* in the pericarp of transgenic line was 6-fold decreased (Figure 1B).

Because the fruits of this line failed to produce well developed seeds even when pollinated with the pollen of wild-type plants, we could not obtain a next generation of p*TPRP-amiCDKB1* transformants. In this case only one line (T0) was analysed in detail.

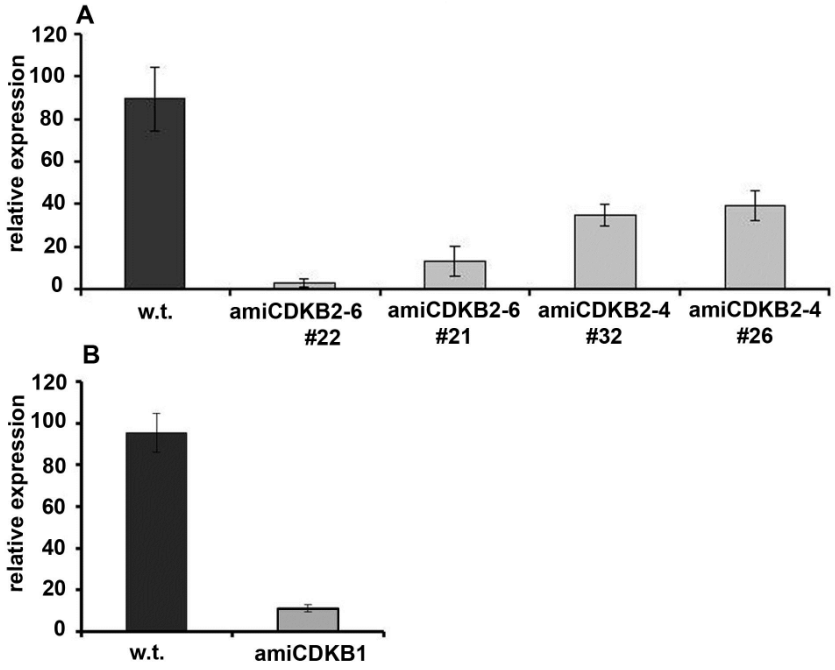


Figure 1. Relative expression of *CDKB2* (**A**) and *CDKB1* (**B**) in pericarp of transgenic lines 4 days after anthesis.
For the *amiCDKB2* construct, two progeny plants each from parental (T0) lines 4 and 6 were analysed. In the case of the *amiCDKB1* construct only one line with decreased expression of *CDKB1* was available in the T0 generation, and no viable seeds, and thus no T1 plants could be obtained.
qRT-PCR performed on samples collected 5-6 days after anthesis. Data are means of two biological replications ± SD (standard deviation).

Plants with decreased *CDKB2* expression had reduced fruit sizes (Figure 2 B,D and Figure 3 B,D) compared to the wild type (Figure 2 A,C). The timing of fruit development was similar to that of the wild-type and took approximately 35-40 days from pollination to the ripe stage. All fruits had well developed seeds and locular gel tissue (Figure 2 D).
Fruits of the transgenic plant with decreased activity of *CDKB1* were clearly smaller, and had a remarkable pointy tip at the stylar end (Figure 2 F,H). All fruits were stenospermocarpic. Following fertilization with pollen from the same plant or from wild-type Ida Gold plants the embryos of transgenic fruits began to grow, but further development was aborted, which lead to seemingly seedless fruits. Despite the arrest of normal seed development the gel was well developed and no empty locular cavities were observed (Figure 2 H,J).

Figure 2. Phenotypes of *CDKB* knock-down lines.

A, C, E, G. Wild-type Ida Gold
B, D - *pTPRP-amiCDKB2*
F, H - *pTPRP-amiCDKB1*
Scale bar indicates 10 mm

I. Seeds of wild-type and **J.** Aborted seed development in *amiCDKB1* fruits
Scale bar indicate 1 mm

Several physiological parameters of fruits with reduced expression of *CDKB1/CDKB2* are presented in Figure 3. Fruits of transgenic plants for both constructs showed in most cases significant reduction in weight with the exception of fruits from plant #6-22 (#4-32 $p<0.05$; #4-28 $p<0.005$; #6-21 $p<0.05$; #6-22 $p=0.21$, Student's t-test). Analysed fruits had a significant reduction in fruit size (diameter) according to a Student's t-test (#4-32 and #4-28 $p< 0.005$; #6-21 and #6-22 $p<0.05$) and significant reduction in pericarp thickness in all cases ($p<0.005$) (Figure 3F). A reduction in pericarp thickness of *amiCDKB1* fruits ($p<0.005$) was correlated with decreased weight and size (diameter) as well (Figure 3 A,C). Fruit firmness (as measured with a Fruit pressure tester in the ripe stage) was increased in fruits with down regulated *CDKB2* ($p< 0.05$) (Figure 3H). In contrast, the firmness of fruits with decreased *CDKB1* expression did not significantly change ($p=0.2$) compared to the wild-type (Figure 3G). We observed reduced cracking of fruits with downregulation of *CDKB2*.

In contrast fruit cracking was not changed in *amiCDKB1* plants compared to the control plants (data not shown).

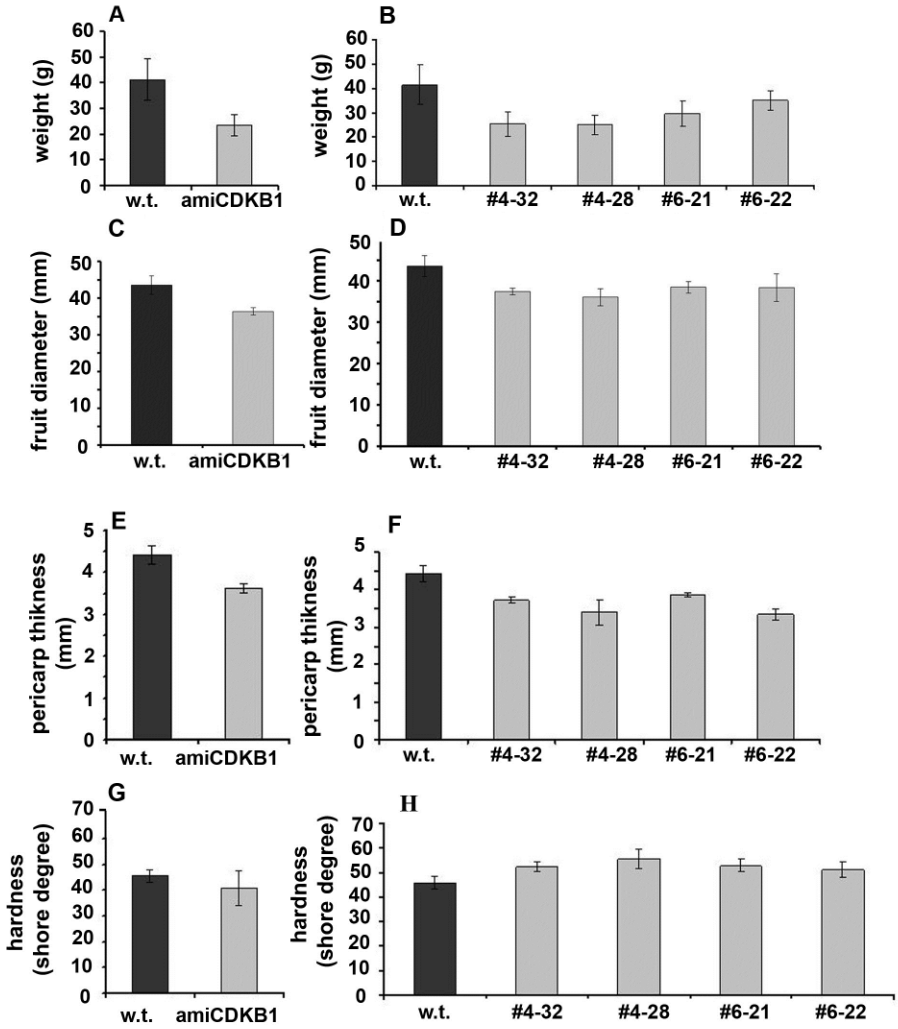


Figure 3. Physiological parameters of transgenic fruits as compared to the wild-type.

A, B. Fruit weight of *TPRP-amiCDKB1* and of *TPRP-amiCDKB2* plants, respectively.
C, D. Fruit diameter of *TPRP-amiCDKB1* and of *TPRP-amiCDKB2* plants.
E, F. Pericarp thickness of amiCDKB1 and of *amiCDKB2* plants.
G, H. Fruit firmness of *TPRP-amiCDKB1*and of *TPRP-amiCDKB2* plants.
In each case means and standard deviation for 6-9 fruits are shown.

We quantified cell size, cell number and cell layers in the pericarp of tomato fruits at breaker stage. The detailed analysis of pericarp cell parameters is presented in Figure 4 and Table 1.

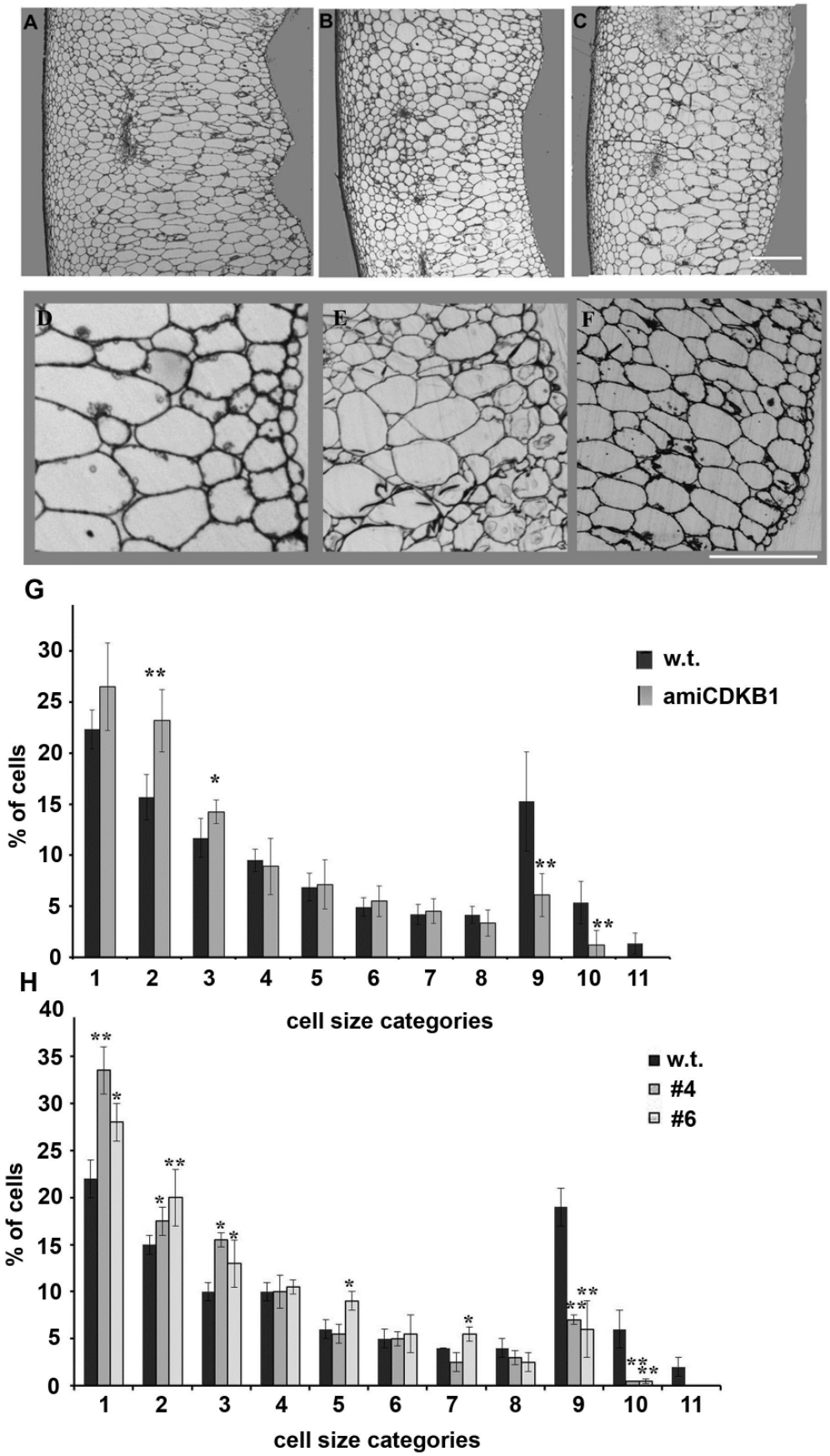

A
B
C
D
E
F
G
% of cells
w.t.
amiCDKB1
cell size categories
H
% of cells
w.t.
#4
#6
cell size categories

Table 1. Quantification of the number of cells per surface unit and of number of cell layers in pericarp sections of wild-type and transgenic fruits harvested at the breaker stage.

The data represent the means ± standard error of 6-10 measurements each for 3 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.

line	cells/mm^2 pericarp	Number of cell layers in pericarp
w.t.	19 ± 1.5	23 ± 1
#4-26	32 ± 2.0 (p<0.005)	21 ± 2 (n.s.)
#4-32	27 ± 2.0 (p<0.05)	21 ± 1 (n.s.)
#6-21	27 ± 5.0 (p<0.05)	22 ± 1 (n.s.)
#6-22	28 ± 1. 5 (p<0.05)	23 ± 2 (n.s.)
amiCDKB1	24 ± 3.0 (p<0.05)	18 ± 1 (p<0.05)

The microscopic analysis of the pericarp showed in both knock down lines thinner pericarp with decreased cell sizes compared to the wild-type (Figure 4 A-F). The pericarp cells of *amiCDKB1* fruits had irregular shapes and were surrounded by a lot of intercellular spaces (Figure 4 E). The overall cell size reduction in transgenic fruits from the *TPRP-amiCDKB1* lines corresponds to a significant change of cell number in category 2 (p<0.005 Student's t-test), category 3 (p<0.05), category 9 (p<0.005) and category 10 (p<0.05). Category 11 contained only cells from the wild-type. Furthermore, the microscopic analysis showed that the number of cells per mm^2 appeared to be significantly higher in the pericarp of transgenic fruit with down regulated *CDKB1* (Table 1). The reduced expression of *CDKB2* resulted in decreased size of pericarp cells and the percentage of cells in different categories was significant different in category 1 (#4 p<0.005 and #6 p<0.05), in category 2 (#4 p<0.005 and #6 p<0.05), in category 3 (#4 for p<0.005 and #6 p<0.05), category 5 for #6 (P<0.05), in category 7 for line #6 (p<0.05), in category 9 (for both lines p<0.005) and in category 10 (for both lines p<0.005).

Figure 4. Microscopic analysis of transgenic fruits with downregulation of *CDKB1* and *CDKB2*.

A,B,C. Microscopic cross-section through a pericarp of a control, a *TPRP-amiCDKB1* and a *TPRP-amiCDKB2* fruits respectively. Scale bar = 1mm

D,E,F. Enlargements of pericarp area of a control fruit, a *TPRP-amiCDKB1* fruits and a *TPRP-amiCDKB2* fruits respectively. Scale bar =0,1 mm

G, H. Cell sizes in pericarp of *TPRP-amiCDKB1* and *TPRP-amiCDKB2* fruits respectively.

In both cases cells were pooled into 11 different size categories. Cells in category 1 have an average size of up to 4 * 10^{-3} mm^2 and the maximum cell size in each subsequent category has doubled. Standard deviations are indicated for a minimum of 4 measurements from 3 different fruits for each bar. Statistical significance represents * P<0,05 **P<0,005 according Student's t-test

All fruits were harvested at the breaker stage.

Category 11 with the largest cells contained cells only from the wild-type. In the case of *TPRP-amiCDKB2* lines the decrease in the number of cell layers was minor.

We analysed the endoreduplication levels in the pericarp cells, as an independent marker for cell-cycle activity. After the measurements of ploidy levels in tomato mesocarp using flow cytometry we calculated the endoreduplication index, which is the mean number of endoreduplication cycles undergone by each nucleus (Figure 5). In the case of fruits with reduced expression of *CDKB2* endoreduplication was elevated significantly ($p<0.05$) compared to the wild-type. In the case of downregulation of *CDKB1* we did not observe any difference in the endoreduplication index as compared to the control ($p=0.1$). In the case of fruits with downregulation of *CDKB2* the EI was significantly ($p<0.05$) higher than for the control.

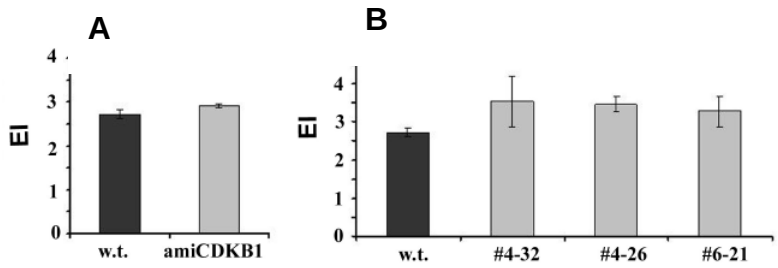


Figure 5. Endoreduplication index (EI) of mesocarp cells of transgenic fruits.

A. Wild-type and fruits from *TPRP-amiCDKB1* line (T0).
B. Wild-type and fruits from *TPRP-amiCDKB2* lines (T1) (fruits from #6-22 were not analysed).
Bars indicated standard deviations, n=3

The most remarkable feature of the transgenic plants was the lack of any abscission of fruits with reduced expression of *CDKB1* or *CDKB2*. The fruits desiccated while remaining on the truss, in contrast to fruits from Ida Gold wild-type, which normally fall off easily even before reaching the ripe stage (Figure 6).

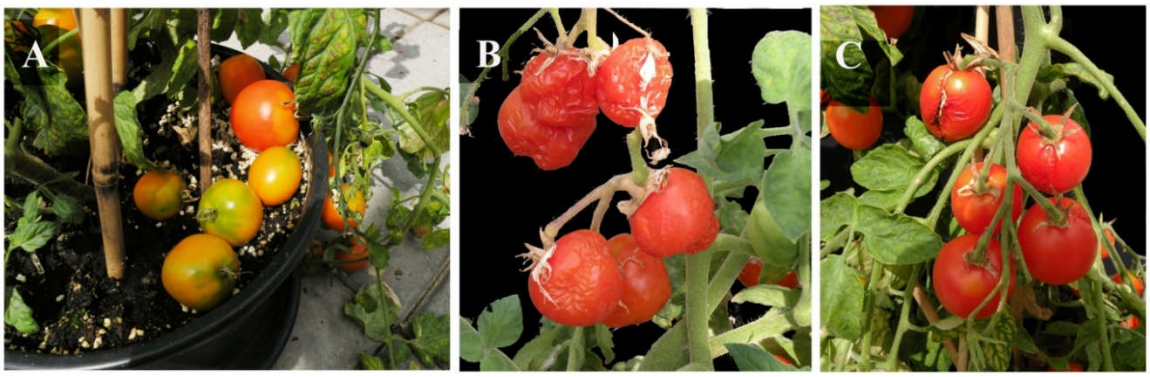


Figure 6. Defective abscission of fruits from transgenic plants.
A. Wild-type Ida Gold, whose fruits are often falling before they reach ripe stage.
B,C Truss of *TPRP-amiCDKB2* line and *TPRP-amiCDKB1* respectively
Fruits from the lines with downregulation of *CDKB1/CDKB2* activity were desiccating on the trusses.

Because abscission of fruits is regulated by changes in the levels of ethylene, abscisic acid (ABA) and auxins, and the proper activity of CDKB2 is required for appropriate hormone signaling (Andersen *et al*., 2008) we did a preliminary assessment of hormone levels in seeds of *amiCDKB2* lines. The results are presented in Figure 7. In seeds of the transgenic fruits we observed increased amounts of auxin and its conjugates IAA-aspartate (IAA-Asp) and indole-3-acetyl-glutamate (IAA-Glu). These normally accumulate in large amounts in cotyledons and mature seeds, forming one of the possible sources of IAA (Bialek *et al.*, 1992). Moreover, IAA-conjugates act as a reservoir for the homeostatic control of IAA concentrations and may function in auxin transport within the plant (citied after Park and Park, 1987).

The level of ABA was slightly decreased in transgenic seeds, but the levels of the ABA conjugate abscisic acid glucose ester (ABAGE) , the ABA-metabolites phaseic acid (PA) and the 2-trans-isomer of ABA (t-ABA) were higher in wild-type seeds compared to transgenic seeds (Figure 7).

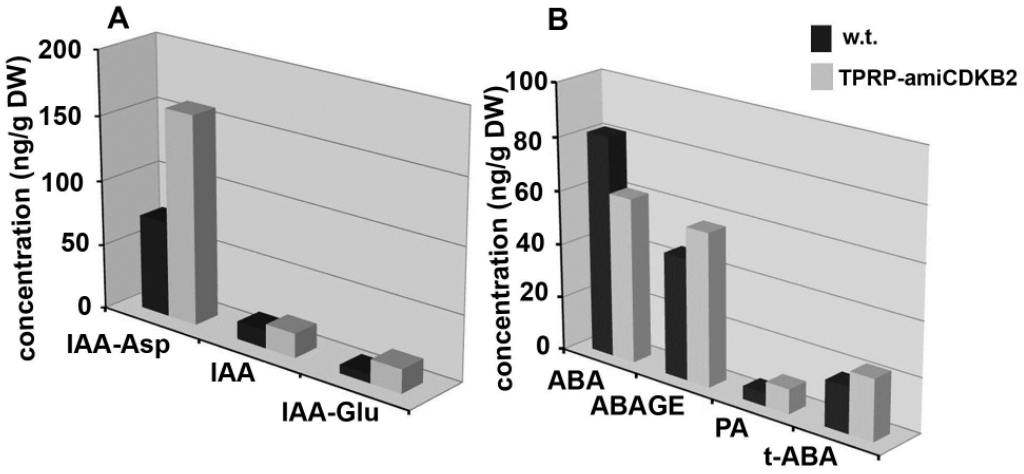


Figure 7. Preliminary analysis of the level of several hormones and precursors in seeds from a *TPRP-amiCDKB2* line.

A. Level of indole-3-acetic acid (IAA), and its conjugates IAA-aspartate (IAA-Asp) and indole-3-acetyl-glutamate (IAA-Glu) in dry seeds of transgenic fruits with reduced *CDKB2* activity.

B. Level of ABA and its conjugate - abscisic acid glucose ester (ABAGE), as well as of ABA-metabolites phaseic acid (PA) and 2-trans-isomer of ABA (t-ABA) in dry seeds of transgenic fruits.

Discussion

Cell size distribution in transgenic lines with *CDKB* downregulation

CDKB1 and CDKB2 belong to the mitotic cyclin-dependent kinases, which operate in the S-G2-M transition in the case of CDKB1 and in the G2-M transition in the case of CDKB2 (Magyar *et al.,* 1997; Umeda *et al.,* 1999).

The kinase activity of CDKB1 and CDKB2 proteins reaches a maximum during mitosis (Porceddu *et al*., 2001; Sorrell *et al*., 2001). In wild-type tomato fruits the exit from the cell division phase of fruit growth correlates with decreased expression of these two genes (see Chapter 2, Figure 4).

Fruits from *amiCDKB1* and *amiCDKB2* lines showed reduced pericarp cell sizes and a slight reduction in cell layers (in the case of amiCDKB1). Therefore, we conclude that the reduced cell sizes in most of the lines predominantly accounts for the observed decrease in fruit size. This is in agreement with a possible role during the mitotic phase and it also is in line with the expression of these *CDKB* genes during early stages of fruit development when mainly cell division takes place (Chapter 3).

In Arabidopsis with aberrant CDKB1;1 function specific defects in stomata guard cell development were observed due to blocking of the cell cycle in the G2 phase (Boudolf *et al*., 2004a), without decrease in cell size. In the studies of Boudolf *et al*. (2004b) and Andersen *et al*. (2008) the reduced expression of *CDKB1* and *CDKB2,* respectively resulted in the premature exit of cells from mitosis. In our transgenic tomato fruits with suppressed expression of *CDKB1* or *CDKB2*, cell division was also (slightly) reduced, indicating that the mitotic cell cycle was suppressed or a premature exit of cells from mitosis had occurred.

The important quality characteristic of ripe fruit firmness was altered in the transgenic lines with suppressed *CDKB2* expression. The smaller cells of *TPRP-amiCDKB2* plants may be responsible for forming a stronger pericarp. The reduced cracking compare to wild-type may also be a consequent of reduced cell sizes forming a more solid pericarp texture.

Increased endoreduplication

In developing tomato fruits M-phase-specific CDK activity is highly reduced during the endoreduplication process (Joubés *et al.*, 1999). We observed increased endoreduplication levels in *amiCDKB2* lines These results suggest a similarity in the mechanisms initiating endoreduplication in tomato and Arabidopsis, where in the latter species downregulation of *CDKB1/B2* leads to elevated ploidy levels (Boudolf *et al*., 2004b, 2009; Andersen *et al*., 2008). Most likely, due to the proposed premature arrest of the mitotic phase, the cells enter the endoreduplication phase earlier in the transgenic lines, resulting in higher ploidy levels.

Surprisingly, in our transgenic lines with decreased expression of *CDKB2*, cell size did not increase with the ploidy level, as would be expected if there was a direct correlation between cell size and ploidy level (Cheniclet *et al*., 2005). In contrast, we observed smaller cells in these lines. Somehow, these increased ploidy levels appeared to be partially uncoupled from cell expansion.

Boudolf *et al*. (2004b) observed a similar phenomenon in Arabidopsis plants overexpressing a dominant-negative mutant version of CDKB1;1.N161 and *CycA2;3*, resulting also in increased endoreduplication levels, while cell numbers and sizes were not affected (Boudolf *et al*., 2004b).

Aberrations in fruit abscission

Fruit abscission is an important mechanism of seed dispersion. For cultivated species such as tomato easy fruit abscission can be seen as a negative feature, because fruits easily drop too early, which makes it difficult to harvest fruits. Cultivar Ida Gold represents a good example of tomatoes with weak abscission zones (Figure 6 A) and fruits often drop in the breaker or turning stage. In *CDKB1* or *CDKB2* knock down plants, we observed a defect in fruit abscission. The abscission zone usually consists of one or more layers of thin-walled parenchyma cells resulting from anticlinal divisions (Salisbury and Ross, 1992). In ripening fruits abscission zone cells enlarge and become highly vacuolated in response to ethylene. Abscission of mature fruit, as that of leaves and flower pedicels, is activated by ethylene and inhibited by hormones such as auxins, gibberellins, and cytokinins. Increased auxin levels can inhibit cell separation in the abscission zone, due to blocking the enlargements and vacuolization of cells, and inhibiting cell wall-degrading enzymes. These cells are less sensitive to ethylene (Ray, 1960; Sexton and Roberts, 1982; Taylor and Whitelaw, 2001). ABA, an abscission-accelerating hormone that is commonly associated with growth retardation and senescence, has elevated levels in fruits and senescent plant organs. Both the levels of these hormones and the sensitivity to them, as determined by the concentrations and affinities of receptors in the abscission zone, are important determinants for progression of abscission (Taylor and Whitelaw, 2001).

Although *CDKB2* is hardly expressed at the later stages of fruit development we observed that it is still expressed in seeds and vascular bundles in mature fruits of reporter lines expressing CDKB2::GUS (Chapter 2, Figure 10). The main source of auxin production in tomato fruits are developing seeds (Mapelli *et al*., 1978; Gillaspy *et al*., 1993). Based on the preliminary results on the levels of ABA and IAA in seeds of *TPRP-amiCDKB2* transgenic fruits we hypothesize that the down-regulation of *CDKB2* in the developing seeds of the *amiCDKB2* plants is related to the lack-of-abscission phenotype due to increased IAA content; and possibly also decreased ABA content, which we observed in transgenic seeds.

Stenospermocarpic fruits of *TPRP-amiCDKB1*

In the *amiCDKB1* line the seeds in all fruits failed to develop fully, which caused a stenospermocarpic phenotype. This phenomenon is well known in some commercial varieties of grape, where double fertilization takes place but the embryo aborts and seeds fail to develop fully (Ledbetter and Ramming, 1989). In the case of grapes the proper synthesis of amino acids, lipids and carbohydrates is one of the causes of this embryo abortion Stenospermocarpy usually does not occur in tomato. In research of Hanania *et al*. (2007), stenospermocarpic tomato fruits were obtained through the agro-injection of a TRV2 vector carrying a fragment of the gene encoding chloroplast chaperonin 21 (ch-Cpn21). This protein is essential for seed development, and its fruit-specific silencing led to embryo abortion in developing fruits. The other tissues in obtained fruits were well developed. We did not analysed the development of embryo and we do not know in which manner the downregulation of *CDKB1* was involved in improper seed development. Nonetheless, the absence of properly developed seeds might explain the defect in abscission because of possible changes in hormone signaling originating from the seeds in fruits expressing the *TPRP-amiCDKB1* construct. This statement needs to be verified by measurements of the active hormone levels in fruits and in the case of *TPRP-amiCDKB1* plants using more transgenic lines for investigation.

We hypothesized that the transgenic lines have perturbed hormone levels and/or signaling, which in turn influenced the abscission of the ripe fruits. How the cell cycle genes *CDKB1* and *CDKB2* are related to ABA and IAA signalling (production, transport or action) remains unclear, but is an attractive subject for future studies.

Material and methods

Transgenic plants

Plants of cv. Ida Gold were transformed by co-cultivation of cut cotyledons according to the protocol described in chapter 3. Artificial microRNA constructs were made according to an adapted version of the protocol of Schwab et al. (ref, and http://wmd3.weigelworld.org/). Briefly, an artificial pre- miRNA was produced by PCR-mutagenesis of the mature miRNA/miRNA* part of the Arabidopsis *pre-miR319a* gene as cloned in vector RS300. For the final amplification of the mutagenized *pre-miRNA* gene we adapted the primers A and B for Gateway cloning by adding *attB1* and *attB2* sequences, respectively. Mutagenic primers I-IV for each gene were designed using the web utility (http://wmd3.weigelworld.org/), and PCR amplification was performed according to the protocol.

The final mutagenized pre-miRNA genes were cloned by BP-recombination into pDONR207 (InVitrogen) to give Entry vectors CZN0456 and CZN0457, for tomato *CDKB1* and *CDKB2*, respectively. Subsequently the pre-miRNA fragements were cloned by LR-recombination into vector pARC983 (see chapter 3), placing them downstream of the *TPRP/TFM7* promoter in that vector, giving plasmids CZN0464 and CZN0465, respectively. Mutagenic primers for both genes as well as for adapted amplification primers A and B are listed in Table 2.

Table 2. Primers used for construction of amiRNA constructs of CDK genes. [1]Primer type refers to the protocol of Schwab et al. (http://wmd3.weigelworld.org/). [2]For primers I-IV the mutagenic part comprising the mature miRNA and miRNA* sequences is shown in capital letters.

Primer Number	Type[1]	Target gene	Primer Sequence[2]
PDS1740	A	generic	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCCCAAACACACGCTCGGA
PDS1741	B	generic	GGGACCACTTTGTACAAGAAAGCTGGGTCCCCATGGCGATGCCTTAA
PDS1764	I miR-s	CDKB1	gaTTGCCTTCTAACCATCTCCGCtctctcttttgtattcc
PDS1765	II miR-a	CDKB1	gaGCGGAGATGGTTAGAAGGCAAtcaaagagaatcaatga
PDS1766	III miR*-s	CDKB1	gaGCAGAGATGGTTACAAGGCATtcacaggtcgtgatatg
PDS1767	IV miR*-a	CDKB1	gaATGCCTTGTAACCATCTCTGCtctacatatatattcct
PDS1768	I miR-s	CDKB2	gaTCATAATGCAACATCTCACGGtctctcttttgtattcc
PDS1769	II miR-a	CDKB2	gaCCGTGAGATGTTGCATTATGAtcaaagagaatcaatga
PDS1770	III miR*-s	CDKB2	gaCCATGAGATGTTGGATTATGTtcacaggtcgtgatatg
PDS1771	IV miR*-a	CDKB2	gaACATAATCCAACATCTCATGGtctacatatatattcct

Plant material

Tomato plants *Solanum lycopersicum* cv. Ida Gold and obtained transgenic lines were grown in a greenhouse under a 16-h of light and 8-h of dark conditions. Supplementary lights (600 Watt high pressure sodium lights) turned on below 200 W/m^2 and turned off above 300 W/m^2 solar irradiation. Temperature was kept above 20^{0}C during the light period and 17^{0}C during the dark period controlled with the PRIVA Integro version 724 system. Plants were watered daily and given fertilizer weekly.

Collection of plant material

To avoid the differences in source-sink balance affected by fruit number and position (Bertin *et al*., 2001) we left at each truss 6 fruits, the rest pollinated flowers were removed. The first developing truss at each plant was removed.

For mRNA isolation in experiment of expression and histological analysis pattern during the whole course of early fruit development we collected the second and third fruit from the second truss on the plant.

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

RNA was isolated and reverse transcribed to cDNA following the protocol described in chapters 2 and 3. Also for the real-time quantitative PCR, the same conditions were used als mentioned in chapter 2. Real-time –quantitative RT-PCR (qRT-PCR) primers (Table 3) were designed using a computer program (Beacon Designer Software, Premier Biosoft International, CA, USA), primers for *LeACT2/7* were kindly provided by dr. De Jong. 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 repeats is depicted with the SD.

Table3. #Primers sequences used for qRT-PCR

Gene name	Primer sequences
TOM 51	5'- TCACACCATCACCAGAGTCC-3' 5'- GCTGTGCTTTCCTTGTATGC-3'
LeACT 2/7 (actin 2/7)	5'-CCGTTCAGCAGTAGTGGTG-3' 5'-GGACTCTGGTGATGGTGTTAG-3'
Le S18	5'- AGCCTTGCGACCATACTCC-3' 5'- AGACGAACAACTGCGAAAGC-3'
LeCDKB1	5'-ACGATGTAGAGAGAATGAGATAGC3' 5'-ATGGAGAAATACGAGAAATTGGAG-3'
LeCDKB2	5'CGGAGAGTAGTTGGAGGAA-3' 5'-ATGCTGGTAAGAGTGTATCGG-3'

Histological analysis

Fruits were analysed at different stages after anthesis. Fruits were cut along the equator to remove seeds and pulp. The parameters weight, diameter (height and width) 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

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 (Dujmović *et al*., 2005; Brewer *et al*., 2006; Gonzalo *et al*., 2009).

Endoreduplication index

Nuclei were prepared from the pericarp of orange ripe fruit. 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 100mm nylon mesh and the remaining sample was reextracted with the same solution. The combined filtrates were analysed with a CA-II cell analyzer (Partec). After flow cytometry was done the EI was calculated from the number of nuclei of each represented ploidy level multiplied by the number of endoreduplication cycles necessary to reach the corresponding ploidy level EI = (1*4C + 2x8C+ 3*16C+4x32C + 5*64C + 6*128C + 7*256C)/100 (Boudolf et al., 2009).

Hormones measurements

The procedure for quantification of hormones and metabolites was performed according procedure by Chiwocha *et al*. (2003, 2005). Samples were injected onto a Genesis C18 HPLC column (100x2.1 mm, 4 um, Chromatographic Specialties, Brockville, ON, Canada) and separated by a gradient elution of water against an increasing percentage of acetonitrile that contained 0.04% acetic acid. Calibration curves were generated as described by Ross *et al.*, (2004). The QC samples, internal standard planks and solvent blanks were also prepared and analysed along each batch of tissue samples.

Acknowledgments

We thank Nunhems B.V. Nederland for generating transgenic tomato lines and André Emons for taking care about plants.

7

Ectopic expression of *LeCycD3;3* increases cell division rates and affects the endocycle in tomato fruit pericarp

Anna Czerednik, Marco Busscher, Peter de Groot, Ruud A. de Maagd and Gerco C. Angenent

Abstract

In this chapter we described the effect of *LeCycD3;3* overexpression on cell size and number as well as on the endocycle during tomato fruit development. Due to ectopic expression of tomato *CycD3;3* cell numbers and cell layers in the pericarp were significantly increased, while the size of the cells was decreased. The reduction of the cell size was correlated with decreased ploidy levels. The increased number of smaller cells had a positive effect on the fruit firmness, which in transgenic plants was significantly increased.

Introduction

D3-type cyclins (CycD3) play key roles in the G1-to-S -phase transition, which is the first main control point of the cell-cycle regulation (De Veylder *et al.,* 2003). In mammalian cells, D-type cyclins initiate the phosphorylation of the retinoblastoma related protein, a tumour suppressor protein and a key regulator of the start of DNA replication (Weinberg *et al.,* 1995). In a similar manner in plants the cell-cycle active complex CDKA-CycD3 is able to phosphorylate RBR (Menges *et al.,* 2006).

In non-dividing cells, RBR protein is bound to the E2F-DP transcription factor, which regulates expression of many genes required for cell cycle progression, particularly for S-phase entry and for DNA replication. The targeted phosphorylation by CDKA-CycD3 is dependent on a specific RBR-binding motif present near the N terminus of both animal and plant D-type Cyclins, consisting of the amino acid sequence LxCxE (where x represents any amino acid). Phosphorylation of RBR by CDK-CycD complexes results in its dissociation from promoter-bound E2F-DP complexes, and as a consequence E2F-DP is able to activate transcription of its target genes and the cell progress into S-phase. Up till now it has been proposed that D3-type gene expression is the rate limiting factor for G1-S transition, cell division and, in certain cases, plant organ growth (reviewed in Menges, 2007). Plant and mammalian D-type cyclins share only 9-14% of homology across the region corresponding to the cyclin core motif, but both contain the conserved retinoblastoma protein (RB)-binding motif and have a similar role in a pathway that is conserved in the cell-cycle of all eukaryotes (Nakagami *et al.,* 2002; Menges *et al.,* 2007). Additionally, plants D3-type cyclins play an important role in response to internal factors. CycD3 mRNA level increases in the presence of sucrose (Healy *et al.,* 2001) and CycD3;1 is activated as response to the presence of cytokinins (Dewitte *et al.,* 2007). In Arabidopsis, there are 10 genes encoding D-type cyclins (CYCD), and homologs have been identified in a variety of other species, like tomato, tobacco, rice, snapdragon, *Helianthus tuberosus*, and maize (Menges *et al.,* 2007; Nieuwland *et al.,* 2007).

There are three D3-type cyclins in *Arabidopsis thaliana*, which are expressed in overlapping, but distinct patterns in developing lateral organs, shoot meristems and young inflorescences. Like Arabidopsis D3-type family cyclins, tomato possesses three genes encoding D3-type cyclins, which may have different functions in plant development, and which have been shown to be expressed during both the cell division and cell expansion/endoreduplication phases in fruits (Kvarnheden *et al.,* 2000; Joubès *et al.,* 2001). Their exact functions in fruit development have not been studied so far. In Arabidopsis the *CycD3;1* and *CycD3;3* genes are expressed at higher levels than other D-type cyclin genes, suggesting that they are the most important in G1 and G1/S control for cell cycle re-entry. Moreover, in *CycD3;1* overexpression experiments with Arabidopsis it appeared that this gene may affect cell differentiation (Dewitte *et al.,* 2003). Taking these study in consideration and because of the possible high similarity in the function between Arabidopsis and tomato D3-type cyclins, we concentrated our research on the function of *CycD3;3*. We overexpressed this gene under control of a fruit specific promoter and analysed the influence on cell number and size in the growing tomato fruit.

qRT-PCR expression analysis revealed that *LeCycD3;3* has the highest expression among the tomato D-type cyclin genes analysed with a peak in the cell division phase of fruit development (Chapter 2 of this thesis). We found that overexpression of this particular cyclin gene directly affected cell size and number in tomato pericarp, and decreased the endoreduplication level with an effect on fruit firmness.

Results

To study the role of *CycD3;3* in developing tomato pericarp and to assess the possibility to use this gene to manipulate cell sizes and/or number in tomato fruit we transformed tomato cv. Ida Gold with the pTPRP-CycD3;3 construct. From this transformation we obtained 40 transgenic plants and selected the 6 lines with the highest expression levels (Figure 1A). Fruits of high-expressing transformants from the lines 30, 32, 130 and 131 had clear morphological changes in fruit appearance, namely slightly thinner pericarp and less seeds than wild-type fruits. Closer inspection revealed that the majority of ovules, whether fertilized or not, degenerated before reaching maturity. Pollination with wild-type plant pollen gave the same results, showing that the reduction in seed number was not due to a reduction in pollen quality.

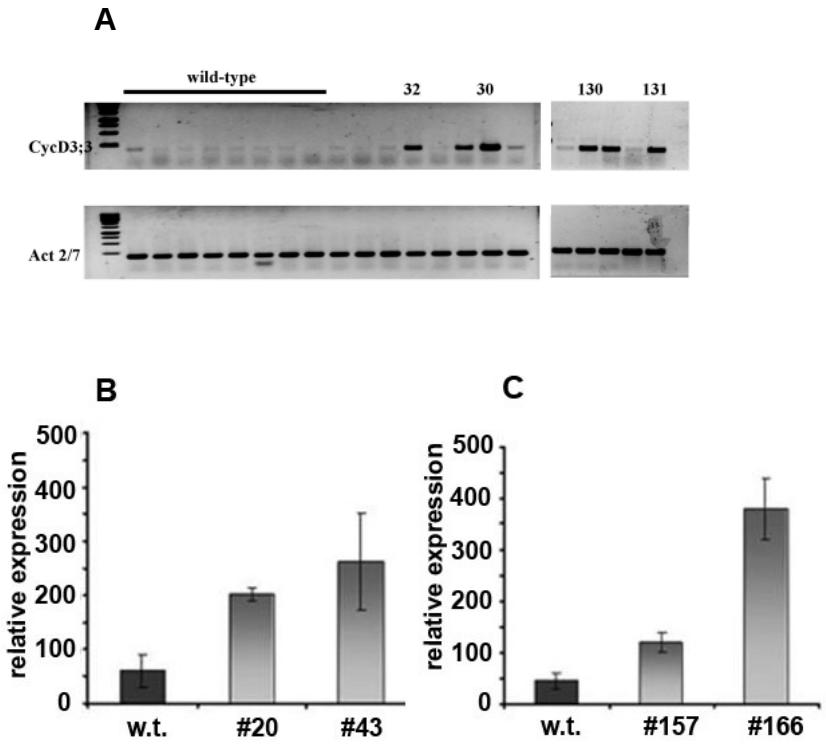


Figure 1. Relative expression level of *CycD3;3* in pericarp of transgenic lines in T0 and T1 generations.
A. Expression in T0 generation determined by semi-quantitative PCR (28 cycles)
B. and **C.** Expression of CycD3;3 in T1 generation determined by qRT-PCR in duplicate 5 days after anthesis. **B**-plants #20 and #43 represents line 30, **C**-plants #157 and #166 represents line 131; wild-type (w.t.) – Ida Gold, used as a control.

For further investigation we selected T1 offspring of lines 30 and 131, and two high overexpressing plants from each line were selected for phenotypic analysis: #20 and #43 for line 30; and #157 and #166 for line 131.

The level of *CycD3;3* expression in the fruit pericarp of transgenic plants was 4-8-fold higher than in non-transgenic control fruits at the same stage (Figure 1 B,C). In Figure 2 the obtained phenotype (B, D) and the wild-type control (A,C) are presented.

We analysed several physiological parameters from collected ripe fruits (Figure 3). For all the results obtained statistical analysis with Student t-test was performed. All analysed transgenic fruits had slightly reduced weight (Figure 3A), but the differences were significant only in the case of plant #20 according the Student t-test (#20 $p < 0.05$; #43 p=0.23; #157 p=0.07; #166 p=0.37). The diameter of transgenic fruits (Figure 3B) was comparable to control (#20 p=0.53, #43 p=0.04; #157 p=0.20; #166 p=0.99) and the pericarp thickness (Figure 3C) was not significantly decreased (#20 p=0.75; #43 p=0.42; #157 p=0.10; #166 p=0.78). The effect of overexpression on the seed number in the T1 generation was less severe than we observed in the T0 generation, which could be partially the effect of the tissue culture in T0.

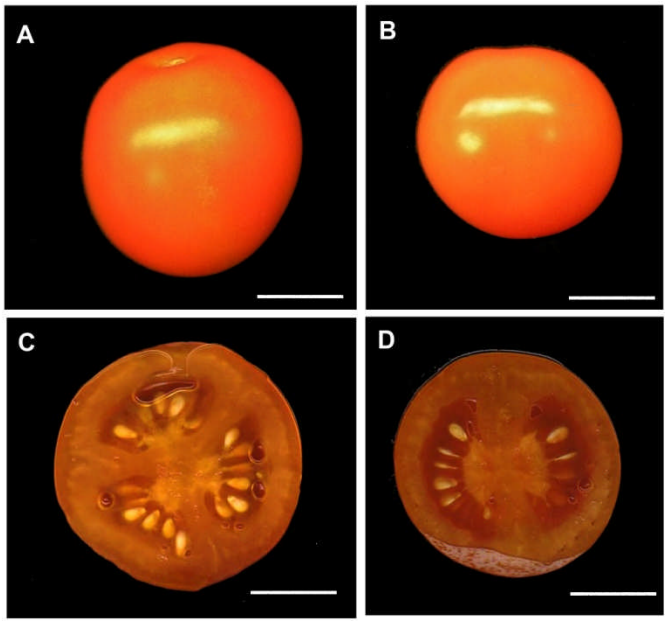


Figure 2. Phenotypes of control and TPRP-CycD3;3 fruits

A, C. A fruit of non-transgenic Ida Gold plant

B, D. A fruit of transgenic pTPRP-CycD3;3 plant

Bar = 20mm

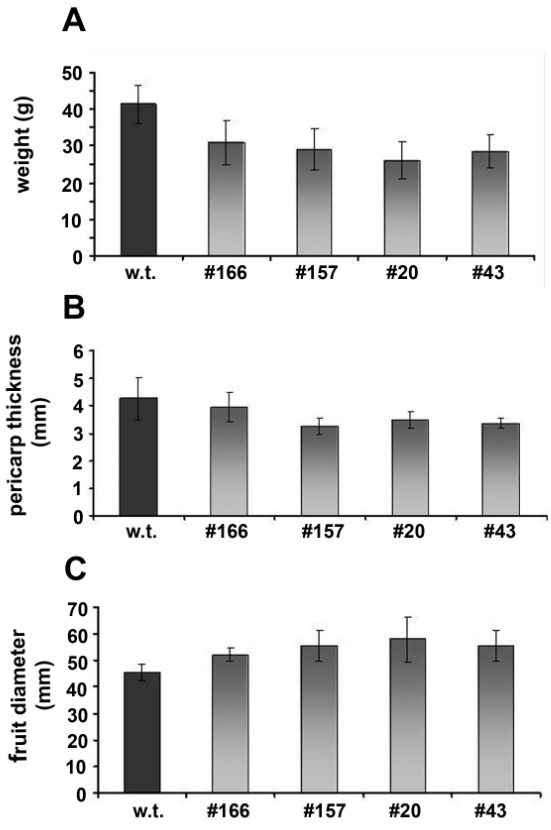


Figure 3. Physiological parameters of transgenic fruits as compared to the wild-type.

Fruits were collected at the ripe stage. The data are means ± standard deviation of 6-12 fruits. The parameters were analysed with Student t-test.

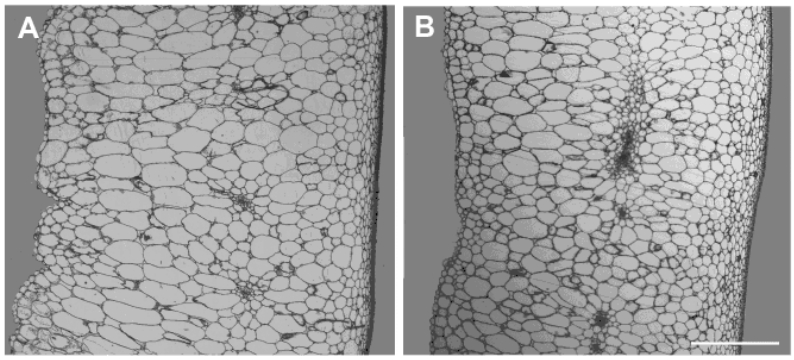
A. Fruit weight (g). The differences between wild-type and transgenic fruits are significant only for plant #20 ($p<0,05$).

B. Diameter (mm) of fruits were analysed with Tomato Analyzer software. Differences are not significant.

C. Pericarp thickness (mm) was analysed with Tomato Analyzer software. Differences are not significant.

Microscopic analysis of transgenic plants

We did not observe any significant changes of fruit morphology or duration of fruit development from anthesis until the mature green stage in transgenic lines. However, the histological analysis of pericarp cross-sections showed remarkable differences in cell sizes and numbers (Figure 4, Table 1), which suggest differences in cell division and expansion activity between the wild-type and transgenic fruits.



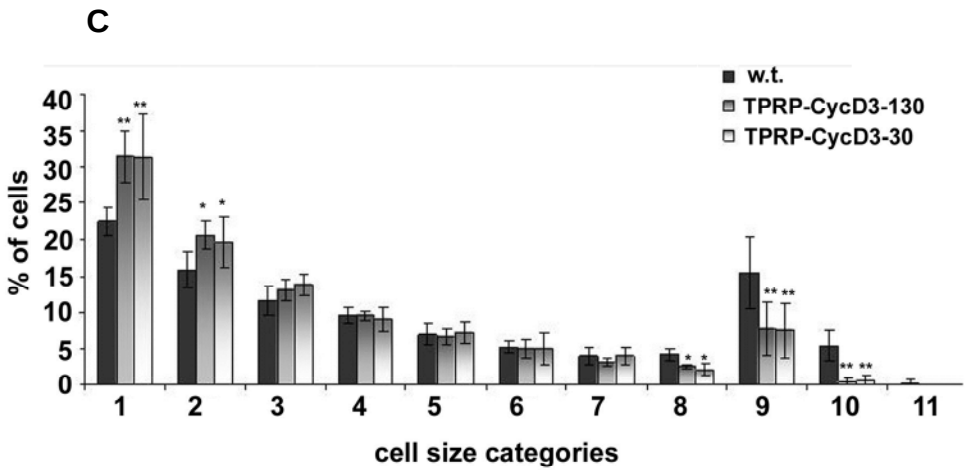


Figure 4. Phenotypic changes in pericarp due to overexpression of *CycD3;3.*
(A), (B) Microscopic cross-section through a pericarp of a control fruit **(A) and** through a pericarp of a *CycD3;3* overexpressing fruit **(B).** Bar=1mm
(C) Percentage of cells grouped into 11 different size categories. Cells in category 1 have an average size of up to $4*10^{-4}$ mm ^{2} and the maximum cell size in each subsequent category has doubled. Standard deviations are indicated for a minimum of 4 measurements from 3 different fruits for each bar.
Statistical significance indicate * P<0,05 **P<0,005.

We quantified cell size and number, and the number of cell layers in the pericarp of fruits at breaker stage.

The overall cell size reduction in transgenic fruits corresponds with a significant change in the number of cells in category 1 ($p<0.005$, Student t-test), category 2 ($p<0.05$), category 8 ($p<0.05$), category 9 ($p<0.005$) and category 10 ($p<0.005$). Category 11 representing the largest cells, contained only cells from the wild-type. Furthermore, the microscopic analysis showed that the number of cells per mm^2 and number of cell layers were significantly higher in the pericarp of transgenic fruit, overexpressing *CycD3;3* (Table1).

Table 1. Quantification of number of cells per surface unit or number of cell layers in the pericarp of mature wild-type and transgenic fruits, collected at breaker stage.

The data represent the means ± standard deviation of five-six measurements from 3 different fruits for each bar. For all measurements, the differences between wild-type and transgenic lines were tested for statistical significance. The p-values (Student's test) are indicated.

Line	Cells /mm2	Cell layers
Wild-type	15,4 ± 2,2	23 ± 1
#166	28,1 ± 5,9 (p<0.005)	28 ± 3 (p<0.05)
#157	26,7 ± 1,5 (p<0.005)	27 ± 1 (p <0.05)
#20	31,5 ± 0,5 (p<0.005)	27 ± 1 (p<0.05)
#43	26,8 ± 4,7 (p<0.005)	26 ± 1 (p<0.05)

Because an increase of pericarp cell number and a decrease in cell size might change fruit firmness (Chaïb *et al.,* 2007; Guillon *et al.,* 2008), we performed the measurements of this quality marker, using a nondestructive method with a fruit pressure tester. In agreement with Chaïb *et al.,* (2007) the ripe transgenic fruits had significantly increased firmness compared to the wild-type control (Figure 5).

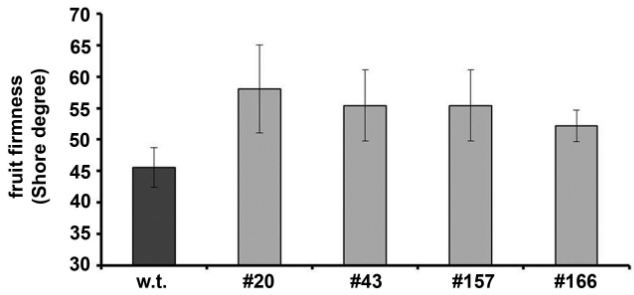


Figure 5. Firmness of transgenic fruits and wild-type.
The data represent the means shore degrees measured on 6-12 ripe fruits and the standard deviations are indicated. For all measurements the differences between wild-type and transgenic fruits were statistically significant. Student t-test: #20 $p<0.05$; #43 $p<0.005$; # 157 $p<0.05$; #166 $p< 0.005$.

Analysis of ploidy level of tomato pericarp cells

In tomato fruit, the cell size is tightly correlated with the ploidy level (Cheniclet *et al.,* 2005, Chevalier *et al.,* 2011). Thus, a clear reduction in cell size, as we observed in the pericarp of pTPRP-CycD3;3 transgenic lines, an obvious next step was to look for a correlation with changes in ploidy level. We performed measurements of ploidy using flow cytometry and the Endoreduplication Index (EI) was calculated. In transgenic fruits overexpressing *CycD3;3* the reduction in cell size was correlated with reduced endoreduplication levels (Figure 6 A). The percentage of cells with 32C and 64C was higher in wild-type, and only in the wild-type we observed cells with 128 copies of the genome. The ploidy levels were used to calculate the endoreduplication index (EI, according to Boudolf *et al.,* 2009; Material and Methods), which was significantly reduced in the pericarp of all analysed transgenic fruits ($p<0.005$) (Figure 6B).

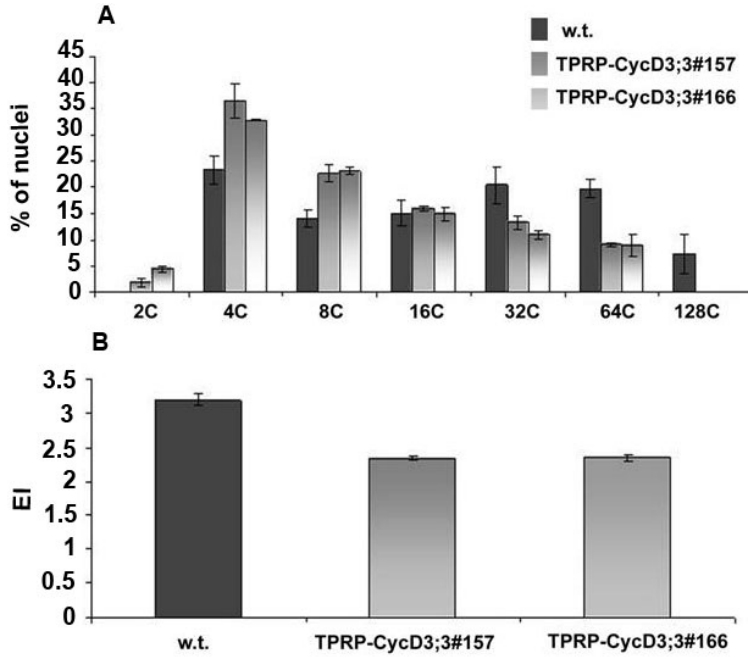


Figure 6. Endoreduplication in pericarp of fruits overexpressing *CycD3;3*.
A. Changes in DNA copy number
B. Endoreduplication index (EI). For all measurements the differences between control and transgenic fruits were statistically significant ($p<0.005$, Student's t test). The data are means of measurements on three transgenic fruits from two plants (#157, #166) and were performed in duplicate for each fruit.

Discussion

Overexpression of *CycD3;3* reduces cell size and increases cell number

The final cell number in a plant organ is primarily determined during the phase of mitotic activity, while the subsequent growth into a mature size organ mainly relies on cell expansion accompanied by endoreduplication. The D3-type cyclins in Arabidopsis are thought to contribute to the control of cell number in developing leaves by regulating the duration of the mitotic phase and timing of the transition to expansion (Dewitte *et al.,* 2007). Downregulation of all D3-type cyclins in Arabidopsis negatively affected the duration of the mitotical cell cycle phase, and led to cells prematurely entering into the endocycle phase. This results in reduced cell numbers in mature leaves and petals of Arabidopsis, but is compensated by an earlier onset of expansion and endoreduplication, giving rise to mature organs of normal size (Dewitte *et al.,* 2007).

The effect of ectopically expressed *CycD3;3* in plants, including Arabidopsis, have never been studied previously. Studies with *CycD3;1* ectopic expression and *CycD3;1* down regulation has been reported, but this was done with high-level constitutive expression, which in some cases may mask the exact view on developmental changes in particular plant organs.

We obtained transgenic tomato plants with increased expression of *CycD3;3* in fruits under control of the fruit-specific TPRP promoter, in which fruits were clearly affected in average cell size and in cell numbers in the pericarp. The duration of fruit development was not affected, and fruit size and pericarp thickness were only slightly or not significantly changed in comparison to the wild-type. The cell number in the pericarp of transgenic fruits was clearly elevated but their average cell size was decreased.

Most likely, during pericarp development of fruits with increased *CycD3;3* expression, cell divisions continued while in wild-type pericarp mitosis had ceased at that stage. These results support the suggestion of Dewitte *et al.,* (2007) about the role of *CycD3* in the specific promotion of the mitotic cell cycle over the endocycle. This is in line with Arabidopsis experiments, where ectopic expression of *CycD3;1* resulted in ectopic divisions in leaves and other tissues (Dewitte *et al.,* 2003). However, in Arabidopsis this led to hyperplasia of leaves and other plant organs, which was not the case in our transgenic tomato fruits.

CycD3 may act as a mitotic cyclin, boosting G2/M kinase activity and hence directly enhancing progress from G2 into mitosis. The ability of CycD3 to associate with a mitotic cyclin-dependent kinase has been detected in plant cell extracts using immunoprecipitation and proteomics approaches (Healy *et al.,* 2001; Boruc *et al.,* 2010; Van Leene *et al.,* 2010).

Endoreduplication

In plants a correlation between final cell size and nuclear ploidy level is often observed, also in tomato (Joubès and Chevalier, 2000; Cheniclet *et al.,* 2005; Vlieghe *et al.,* 2005; Chevalier *et al.,* 2011) However some exceptions from this rule are known (Beemster *et al.,* 2002; Vilhar *et al.,* 2002; Bertin *et al.,* 2003; Leiva-Neto *et al.,* 2004; Bertin 2005; Bourdon *et al.,* 2009). Our results with the *LeCycD3;3* overexpression plants are in agreement with the correlation hypothesis, as a reduced size of pericarp cells was nicely correlated with decreased endoreduplication levels. Such inhibition of the endocycle was also observed in Arabidopsis leaf cells with increased expression of *CycD3*. Conversely, the downregulation of all D3-type cyclins resulted in increased ploidy level (Dewitte *et al.,* 2007), confirming the involvement of D3-type cyclins in the regulation of the endocycle process. We suggest that the mechanism of reduced endoreduplication and the strong correlation with decreased cell sizes is similar between Arabidopsis and tomato. High mitotic activity due to overexpression of *CycD3;3* results in the shortening of the cell expansion phase and cessation of endocycle duration.

Increase of fruit firmness

Fruit firmness is one of the important indicators of fruit quality (Batu, 1998; Chaïb *et al.,* 2007). Firmness has effects on the fruit shelf life and reduces losses during mechanical harvesting, thus influencing the commercial value (Du *et al.,* 2006; Chaïb et al., 2007). In addition, fruit firmness correlates with the outer appearance of tomatoes, such as color, shape and sense of firmness at time of purchase or during preparation and consumption (Gormley and Egan, 1978; Batu, 1998; Chaïb *et al.,* 2007). Fruit texture of ripening tomatoes depends on the (dis)assembly of cell wall polymers (Jackman and Stanley, 1995; Rose and Bennett, 1999), on cell turgor pressure (Lin and Pitt, 1986; Shackel *et al.,* 1991; Konstankiewicz and Zdunek, 2001), and on anatomical features. The increased number of cells together with a decrease in size is positively correlated with pericarp skin toughness and elasticity, the sugar content and resistance to cracking (Waldron, 2004; Chaïb *et al.,* 2007; Prudent *et al.,* 2009).

In our transgenic fruits firmness was significantly increased, which may well be a consequence of a higher proportion of small cells in the subepidermal area of the pericarp, rather than pericarp thickness. The smaller cells positively influence the capacity to store soluble dry matter (Prudent *et al.,* 2009) and form more solid texture comparing to bigger cells.

A positive correlation between firmness and a heterogeneous cell distribution in the fleshy part of tomato pericarp was found, and skin toughness was correlated with the presence of small cells in the tomato peel. We suggest that the elevated proportion of small cells due to prolonged cell division in fruits overexpressing *CycD3;3* might be one of the reasons for the increased pericarp firmness.

Future perspective

Our results gave insights into the effects of elevated *CycD3;3* expression on cell size and cell number of tomato fruits The clear phenotype on the cellular level are the basis for detailed investigations on quality values such as shelf life duration, resistance to cracking and resistance to pathogens.

Material and methods

Plant material

Tomato plants, cv. Ida Gold 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^2 and turned off above 300 W/m^2 solar irradiation. Temperature was kept above 20^{0}C during the light period and 17^{0}C during the dark period controlled with the PRIVA Integro version 724 system. Plants were watered daily and given fertilizer weekly.

Construction binary vectors for transformation

To generate the fruit-specific CycD3;3, overexpression lines, the coding sequence of *LeCycD3;3* (accession number AJ002590) was cloned into the pENTR/D-TOPO entry vector (Invitrogen) using the primers F - 5'- CACCATGTCTCACCATTATCAAGAACAAGAAC -3' and R-5'- GTGGATCACTGTTATTGGACATTATAC -3'. Obtained clones were recombined with an overexpression vector, pARC983, containing the TPRP-promoter driving the expression of a Gateway cassette, in which a gene or ORF of choice was recombined in vitro using LR clonase.

The right connection of TPRP promoter and *CycD3;3* was confirmed by PCR using the pair of primers: F-5'-TCATTATATTTAACAATCCCACTTGATG-3' and
R-5'-AGAAATTCCAAAGATAGACAGTGCTTC-3'

Transformation of tomato

Transgenic tomato plants were generated by *Agrobacterium tumefaciens* -cocultivation of seedling cotyledons, as described in De Jong et al. (2009).

Plants were selected on kanamycin-containing medium and then checked by PCR with primers specific partially for TPRP-promoter and gene of interest on genomic DNA. Subsequently, lines were tested for ploidy, as only diploid lines were used for further analysis.

Harvesting plant material

To avoid the differences in source-sink balance affected by fruit number and position (Bertin *et al.,* 2001) we left 6 fruits at each truss, the additional pollinated flowers were removed. The first developing truss from each plant was removed.

For mRNA isolation we collected the third and fourth fruit from the truss on the plant.

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

RNA was isolated and reverse transcribed to cDNA following the protocol described in chapters 2 and 3. Also for the real-time quantitative PCR, the same conditions were used als mentioned in chapter 2. Real-time –quantitative RT-PCR (Q-PCR) primers were designed using a computer program (Beacon Designer Software, Premier Biosoft International, CA, USA). Primers for *CycD3;3* : F 5'-CTTGTTGCTGTTACTTGTCTTTC-3'
R 5'-AATGGTGTTACTGGATTCATCTTC-3'. As control genes *Actin 2/7*
F 5'-GGACTCTGGTGATGGTGTTAG-3' and R 5'- CCGTTCAGCAGTAGTGGTG-3' and
Le18S F 5'-AGACGAACAACTGCGAAAGC-3' and R 5'-AGCCTTGCGACCATACTCC-3' were used. Relative mRNA levels were calculated following the Bio-Rad outlined methodology based on Vandesomple et al. (2002) and corrected for PCR efficiencies. The average of two biological repeats is depicted with the SD.

Histological analysis

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

Ploidy analysis and Endoreduplication Index (EI)

Nuclei were prepared from the pericarp of orange ripe fruit. Two types of tissue were analysed - subepidermal layer and mesocarp tissues. 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 100mm nylon mesh and the remaining sample was reextracted with the same solution. The combined filtrates were analysed with a CA-II cell analyser (Partec).

After flow cytometry was done the EI was calculated from the number of nuclei of each represented ploidy level multiplied by the number of endoreduplication cycles necessary to reach the corresponding ploidy level EI = (1*4C + 2x8C+ 3*16C+4x32C + 5*64C + 6*128C + 7*256C)/100 (Boudolf *et al.,* 2009).

Measurements of fruit characteristics

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

8

Concluding remarks and perspectives

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Tomato fruit growth depends on the successful pollination, after which rapid cell division of the fruit tissues takes place and followed by cell expansion, which is associated with endoreduplication, when most of the cells become polyploid (Gillaspy *et al*., 1993; Bergervoet *et al*.,1996; Joubès and Chevalier, 2000; Chevalier *et al*., 2011). At the end of the cell expansion period the fruit reaches the final size and ripening starts. The latter process has been intensively studied, and the changes that affect fruit aroma, color and biochemical compositions are well understood (Giovannoni, 2001, 2004). Nonetheless, most qualitative markers of the ripe fruit are determined during the first developmental stages after pollination, and perturbations during the early development may have crucial influence on the quality characteristics, such as texture, taste, shelf life, palatability, viscosity and cracking resistance (Bohner and Bangerth, 1988; Catala *et al*., 2000; Tanksley, 2000; Cong *et al*., 2002; Liu *et al*., 2003). Based on these observations we hypothesized that an increase in number of smaller cells in tomato pericarp may influence important quality markers. Because the driving forces of cell division and growth is the cell cycle, we studied several cell cycle key regulators operating at the early stages of tomato fruit development. We looked for possibilities to manipulate the ratio between cell size and cell number in tomato pericarp, and the final aim of our research was to give breeders clues how to improve the quality of tomato fruits and make them more attractive to consumers.

In the last ten years the knowledge about the role of key cell cycle regulators in plants has increased considerably, specially in Arabidopsis (Cockcroft *et al*., 2000; Dewitte *et al.,* 2003; Boudolf *et al*., 2004a, 2004b; Qi and John, 2007; Andersen *et al*., 2008; Adachi *et al.,* 2009; Boudolf *et al*., 2009; Imai *et al*., 2006). In tomato, only the expression characteristics of the core cell cycle genes and the regulation of endoreduplication have been studied (Joubès, 1999, 2000; Chevalier *et al*., 2011; Kvarnheden *et al*., 2000; Bisbis, *et al*., 2006; Gonzalez *et al*., 2007). Many aspects of tomato fruit growth are different from Arabidopsis siliques development and this makes the research on the cell cycle regulators in tomato fruit scientifically interesting and important for practical use in breeding programs.

In tomato, the expression characteristic of cell cycle genes was investigated before (Joubès 1999, Kvarnheden *et al*., 2000). We characterized the expression of a large set of cell cycle regulators throughout fruit growth from anthesis to mature full grown stage and we studied their role in tomato fruit development by manipulating their expression using the fruit specific promoter pTPRP (**chapter 2).**

The transgenic fruits we obtained had clear aberrations in pericarp cell composition due to the overexpression or downregulation of cell cycle regulators CDKs and Cyclins: *CDKA1* (**chapter 3** and **chapter 4**), *CDKB1* and *CDKB2* (**chapter 3** and **chapter 6**); *CycA2* (**chapter 5**) and *CycD3;3* (**chapter 7**).

Role of CDKA1 in tomato fruit development

CDKA in plants is a homologue of *cdc2* in yeast, which is conserved throughout eukaryotes and acts as a key regulator of the cell cycle (Dicommun et al., 1991). *CDKA* is constitutively expressed through the cell cycle in G1-S and G2-M transition and has dual functions in both S and M phase progression (De Veylder *et al.,* 2002). The CDKA/CycD3 complex positively regulates G1/S transition through the phosphorylation of RBR and the activation of the E2F/DP complex (Nakagami *et al*., 1999, 2002; Uemulkai *et al*., 2005). CDKA is involved in cell proliferation and maintains cell division competence in different plant tissues, also in non-dividing tissues (Hemerly *et al*., 1993; Segers *et al*., 1997; Joubès *et al*., 1999). The specific role of *CDKA1* in cell differentiation has been discovered recently in *Arabidopsis thaliana* by Adachi *et al.* (2009), who found that CDKA1 coordinates cell differentiation of different cell layers of Arabidopsis leaves, indicating that cell division is apparently controlled in the epidermis and affects the underlying tissues.

In tomato two CDKA genes - *CDKA1* and *CDKA2,* which share 94 % of homology, are recognized so far (Joubès *et al*., 1999). Several studies on the function of *CDKA* using missexpression lines of Arabidopsis and tobacco gave us the idea that it should be possible to manipulate the correlation between cell division and cell expansion in tomato fruit during early development (Hemerly *et al*., 1995; 2000; Imajuku *et al*., 2001;Verkest *et al*., 2005a; Verkest *et al*., 2005b; Iwakawa *et al*., 2006). We report in chapter 4 that fruit-specific overexpression of *CDKA1* results in a significant increase of the pericarp thickness and placenta. This was due to an increased number of cells in these tissues, despite a decrease in cell size. In line with this, the suppression of *CDKA1* expression in tomato fruit reduced the cell numbers. In addition to a reduction of the exocarp and peel thickness, the fruits showed a faster desiccation and a reduction of their shelf life when compared to wild-type (**chapter 3**).

Surprisingly, the *CDKA1* expression was downregulated in fruits with overexpression of *CDKB1* and *CDKB2*, suggesting a link between these different CDK types. Furthermore, plants overexpressing *CDKB1* and *CDKB2* with reduced exocarp and peel thickness phenocopied plants with downregulated levels of *CDKA1* (see **chapter 3**).

Effects of CDKB1 /CDKB2 overexpression and downregulation on tomato cell distribution

B-type CDKs belong to the group of cell cycle regulators that are present only in plants and are responsible for specific plant processes. In tomato two B-type CDKs were identified, *CDKB1* and *CDKB2*, both are the mitotic CDKs (Joubès *et al*., 2000).

We studied the effect of overexpression and downregulation of *CDKB1* and *CDKB2* on cell size and numbers in tomato. The thickness of the pericarp in TPRP-CDKB1 and TPRP-CDKB2 overexpression lines was significantly reduced due to smaller cells and reduced cell layers (**chapter 3**). The downregulation of *CDKB1* and *CDKB2* resulted in reduced cell sizes and in the case of amiCDKB1 lines also a significant reduction of the number of cell layers was observed (**chapter 6**). We propose that downregulation of *CDKB2* and *CDKB1* resulted in earlier exit from the mitotic phase and subsequent faster entry in the expansion phase**.** This is in line with the increased ploidy levels that we observed in these transgenic lines and it also is in agreement with the expression of these *CDKB* genes during early stages of fruit development when mainly cell division takes place (**chapter 3**).

It was reported by Andersen *et al*. (2008) that *CDKB2* knock-down in Arabidopsis plants caused an inability of cells to properly respond to hormone. In our study with *CDKB1* and *CDKB2* knockdown plants we observed a lack of fruit abscission and a failure of seed development (**chapter 6**). The lack of fruit abscission may be caused by impaired hormone production, which is mainly happening in the developing seed.

Role of Cyclins in the regulation of cell number and size in tomato pericarp

Cyclins are the regulatory partners of the cyclin-dependent kinases, and drive crucial transitions in the cell cycle from G1 to S and from G2 to M. Five families operate in the plant cell cycle and cyclins from the A, B and D types were found in tomato (Nieuwland et al., 2007). We analysed the effect of overexpression of *CycA2* (**chapter 5**) and *CycD3;3* (**chapter 7**) on cell size and numbers in the tomato pericarp.

CycA1 and *CycA2* are typical mitosis cyclins (Menges *et al*., 2005), while *CycA3* is expressed from G1/S to S-phase. Fruit specific overexpression of *CycA2* in tomato resulted in significant decrease of the cell volume and of the fruit size, compared to control plants (**chapter 5**). Further analysis of fruit development led to the suggestion that in the pericarp cells of the transgenic fruits both phases - division and expansion - were shortened.

CycD3;3 belongs to the group operating in G1-S phase and regulates G1-S transition. In Arabidopsis CycD3 interacts with CDKA providing the major part of CDK activity in the G1-S check point and regulates the phosphorylation of RBR (Menges *et al*., 2006; Nieuwland *et al*., 2007). D3-type cyclins contribute to the control of cell number in Arabidopsis leaves, and may affect the duration of the mitotic cell cycle and entrance in the endocycle (Dewitte *et al*., 2007). We found that overexpression of *CycD3;3* leads to more cell divisions and inhibits cells from leaving the mitotic cell cycle, which indicated that *CycD3;3* to be one of the limiting factors of the mitotic cell cycle duration and cell proliferation (**chapter 7**).

The decreased expansion of cells had particularly effect on the pericarp thickness, which in spite of the increased cell number was still reduced in thickness. This phenotype at the cellular level is an interesting starting point for further detailed studies on other fruit characteristics and quality traits, such as shelf life, resistance to cracking and to pathogens.

Endoreduplication in tomato fruits with altered expression of cell cycle regulators

During tomato fruit development cells become highly polyploid, due to DNA replication without cell division resulting in nuclei with high C-values, which can be up to 256C (Bergervoet *et al*., 1996; Joubès *et al*., 1999; Cheniclet *et al*., 2005; Bertin *et al*., 2007). Endoreduplication in tomato is correlated with cell expansion and contributes as a major determinant for the final fruit size (Chevalier *et al*., 2011). Endoreduplication is supposed to correlate with cell volume, and we found this clear correlation when overexpressing *CycD3;3*, *CDKB2, CDKB1* and *CDKA1* in tomato fruits: there decrease in cell size was associated with decreased ploidy levels. Strikingly, in *CycA2* overexpression lines, the decreased cell size was not correlated with changes in endoreduplication. A similar mechanism could also operate in the cells of fruits with reduced *CDKB2* expression levels in these fruits, elevated endoreduplication was surprisingly observed in pericarp cells with reduced size. However, in *amiCDKB1* lines the reduced cell size had less connection with ploidy level, which was not changed when compared to wild-type.

There are more reports showing that endoreduplication does not correlate with cell volume (Beemster *et al*., 2002; Vilhar *et al*., 2002; Bertin *et al*., 2003; Leiva-Neto *et al*., 2004; Bertin 2005; Bourdon *et al*., 2009). The results of Boudolf (Boudolf *et al*., 2004b) suggest that changes in the length of cell division or cell expansion phases does not directly lead to changes in endoreduplication and changes in endoreduplication level could already be observed when cell number and size are indistinguishable between wild-type and transgenic lines. We suggest that the increase in DNA copies may derive from a shorter period of cell division triggered by suppression of *CDKB2*, or a shorter period of cell expansion in case of *CycA2* overexpression lines.

Reflection on this study

This project was supported by the Technology Foundation STW (project NPB.6705) and potential users of our results are several Dutch breeding companies. Our objective was to investigate how cell number and sizes in the tomato fruit can be manipulated to improve fruit quality.

We found several candidate genes for this purpose, which are useful for further investigation. In our research we faced several problems, which should be taken into account in future study on tomato quality.

We used as genetic background for transformation the cultivars M82 and Ida Gold. M82 is a known inbred variety for processing, which is often used in tomato research. Ida Gold is an outdoor tomato variety, which is also used as ornamental variety. The big advantage of Ida Gold is a more effective transformation and better formation of callus, as compare to M82, therefore, the time from transformation to rooted plants was about 6 weeks shorter for Ida Gold.

Both cultivars used are characterized by determined growth. When propagating by cuttings changes in plant architecture occurred, which may also affect fruit development. Cultivar M82 has oval-shaped fruits with a thick pericarp. In this variety it was easier to monitor the changes in pericarp thickness and changes in placenta size, but due to its irregular fruit shape it was more difficult to quantify cellular parameters. M82 tomato is sensitive to so-called “blossom-end” disease, which is often the result of poor calcium uptake. This severely affected the research on fruit growth. The Ida Gold is easier to transform and this variety is resistant to blossom-end. Nonetheless, this variety has a weak pedicels, which often resulted in abscission before fruits were ripe. On the other hand this feature offered us the opportunity to observe the aberrations in fruit abscission in the case of *amiCDKB2* and *amiCDKB1* transgenic lines. In conclusion, we advice for research on fruit quality a more useful cultivar, e.g. Money Maker, which has indeterminate growth and round-shape uniform fruits.

Despite these technical problems this research shed light on the role of cell cycle genes in tomato fruit growth. Changes in expression of key cell cycle regulators affected the delicate balance between cell size and cell number, and consequently the texture of the pericarp. We observed a correlation between an increase of firmness and cell sizes in the pericarp, particularly in the layers located directly under the cuticle. These novel phenotypes need further investigation on these quality characteristics.

Although not conclusive we hypothesize that the missexpression of *CDKB2*, *CDKB1* and *CDKA1* in tomato seeds leads to impaired hormone production and/or signaling. We recommend these lines for further study on the link between cell cycle genes and hormone signaling, which may help the breeders to prevent early fruit abscission.

Altogether, the phenotypes obtained can be a good starting point for further research on fruit texture and other characteristics for improving tomato fruit quality.

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Summary in English and in Dutch

Fruit development progresses through a number of stages from ovary development, fruit set, cell division, cell expansion to ripening. The phase of cell division is an essential determinant of fruit development, as it fixes the final number of cells inside the fruit and therefore determines to a large extent the final fruit size. The cell division phase is followed by cell expansion leading to an enormous increase in the average cell volume. During this process cells enlarge up to 20-fold and become polyploid due to multiple rounds of endoreduplication. The tight control of cell division and expansion is a crucial determinant of the final size, shape and texture of the fruit.

For proper development and differentiation of plants it is essential that cell division and cell growth are precisely controlled. One level at which such control exists is the cell cycle, i.e. entry of cells into the cycle and their progress through mitosis followed by cytokinesis. The main focus of these thesis was to study the role of cell cycle genes in fruit growth with the ultimate goal to test the hypothesis that a change in the ratio between cell division/expansion even though no effect on total fruit size, may have substantial effects on various quality traits of the full grown ripe tomato.

In these thesis the results of research on the modifications by overexpression or knocking-down of candidate genes, which are involved in the regulation of cell division and cell expansion are presented. Several major tomato cell cycle regulators were isolated and their expression patterns in the pericarp throughout fruit development were studied (**chapter 2**). In addition the development of the pericarp with respect to cell number and size were analysed by histology. It was observed that the phases of cell division and cell expansion are not completely separated in time and cell expansion occurs already from the earliest stages onwards and cell division continues till the mature stage, in particular in the exocarp (**chapter 2**).

For the modification of gene expression in a fruit specific manner a fruit specific promoter was necessary. We selected the TPRP promoter and its activity using transgenic plants expressing the β-glucoronidase (uidA) reporter gene under the control of the TPRP promoter (pTPRP-GUS plants) was investigated (**chapter 2**).

Using the TPRP fruit specific promoter the overexpression or knocking-down of candidate genes, which are involved in the regulation of cell division and cell expansion were analysed.

The overexpression of *CDKB1* and *CDKB2* resulted in a decreased fruit size due to reduced numbers and sizes of cells in the pericarp (**chapter 3**). The fruits desiccated earlier than control fruits and had a reduced shelf-life. The down-regulation of *CDKA1* presented in the same study resulted 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 the *CDKB1* and

CDKB2 overexpression mutants, suggesting opposite roles of these types of CDK proteins in tomato pericarp development (**chapter 3**).

In **chapter 4** the analysis of fruits overexpressing *CDKA1* was presented. The obtained transgenic fruits had increased pericarp thickness and size of the septum and placenta, which was caused by an increase in the number of cells, despite of the slight decrease in cell size.

In **chapter 5** the action of *CycA2* in tomato fruit development and specifically the duration of cell division in the pericarp were analysed. The overexpression of CycA2 resulted in a reduction in cell size and number in the pericarp. *CycA2* might be involved in the regulation of cell division duration and have a role in cell expansion, although the involvement of *LeCycA2* in the cell expansion process was not fully understood.

A further analysis of *CDKB1* and *CDKB2* functions in tomato fruits was done on transgenic fruits in which these genes were down-regulated using an artificial microRNA approach (**chapter 6**). The downregulation of *CDKB1* and *CDKB2* yielded fruits with a reduced weight and decreased cell size in the pericarp. Both types of transgenic plants were impaired in the abscission of ripe fruits, which could not directly explained by the role of these *CDK* genes in the cell cycle.

Chapter 7 presents the study of the overexpression of *CycD3;3*. In the obtained transgenic fruits the compensation effect of cell division and cell expansion was observed, because the decreased cell size was correlated with an increasing cell number.

In conclusion the study in this thesis shows that changes in expression of key cell cycle regulators affected the delicate balance between cell size and cell number, and consequently the texture of the pericarp. The obtained phenotypes are a nice starting point for further research on fruit texture and other characteristics for improving tomato fruit quality.

De vruchtvorming van tomaat kan verdeeld worden in een aantal stadia: de ontwikkeling van het ovarium, de vruchtzetting na bestuiving en bevruchting, gevolgd door celdeling en celstrekking en uiteindelijk de vruchtrijping. De celdeling is het stadium waarin het uiteindelijke aantal cellen binnen de vrucht wordt vastgesteld. Dit proces speelt een belangrijke rol in het bepalen van de grootte van de vrucht. De celdeling wordt gevolgd door celstrekking waardoor het gemiddelde volume van de cellen tot 20-keer groter kan worden. Gedurende dit proces van celgroei, worden de cellen polyploid door endoreduplicatie zonder dat er celdeling plaats vindt.

Een nauwkeurige controle van celdeling en celstrekking is cruciaal voor de uiteindelijke grootte, vorm en textuur van de vrucht.

Celdeling en strekking wordt o.a. gestuurd tijdens door de celcyclus waarin DNA duplicatie, mitose en cytokenese plaats vinden.

Het onderzoek van dit proefschrift richt zich vooral op de rol van genen die betrokken zijn bij de celcyclus bij de groei van de vrucht met als ultieme vraagstelling of veranderingen in de verhouding tussen celdeling en celstrekking kunnen leiden tot veranderingen in verschillende kwaliteitskenmerken van de rijpe tomaat.

In dit proefschrift presenteer ik de resultaten van het onderzoek waarin de expressie van kandidaatgenen, die betrokken zijn bij de regulatie van celdeling en celstrekking, waren veranderd door overexpressie en gene-silencing. Een aantal belangrijke celcyclus regulators behorende bij de Cycline en Cycline dependent kinases (CDK) klassen waren geïsoleerd en de expressiepatronen in de pericarp tijdens de vruchtontwikkeling zijn bestudeerd (hoofdstuk 2). Tegelijkertijd is de ontwikkeling van de tomaat histologisch geanalyseerd en aantallen en grootte van de cellen in de pericarp bepaald. Uit deze analyse was te concluderen dat de stadia van celdeling en celstrekking niet compleet van elkaar in tijd gescheiden kunnen worden. Celstrekking kan al in een vroeg stadium plaats vinden en celdeling gaat door tot het volgroeide stadium, vooral in de exocarp (hoofdstuk 2).

Voor dit onderzoek hebben we de vrucht-specifieke promotor TPRP gebruikt voor specifieke genexpressie in de vrucht. In hoofdstuk 2 is deze promotor verder onderzocht met behulp van transgene planten die het reporterconstruct met het β–glucoronidase (uidA) reporter gen gefuseerd aan de TPRP promotor (pTPRP-GUS planten) tot expressie brachten. De promoter bleek inderdaad vrucht-specifiek te zijn en gedurende een groot deel van de vruchtontwikkeling actief te zijn.

Dezelfde promotor werd gebruikt voor overexpressie of silencing van kandidaat-genen, die betroken zijn bij de regulatie van celdeling en celstrekking.

De overexpressie van *CDKB1* en *CDKB2* resulteerde in een afname van vruchtgrootte door een vermindering van aantal en grootte van de cellen in de pericarp (hoofdstuk 3).

De vruchten waren ook eerder uitgedroogd na het oogsten in vergelijking tot controle vruchten en hadden een kortere houdbaarheid. De verlaging van de expressie van *CDKA1*gaf een vergelijkbaar fenotype. In deze vruchten waren minder cellagen in de exocarp waargenomen en vond er eerdere uitdroging plaats door een dunnere schil. In de *CDKB1/CDKB2* overexpressieplanten kwam het *CDKA1* gen lager tot expressie, waaruit gesuggereerd kon worden dat deze twee typen *CDK* genen een tegenovergestelde functie hebben in de ontwikkeling van de pericarp (hoofdstuk 3).

In hoofdstuk 4 wordt de analyse van vruchten met overexpressie van *CDKA1* gepresenteerd. De transgene vruchten hadden een dikkere pericarp en een grotere placenta dan wildtype. Dit werd veroorzaakt door een toename van celaantallen ondanks een lichte afname van de celgrootte.

In hoofdstuk 5 wordt het onderzoek over de rol van het *CycA2* gen in de tomaatontwikkeling beschreven. Overexpressie van *CycA2* leverde vruchten op met minder en kleinere cellen. De totale duur van de vruchtontwikkeling van vruchtzetting tot volgroeit was ook duidelijk verminderd, met name was dat het geval voor de duur van de celdelingfase.

Voor een verdere analyse van *CDKB1* en *CDKB2* werden transgene vruchten geproduceerd waarin deze genen waren geremd met behulp van de microRNA aanpak (hoofdstuk 6). Vruchten van deze amiRNA lijnen hadden een gereduceerd gewicht en cellen in de pericarp waren kleiner dan in wild type. Opmerkelijk was dat in deze planten nauwelijks abscissie van rijpe vruchten plaats vond, een observatie die niet direct te verklaren is.

In hoofdstuk 7 zijn de resultaten gepresenteerd van het onderzoek met planten die het *CycD3;3* gen tot overexpressie brachten. In de transgene vruchten vond een zogenaamd compensatie effect van celdeling en celstrekking plaats: de afname in celgrootte werd gecompenseerd door een toename in celaantallen, waardoor de vrucht een normale omvang had.

Uit het onderzoek beschreven in dit proefschrift blijkt dat veranderingen in de expressie van belangrijke celcyclus regulatoren de delicate balans tussen celgrootte en celaantal kan verstoren en daardoor de textuur van de pericarp kan veranderen.

De fenotypen die waren verkregen in dit onderzoek zijn een mooi beginpunt voor een verdere studie naar vruchttextuur en andere kenmerken voor een verbetering van de tomaatkwaliteit.

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Curriculum vitae

Publications

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Anna Czerednik was born on the 16st May 1969 in Leningrad, Russia. From 1976 to 1986 she followed her School Education at Baranovichi in Belarus and graduated with distinguish. Between 1989 - 1994 she studied soil science and agronomy at the Faculty of Biology and Soil Science at the University of Sankt-Petersburg in Russia. Her major interest was in agricultural microbiology. She made her diploma thesis (equivalent of MSc) at the Department of Biological Nitrogen in the Institute of Agricultural Microbiology. In her diploma project she studied the effect of heavy metals on yield of biomass and assimilation of nitrogen by alfalfa after inoculation with symbiotic and free-living microorganisms.

Directly after graduation Anna obtain a scholarship from the Polish Ministry of Education and began to work on her PhD thesis at the Department of Plant Physiology at the Warsaw Agricultural University. This project aimed to study photosynthetic productivity of traditional and new morphotypes of triticale (*Triticosecale* Wittmack) and pea (*Pissum sativum* L.) in the canopy. The main goal was to estimate the influence of difference in the architecture of plants on absorption of photosynthetically active radiation by the canopy and possible changes in the productivity of plants. In July-September 2007 Anna participated in the project "The influence of photosynthetically active radiation on nitrogen changing in the canopy of quinoa (*Chenopodium quinoa*) at different plant sowing density" at the University Hohenheim (Stuttgart, Germany). She defended her PhD thesis 6th June 1999.

In 1998 -1999 she worked at the Danko Plant Breeding company in Poland as a group leader of cereal technology laboratory.

From September 1999 to June 2001 Anna worked as an assistant at the Department of Biochemistry at the Warsaw Agricultural University, where she was involved in didactics.

From July 2001 to January 2004 Anna worked at the Institute of Botany and Molecular Genetics at the University of Aachen, Germany. The topic of her project was "Light inducible expression of enzymes necessary for C4-type CO2 fixation in C3 plants". During this fellowship Anna obtained intensive training in molecular biology and plant biotechnology.

After her marriage Anna moved to The Netherlands and worked first several months at the Department of Molecular Plant Physiology at the University Utrecht. In August 2005 she obtained a position as a junior researcher (AIO) at the Radboud University Nijmegen. She was focusing on the early stages of tomato fruit development and their role in fruit production and quality. The results of this research are described in this thesis. She also presented these results at several international scientific conferences, such as the XV Congress of the Federation of European Societies of Plant Biology in Lyon, France (2006), the Keystone Meeting of Plant Cell Biology in Coeur d'Alene, USA (2007), The 5th Solanaceae Genome Workshop in Cologne, Germany (2008) and The 6th Solanaceae Genome Workshop in New Delhi, India (2009), where she held an oral presentation. During her PhD she took several courses including radiation hygiene, presentation skills, bioinformatics and didactics.

In February 2011 Anna started to work at the Department of Plant Ecophysiology at the University of Utrecht. There she studies plant stress during *in vitro* propagation.

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