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Molecular and Biochemical Characterization of the Involvement of Cyclin-Dependent Kinase A during the Early Development of Tomato Fruit[1]

Jérôme Joubès, Thi-Hai Phan, Daniel Just, Christophe Rothan, Catherine Bergounioux, Philippe Raymond, and Christian Chevalier*

Unité de Physiologie Végétale, Institut de Biologie Végétale Moléculaire, Centre de Recherche Institut National de la Recherche Agronomique-Bordeaux, BP 81, 33883 Villenave d'Ornon cedex, France (J.J., D.J., C.R., P.R., C.C.); and Institut de Biotechnologie des Plantes, Université Paris-sud, Bâtiment. 630, 91405 Orsay cedex, France (T.-H.P., C.B.)

Following fruit set, the early development of tomato (*Lycopersicon esculentum* Mill.) fruit comprises two distinct phases: a cell division phase and a consecutive phase of cell expansion until the onset of ripening. In this study, we analyzed cytological and molecular changes characterizing these early phases of tomato fruit development. First we investigated the spatial and temporal regulation of the mitotic activity during fruit development. The DNA content of isolated nuclei from the different fruit tissues was determined by flow cytometry analysis. The results confirm the data of mitotic activity measurements and show that cell differentiation, leading to expanded cells, is characterized by endoreduplication. Second, we isolated two cDNAs, named Lyces;*CDKA1* (accession no. Y17225) and Lyces;*CDKA2* (accession no. Y17226), encoding tomato homologs of the cyclin-dependent kinase (CDK) p34^{cdc2}. Tomato *CDKA* gene expression was followed at both the transcriptional and translational levels during fruit development. The transcripts for Lyces;*CDKA1* and Lyces;*CDKA2* and the corresponding CDKA proteins are predominantly accumulated during the phase of cell division between anthesis and 5 d post anthesis (DPA). In whole fruits, the maximum CDK activity was obtained between 5 and 10 DPA. The determination of the kinase activity using protein extracts from the different fruit tissues was in agreement with mitotic activity analysis. It showed the particular disappearance of the activity in the gel tissue as early as 15 DPA. The overall data of CDK activity measurements suggest a strong post-translational regulation of CDK at the temporal and spatial levels during early tomato fruit development.

Most of the studies dealing with fruit development have mainly focused on ripening, the ultimate developmental phase of fleshy fruits. As a consequence, little is known about the regulatory mechanisms governing the earliest developmental stages by which various floral organs differentiate into fruit.

The fruit of tomato (*Lycopersicon esculentum* Mill.) is a berry that consists of placental tissue bearing the seeds and a pericarp surrounded by an epidermis. Following fertilization and fruit set, the early development of tomato fruit can be divided into two distinct phases (Gillaspy et al., 1993). During the first phase, which lasts for about 7 to 10 d after fertilization, a very active period of cell division occurs inside the ovary. The pericarp develops into multiple layers of large, thin-walled cells enclosing many intercellular spaces. At the end of the cell division period begins the second phase, which is characterized by fruit growth mostly by cell expansion. The parenchymatous tissue of the placenta grows around the funiculi until it completely encloses the developing seeds, and at the end of development the cells of this parenchyma are thin-walled, giant cells that form a jelly-like homogenous tissue. In the whole process of tomato fruit development, the phase of cell division is an essential determinant of fruit organogenesis, as it fixes the final number of cells inside the fruit and therefore determines at least in part the final size of the fruit (Bohner and Bangerth, 1988; Ho, 1996).

In the last decade, our knowledge concerning cell division and its regulation in plants has been considerably enriched. The molecular analysis of the plant cell cycle progression has revealed that cell cycle regulators are universally conserved despite the expected singularities in the control mechanisms of development among phylogenetic kingdoms (Doerner, 1994; Jacobs, 1995). Key regulators controlling the progression through cell cycle checkpoints assemble in a multicomponent complex composed of a catalytic subunit, a member of the Ser/Thr protein kinase family called cyclin-dependent kinase (CDK), and a regulatory subunit of the cyclin family. The kinase activity of CDKs is dependent on the phosphorylation/dephosphorylation status of the kinase itself, on the availability and binding of the cyclin, and on CDK inhibitors and/or regulatory factors (Lees, 1995).

At present, three distinct classes of plant cyclins have been defined according to sequence similarities with animal homologs, namely the mitotic cyclins of the A- and B-type and G1 cyclins of D-type (Renaudin et al., 1996). Based on multiple sequence alignments between the 30 or so CDKs identified so far in plants (Burssens et al., 1998), it has been proposed that two distinct classes can be clearly defined and named CDKA and CDKB (Mironov et al., 1999). The CDKA family regroups functional homologs of

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* Corresponding author; e-mail chevalie@bordeaux.inra.fr; fax 33–556–84–32–45.

the yeast $p34^{cdc2/CDC28}$ protein and are characterized by the presence of the PSTAIRE motif, which is essential for cyclin binding (Ducommun et al., 1991). CDKA appears to be constitutively expressed throughout the cell cycle (Segers et al., 1997).

The CDKB proteins present unique features that indicate that these kinases may represent examples of mitotic kinases with putative plant-specific functions for entry into or progression through the M phase (Burssens et al., 1998). A third class of CDK may be putatively defined, as they exhibit closer phylogenetic relationships with two related human proteins involved in the control of the G1 phase (Renaudin et al., 1996). Thus, they are thought to represent G1-specific CDKs (Burssens et al., 1998). As hypothesized by Magyar et al. (1997), the variability observed in the cyclin-binding motif of the different types of CDKs may have a functional significance, i.e. a role in the selectivity of the cyclin partner as observed for animal cells (Sherr, 1993). However, despite the ever-growing number of cloned genes related to cell cycle control in plants, we still lack information concerning the identification of the cyclin and CDK partners that preferentially interact at defined phases of the cell cycle and their direct implication in developmental processes.

In this study, we aimed to document the early fruit development of tomato using cytological and molecular approaches. First, we measured mitotic indexes and DNA synthesis as determined by flow cytometry during the different phases of fruit development. We showed that differentiating cells in pericarp and gel tissues during the expansion phase become gradually more polyploid as the mitotic activity decreases, unlike in the epidermis. Second, we followed both at the transcriptional and protein levels the expression of CDKA during fruit development and correlated the histone H1 kinase activity of the tomato CDKA with cytological data.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Cherry tomato (*Lycopersicon esculentum* Mill. cv West Virginia 106) plants were grown in a growth chamber in a 15-h (25°C) day/9-h (20°C) night cycle with a light intensity of 400 μmol m^{-2} s^{-1}. Tomato fruits were harvested at different stages of their development, determined according to the number of days after anthesis and the fruit diameter. Prior to protein or RNA extraction, they were frozen quickly in liquid nitrogen and stored at −80°C.

DNA Staining, Nuclei Isolation, and Flow Cytometric Analysis

Mitotic indexes were determined on dissected tissues of tomato fruits harvested at various stages of development. The fruit tissues were placed on a glass slide and immersed in a drop of 4′,6-diamino-2-phenylindole (DAPI) at a concentration of 5 μg mL^{-1} for 15 min. The tissues were then washed in distilled water and squashed. Stained DNA was then visualized by epifluorescence microscopy using the ×20 and ×40 objectives of a microscope (Eclipse-E 800, Nikon, Tokyo).

Nuclei were prepared from tomato fruits harvested at different developmental stages in Galbraith's buffer (Galbraith et al., 1983). The fruits were briefly rinsed and transferred to a Petri dish. Whole fruits or dissected tissues (except for locular tissues) were chopped with a razor blade in 1 mL of ice-cold Galbraith's buffer. For the nuclei isolation from locular tissue, protoplasts were prepared according to the method described by Planchais et al. (1997). Dissected locular tissues were incubated for 20 min at 37°C in a mixture of 2% (w/v) cellulase (Onozuka R10, Yakult Honsha, Tokyo), 0.1% (w/v) pectolyase (Sigma, St. Louis), and 12% (w/v) mannitol, pH 5.6.

After centrifugation and washes, nuclei were released by incubation in Galbraith's buffer. Finally, the suspensions of nuclei were conserved at 4°C in 1% (w/v) formaldehyde and 5 mM β-mercaptoethanol. The samples were then filtered through a nylon filter (pore size, 30 μm) (Tetko, New York). The nuclei in the filtrate were stained directly with 5 μg mL^{-1} DAPI for fluorescence microscopy using the ×40 objective of a microscope, or with 2 μg mL^{-1} of bisbenzimide Hoechst 33342 (Sigma) for flow cytometry. Cytometric analysis was performed on 10^4 nuclei with a Fluorescence-Activated Cell Sorter vantage flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) according to the conditions described in Perennes et al. (1993). Histograms were processed with DNA Fit (Becton-Dickinson). Care was taken to eliminate both debris and doublets through light-scatter and pulse-shape analysis.

Extraction of Total RNA

Total RNA from fruits or various organs of tomato plants was extracted using the hot phenol method (Verwoerd et al., 1989) with slight modifications, as described previously (Chevalier et al., 1995). After extraction, total RNA from tomato tissues was dissolved in DEPC-treated water.

cDNA Library Screening

A cDNA library was constructed with poly(A^+) mRNA prepared from total RNA extracted from tomato fruits at the cell division stage, using the poly(A^+) spin mRNA isolation kit (New England Biolabs, Beverly, MA). Double-stranded cDNAs were synthesized from 5 μg of poly(A^+) mRNA, ligated into Uni-ZAP XR vector using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA), and packaged into bacteriophage using packaging extracts (Gigapack II, Stratagene) to produce the cDNA library stock, following the manufacturer's instructions. Recombinant Uni-ZAP XR packaged phages were plated on *Escherichia coli* XL1-Blue cells. The cDNA library comprised 5.5×10^6 recombinant plaques.

To screen the cDNA library for CDKA-encoding cDNAs, a specific probe was generated from total RNA by reverse transcription (RT) followed by PCR amplification. Two degenerate oligonucleotides were synthesized, corresponding to conserved amino acid sequences in plant homologs

of $p34^{cdc2}$. These oligonucleotides correspond to the peptides GEGTYGVV and GCIFAEM (at positions 11 to 18 and positions 191 to 197 of the consensus amino acid sequence) and harbor the following sequences: 5′-GGI GAR GGI ACI TAY GGI GTI GT-3′ and 5′-CAT YTC IGC RAA DAT RCA ICC-3′. The latter oligonucleotide was used to prime the RT reaction in the presence of 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Cleveland). Following RT, a 654-bp cDNA product was PCR amplified between the two degenerate primers and cloned into pGEM-T vector (Promega, Madison, WI). The identity of the amplified cDNA fragment was confirmed by determining its nucleotide sequence using the dideoxy chain-termination method (Sanger et al., 1977). The amplicon was then used as a probe to screen the cDNA library afer labeling with [α-^{32}P]dCTP using the Prime-It II random primer labeling kit (Stratagene). About 300,000 plaques from the library were first screened. After three rounds of screening, the positive clones were isolated and rescued from the Uni-ZAP XR vector using the R408 helper phage following the manufacturer's instructions (Stratagene), and the nucleotide sequence of the inserts was determined.

Southern-Blot Analysis

Genomic DNA was isolated from tomato leaves according to the method of Dellaporta et al. (1983). DNA (10 μg) was digested with *Bam*HI, *Hin*dIII, *Eco*RI, and *Xba*I restriction enzymes, separated on a 1.0% agarose gel, and blotted onto a Hybond-N membrane (Amersham, Les Ulis, France). Prehybridization and hybridization with cDNA probes labeled with [α-^{32}P]dCTP by random priming were performed at 65°C according to a standard method (Sambrook et al., 1989).

Northern Analysis

Total RNA was size-fractionated by 6.6% (v/v) formaldehyde-1.2% (w/v) agarose gel electrophoresis, transferred to Hybond-N (Amersham) membranes by capillary action, and hybridized to random-primed labeled cDNA probes. Hybridizations were performed at 65°C according to standard procedures (Sambrook et al., 1989). A rice rDNA probe was used as a control for relative loadings of RNA in each lane.

Estimation of Relative Transcript Levels of Lyces;*CDKA1* and Lyces;CDKA2 with RT-PCR

Total RNA (2 μg) treated with DNase RQI (Promega) were reverse-transcribed using 0.05 μM oligo-dT as a primer and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies). Specific amplification for Lyces;*CDKA1* and Lyces;*CDKA2* cDNAs were obtained using as the 5′ primer: cdc2A-S, 5′-GCTTATTGTCATTCTCATAGAGTTCTT-3′, in combination with the respective 3′ primers: cdc2A-1-AS, 5′-CTGGATGAAGGGGCAGACAATCACGG-3′, and cdc2A-2-AS, 5′-GAAGATGCAGGTGCCTCGATTCATGG-3′. As a control of RT-PCR expression pattern, a 525-bp cDNA fragment for histone H1 was amplified using the following set of primers: 5′ primer H1-S, 5′-GGCCACTGAAGAACCAGTCATCG-3′, and 3′ primer H1-AS, 5′-GCCTTGGCAGCGGGCTTTGCCTTGGC-3′. PCR reactions were performed using 1/100 of the RT reaction in the presence of 0.02 μM of each primer.

After an initial denaturation step of 5 min at 95°C, the reaction program was as follows: 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C for 20 cycles, and a final step of 5 min at 72°C. The amount of first-strand cDNA and the number of cycles used allowed the reaction to be in the linear range of PCR amplification. As a control for DNA contamination, a PCR reaction was performed using the H1 primers in the absence of any added DNA. For each RT-PCR product to be tested, a specific amplification was performed using recombinant plasmids harboring the cDNA of interest. The RT-PCR products were separated on a 1.2% (w/v) agarose gel, blotted onto a Hybond N^+ membrane (Amersham), and hybridized at 65°C with the appropriate cDNA probes.

Protein Extraction, $p9^{CksHs1}$-Sepharose Affinity Binding, and Histone H1 Kinase Assays

Fruits harvested at different developmental stages or dissected tissues of fruits 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 consisting of 25 mM Tris-HCl (pH 7.5), 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 15 mM $MgCl_2$, 15 mM EGTA, 5 mM NaF, 1 mM DTT, 1 mM PMSF, 10 μg mL^{-1} leupeptine, 10 μg mL^{-1} soybean trypsin inhibitor, and 100 μM benzamidine. The cell debris were discarded after a 15-min centrifugation at 18,000*g* and 4°C.

$p9^{CksHs1}$ was purified from an overproducing strain of *E. coli* and conjugated to CNBr-Sepharose 4B (Pharmacia Biotech, Uppsala) according to the method of Azzi et al. (1992). A 50-μL aliquot of packed-$p9^{CksHs1}$ protein-Sepharose beads was washed with bead buffer (50 mM Tris [pH 7.4], 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% [v/v] Nonidet P40, 10 μg mL^{-1} leupeptine, 10 μg mL^{-1} soybean trypsin inhibitor, and 100 μM benzamidine) and mixed with the protein extract. The tubes were kept under constant rotation at 4°C overnight. After a brief centrifugation at 10,000*g* and removal of the supernatant, the beads were carefully washed three times with bead buffer, once with kinase buffer, and then used for histone H1 kinase assay. The kinase reaction was initiated by resuspending the pellet of beads with 30 μL of the reaction mixture containing 1 mg mL^{-1} histone H1 as the substrate and 2.5 μCi of [γ-^{32}P]ATP according to the method of Magyar et al. (1993). Assays were terminated by transferring the tube into ice. After a brief centrifugation at 10,000*g*, 10 μL of 4× Laemmli sample buffer was added to the supernatant. Samples were analyzed by the procedure of Laemmli (1970) using SDS-PAGE on a 15% polyacrylamide gel followed by Coomassie Blue staining to visualize the histone H1 and autoradiography to detect histone H1 phosphorylation. Intensities of silver grains on the autoradiogram

were analyzed and quantified using the ImageMaster VDS from Pharmacia Biotech coupled to its LabScan 2D Software.

Western Analysis

After quantification by the method of Bradford (1976) using a protein assay (Bio-Rad, Hercules, CA) and γ-globulin as a standard, protein samples were separated in 15% (w/v) SDS-PAGE. Immunoblots of total proteins were performed by loading equal amounts of protein. For immunoblots of proteins bound to the $p9^{CksHs1}$-Sepharose beads, the pellet of beads was resuspended with 30 μL of 4× Laemmli sample buffer and loaded in the SDS-PAGE gel. After electrophoresis, proteins were transferred to 0.1-μm nitrocellulose sheets (Schleicher & Schull, Keene, NH) in a semidry blotter (Millipore, Bedford, MA) for 45 min at 2.5 V cm^{-2}. The filters were blocked with Tris-buffered saline-Tween, 3% (w/v) bovine serum albumin for 2 h at room temperature. The membranes were incubated overnight at 4°C with a monoclonal anti-PSTAIRE antibody (Yamashita et al., 1991). After three 15-min washes with Tris-buffered saline-Tween, the membranes were treated with an anti-mouse antibody conjugated to peroxidase (Sigma). Detection of target proteins was performed by chemiluminescence using the ECL western-blotting system (Amersham).

RESULTS

Fruit Development

During development, the growth of cherry tomato fruits was followed by measuring the fruit diameter increase from anthesis to the red-ripe stage and by determining mitotic indexes in the corresponding fruits dissected into epidermis, pericarp, and gel tissues (Fig. 1). In accordance with published data (Bohner and Bangerth, 1988; Gillaspy et al., 1993), the fastest period of growth occurred between 2 and 25 DPA. The evolution of mitotic indexes in the different tissues of the fruit showed that a 3-fold increase in the number of mitotic figures occurs both in the epidermis and the pericarp between anthesis up to 10 DPA, i.e. during the cell division phase of early development. Compared with the values for epidermis and pericarp, the mitotic index in the gel tissue decreased from 5 to 10 DPA. Before 5 DPA, the placental tissue giving rise to the gel is not differentiated enough to be separated from the pericarp. In fruits harvested between 10 and 25 DPA, i.e. during the cell expansion phase, the mitotic index of cells from the pericarp and the gel tissue decreased, while it remained elevated in epidermal cells. In the gel tissue, the mitotic index starts to decrease at the beginning of the cell expansion phase (at 10 DPA). After 25 DPA, the maturation process started and the growth of fruits was almost stopped. During ripening, the mitotic index reached its lowest value in pericarp and gel tissue, while it remained 3-fold higher in the epidermis.

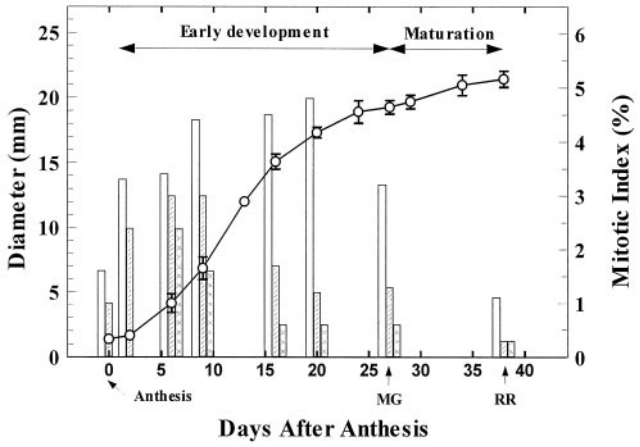

Figure 1. Development of cherry tomato (cv West Virginia 106) fruits. The growth curve was established by measuring daily fruit diameters from anthesis to the red-ripe stage directly on fruits within a truss. Data are the average of 10 distinct measurements. The mitotic index (bars) was determined in the various fruit tissues according to development. Dissected tissues of tomato fruits harvested at various developmental stages were stained with DAPI (see "Materials and Methods") and squashed prior to microscopic observation. A total of 1,000 nuclei were counted for each developmental stage. White bars, Epidermis; hatched bars, pericarp; cross-hatched bars, gel.

Nuclei Analysis

The observation of DAPI-stained fruit tissues showed a high heterogeneity in nuclear size among the different tissues. Therefore, we investigated the analysis of nuclear DNA content in cells of developing tomato fruits (Fig. 2). For the earliest stages (anthesis and 2 and 5 DPA), we used whole fruits for nuclei preparations, as fruits were too small to separate the different tissues. From 10 DPA to the red-ripe stage, the fruits were dissected into the epidermis and pericarp and gel tissue was separated from the seeds.

In very young developing fruits (from anthesis to 5 DPA), DAPI-stained nuclei appeared as dense, spherical structures of homogenous size (Fig. 2A). In the epidermis, whatever the developmental stage, the size and shape of nuclei did not change and were quite similar to nuclei of young fruits. On the contrary, the size of pericarp and gel nuclei increased considerably during fruit development. As shown in Figure 2B, the observed increase in nucleus size was correlated with measurements of DNA content by flow-cytometric analysis. Nuclei of very young developing fruits displayed essentially two large peaks at the 2C and 4C DNA levels, accounting for 46% (2C) and 50% (4C) of total nuclei at anthesis, and 37% (2C) and 58% (4C) of total nuclei at 2 DPA.

Because tomato is a diploid species ($2n = 24$), the 2C DNA level corresponds to the diploid state of the genome found in the G1 phase, while the 4C DNA level results from the S-phase doubling of chromatids found in the G2 phase, and thus is an indicator of the capacity of cells to enter mitosis (Bergounioux et al., 1992). Therefore, the major 2C and 4C peaks suggest that the tissue is in a dividing state. However, the 4C value may not be the G2 intermediate state of ploidy, but may represent a multiploid form of the genome. In vivo DNA labeling experiments based on the incorporation of 5-bromo-2′-deoxyuridine indicated that in

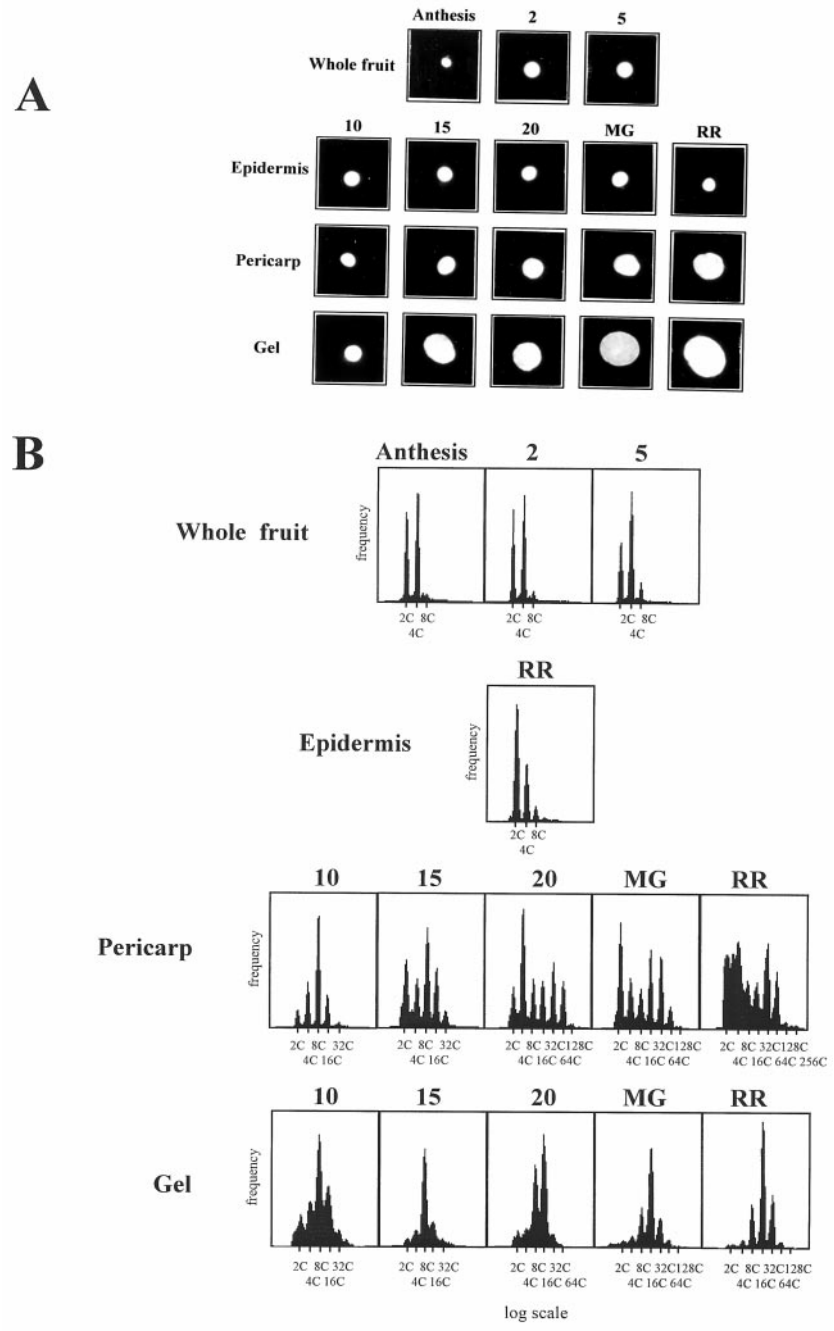

Figure 2. Endoreduplication during tomato fruit development. A, Increase in nuclear size during fruit development. Fruits were harvested at the following developmental stages: anthesis, 2, 5, 10, 15, and 20 DPA, mature-green (MG), and red-ripe (RR) stages. Nuclei were prepared as described in "Materials and Methods" and stained with DAPI. B, Flow-cytometric analysis of DNA content of purified nuclei from various fruit tissues in the course of development.

very young fruits at anthesis and 2 DPA, there is one population of nuclei at the G1 phase (2C) and another one at the G2 phase (4C), and definitely not with two populations of G1 nuclei with 2C and 4C DNA levels (data not shown). Later in development, a faint peak at a polyploid 8C level can be detected in nuclei from fruits harvested at 5 DPA, accounting for 10% of total nuclei, while the 2C and 4C peaks corresponded to 26% and 64%. In accordance with DAPI staining, the analysis of nuclei from the epidermis of tomato fruits at the red-ripe stage gave similar results to that of nuclei from fruits up to 5 DPA. They displayed predominantly 2C and 4C DNA levels and a small 8C peak. In nuclei isolated from the pericarp and gel tissue, the flow cytometry profiles displayed additional peaks of higher DNA content appearing throughout fruit development, from 10 DPA to the red-ripe stage. However, nuclei at the 2C and 4C DNA level were still detectable in the pericarp up to the red-ripe stage. Unlike the pericarp, the number of nuclei at the 2C and 4C DNA level in the gel tissue decreased dramatically (after 10 DPA), and became almost undetectable at the onset of maturation (the mature-green stage), accounting for less than 3% of total nuclei.

Isolation of Tomato cDNAs Homologous to cdc2

As demonstrated above, the determination of mitotic indexes and nDNA levels during fruit development indicated that the mitotic activity was quite different according to the developmental stage and with the type of tissue taken into account. Therefore, we aimed to correlate these results with molecular data by investigating the expression of the key cell cycle regulator CDKA at the transcriptional, translational, and protein kinase activity levels. In a first attempt to follow the expression of *cdc2*-related genes, we used a RT-PCR strategy to isolate tomato cDNAs encoding CDKA, as described in "Materials and Methods."

We created four cDNA clones presumed to be homologous to *cdc2*. After complete sequencing of the inserts, the four cDNAs (1,457, 1,311, 1,269, and 1,178 nucleotides in length) were shown to harbor a 885-bp long open reading frame encoding a 294-amino acid product with a M_r of 33.7. Based on the sequence analysis (data not shown), the four cDNAs fell into two distinct groups. The three longest cDNAs encode an identical translation product and share 100% homologous 3′-UTR sequences—although different in length. Therefore, these three cDNAs might originate from the same gene, which was named Lyces;*CDKA1*. The fourth, 1,178-bp-long cDNA showed marked differences with the three others in the ORF and the 5′- and 3′-UTR sequences. Therefore, this cDNA reflected the occurrence of a second gene, Lyces;*CDKA2*. The cDNAs share 83.2% identity in their overlapping sequences, and the two predicted peptides share 94.6% identical residues (Fig. 3).

Amino acid sequence alignments of the Lyces;*CDKA1*- and Lyces;*CDKA2*-predicted proteins with different plant CDKAs (Fig. 3) revealed a high degree of identity (84%–95%). The predicted proteins contain functionally important regions characteristic of CDKA, such as the cyclin-binding domain (residues 44–56) containing the PSTAIRE hallmark; the T-loop area (residues 147–172) centered around Thr-161, whose phosphorylation stabilizes the cyclin binding; the T-loop flanking Asp-146 involved in the positioning of bound ATP required for kinase activity; and the SUC/CKS-binding motif (residues 207–244). Moreover, they contain both of the two functionally important phosphorylation sites, Thr-14 and Tyr-15, in the N terminus. This extensive structural similarity to various plant CDKA proteins supports the identification of the proteins encoded by the cDNAs Lyces;*CDKA1* and Lyces;*CDKA2* as being CDKA from tomato.

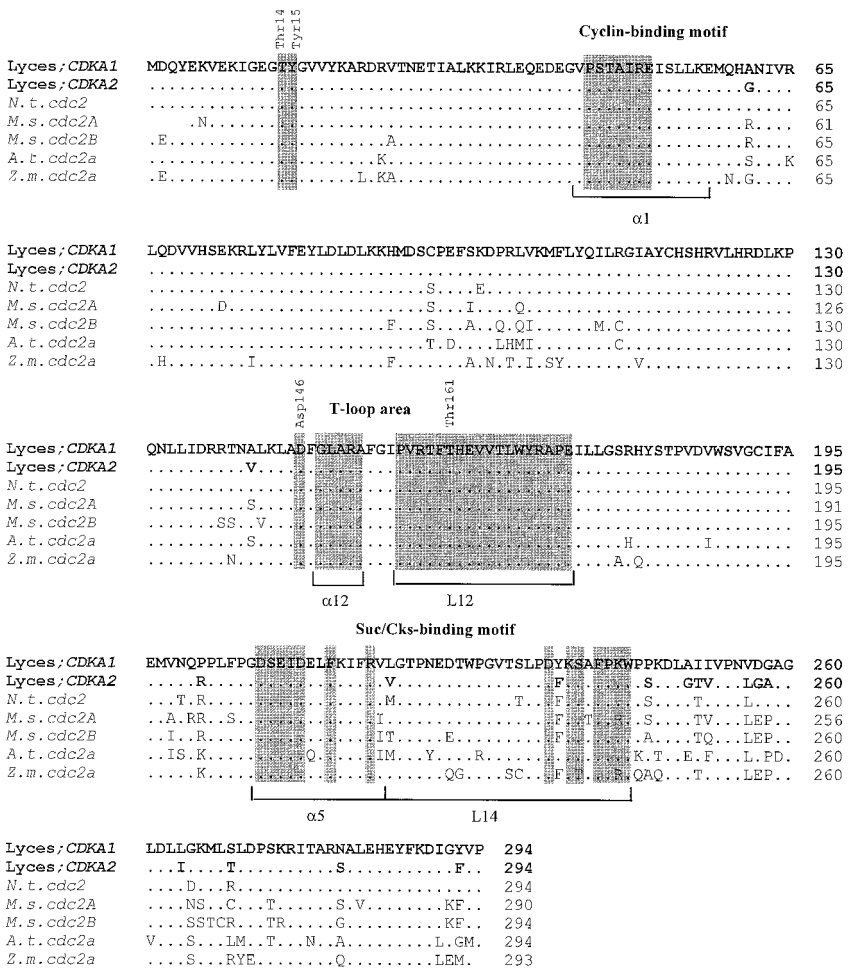

Figure 3. Amino acid sequence alignment of Lyces;*CDKA1* and Lyces;*CDKA2* encoded proteins with the sequences of various plant CDKAs. Derived sequences from Lyces;*CDKA1* and Lyces;*CDKA2* were aligned with sequences of cdc2Nt1 from tobacco (*N.t. cdc2*) (Setiady et al., 1996), cdc2MsA (*M.s. cdc2A*) and cdc2MsB (*M.s. cdc2B*) from alfalfa (Hirt et al., 1993), cdc2a from Arabidopsis (*A.t. cdc2a*) (Ferreira et al., 1991), and cdc2a from maize (*Z.m. cdc2a*) (Colasanti et al., 1991). Identical amino acids are represented by dots.

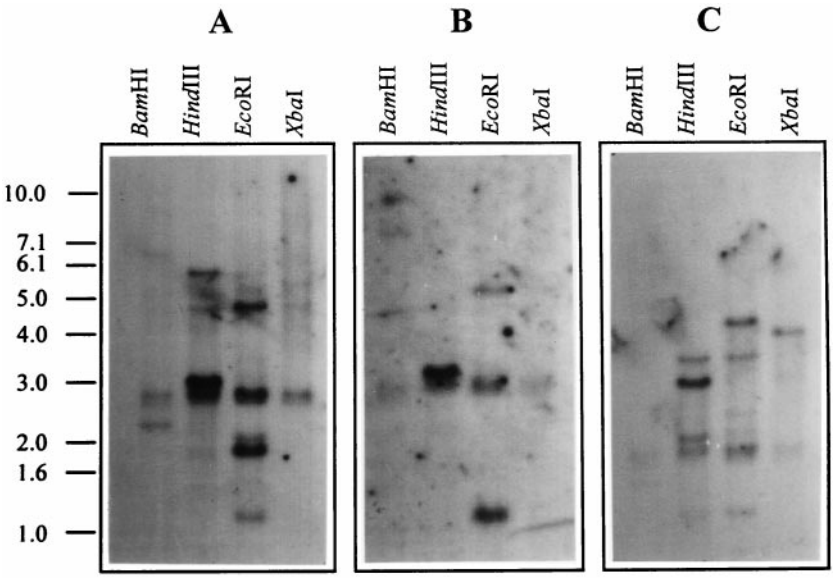

Figure 4. Southern-blot analysis of *CDKA* genes in tomato. Tomato genomic DNA (10 μg) was digested with the following restriction enzymes: *Bam*HI, *Hin*dIII, *Eco*RI, and *Xba*I, resolved by gel electrophoresis, transferred to nylon membrane, and successively probed with the following ^{32}P-labeled cDNA fragments: A, Full-length Lyces;*CDKA1* cDNA; B, 3′-specific Lyces; *CDKA1* and cDNA fragment; C, 3′-specific Lyces;*CDKA2* cDNA fragment.

Genomic Southern-Blot Analysis of Lyces;*CDKA1* and Lyces;*CDKA2* cDNA Clones

Total tomato DNA was digested with the restriction enzymes *Bam*HI, *Hin*dIII, *Eco*RI, and *Xba*I. The full-length insert of Lyces;*CDKA1* was first used as a probe to hybridize the restricted tomato DNA (Fig. 4A). For each digestion, a complex pattern of hybridization was revealed after autoradiography, resulting from the hybridization of the probe with both the corresponding Lyces;*CDKA1* and Lyces;*CDKA2* genes. The blot was stripped and reprobed successively with specific cDNA probes encoding the 3′-UTR of Lyces;*CDKA1* and Lyces;*CDKA2*, respectively (Fig. 4, B and C). As suggested by the ever-apparent complexity of the two specific hybridization patterns, both Lyces;*CDKA1* and Lyces;*CDKA2* seem to be encoded by at least two genes in the tomato genome.

Expression of CDKA mRNAs during Fruit Development and in Various Plant Organs

The expression of CDKA transcripts at various stages of fruit development and in various plant organs was analyzed by northen-blot experiments (Fig. 5A). The expression pattern shown in Figure 5A was obtained using the Lyces;*CDKA1* full-length cDNA as a probe. In whole fruits, mRNA for *CDKA* genes were highly expressed from anthesis to 5 DPA. From 10 DPA to the onset of ripening (the mature-green stage), the level of mRNA decreased steadily until the red-ripe stage. The expression of *CDKA* genes could be detected in young leaves, roots, and suspension-cultured cells of tomato, i.e. in organs harboring meristematic activities or actively dividing cells. In non-dividing tissues such as old leaves and stems, the level of tomato *CDKA* transcripts was very low.

To discriminate the relative transcript levels of Lyces;*CDKA1* and Lyces;*CDKA2*, we used a RT-PCR assay (Fig. 5B). Specific PCR amplification of each cDNA was performed using a 3′ primer in the 3′-UTR sequence, combined with a 5′ primer corresponding to a sequence present at the C terminus of the protein, thus delimiting a 560-bp fragment. To visualize the respective amplified fragments, specific probes for each of the two isolated cDNAs were used to detect the separated RT-PCR reaction products. No difference was observed in the hybridization patterns between Lyces;*CDKA1* and Lyces;*CDKA2* gene expression. Furthermore, their expression pattern was similar to that obtained in the northern blot assay using the full-length Lyces;*CDKA1* probe, i.e. a preferential expression in young developing fruits (from anthesis to 5 DPA) and in actively dividing tissues. The amplification of a histone H1 cDNA fragment used as a control for the RT-PCR reaction and as a marker of cycling cells revealed a similar pattern of expression. These results suggest that Lyces;*CDKA1* and Lyces;*CDKA2* were equally and concomitantly expressed during early fruit development.

Analysis of CDKA Protein Level and Associated Histone H1 Kinase Activity in Whole Fruits

Total proteins were prepared from fruits harvested at various stages of development, and the evolution of the CDKA protein level and associated histone H1 kinase activity during fruit development was measured (Fig. 6). The level of CDKA proteins probed with an anti-PSTAIR antibody was shown to increase from anthesis to 5 DPA, when it reached its maximum (Fig. 6a). It then decreased to become almost undetectable at the maturation stages. Thus, the pattern of CDKA protein expression followed that of mRNAs.

To monitor protein kinase activity, the CDKA-cyclin complexes were purified by p9^{CksHs1}-affinity chromatography. Equal amounts of protein extracts (250 μg) were bound to the p9^{CksHs1}-Sepharose matrix and used for in vitro phosphorylation assay using histone H1 as a sub-

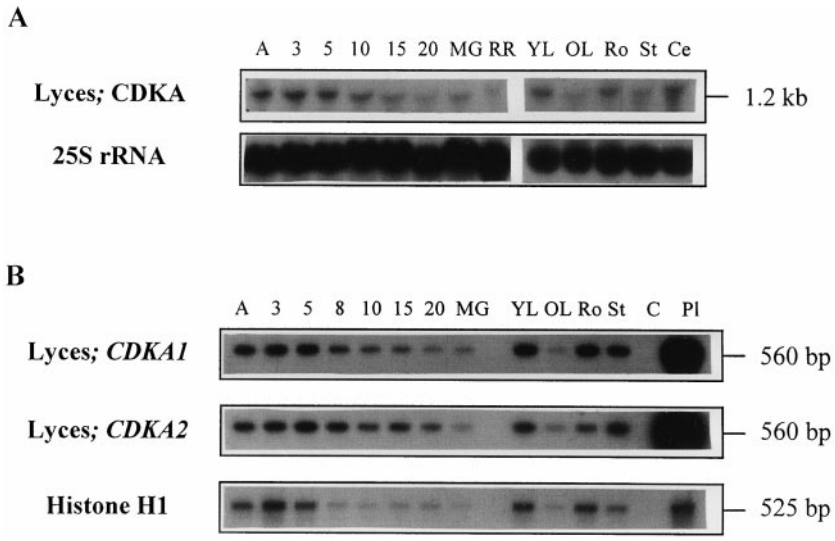

Figure 5. Expression analysis of the *CDKA* genes in tomato. A, Northern-blot analysis. Total RNA (50 μg per track of the gel) isolated from fruits harvested at the following developmental stages: anthesis (A), 2, 5, 10, 15, and 20 DPA, mature-green (MG) and red-ripe (RR) stages, or from different plant organs (YL, young leaves; OL, old leaves; Ro, roots; St, stems; Ce, suspension-cultured cells) were probed successively with ^{32}P-labeled Lyces;*CDKA1* cDNA insert, and with a rice rDNA probe. B, RT-PCR analysis. RT-PCR experimental conditions were as described in "Materials and Methods." Abbreviations for sources of reverse-transcribed total RNA are the same as in A. PCR controls were performed using no cDNA matrix (C), and the plasmid containing the cDNA of interest (Pl). Specific amplification of cDNA fragments for Lyces;*CDKA1* and Lyces;*CDKA2* were detected after gel electrophoresis, Southern blotting, and hybridization to Lyces;*CDKA1* and Lyces;*CDKA2* ^{32}P-labeled probes. As a RT-PCR control for cell division-preferential expression, we amplified a 525-bp cDNA fragment encoding histone H1.

strate. The level of bound CDKA was visualized by western blotting using the anti-PSTAIR antibody (Fig. 6b). The pattern of bound CDKA was similar to that of free CDKA revealed in Figure 6A relative to the starting amount of total proteins, which was 5-fold more in this case. Therefore, we could detect the CDKA protein in mature-green and red-ripe fruits. In whole fruits the activity of the CDKA-cyclin complexes detected by the histone H1 phosphorylation assay (Fig. 6c) revealed maximum histone H1 kinase activity between 5 and 10 DPA. The activity then decreased and became very low at the maturation stages, although CDK-cyclin complexes could be bound to the p9^{CksHs1}-Sepharose matrix.

Analysis of CDK Histone H1 Kinase Activity in the Different Fruit Tissues during Development

Because the mitotic activity during early development differs from one tissue to the other, we tested CDK histone H1 kinase activity in different parts of the fruit. Like the previous experiment (Fig. 6), we analyzed the level of CDKA proteins after binding to the p9^{CksHs1}-Sepharose matrix and the histone H1 kinase activity (Fig. 7A, top and bottom, respectively). The relative protein and kinase activity levels were estimated by measuring silver grain intensities using image scanning software (Fig. 7B).

In the epidermis, the CDKA protein level increased up to 15 DPA, then decreased and became undetectable at the red-ripe stage. In pericarp, it decreased gradually throughout development, and was barely detectable at the mature-green stage. The profile of CDKA protein accumulation in gel appeared to be very similar to that in epidermis. However, the amount of CDKA protein in the gel was very low compared with that in the epidermis (5-fold less): the 2-fold lower signal for gel protein was obtained with a 2.5-fold higher quantity of proteins used for the assay (250 μg instead of 100 μg). The levels of histone H1 kinase activity in the three tested tissues were determined after substraction of the autophosphorylation value (Fig. 7A, lane C). In the epidermis, the histone H1 kinase activity was maximum at 15 DPA, then decreased and disappeared at the red-ripe stage. In the pericarp, it was maximum at 10 DPA but at a level 3-fold less than the maximum in epidermis. It then dropped at 20 DPA and seemed to slightly increase during maturation. In gel tissue, the histone H1 kinase activity was maximum between 10 and 15 DPA. After 15 DPA, the activity decreased dramatically until it disappeared. A very low increased level was then detected at the red-ripe stage.

DISCUSSION

As mentioned by Gillaspy et al. (1993), the study of early fruit development may help in providing information about the regulatory mechanisms that link division, growth, and differentiation of plant cells. In the present work, we aimed to correlate cytological analysis with molecular analysis of the expression of CDKA, the key cell-cycle regulator, during tomato fruit development.

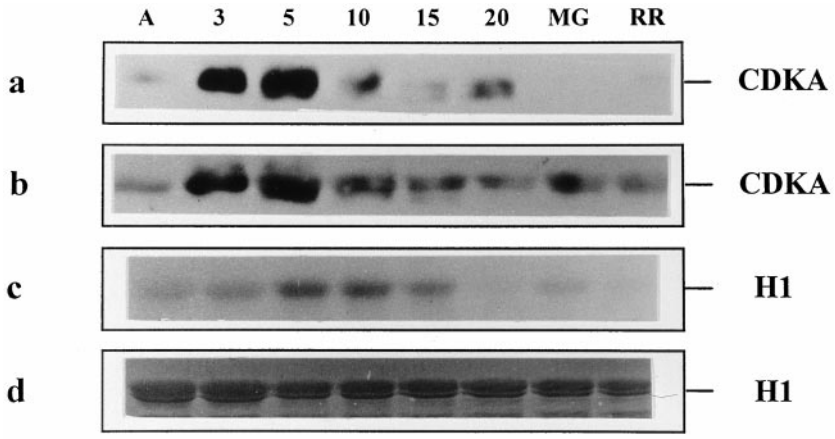

Figure 6. Analysis of the amount of CDKA protein and CDK-associated histone H1 kinase activity during tomato fruit development. Proteins were extracted from fruits harvested at the following developmental stages: anthesis (a), 2, 5, 10, 15, and 20 DPA, MG and RR stages. a, Immunological detection of CDKA proteins using an anti-PSTAIR monoclonal antibody (Yamashita et al., 1991). Equal amounts (50 μg) of total proteins were assayed. b, Immunological detection of $p9^{CksHs1}$-bound CDKA proteins using the anti-PSTAIR antibody. Equal amounts (250 μg) of total proteins were used for binding to the $p9^{CksHs1}$-Sepharose matrix. After completion of the histone H1 phosphorylation assay, the subsequent western-blot analysis was as described in "Materials and Methods." c, Histone H1 kinase phosphorylation activity of $p9^{CksHs1}$-bound CDKA proteins. Protein samples were the same as in b. The time exposure for autoradiography was 48 h. d, Coomassie Blue staining of the electrophoresis gel area showing histone H1 as a control of equal substrate quantity used per phosphorylation reaction.

Cell Division in Early Tomato Fruit Development: Temporal and Spatial Regulation

By measuring the mitotic index and the nuclear DNA content inside the different tissues of the fruit (Figs. 1 and 2), we show that the distribution of mitotic activity inside tomato fruits is not only temporally, but also spatially determined according to the tissue considered. Our results confirm that cherry tomato fruit growth is mainly sustained after anthesis by cell divisions up to 10 DPA, especially in pericarp. Varga and Bruinsma (1986) determined that between 3 and 14 DPA, the number of cell layers in pericarp increases from 14 to 30. In accordance with the observations of Gillaspy et al. (1993), divisions still occur in the epidermis very late in fruit development, unlike pericarp and gel tissue, and even occur even when maturation has started (our data, Figs. 1 and 2). As the fruit grows mainly by cell expansion from 10 DPA to the onset of maturation, the epidermis, which is composed of four layers of collenchymous tissue and an outer single cell layer of epidermal cells (Varga and Bruinsma, 1986), has to maintain a significant mitotic activity to follow the growth force imposed by the inner expanding tissues such as pericarp and placental tissue. As a consequence, the flow cytometry profile for epidermal cell nuclei reveals a high similarity with those obtained for very young "dividing" fruits.

Cell Expansion in Early Tomato Fruit Development: Involvement of DNA Endoreduplication

Our flow cytometry data show that during development (from 10 DPA to the maturation stages), pericarp and gel tissues are characterized by an increasing nuclear ploidy. This endopolyploidy originates from an endonuclear chromosome duplication leading to the production of chromosomes bearing 2^n chromatids without changing the chromosome number, a process called endoreduplication (D'Amato, 1964). In higher plants, and especially in angiosperms, endoreduplication is very common (Galbraith et al., 1991) and it is generally assumed that this is the most prevalent process for increasing the nuclear ploidy (D'Amato, 1984). A strong correlation is observed between endoreduplication and cell differentiation in eukaryotic organisms, and especially in plants (Nagl, 1976, 1978). Furthermore, there is a clear relationship between endopolyploidy and cell size (Melaregno et al., 1993), and, consequently, cell expansion such as that in elongating organs (Gendreau et al., 1997, 1998).

In cherry tomato fruits, our results indicate that the increase in nDNA levels resulting from endoreduplication is concomitant with the start of the growing period mainly by cell expansion (around 10 DPA), together with the mitotic arrest. This is particularly true for the placental locular tissue composed of large and hypervacuolarized cells. In accordance with the determination of mitotic index, the flow cytometry profiles for gel tissue show that cell divisions cease after 10 DPA, as we could hardly detect nuclei with 2C and 4C DNA levels for the oldest stages. Even after 10 DPA and until the maturation stages, the pericarp still displays 2C and 4C nuclei together with highly polyploid nuclei. This ladder of C values probably reflects the gradient of cell division and expansion that occurs inside the pericarp: indeed, mitotic activity is restricted to the outer pericarp cell layer, and the closer to the locular cavities the bigger the cells (Gillaspy et al., 1993). On the contrary, the epidermis, which is composed of small cells, displays at the red-ripe stage a flow cytometry profile characterized by

a high proportion of nuclei at the 2C and 4C levels and a very faint 8C peak. Therefore, the appearance of endoreduplication is impaired by the maintenance of mitotic activity in the epidermis throughout development. This supports the idea that mitotic and endoreduplication cycles are mutually exclusive (Traas et al., 1998).

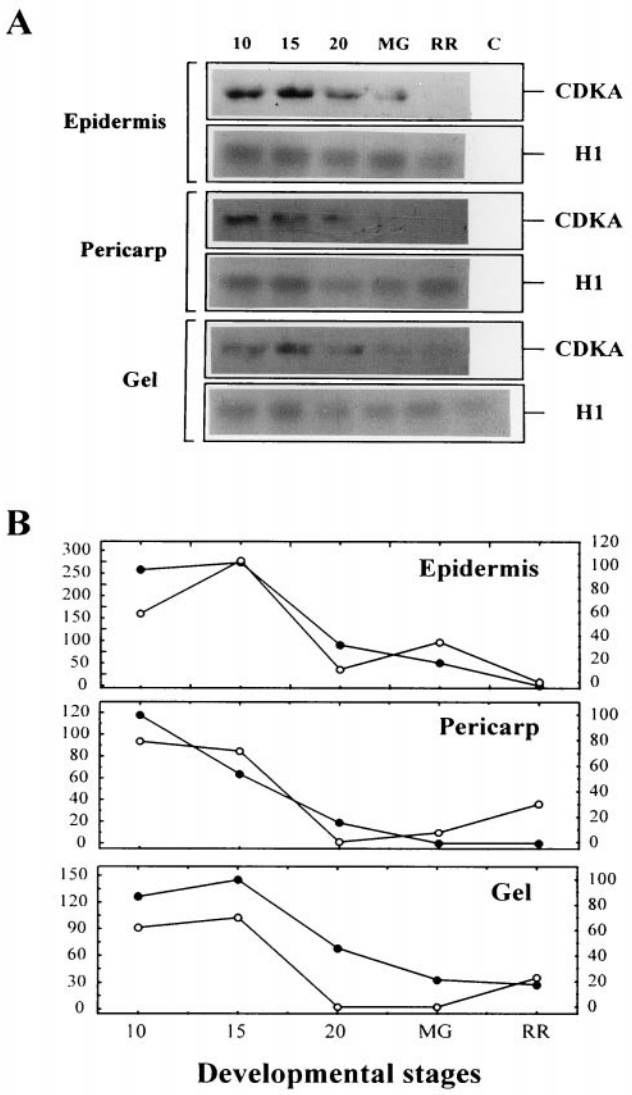

Figure 7. Spatial and temporal analysis of CDK histone H1 kinase activity in tomato fruit tissues. A, CDKA protein and kinase activity levels as detected by western blot and phosphorylation assay autoradiography. Fruits were harvested at 10, 15, and 20 DPA, mature-green (MG) and red-ripe (RR) stages, and dissected into epidermis, pericarp, and gel tissue prior to protein extraction. Proteins (100 μg) from epidermis and pericarp were used for binding to the $p9^{CksHs1}$-Sepharose matrix, and 250 μg for gel tissue. The immunodetection of CDKA proteins (top) and the autoradiogram of phosphorylated histone H1 (bottom) were obtained as described in Figure 6. To perform a histone H1 autophosphorylation assay (lane C), $p9^{CksHs1}$-Sepharose beads were incubated in the presence of histone H1 and [γ-^{32}P]ATP without prior mixing with fruit tissue proteins. The time exposure for autoradiography was 48 h. B, Image scanning quantification of protein (●) and kinase activity (○) levels. Values are expressed as arbitrary units for silver grain intensities. For each histone H1 kinase assay with the proteins of different tissue source, silver grain intensities were measured. The autophosphorylation signal was then subtracted from all values in order to plot the data.

Our results suggest that, as in Arabidopsis (Galbraith et al., 1991; Melaregno et al., 1993), the endoreduplication process in tomato fruits is developmentally regulated according to the age of the organ. Recently, Bergervoet et al. (1996) reported the determination of the DNA content in isolated nuclei from tomato fruit pericarp. Although these authors used a different tomato cultivar (cv Typico) of a much larger size than cherry tomato, our results are strikingly similar to theirs. At the corresponding stages of fruit development, the overall pattern of ploidy increase and the C values evolve identically in the small cultivar (cv West Virginia 106) and in the large cultivar (cv Typico). Therefore, assuming that cell size is linked to the nucleus size according to the cytonuclear ratio hypothesis of Nagl (1978), the comparison of our data with those of Bergervoet et al. (1996) supports the idea that the final size of a fruit is determined right before the start of the cell expansion period (Ho, 1996). As shown by Bohner and Bangerth (1988), the cell number inside the ovaries as early as anthesis may be the determining factor of final fruit size.

CDKA Belongs to a Multigene Family in Tomato That Is Highly Expressed during Early Fruit Development

To investigate potential regulatory mechanisms governing fruit development, the cyclin-dependent kinase CDKA is an obvious candidate to monitor, as it controls the progression from the G1 to the S phase (before DNA synthesis) and from the G2 to the M phase (before mitosis) (Mironov et al., 1999).

We isolated two different cDNAs encoding two highly homologous members of the CDKA family (Fig. 3). As shown in Southern blots (Fig. 4), each of these cDNAs are putatively encoded by more than one copy in the genome of tomato. Preliminary results of localization on a tomato genetic map seem to indicate that Lyces;*CDKA1* and Lyces;*CDKA2* could be mapped on chromosomes VIII and XI and on chromosomes VI and XII, respectively (M. Causse, personal communication), confirming the presence of multiple copies of each of these genes in the tomato genome.

Using the *CDKA* cDNAs and a specific antibody against the PSTAIRE hallmark, we could combine these molecular probes for CDKA using a biochemical approach to measure *CDKA* gene expression, the protein amount, and the associated kinase activity during fruit development. Tomato *CDKA* transcripts are predominantly expressed in dividing organs (Fig. 5), and the pattern of protein accumulation in whole fruits parallels that of the mRNA (Fig. 6). In older fruits, CDKA transcripts and proteins were still detected, suggesting that this expression could be associated with the remaining cell divisions occurring in the epidermis. However, transcription and translation of *cdc2a* genes are also observed in non-dividing, differentiated tissues (such as mature leaves and stems, as shown in Fig. 4) (Bergounioux et al., 1992; Hemerly et al., 1993; Fobert et al., 1996; Magyar et al., 1997), which led to the proposal that the expression of CDKA transcripts and proteins is corre-

lated with the competence of cells to divide rather than with division itself (Hemerly et al., 1993; Mironov et al., 1999).

The M-Phase-Associated H1 Kinase Activity of CDKA Is Differentially Regulated during Early Tomato Fruit Development

We further analyzed the implication of CDKA in tomato fruit development by monitoring the M-phase-associated histone H1 kinase activity of CDK complexes isolated using the $p9^{CksHs1}$-Sepharose matrix (Dunphy and Newport, 1989). In whole fruits CDKA is present in the $p9^{CksHs1}$-bound complex isolated from every developmental stage (Fig. 6). The maximum histone H1 kinase activity was obtained between 5 and 10 DPA, while the maximum levels of transcription and translation (Figs. 5 and 6) were obtained between 3 and 5 DPA. Thus, a high level of CDKA protein at 3 DPA is associated with a relatively low kinase activity level, which suggests that an activation mechanism of the CDKA may account for its increased kinase activity. As we did not perform selective immunoprecipitation prior to the histone H1 phosphorylation assay, we cannot exclude the possibility that the immunodetection and activity assay are dealing with completely different proteins and, consequently, that the activity we measured comes from different forms of CDK complexes that may be retained on the $p9^{CksHs1}$ affinity matrix. However, De Veylder et al. (1997) showed that $p9^{CksHs1}$ exclusively binds Cdc2aAt, the representative member of CDKA in Arabidopsis. Furthermore, even though in some cases an active kinase has been isolated from S-phase cells in plants by interaction with $p13^{suc1}$ (the yeast homolog) (Magyar et al., 1993), the CDK present in $p9^{CksHs1}$- or $p13^{suc1}$-bound complexes from S-phase cells or differentiated cell extracts exhibits low, if any, histone H1 kinase activity (Colasanti et al., 1991; Perennes et al., 1993; Grafi and Larkins, 1995; Bögre et al., 1997). Thus, the histone H1 kinase activity we detected in fruit extracts may indeed correspond to the mitosis-associated form of the CDKA complex.

The CDKA histone H1 kinase activity was determined in the different fruit tissues (Fig. 7) to associate molecular data with the cytological study of fruit development. In the three studied tissues, the overall pattern of kinase activity level paralleled that of CDKA protein accumulation. Furthermore, it correlated with the estimated mitotic index determined for the dissected tissues. In epidermis showing the highest mitotic index values (Fig. 1), a much higher kinase activity level was measured compared with pericarp and gel tissue. After the onset of maturation, kinase activity was still observed in epidermis and pericarp, which fully agrees with the persistence of cell divisions inside the epidermis and pericarp during the latest developmental stages (Figs. 1 and 2). In both the pericarp and gel of fully ripened fruits (the red-ripe stage), the kinase activity level increased. However, the significance of this small increase in activity is questionable, since the mitotic index was lowest at the red-ripe stage in these tissues, and the activity could be due to an artifactual effect of protein extract from red-ripe fruits. Interestingly, the CDKA protein level in gel tissue, although diminishing, could be still detected up to the red-ripe stage, while the kinase activity was dramatically affected after 15 DPA. In this particular tissue, this may be related to a strong post-translational regulation of the CDKA activity that involves an inhibitory mechanism.

CDK phosphorylation/dephosphorylation, lack or degradation of the corresponding mitotic cyclin, and inactivation by a CDK inhibitor may be responsible for the decrease in the mitosis-associated histone H1 kinase activity. In the development of maize endosperm, which is characterized by the inhibition of mitosis and subsequent endoreduplication, Sun et al. (1999) showed that the Wee1 homolog from maize is up-regulated, suggesting that its potential phosphorylation activity on CDKA Thr-14 and Tyr-15 residues influences CDK activity. The presence of a CDK inhibitor might be a determinant of cell division arrest in the developing gel tissue by interacting with the M-phase kinase (CDKA/cyclin B complex).

Grafi and Larkins (1995) demonstrated that endoreduplication in the development of maize endosperm proceeds as a result of both the inactivation of the M-phase kinase with an inhibitor and the induction of S-phase-related kinases. Similarly, in synchronized alfalfa cells, Bögre et al. (1997) showed that the cdc2 kinase present in the $p13^{suc1}$-bound complex isolated from S-phase extracts was inactivated by an inhibitory protein. The use of drugs known to inhibit protein kinases was shown to induce endoreduplication in mammalian and plant cells (Usui et al., 1991; Nagl, 1993). We can hypothesize that an inhibitory molecule may be present in the differentiated (mitotically inactive), endoreduplicating cells from the gel tissue after 15 DPA, which is similar to what occurs in maize endosperm. Preliminary results support this speculation (J. Joubès and C. Chevalier, unpublished data), and the role of this potential inhibitor is currently being investigated.

CONCLUSIONS

Recently, the discovery and the characterization of the first plant CDK inhibitor were reported in a study using Arabidopsis (Wang et al., 1997, 1998), arguing for conserved regulatory mechanisms in the cell cycle control between plants and animals. By analogy to animals, the progression from the G1 to the S phase in plant cells is restricted by a checkpoint control involving D-type cyclins and Rb-like proteins (Murray et al., 1998). Evidence was provided for the implication of this G1 to S transition control in maize endosperm-associated endoreduplication (Grafi et al., 1996) and in cell differentiation in maize leaves (Huntley et al., 1998). Moreover, the G1 to S transition checkpoint in plants seems to be under the control of growth factors. Indeed, Soni et al. (1995) demonstrated that Arabidopsis cyclin δ2 (Arath;*CycD2*;1) and cyclin δ3 (Arath;*CycD3*;1) are induced by Suc and cytokinin, respectively, and postulated that auxin might be responsible for the induction of the expression of the kinase subunit of the CDK complex. In tobacco protoplasts, an auxin-only signal induces endoreduplication and cell expansion (Valente et al., 1998). It has been observed in planta that treatment of apricot fruits with auxin results in the increase of the meso-

carp volume due to cell enlargement and endopolyploidy (Bradley and Crane, 1955). Therefore, early fruit development offers an interesting model for studying plant organogenesis, especially in the regulation of cell division and cell expansion phenomena. As fruit is a sink organ, deeper attention should be given to the nutritional and hormonal regulations of the different cell-cycle regulators.

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