

The cell cycle-associated protein kinase WEE1 regulates cell size in relation to endoreduplication in developing tomato fruit

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Summary

Tomato fruit size results from the combination of cell number and cell size which are respectively determined by cell division and cell expansion processes. As fruit growth is mainly sustained by cell expansion, the development of pericarp and locular tissues is characterized by the concomitant arrest of mitotic activity, inhibition of cyclin-dependent kinase (CDK) activity, and numerous rounds of endoreduplication inducing a spectacular increase in DNA ploidy and mean cell size. To decipher the molecular basis of the endoreduplication-associated cell growth in fruit, we investigated the putative involvement of the WEE1 kinase (Solly;WEE1). We here report a functional analysis of Solly;WEE1 in tomato. Impairing the expression of Solly;WEE1 in transgenic tomato plants resulted in a reduction of plant size and fruit size. In the most altered phenotypes, fruits displayed a reduced number of seeds without embryo development. The reduction of plant-, fruit- and seed size originated from a reduction in cell size which could be correlated with a decrease of the DNA ploidy levels. At the molecular level downregulating Solly;WEE1 *in planta* resulted in the increase of CDKA activity levels originating from a decrease of the amount of Y15-phosphorylated CDKA, thus indicating a release of the negative regulation on CDK activity exerted by WEE1. Our data indicated that Solly;WEE1 participates in the control of cell size and/or the onset of the endoreduplication process putatively driving cell expansion.

Keywords: cyclin-dependent kinase, cell cycle, cell size, endoreduplication, *Solanum lycopersicum*, WEE1.

Introduction

The progression within the four phases of the plant cell cycle is regulated by a class of conserved heterodimeric protein complexes (Inzé and De Veylder, 2006). These complexes consist of a catalytic subunit referred to as cyclin-dependent kinase (CDK) and a regulatory cyclin subunit whose association determines the activity of the complex, its stability, its localization and substrate specificity. The kinase activity of the complexes is dependent on the availability and binding of the cyclin regulatory subunit, CDK inhibitors and/or regulatory factors, but also importantly on the phosphorylation/dephosphorylation status of the kinase itself (Inzé and De Veylder, 2006).

In fission yeast (*Schizosaccharomyces pombe*), the SpWEE1 kinase and the SpCDC25 phosphatase are acting

at the G2/M boundary. Following the DNA replication in S phase, the activity of the CDK CDC2 is inhibited by phosphorylation on the Y15 residue mediated by the activity of the WEE1 kinase during G2 (Russell and Nurse, 1987). At the G2/M boundary, CDC2 must be then dephosphorylated on Y15 within the CDC2/CycB complex through the action of CDC25 in order to drive the cell through mitosis (Russell and Nurse, 1986; O'Farrell, 2001). In *S. pombe* CDC25 and WEE1 overexpressors give rise respectively to a short-cell phenotype and a long-cell phenotype as the G2 phase length is affected (Russell and Nurse, 1986, 1987). The short-cell phenotype was even reproduced in plant systems as the result of SpCDC25 overexpression (Bell *et al.*, 1993; McKibbin *et al.*, 1998;

Orchard *et al.*, 2005; Wyrzykowska *et al.*, 2002; Zhang *et al.*, 2005).

The existence of a SpCDC25 homologue in higher plants remains to be fully demonstrated, although a putative candidate named Arath;CDC25 was identified in Arabidopsis. Arath;CDC25 was able to dephosphorylate plant CDKs and activate Arabidopsis CDK activity (Landrieu *et al.*, 2004). However since it has been demonstrated to be an arsenate reductase its true implication in the cell cycle was questioned (Bleeker *et al.*, 2006). Plant homologues to SpWEE1 have been identified in maize (Sun *et al.*, 1999), Arabidopsis (Sorrell *et al.*, 2002) and tomato (Gonzalez *et al.*, 2004), and the overexpression of maize and Arabidopsis WEE1 in *S. pombe* induced the expected long-cell phenotype. Altogether these data indicated the functionality of the WEE1/CDC25-like phosphoregulators of CDK activity in plants, and suggested that they may also act in the control of cell size.

Under the control of developmental programs or in response to environmental constraint cells are able to modify the classical cell cycle into the endoreduplication cycle or endocycle that lacks mitosis (Joubès and Chevalier, 2000; Larkins *et al.*, 2001). The molecular mechanisms underlying endoreduplication remain still poorly understood. However the involvement of key cell cycle regulators in the commitment towards endoreduplication starts to be more and more documented. From the literature two reports shed light on the implication of CDKs in the control of the endoreduplication cycle. Leiva-Neto *et al.* (2004) demonstrated that CDKA activity plays a role in the endocycle, since the specific overexpression of a dominant negative form of CDKA could inhibit endoreduplication in maize endosperm cells. The activity of the mitosis-specific CDKB1;1 was shown to prevent the exit from the cell cycle and the subsequent commitment to endoreduplication (Boudolf *et al.*, 2004).

Fruit organogenesis results from the relationship between cell division and cell expansion (Bohner and Bangert, 1988), and the control of these two developmental phenomena is a crucial determinant of the final size and shape of fruits (Tanksley, 2004). Tomato fruit size thus depends upon mitotic activities which set the cell number, but also upon cell expansion which determines the relative cell size inside fruit. Following pollination and fertilization a very active period of cell division occurs inside the ovary and drives fruit growth for about 7–10 days (Gillaspy *et al.*, 1993). Thereafter and until ripening, fruit growth to almost full size is principally obtained through cell expansion. In tomato fruit cell size can reach spectacular levels such as hundreds or thousands of times the initial one (e.g. >0.5 mm in diameter inside pericarp) (Cheniclet *et al.*, 2005). These very large cells are found within the pericarp (fleshy part) and the locular (gel) tissue in tomato fruits which are both characterized by the arrest of mitotic activity and the inhibition of CDK activity during the cell expansion phase of tomato fruit development (Joubès *et al.*, 1999; 2000; 2001). This dual impairment is

concomitant with numerous rounds of endoreduplication that induce a spectacular increase in DNA ploidy and a consequent hypertrophy of the nucleus, leading to DNA contents up to 256 C or even 512 C (Bergervoet *et al.*, 1996; Cheniclet *et al.*, 2005; Joubès *et al.*, 1999) that cannot be observed in other model plants such as Arabidopsis or maize (Melaragno *et al.*, 1993; Vilhar *et al.*, 2002). This increase in the ploidy level influences obviously the final size of the cell, and a clear correlation exists between the mean cell size in the pericarp of various tomato genotypes and the mean ploidy level of developing fruit that can explain the observed gradation in cell size in tomato fruit (Cheniclet *et al.*, 2005).

The inhibition of M-phase CDK activity related to the impairment of mitosis during endoreduplication (Boudolf *et al.*, 2004; Grafi and Larkins, 1995) can originate from the selective degradation of M-phase-specific cyclins via the activation of the anaphase-promoting complex (Kondorosi and Kondorosi, 2004) or from the binding of specific CDK inhibitors to the CDK/Cyclin complex (Bisbis *et al.*, 2006; Coelho *et al.*, 2005; De Veylder *et al.*, 2001; Schnittger *et al.*, 2003; Verkest *et al.*, 2005). Additionally the inactivation of the CDK activity by phosphorylation mediated by the WEE1 inhibitory kinase has been postulated to contribute to the endoreduplication process in maize endosperm (Sun *et al.*, 1999) as well as in the endoreduplicating tissues of developing tomato fruit (Gonzalez *et al.*, 2004).

Up to now a single report dealing with a functional analysis of WEE1 in plants appeared in the literature. De Schutter *et al.* (2007) demonstrated that WEE1 knock-out mutants in Arabidopsis grew perfectly well under non-stress conditions: neither cell division nor endoreduplication was affected in these mutants, thus indicating that WEE1 is not rate-limiting for cell cycle progression under normal growth conditions. However WEE1 was shown to be a critical target of the DNA replication and DNA damage checkpoints which operates in the G2 phase by arresting the cell cycle in response to DNA damage.

Since the work of De Schutter *et al.* (2007) appears conflicting with the proposed role for WEE1 in the onset of endoreduplication, we aimed at performing another functional analysis of the WEE1 kinase in a highly endoreduplicating cell context, i.e. during tomato fruit development. We here provided evidence that WEE1 is involved in the determination of endoreduplication and thus participates in the control of cell size during tomato fruit development through the negative regulation of CDK activity.

Results

Down-regulating WEE1 alters fruit growth and DNA ploidy profiles in tomato primary transformant plants

To address the role of Solly;WEE1 (for *Solanum lycopersicum* WEE1, previously called LeWEE1; Gonzalez *et al.*, 2004) in

plant development, transgenic plants were generated that overexpressed a fragment of the *Solly;WEE1* cDNA in an antisense orientation under the control of the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter. These plants will be referred to hereafter as *Pro35S:Slwee1^{AS}*.

Forty eight transgenic lines expressing the transgene were obtained. None of them displayed any significant vegetative alteration compared to wild-type (WT) plants (data not shown). However, following fruit development, we could classify the primary T0 *Pro35S:Slwee1^{AS}* transformants into three distinct classes based on mature fruit size (Figure 1a). Nine lines produced fruits similar to WT ones (normal size, class I) with a mass > 4 g. Fifteen plants displayed slightly smaller fruits (medium size, class II) weighing between 2.13 and 3.91 g. Interestingly, 24 plants over a total of 48 produced fruits with a weight not exceeding 2 g (small size, class III) (fruit mass between 0.49 and 1.94 g). The gradation in the observed fruit phenotypes was correlated with distinct effects of the transgene on the expression of endogenous *Solly;WEE1* in the different T0 *Pro35S:Slwee1^{AS}* lines (Figure 1a). In all transformant lines, the *Solly;WEE1* transcript levels were lower than the transcript level found in untransformed plants. Interestingly, the lowest expression of endogenous *Solly;WEE1* (from 20% to 46%) was observed in *Pro35S:Slwee1^{AS}* lines which produced fruits with the most altered size (class III).

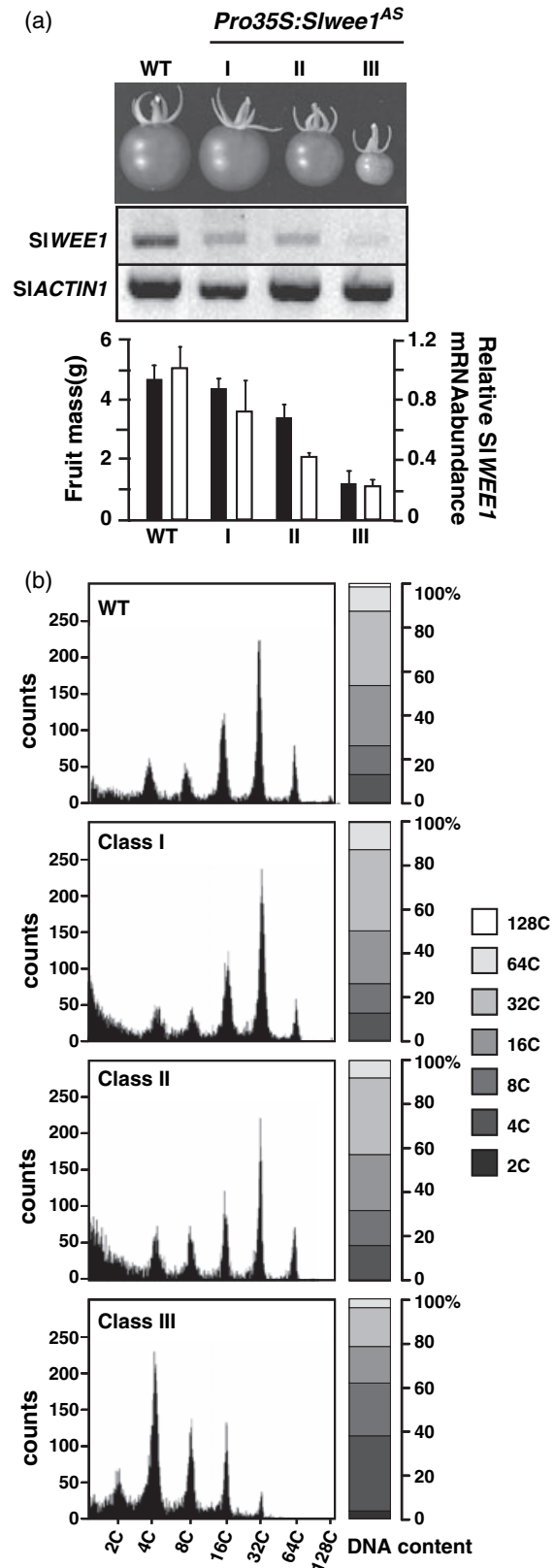
In the class III small fruits, the number of seeds was reduced (0–10 seeds per fruit) while it was only slightly affected in fruits from the two other classes of plants. Furthermore seeds originating from class III fruits were smaller than WT seeds (data not shown) and unable to germinate. Pollen germination tests indicated that T0 *Pro35S:Slwee1^{AS}* plant pollen grains were not significantly different from that of WT pollen grains in their capacity to germinate (data not shown). In addition, hand-pollination of flowers of the most affected T0 *Pro35S:Slwee1^{AS}* lines with pollen from WT flowers did not restore a normal fruit size.

Figure 1. Phenotypic and molecular analysis of fruits harvested from *Pro35S:Slwee1^{AS}* primary transformants.

Mature fruits (red ripe stage) were compared with untransformed control fruits (WT) of the same age.

(a) All fruits of a given class of size were harvested from the 48 *Pro35S:Slwee1^{AS}* primary transformants and weighed. The compiled data are represented in black histograms. The *Solly;WEE1* expression was determined in representative fruits of the three classes of size by semi-quantitative real-time polymerase chain reaction analysis. The accumulation of the transcripts was quantified by image scanning of the autoradiogram and the *Solly;WEE1* mRNA relative abundance was normalized to *Solly;ACTIN1* and expressed as a ratio of arbitrary units for pixel intensities (white histograms). Relative quantification data were the mean value (\pm SD) of three repeats.

(b) Ploidy level distribution of fruit pericarp at 25 DPA of wild type and one representative fruit of each classes of size. Histograms represent average data of three independent measurements.



Taken together, these data showed that the reduction of seed number and fruit size did not originate from a default in pollen viability.

A flow cytometry analysis of nuclei prepared from the pericarp tissue of 25 DPA-fruits revealed that 16 C, 32 C and 64 C DNA levels were predominant, accounting for respectively 27%, 34% and 11% of total nuclei (Figure 1b), while nuclei at the 2 C DNA level were almost undetectable. The ploidy profiles of pericarp nuclei prepared from T0 *Pro*_{35S}:*Slwee1*^{AS} 25 DPA-fruits of normal (class I) or medium (class II) size were almost similar to that of nuclei from WT fruits. By contrast, nuclei isolated from pericarp of smaller fruits (class III) displayed a striking reduced level of endoreduplication when compared to WT. Nuclei of 16 C and 32 C DNA levels only represented 34% of total nuclei in the pericarp of the *Pro*_{35S}:*Slwee1*^{AS} class III fruits, while the 64 C peak completely disappeared. Concomitantly, the DNA levels corresponding to the 4 C and 8 C peaks were greatly increased, now accounting for 42% and 22% respectively.

Taken together our data indicated that the downregulation of *Solly;WEE1* transcript levels in T0 *Pro*_{35S}:*Slwee1*^{AS} plants resulted in the production of small fruits which displayed a significant reduction in the endoreduplication levels.

*T1 Pro*_{35S}:*Slwee1*^{AS} plants are affected in plant growth, fruit and seed size

Since the class III plants produced very few seeds or seeds unable to germinate, we used seeds obtained from class I fruits to obtain a T1 progeny and further characterize the effect of the inhibition of *Solly;WEE1* expression.

Seeds from three independent T0 transformant lines (lines A, B and C) were sown on selective medium. T1 *Pro*_{35S}:*Slwee1*^{AS} plants obtained from these three T0 transformants were transferred to the greenhouse. By contrast with T0 plants, the T1 lineage displayed an important gradation in whole plant size (as illustrated for the line A progeny, Figure 2a). Some individuals displayed a size comparable to WT plants, while others were smaller. Three month-old control plants reached a size of 231 ± 3.6 cm while T1 progeny plants measured between 145 and 230 cm in height (Figure 2b). The size of the most altered plant (line A3) was reduced to 37% of the size of WT plants. In WT plants, the first inflorescence appeared 45 d after transplanting in the greenhouse when 13 internodes have been formed. Whatever the size of the T1 *Pro*_{35S}:*Slwee1*^{AS} plants may be the internode number at the first inflorescence was always similar to that of WT plants. This first inflorescence emerged after the same period of time in almost all transformant lines, but in some T1 *Pro*_{35S}:*Slwee1*^{AS} plants such as line A3, the first inflorescence formation was considerably delayed and only appeared after 75 d.

Fruits harvested at maturity (Red Ripe stage) from T1 *Pro*_{35S}:*Slwee1*^{AS} plants displayed a similar pattern of fruit

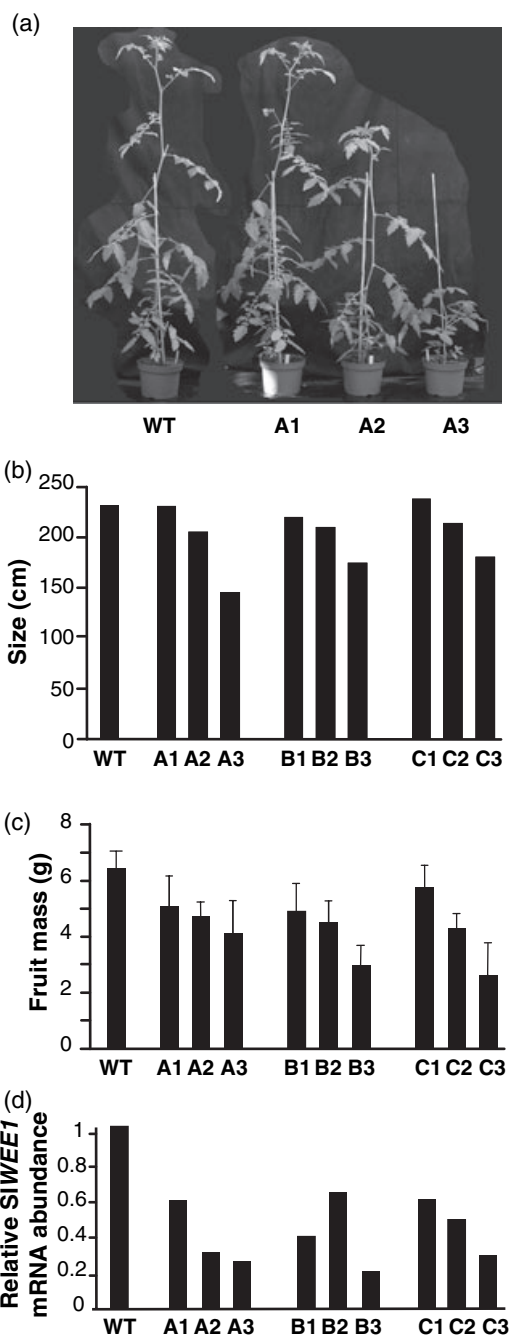


Figure 2. Phenotypic and molecular analysis of T1 *Pro*_{35S}:*Slwee1*^{AS} plants. (a) Growth phenotype of three descendants from Line A (A1, A2, and A3). Three month-old plants were compared with untransformed control plant (WT) of the same age. (b) Plant size determination of three descendants from Line A (A1, A2, and A3), Line B (B1, B2, and B3) and Line C (C1, C2, and C3). Three month-old plants were compared with untransformed control plant (WT) of the same age. (c) Fruit mass measurements of fruits harvested from Line A (A1, A2, and A3), Line B (B1, B2, and B3) and Line C (C1, C2, and C3) descendants. Histograms represent the compiled data from all fruits harvested from the various plants. (d) Semi-quantitative RT-PCR analysis of the *Solly;WEE1* expression in descendants from Line A (A1, A2, and A3), Line B (B1, B2, and B3) and Line C (C1, C2, and C3). The accumulation of the transcripts was analyzed from young leaves and data were quantified as described in Figure 1. Relative quantification data were the mean value of two repeats.

size reduction than T0 plants (Figure 2c). Interestingly, plants with size similar to that of WT ones (lines A1, B1 and C1) produced fruits of almost normal mass (>3.5 g), while the smallest plants (lines A3, B3 and C3) produced the smallest fruits. In the most altered T1 *Pro_{35S}:Slwee1^{AS}* plants, the reduction of fruit mass ranged from 36% to 54% to that of untransformed plant (fruit mass between 2.16 and 3 g). A good correlation was found between the plant and fruit size reduction and the relative *Solly;WEE1* mRNA abundance in the different T1 *Pro_{35S}:Slwee1^{AS}* lines (Figure 2d).

The very small fruits of the T1 progeny contained a reduced number of seeds which could be divided into two distinct populations (as illustrated for line A3 in Figure 3a, upper panel). Seeds belonging to the first one, representing 50%, 10% and 50% of seed population from lines A3, B3 and C3 respectively, were slightly smaller than WT seeds but still able to germinate. Seeds belonging to the second population, representing respectively 50%, 90% and 50% of seed population from lines A3, B3 and C3, were very small and devoid of any developed embryo (Figure 3a lower panel). However, they still contained some endosperm tissue since the presence of starch was revealed using Lugol as an indicative reactive stain. As a consequence the absence of any embryo was not due to a default in fertilization but to a post-fertilization abortion of the primary and secondary embryo. Furthermore, we also observed in some cases that seeds containing an embryo could display a residual endosperm since the characteristic dark-brown staining with Lugol was obtained outside of the cotyledons. This was indicative of a failure in starch translocation from endosperm to the cotyledons during the embryogenesis process. In addition a cytological analysis revealed that these cotyledons displayed a significant decrease in cell area (Figure 3b). Interestingly, when embryo-containing seeds were sown to germinate on MS medium, the obtained progeny (T2 plants) displayed also the 'scale-of size' phenotype (Figure 3c), indicating the heritability of the size alteration. All these plants were smaller than WT ones.

Downregulating Solly;WEE1 alters cell size in tomato plants

A scanning microscopy analysis of stem epidermal cells revealed that size-affected plants (as illustrated for the A3 line, Figure 4a) displayed very small cells compared to that of WT or unaffected transformed plants. A careful analysis of internode stem cell area revealed that the mean cell size in A3, B3 and C3 plants was reduced by 45%, 50% and 49% respectively, when compared to that of WT cells (Figure 4b).

We then investigated the origin of the fruit size reduction as observed previously (Figure 2). As shown in Figure 5a, the pericarp width of fruits harvested from A3, B3 and C3 plants displayed a slight but significant difference when compared to WT, with 15%, 19% and 14% of reduction

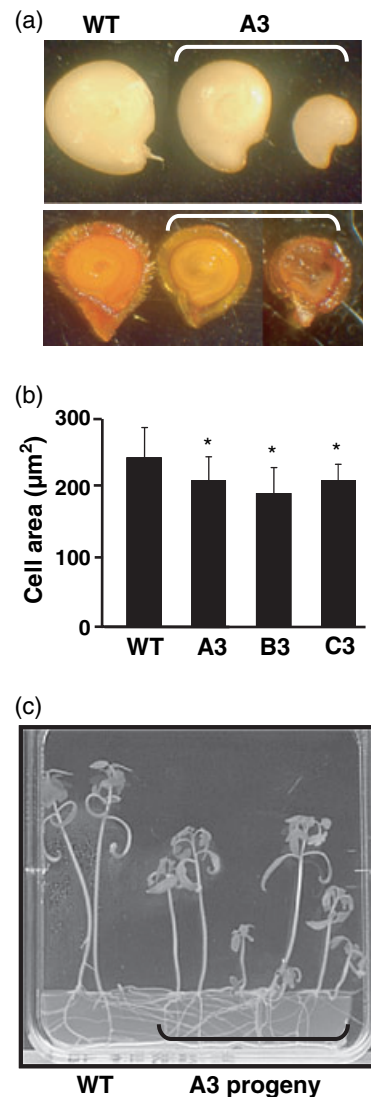


Figure 3. Phenotypic analysis of seeds harvested from T1 *Pro_{35S}:Slwee1^{AS}* fruits.

Seeds harvested from mature fruits (red ripe stage) were compared with untransformed control fruits (WT) of the same age.

(a) Phenotype of line A3 seeds displaying a smaller size than the control seeds (WT). Upper panel: seed morphology showing the representative seeds of the two size populations; lower panel: Lugol staining of the corresponding seeds. (b) Cell area determination at the level of cotyledons in embryo-containing seeds from A3, B3 and C3 lines. Star symbols above bars indicate that Student's tests show that the means of A3, B3 and C3 plants are significantly different ($P \leq 0.01$) in cell areas from that of wild type.

(c) Plantlet growth of the line A3 progeny. WT and line A3 seeds were sown at the same time, and picture was taken on 22-day-old plantlets.

respectively. This relative thinness of the pericarp did not originate from a decreased number of cell layers since line B3 contained an identical number of cells across pericarp to that of WT, and line A3 and C3 only lost one cell layer when compared to WT fruit pericarp (Figure 5b). Thus the *Solly;WEE1* downregulation did not affect the periclinal histogenic cell divisions giving rise to new cell layers which

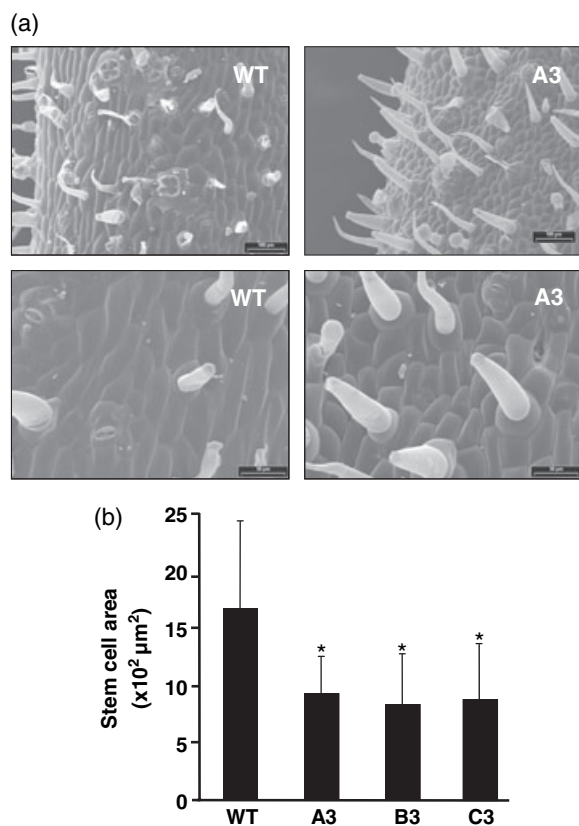


Figure 4. Cell size alteration induced by a downregulation of Solly;*WEE1* in T1 *Pro35S:Slwee1^{AS}* plants.

(a) Electron scanning micrograph of stem epidermal cells of line A3 plant compared with untransformed control plants (WT) (scale bar = 100 μm). The lower panels were taken at a higher magnification (scale bar = 50 μm).

(b) Cell area determination at the level of stem epidermis from lines A3, B3 and C3. Star symbols above bars indicate that Student's tests show that the means of A3, B3 and C3 plants are significantly different ($P \leq 0.01$) in stem cell areas from that of wild type.

account for pericarp width (Cheniclet *et al.*, 2005). A significant reduction in size of cells belonging to layers 5 to 9 from the central part of the pericarp which normally displays the largest cells in WT fruits (Cheniclet *et al.*, 2005) was observed (Figure 5c). This effect on cell size mediated by the Solly;*WEE1* downregulation is thus likely to explain the variation in pericarp width occurring in line A3, B3 and C3 fruits. As another explanation one cannot exclude that the randomly oriented cell divisions inside pericarp cell layers accommodating pericarp growth with fruit growth (Cheniclet *et al.*, 2005) may not be also affected.

The ploidy profiles of 25 DPA-fruits from lines A3, B3 and C3 were determined, and data were expressed as the transgenic-to-WT plant ratio of nuclei numbers for a given ploidy level (Figure 5d). As expected this analysis clearly showed an imbalance in the ratio towards an increase in low DNA levels (2 C to 8 C) and a decrease in high DNA levels (64 C and 128 C), while the 16 C and 32 C DNA levels were almost

unaffected, thus confirming that the effect of Solly;*WEE1* repression on cell size correlated with a reduction of endoreduplication in T1 *Pro35S:Slwee1^{AS}* plants.

Downregulating *WEE1* affects the CDKA phosphorylation status in planta

Shimotohno *et al.* (2006) recently demonstrated that the Arabidopsis *WEE1* could phosphorylate *in vitro* the Tyr15 residue of monomeric CDKA;1. We were able to reproduce these data using a recombinant 6xHis-tagged version of tomato *WEE1*. As shown in Figure 6a, tomato *WEE1* can phosphorylate CDKA;1 *in vitro* in the presence of ATP. A Tyr-phosphorylated CDK migrated with the same electrophoretic mobility as CDKA;1 as revealed by the immunoblot using an anti P-Tyr antiserum. In the absence of ATP, a very slight phosphorylation was observed on CDKA;1 which is likely due to the presence of residual ATP in the kinase pocket of the purified *WEE1*. This is ascertained as the autoradiography did not reveal any phosphorylation in the absence of labelled ATP.

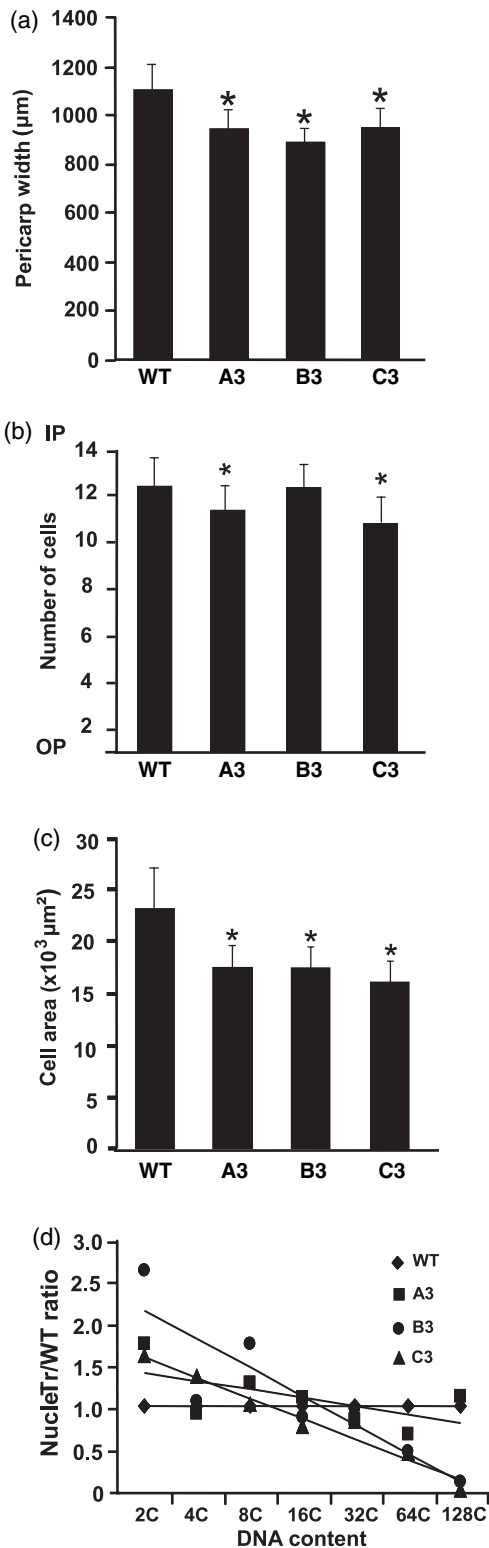
CDKA kinase activities from *Pro35S:Slwee1^{AS}* plant protein extracts were then measured using p9^{CksHs1} affinity purification and histone H1 as a substrate. Phosphorylation assays were performed using total protein extracts from 2 mm-flower buds, since the *Pro35S:Slwee1^{AS}* plants produce size-altered fruits in a too low quantity to prepare protein extracts of a satisfactory amount. As shown in Figure 6b, the CDK Histone H1 kinase activity in flower buds harvested from the three distinct transgenic *Pro35S:Slwee1^{AS}* lines, A3, B3 and C3, was respectively 2.5-fold, 2-fold and 3-fold more important than in WT flower buds. We next performed immunoblotting experiments using the same total protein extracts as described above and the anti-P-Tyr antiserum to check the phosphorylation status of CDKA. A clear decrease in the amount of Tyr-phosphorylated CDKA could be observed for the three distinct transgenic *Pro35S:Slwee1^{AS}* lines when compared to control WT plants (Figure 6c). We could thus demonstrate that the downregulation of *WEE1* in tomato transgenic plants resulted in an increased quantity of dephosphorylated CDKA, i.e. under its active form to drive mitosis.

Effect of a *WEE1* overexpression in synchronized tobacco BY-2 cells

Tobacco BY-2 cultured cells were transformed with the Solly;*WEE1* cDNA as to overexpress the gene (*WEE1^{OE}* cells) (Figure 7).

The size of transformed BY-2 *WEE1^{OE}* cells was significantly larger than control cells (at least 1.3-fold) (Figure 7a), and this alteration in cell size status was correlated with the overall *WEE1* (Solly;*WEE1* plus Nicta;*WEE1*) expression which was enhanced almost 1.5-fold (Figure 7b). In addition,

the CDKA histone H1 kinase activity in *WEE1^{OE}* cells was reduced to 60% when compared to untransformed cells, and the amount of phosphorylated CDKA was increased to a 1.4-fold factor (Figure 7b).



The progression into the cell cycle after synchronization by aphidicolin of the transformed BY-2 *WEE1^{OE}* line was monitored using a flow cytometry analysis (Figure 7c) and semi-quantitative RT-PCR analysis following the expression of Histone H4 and *CDKB2;1* mRNAs as respective markers for S phase and G2-M transition (Figure 7d). After the block release, tobacco BY-2 wild type (WT) cells proceeded normally through the S, G2 and M phases of the cell cycle (Figure 7c). As indicated by Histone H4 expression (Figure 7d), WT cells have completed the S phase 6 h after the block release. *CDKB2;1* expression peaked at 10 h and the maximum of mitotic index was then obtained 12 h after the block release. By 14 h after the removal of aphidicolin, half of the cells were already in the G1 phase of the cell cycle as deduced from both the ploidy profile and mitotic index determination (Figure 7c). In *WEE1^{OE}* cells, the duration of the S phase was similar to WT cells. Strikingly an important delay in the G2-to-M transition was observed as shown by the peak of *CDKB2;1* expression only occurring 14 h after the block release. Consequently the peak of mitosis was attained between 15 and 17 h after removal of aphidicolin, and 50% of cells were in G1 phase by 18–19 h after block release.

Discussion

The first functional analysis of a plant WEE1 kinase was recently provided in Arabidopsis using knock-out mutants (De Schutter *et al.*, 2007). In Arabidopsis neither cell division nor endoreduplication under normal growth conditions are affected when the WEE1 function is totally impaired. However the *WEE1* gene is activated upon loss of DNA integrity: in response to DNA-damaging stresses, the *WEE1* gene is induced in an ataxia telangiectasia-mutated- (ATM-) or Rad3-related- (ATR-) dependent manner, two kinases acting as sensors of double-strand breaks and DNA replication

Figure 5. Phenotypic analysis of fruits harvested from T1 *Pro35S:Slw1^{AS}* plants.

Immature green fruits (25 DPA) were compared with untransformed control fruits (WT) of the same age.

(a) Determination of fruit pericarp width. Star symbols above bars indicate that Student's tests show that the means for A3, B3 and C3 fruits are significantly different ($P \leq 0.01$) from that for WT. Histograms represent the compiled data from at least three fruits harvested from the various plants.

(b) Determination of cell layer number across pericarp. Cross sections of pericarp were prepared and stained with toluidine blue. Star symbol above bars indicates that Student's tests show that the means for A3 and C3 fruits are significantly different ($P \leq 0.01$) from that for WT. OP: outer pericarp; IP: inner pericarp.

(c) Cell area determination in central pericarp (between layer 5 and 9) from A3, B3 and C3 fruits. Star symbols above bars indicate that Student's tests show that the means for A3, B3 and C3 lines are significantly different ($P \leq 0.01$) in stem cell areas from the WT.

(d) Ploidy level distribution in fruit pericarp at 25 DPA of lines A3, B3 and C3 compared to wild type. Data are expressed as the ratio between A3, B3 or C3 nuclei number and WT nuclei number (Nuclei number ratio) for a given class of ploidy.

block respectively. As a consequence the WEE1 kinase activity targets the CDKA/Cyclin complex, resulting in a stop of the cell cycle in the G2 phase until DNA is repaired or replication is completed (De Schutter *et al.*, 2007).

We here provide new data relative to the function of WEE1 *in planta*. Our results showed that the downregulation of the cell cycle-regulated WEE1 kinase induced a small-fruit

phenotype in tomato (Figures 1 and 2), together with an alteration of plant height only observed in the T1 and T2 progenies (Figures 2 and 3). These phenotypes were likely to originate from a reduction in cell size, as short-cell sizes were observed in all the plant organs that were tested (Figures 3b, 4 and 5c). Interestingly affecting *WEE1* expression had little or no effect on mitosis itself in accordance with De Schutter *et al.* (2007), since the number of cell layers across the pericarp of small *Pro_{35S}:Slwee1^{AS}* fruit was not substantially altered when compared to that of normal WT fruit (Figure 5b). This suggests that *Solly*;WEE1 operates post-mitotically through the control of cell elongation. However unlike in *Arabidopsis* *WEE1* mutants, the reduction in *WEE1* expression and correlated cell size observed in *Pro_{35S}:Slwee1^{AS}* tomato plants was accompanied by an alteration of the endoreduplication process in fruit as another heritable trait (Figures 1b and 5d). This observation was in accordance with (i) the high levels of expression of the *WEE1* gene measured during endoreduplication (Gonzalez *et al.*, 2004), and (ii) the established correlation existing between the mean cell size in the pericarp of various tomato genotypes and the mean ploidy level of developing fruit (Cheniclet *et al.*, 2005). This suggests that WEE1 may act as a regulator of cell size and endoreduplication in tomato, a key process involved in fruit growth (Bergervoet *et al.*, 1996; Cheniclet *et al.*, 2005; Joubès *et al.*, 1999). Since *WEE1* transcript levels increase when cells undergo endoreduplication in maize endosperm and tomato fruit (Sun *et al.*, 1999; Gonzalez *et al.*, 2004), it was thus proposed that WEE1 may act on endoreduplication in a species-dependent

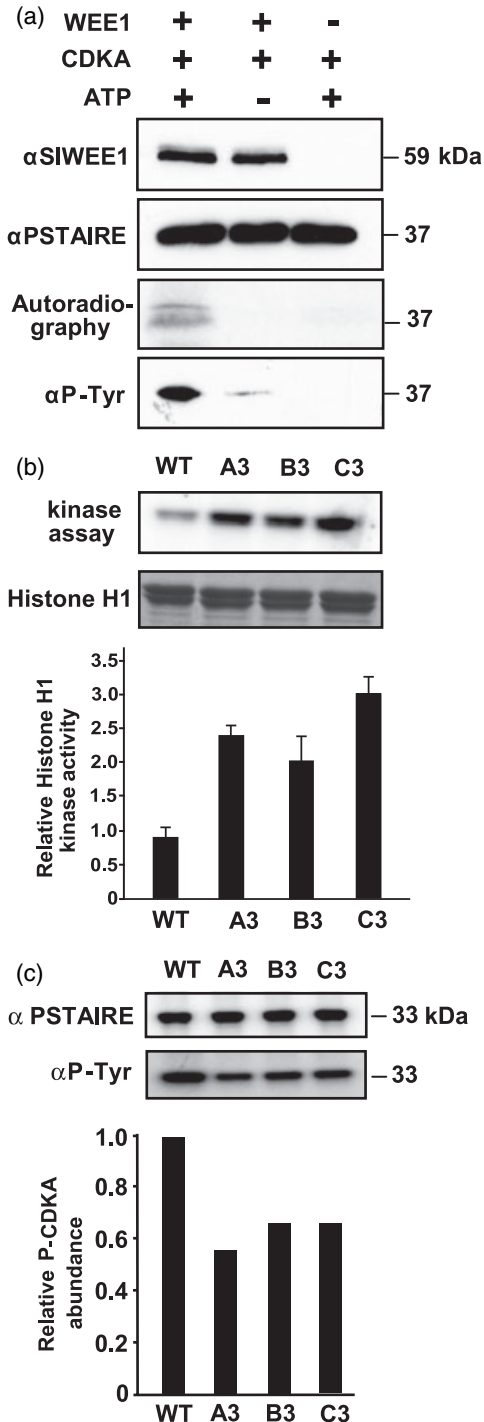


Figure 6. CDKA kinase activity and CDKA phosphorylation status in T1 *Pro_{35S}:Slwee1^{AS}* plants.

(a) Phosphorylation of CDKA;1 by WEE1. The recombinant His-tagged versions of tomato CDKA;1 (37 kDa) and WEE1 (64 kDa) were incubated for 2.5 h at 25°C in the presence of either 7.5 μCi of [³²P]ATP prior to electrophoretic separation and autoradiography or with 300 μM of cold ATP prior to protein gel blotting revealed with the anti-phosphorylated-Tyr (αP-Tyr) antibody. As controls of equal protein quantity used per phosphorylation reaction, CDKA;1 and WEE1 were detected by protein gel blotting using respectively the anti-PSTAIRES (αPSTAIRES) and the specific anti-*Solly*;WEE1 (α*Solly*;WEE1) antisera.

(b) Kinase activity of CDK/cyclin complexes present in 2 mm-flower bud protein extracts bound to p9^{CksHs1}-sepharose beads was assayed in A3, B3 and C3 lines using Histone H1 as a substrate (upper panel). Equal amounts of protein (750 μg) were used in each assay. The Coomassie blue staining of the electrophoresis gel area showing Histone H1 (lower panel) is shown as a control of equal substrate quantity used per phosphorylation reaction. The kinase activity was quantified by image scanning of the autoradiogram, normalized for the quantified amount of Histone H1, and expressed as a ratio of arbitrary units for pixel intensities.

(c) Western blot analysis of p9^{CksHs1}-purified CDKA from 2 mm-flower bud protein extracts using the αP-Tyr antiserum (upper panel). Equal amounts of protein (900 μg) were used in each assay. The level of total CDKA protein in each sample was detected using the αPSTAIRES antibody (lower panel). The amount of phosphorylated CDKA (revealed by αP-Tyr antibody) was quantified by image scanning of the autoradiogram, normalized for the quantified amount of total CDKA, and expressed as a ratio of arbitrary units for pixel intensities.

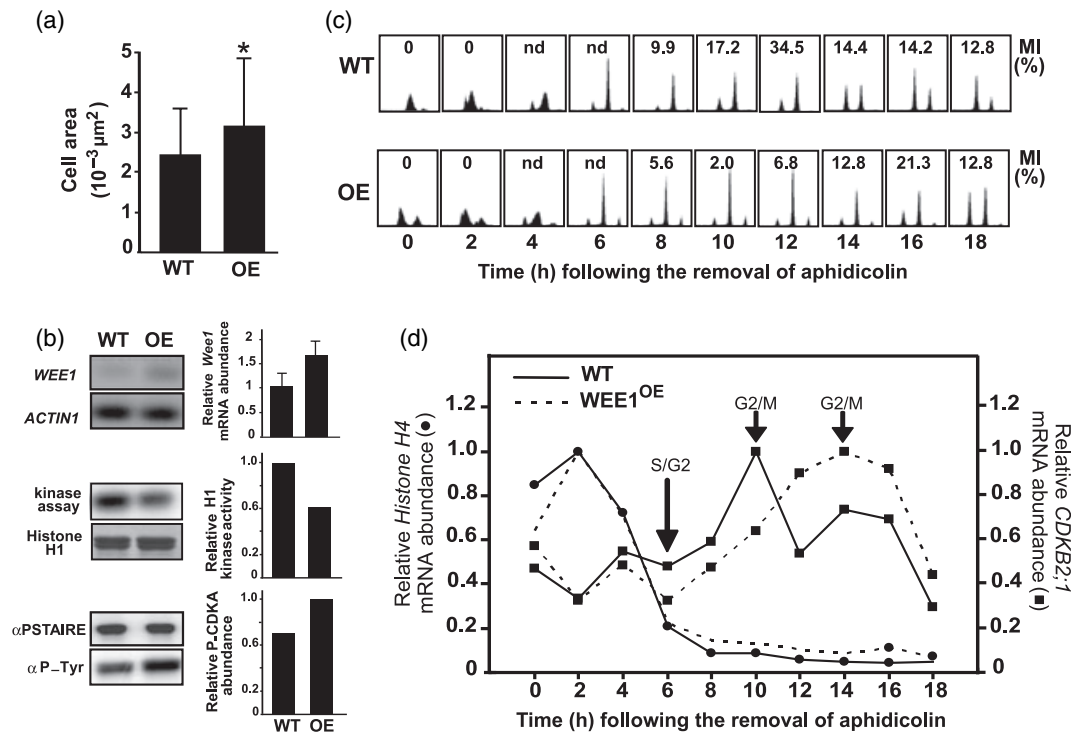


Figure 7. Cell cycle progression of transformed tobacco BY-2 cells upregulated for WEE1 compared to untransformed control cells (WT). Tobacco BY-2 *WEE1*^{OE} (OE) transformed cells were compared with untransformed control cells (WT) at the same age of subculture (4 d). (a) Cell area determination of *WEE1*^{OE} cells compared with untransformed control cells (WT). Star symbol above bars indicates that Student's tests show that the mean for *WEE1*^{OE} cells is significantly different ($P \leq 0.01$) from the WT. (b) Molecular analysis of Tobacco BY-2 *WEE1*^{OE} (OE) transformed cell line. The *WEE1* mRNA expression in the transformed BY-2 cell line was analyzed by semi-quantitative RT-PCR and the *WEE1* mRNA relative abundance was quantified by image scanning of the autoradiogram, normalized to *Nicta*/*Actin1* and expressed as a ratio of arbitrary units for pixel intensities. Data were obtained from three independent experiments. CDK/cyclin Kinase activities present in WT and *WEE1*^{OE} cell protein extracts (500 μg) and the amount of phosphorylated CDKA were determined as described in Figure 6. (c) Cell cycle progression monitored by flow cytometry and by measurement of the mitotic index (MI). BY-2 cells were synchronized with aphidicolin (see Experimental procedures) and samples were taken every hour. (d) Quantification of cell cycle-specific mRNA accumulation. Total RNA to be used for RT was isolated from the same samples as in (c). The real-time polymerase chain reaction analysis was performed as in (b). Specific cDNA probes were prepared for cDNAs coding for Histone H4 as a marker of S phase and NtCDKB2;1 as a marker G2/M transition.

manner (De Schutter *et al.*, 2007). Spectacular levels of endoreduplication can be reached up 512 C in some varieties of tomato (Cheniclet *et al.*, 2005), while the extent of ploidy levels that can be observed in Arabidopsis are limited to 16 C in hypocotyls and cotyledons (De Schutter *et al.*, 2007). As mentioned earlier WEE1 is not only involved in cell size determination in every tomato plant organs, but it is also tempting to speculate that this so-proposed species-dependent function for WEE1 could be closely related to the cell expansion- and endoreduplication-dependent growth of the fruit organ. Furthermore this role of WEE1 in the control of cell size is not exclusive to a putative function at the DNA integrity checkpoints, since endoreduplication as a DNA amplification process may require a sustained WEE1 activity to cope with the DNA damages potentially occurring during the successive rounds of DNA replication. Consequently *WEE1* is actively transcribed in endoreduplicating tissues such as tomato fruit pericarp and gel or maize endosperm (Gonzalez *et al.*, 2004; Sun *et al.*, 1999).

Little is known about the molecular basis of cell size control. In fission yeast, the WEE1 kinase has a well-defined role in delaying entry into mitosis until the cell reaches a critical cell size (Fantes and Nurse, 1977). A similar role has been proposed for SWE1, the WEE1 orthologue in budding yeast (Harvey and Kellog, 2003). We were thus interested in investigating at the cellular level the effect of the WEE1 activity on the progression into the cell cycle and especially on cell cycle phase length. The overexpression of WEE1 in Tobacco BY-2 cells resulted in larger cells (Figure 7a) as a consequence of a lengthening of the G2 phase (Figure 7c,d). This observation was in accordance with *WEE1* being transcribed in early S phase (Gonzalez *et al.*, 2004; Menges *et al.*, 2005), as well as CDK Tyr phosphorylation occurring also predominantly in S phase (Mészáros *et al.*, 2000). Interestingly a recent report from Orchard *et al.* (2005) demonstrated that BY-2 cells expressing SpCDC25 exhibited a reduced mitotic cell size through a shortening of the G2 phase, that is to say the expected opposite effect observed in

WEE1^{OE} cells. Thus the induced short-cell phenotype in *Pro_{35S}:Slwee1^{AS}* plants (Figures 3b, 4 and 5c) may originate from a shorter G2 phase and a coincident premature entry into mitosis. Support for this was provided by the observed increase in CDK kinase activity in *Pro_{35S}:Slwee1^{AS}* plants which originates from the impairment of Solly;*WEE1* kinase activity thus decreasing the amount of Y15-phosphorylated CDKA (Figure 6).

Our previous (Gonzalez *et al.*, 2004) and present data thus indicate that *WEE1* is likely to play a part in the control of the endoreduplication cycle. *WEE1* may not only contribute to trigger the onset of endoreduplication by blocking the M-phase CDK/Cyclin complex (Inzé and De Veylder, 2006), but also *WEE1* may play an important role during the endoreduplication cycle progress. The endocycle is only composed of a DNA synthetic (S) phase and an undifferentiated gap (G) phase (for a review see Kondorosi and Kondorosi, 2004). Within the endocycle a minimal cell size must be required to pass the Start/restriction point in the G phase prior to commit to the following round of DNA replication. Once DNA synthesis is completed, the *WEE1* activity could operate in early G phase (post-S phase of the endocycle) and contribute to inhibit the CDK/cyclin complexes driving the G-to-S transition, preventing a premature entry into the S phase of the following endocycle and thus allowing cell enlargement in response to nucleus hypertrophy as observed during endoreduplication (Joubès *et al.*, 1999). Hence *WEE1* may regulate the length of the G phase during the endocycle. This type of regulation may be extremely important for the cell as to adjust its nuclear-to-cytoplasmic volume ratio (Sugimoto-Shirasu and Roberts, 2003). A similar type of cell size control was demonstrated in yeast as the CDK inhibitor p25^{um1} delays the passage through Start until the required cell mass is reached (Martin-Castellanos and Moreno, 1996). Furthermore reducing *WEE1* expression in *Pro_{35S}:Slwee1^{AS}* tomato fruits affected endoreduplication towards lower DNA ploidy levels (Figures 1b and 5d). This suggests that *WEE1* may also be involved in the G-to-S phase progression within the endocycle, thus contributing to regulate subsequently DNA replication.

Obviously endoreduplication is not the only mechanism contributing to the cell size and organ size control in plants. As reviewed by Mizukami (2001), one of the mechanisms controlling the final organ size is mediated by phytohormone signaling. In tomato most of the cytokinin, auxin and gibberellins originate from the developing seed (Gillaspy *et al.*, 1993). A clear correlation occurs between high cytokinin levels in developing seeds and cell division in the surrounding fruit tissues on the one hand, and high gibberellins levels and cell expansion activity on the other hand (Srivastava and Handa, 2005). Hence during tomato fruit growth, the developing embryo or seed controls the rate and sustenance of both cell division and cell expansion

in fruit which in turn influences the final size of fruit. Downregulating Solly;*WEE1* resulted in a medium to high level of embryo abortion in *Pro_{35S}:Slwee1^{AS}* plants respectively (Figure 3a), and (in the worst cases) in the total absence of seeds giving rise to small parthenocarpic fruits. Since Solly;*WEE1* is highly transcribed in ovules and developing embryos and/or seeds (Gonzalez *et al.*, 2004), the downregulation of Solly;*WEE1* giving rise to embryo abortion is likely to alter hormone production which is ultimately necessary for full fruit growth. Interestingly it seems that the effect of *WEE1* operates according to a certain threshold, since fruits of class I and class II from the T0 progeny were normally seeded though *WEE1* transcript level and fruit size were reduced (Figure 1). Small parthenocarpic fruits developed (fruits of class III) when *WEE1* expression was reduced to 20 to 40% when compared to control plants. In addition the same threshold effect of *WEE1* operates on the endocycle since only class III fruits were affected in their ploidy profile.

In T0 and T1 progenies the absence of a viable embryo was not due to a failure in pollination/fertilization since seeds contained endosperm (Figure 3a). This indicated that a secondary zygote giving rise to the endosperm was formed, started to develop and then stopped its development at an early stage. It is likely that the low level of Solly;*WEE1* transcripts in *Pro_{35S}:Slwee1^{AS}* transformed plants did not prevent but only delayed the vegetative growth. Furthermore it did not prevent the gametogenesis since male gametophytes produced the two gametic nuclei to fertilize the egg cell and the polar nuclei, but impaired the development of zygotic and secondary embryo.

Why such a high percentage (50 to 90%) of very early collapsed embryo appeared is not yet understood. A possible explanation could rely on the high rate of cell division in the embryogenic tissues and the expected effect of downregulating *WEE1* on both DNA repair and/or cell size. Assuming that *WEE1* could be involved in the DNA replication/DNA damage checkpoint (De Schutter *et al.*, 2007), the downregulation of a functional *WEE1* in the *Pro_{35S}:Slwee1^{AS}* embryo following fertilization would prevent the repair of the DNA damages arising during meiotic recombination events and consequently could allow an accumulation of mutations, thus impairing embryo development. Alternatively the impaired control on cell size by *WEE1* would lead to the production of too small cells within both zygotic and secondary embryos after several rounds of consecutive cell divisions, thus becoming unable to divide further and finally collapsing. It was recently reported that Sp*CDC25* expression under the control of the ADP-glucose pyrophosphorylase large subunit promoter disrupted reproduction in wheat (Chrimes *et al.*, 2005). Since it is expected that *WEE1* and *CDC25* act conversely, this remarkable observation supports our data obtained when *WEE1* activity is impaired.

In conclusion, we showed that WEE1 is an important regulator of tomato fruit development as its activity at the interplay between normal cell cycle events and endoreplication has an important impact on organ and cell size determination.

Experimental procedures

Material and growth conditions

Cherry tomato (*Solanum lycopersicum* Mill. cv vva106) plants were grown in a greenhouse under a thermoperiod of 25°C/20°C and a photoperiod of 14/10 h (day/night). Tomato fruits were harvested at various developmental stages determined according to DPA and fruit diameter.

Suspension-cultured tobacco (*Nicotiana tabacum* L. cv BY2) cells (Nagata *et al.*, 1992) were grown in the dark at 26°C on a rotary shaker (130 rpm) in Murashige-Skoog medium (Sigma, St-Quentin Fallavier, France), supplemented with 1.5 mM KH₂PO₄, 3 µM Thiamine, 0.55 mM Inositol, 87 mM Sucrose, and 1 µM 2,4-dichlorophenoxy acetic acid. Cells were subcultured every 7 days by transferring 2 ml into 100 ml of fresh medium.

The *Agrobacterium tumefaciens* strains LBA4404.pBBR1MCS virGN54D and GV3101 were used to transform BY-2 cells and tomato plants respectively.

Plasmid construction

To obtain the *Pro*_{35S}:*Slwee*^{AS} construct, a 776 bp cDNA fragment corresponding to nucleotides 924 to 1699 of the Solly;*WEE1* cDNA (accession no. AM180939; Gonzalez *et al.*, 2004) encoding the C-terminal half of the protein, was amplified by PCR using *Pfu* DNA polymerase (Stratagene, San Diego, CA, USA) and further subcloned in antisense orientation into a modified pPZP212 binary vector containing the 35S CaMV promoter (Hajdukiewicz *et al.*, 1994) and the terminator of the CaMV gene VI from the pDH51 vector (Pietrzak *et al.*, 1986). Blast search analyses using this 776 bp DNA sequence were performed to ascertain that no other sequences from the databases than plant *WEE1* genes could be recognized and putatively hybridize.

To obtain the *Pro*_{35S}:*SIWEE*^{OE} construct, the complete Solly;*WEE1* ORF was amplified and subcloned into the modified pPZP212 binary vector as described above.

To overexpress and purify the recombinant tomato *WEE1* and *CDKA;1* proteins, the coding regions of Solly;*WEE1* (Gonzalez *et al.*, 2004) and Solly;*CDKA;1* (Joubès *et al.*, 1999) were amplified by PCR and cloned respectively into the pSCodon1 vector (Delphi Genetics S.A., Charleroi, Belgium) using the *Nhe*I and *Sal*I sites and into the pET-28a(+) vector (Novagen, Darmstadt, Germany) using the *Bam*H1 and *Xho*I sites. The two recombinant proteins containing a 6xHis tag at the N-terminal were expressed in *Escherichia coli* strain SE1 (Delphi Genetics S.A., Belgium) and purified on Ni-NTA resin (Novagen) using the manufacturer's protocol.

Generation of an anti-Solly;*WEE1* antiserum

A *WEE1*-specific polyclonal antibody was raised in rabbits against a synthetic peptide (N-MMDPDPTRRPSAKGV-C) derived from tomato *WEE1* (Eurogentec, Seraing, Belgium). The antiserum was purified by affinity column against the immunizing peptide.

Tomato plant and tobacco BY-2 cell transformation

The *Pro*_{35S}:*Slwee*^{AS} construct was introduced into *A. tumefaciens* strain GV3101 by electroporation. The transformation of tomato cotyledons was performed as described (Hamza and Chupeau, 1993) and resulted in the obtention of *Pro*_{35S}:*Slwee*^{AS} plants. Plant regeneration, selection and agrobacteria elimination were performed on Murashige and Skoog (MS) medium (Sigma) supplemented with 0.1 mg l⁻¹ of AIA, 1 mg l⁻¹ of BAP, 300 mg l⁻¹ of kanamycin and 300 mg l⁻¹ of Timentin (Duchefa Biochemie B.V., Haarlem, The Netherlands). Transformed plants were grown on hormone free MS medium supplemented with 300 mg l⁻¹ of kanamycin and then transferred into the greenhouse.

To transform BY-2 cells, the *Pro*_{35S}:*SIWEE*^{OE} construct was introduced into *A. tumefaciens* strain LBA4404.pBBR1MCS virGN54D by electroporation. Co-incubations of BY2 cell suspension (4 ml) at an exponential phase of growth (3–4 d after subculture) with various volumes (10, 20, 50, 100, 200 µl) of a 48 h *A. tumefaciens* pre-culture diluted to an OD₆₀₀ = 1 with MS medium were performed in plate culture for 72 h in the dark with gentle agitation. The BY2 cells were then washed three times with 10 ml of fresh culture medium and resuspended in 3 ml. Two ml of cells were then plated onto agar-MS medium containing 250 µg ml⁻¹ Timentin and the selective antibiotic (100 µg ml⁻¹ Kanamycin). After 4 weeks, isolated calli were picked and transferred onto new plates. Individual calli were then maintained on agar medium or resuspended in liquid medium to obtain cell suspensions of transformed cells.

Expression analysis by semi-quantitative RT-PCR

Extraction of total RNA from leaves of tomato plants or BY-2 cells suspensions and semi-quantitative RT-PCR assays were performed as previously described (Gonzalez *et al.*, 2004). The relative transgene transcript level was analysed by using a TermVI-specific oligonucleotide (TATGCTCAACACATGAGCCG) combined to W1i3 (CATCATTGCCTTGAGTAGATTCTG) (for *Pro*_{35S}:*Slwee*^{AS} plants) or to W51 (CTCATTAGATGTAAAGCCAG) (for *Pro*_{35S}:*SIWEE*^{OE} BY-2 cells). The RT-PCR protocol and primers used as controls were as described previously (Gonzalez *et al.*, 2004).

Protein extraction, kinase assays and immunoblotting

Flower buds (2 mm stage) were frozen in liquid nitrogen prior to protein extraction. They were ground to a fine powder and stored at -80°C. For protein extraction, 100 mg of frozen powder was thawed in 1 ml of extraction buffer [50 mM Tris-HCl (pH 7.5), 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 15 mM MgCl₂, 15 mM EGTA, 5 mM NaF, 2 mM DTT, 1 mM PMSF, 0.1 mM vanadate and cocktail inhibitor (Sigma)]. The cell debris was discarded after a 15 min centrifugation at 18 000 g at 4°C.

The *in vitro* phosphorylation of *CDKA;1* by *WEE1* was carried out by incubating purified recombinant Solly;*CDKA;1* (20 ng) and Solly;*WEE1* (100 ng) for 2.5 h at 25°C in a 40 µl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, and 0.1% Triton X-100 in the presence of 300 µM ATP supplemented with 7.5 µCi of [γ-³²P] ATP to visualize phosphorylated *CDKA;1* after electrophoretic separation and autoradiography. Radiolabelled ATP was removed in the reaction mixture to visualize phosphorylated Tyr residue in *CDKA;1* by immunoblotting using a monoclonal anti-P-Tyr antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). As controls of equal protein

quantity used per phosphorylation reaction, CDKA;1 and WEE1 were detected using a polyclonal anti-PSTAIRE (α PSTAIRE) antibody and a specific anti-Solly;WEE1 (α Solly;WEE1) antiserum respectively. The immunoblots were revealed using anti-rabbit IgG horseradish peroxidase-linked antibodies (Chemicon International Inc., Chandlers Ford, UK) and BM Chemiluminescence Blotting Substrate (Roche Diagnostics, Meylan, France).

Purification of CDK/Cyclin complexes using p9^{CksHs1} protein-Sepharose beads and Histone H1 kinase activity assays were as described (Bisbis *et al.*, 2006). Intensities of silver grains on the autoradiogram were analyzed and quantified using the QUANTITY-ONE software.

Cytological methods

Morphometric analyses of cell size from seeds, stem epidermis and pericarp were performed. Longitudinal sections of seeds were prepared and fixed in 4% (w/v) formaldehyde, 50% (v/v) ethanol, 5% (v/v) acetic acid for 4 h at room temperature. The fixative was replaced each hour and vacuum was applied for 15 min. Samples were then treated as described by Gabe (1968). Sections (8 μ m thick) were stained with 0.1% (w/v) toluidine blue and slides were analysed with a microscope (Zeiss Axioplan, Oberkochen, Germany). Morphometric analyses were performed by using a gauge of $2.5 \times 10^3 \mu\text{m}^2$. Cell number and size (assimilated to a square) were evaluated in the cotyledon abaxial side close to the shoot apical meristem. Approximately 30 to 50 gauges were analyzed for each genotype. To visualize the size of epidermis cells, the stem surface was sprayed with liquid collodion at the level of the internode of the third flower truss. After drying, the collodion was peeled off and placed in a drop of water and covered with a cover-slip. Images were taken on an average of 100 cells for each genotype using a microscope (Zeiss Axioplan) and then analysed with IMAGEPRO-PLUS software (Media Cybernetics, Silver Spring, MD). Pericarp slices were prepared as described by Cheniclet *et al.* (2005), and the number of cell layers and cell size were determined accordingly. For all morphometric measurements data were statistically analyzed and Student's test was used to evaluate the significance of the results.

Scanning electron microscopy was performed on stem epidermis fragments. Fragments were dissected and fixed overnight in 3% glutaraldehyde in 0.025 M sodium phosphate (pH 7.0) at 4°C. After rinsing and dehydration in a graded ethanol series, they were critical point dried in liquid carbon dioxide. They were then mounted on stubs, coated with gold and viewed with a scanning electron microscope (Philips, Eindhoven, The Netherlands) at an accelerating voltage of 3 ± 10 kV.

Flow cytometry analysis

Ploidy profiles of isolated nuclei from 25 DPA tomato pericarp were determined as described (Cheniclet *et al.*, 2005). Flow cytometry analysis of isolated nuclei from tobacco BY-2 protoplasts was performed as described (Porceddu *et al.*, 2001).

Synchronization of tobacco BY-2 cell suspension culture

Tobacco BY-2 cell suspension was cultured and synchronized with aphidicolin as described (Porceddu *et al.*, 2001). Mitotic index was determined by UV light microscopic analysis of 500 cells stained with $0.1 \mu\text{g ml}^{-1}$ 4',6-diamino-2-phenylindole (Sigma) in the presence of 0.2% (v/v) Triton X-100.

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