The jelly-like locular (gel) tissue of tomato fruit is made up of large thin-walled and highly vacuolized cells. The development of the gel tissue is characterized by the arrest of mitotic activities, the inhibition of cyclin-dependent kinase A (CDKA) activity, and numerous rounds of nuclear DNA endoreduplication. To decipher the molecular determinants controlling these developmental events, we investigated the putative involvement of CDK inhibitors (p27Kip-related proteins, or KRPs) during the endoreduplication process. Two cDNAs, LeKRP1 and LeKRP2, encoding tomato CDK inhibitors were isolated. The LeKRP1 and LeKRP2 transcript expression was shown to be enhanced in the differentiating cells of the gel tissue. At the translational level, LeKRP1 was shown to accumulate in the gel tissue and to participate in the inhibition of the CDK-cyclin kinase activities occurring in endoreduplicating cells of the gel tissue. We here propose that LeKRP1 participates in the control of both the cell cycle and the endoreduplication cycle.

Although endoreduplication is a widespread phenomenon in higher plants, its control and role during plant development are still poorly understood. The endoreduplication cycle or endocyte corresponds to a modified cell cycle lacking mitosis, and as such it utilizes several key cell cycle regulators, including those controlling the G1/S transition (4). Increasing our knowledge of the cell cycle control may lead to the elucidation of the molecular mechanisms underlying endoreduplication. The progression within the plant cell cycle is regulated by a class of heterodimeric protein complexes common to all eukaryotic cells (5). 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, and its localization and substrate specificity. The activity of the complexes is governed by phosphorylation/dephosphorylation events, availability of the protein partners (synthesis and controlled degradation of the cyclin moiety), and the binding of other proteins such as inhibitors or regulatory factors.

The involvement of CDKs in the control of endoreduplication has been recently demonstrated in maize endosperm cells (6) and in Arabidopsis leaf cells (7). Furthermore, this control is associated with the inhibition of M-phase CDK activity. This has been observed in various plant cells or organs displaying an increase in the DNA ploidy level through endoreduplication, e.g. maize endosperm cells (8), developing tomato fruit tissues (2), and Arabidopsis leaf cells (7). The three mechanisms for the control of CDK complex activity are likely to occur during endoreduplication. The WEE1 inhibitory kinase may play a role in endoreduplicating cells of the maize endosperm (9) and tomato fruit (10). The selective degradation of M-phase-specific cyclins via the activation of the anaphase-promoting complex is likely to be involved in the control of endoreduplicating cells of alfalfa root nodules (11, 12). The involvement of CDK inhibitors in the control of endoreduplication has been more difficult to demonstrate. Their in planta overexpression always resulted in reduced endoreduplication because they are probably able to target a broad spectrum of CDK complexes, including the G1/S-specific ones (13, 14). Recently, Verkest et al. (15) showed that a weak overexpression of the Arabidopsis CDK inhibitor KRP2 inhibited only the mitotic cell cycle-specific CDKA;1 kinase complexes, whereas the endoreduplication cycle-specific CDKA;1 complexes were unaffected. This led to an expected increase in the DNA ploidy level. This study demonstrated for the first time the involvement of a CDK inhibitor in the control of the transition from mitosis to endoreduplication.
Tomato fruit is a good model for study of the interconnection between normal cell cycle events and endoreduplication, as its nuclei become highly polyploid during fruit growth, especially in pericarp and locular tissue cells (Fig. 1C) (2). In these tissues, the ploidy can reach levels up to 256 or even 512 C (where C is the DNA content of the haploid genome) that cannot be observed in other model plants such as Arabidopsis or maize (16, 17). At the molecular level, we have shown that the arrest of cell division and subsequent endoreduplication during fruit development are associated with the inhibition of the CDK-cyclin complex (2, 18, 19).

Here we investigated the molecular basis of such mitosis impairment, focusing on the putative involvement of CDK inhibitors. Two cDNAs, LeKRP1 and LeKRP2, encoding tomato CDK inhibitors were isolated. Their expression was associated with differentiating cells of the gel tissue. These data provide further information on the putative role of KRP in the control of endoreduplication in plant cells.

**EXPERIMENTAL PROCEDURES**

**cDNA Library Screening**—A cDNA library was constructed with poly(A)⁺ mRNA extracted from the gel tissue of 20-DPA tomato fruits using the ZAP Express cDNA synthesis kit and Gigapack III Gold cloning kit (Stratagene, La Jolla, CA). The cDNA library comprised 1.1 × 10⁶ recombinant plaques. The isolation of full-length cDNA was obtained by screening the library as described previously (2).

**Estimation of Relative Transcript Levels with RT-PCR**—The relative transcript levels of each cDNA were determined by semiquantitative RT-PCR assays as previously described (2, 18). The specific pairs of primers used for the amplification of the cDNAs were CAACATTCTAGACCCCTGGTTCC and GTCTAGAGATGAAACGAGGC for LeKRP1 and GCTGAGCGACAAACAACACGCC and GACATTGGCTATGT-CACCTTG for LeKRP2. Primers for LeCYCA2.1 and LeActin1 were as described previously (18). RT-PCR experiments were repeated three times.

**In Situ Hybridization**—Vegetative meristems and flower buds at different developmental stages were fixed according to previously described procedures (19). The LeKRP1 and LeKRP2 templates used for riboprobe synthesis were amplified by PCR using the specific primers described above and cloned into the pGEM-T vector (Promega). Synthesis of digoxigenin-11-UTP-labeled riboprobes, hybridization, washes, and immunological detection of the hybridized probes were carried out as previously described (19).

**Purification of His-LeKRP1 Recombinant Protein and Antibody Preparation**—The open reading frame of LeKRP1 cDNA was inserted into pET28a vector between the BamHI and XhoI sites (Novagen, Madison, WI) to yield the recombinant plasmid pET28a-LeKRP1 and cloned into Escherichia coli strain BL21 (DE3) cells. The induction of the recombinant histidine-tagged LeKRP1 protein and subsequent purification by Ni-NTA column affinity chromatography were performed according to the manufacturer’s instructions (Novagen). The His-LeKRP1 recombinant protein was dialyzed against the following buffer, 50 mM Tris-HCl, pH 7.5, 15 mM MgCl₂, 5 mM EDTA, and 1 mM dithiothreitol, and used for rabbit immunization to yield a polyclonal antibody (Eurogentec, Herstal, Belgium). Total IgG was further purified on protein A-agarose using a 1-ml column of HiTrap™ Protein A high performance (Amersham Biosciences).

**Protein Extraction, Immunoprecipitation, and Immunodepletion**—Epidermis and gel tissues were dissected from tomato fruit and flash frozen in liquid nitrogen prior to protein extraction. They were then ground into a fine powder and stored at −80 °C. For protein extraction, 100 mg of frozen powder was thawed in 1 ml of radioimmune precipitation assay extraction buffer (20 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 5 mM EDTA, 2 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM NaF, 0.2% (v/v) Nonidet P-40, and 0.1% anti-protease mixture). The cell debris was discarded after a 15-min centrifugation at 18,000 × g at 4 °C.

For immunoprecipitation, 100 μg of total protein from gel tissue in radioimmune precipitation assay buffer was precleared with 20 μl of 25% (v/v) protein A-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4 °C on a rotating wheel. After a brief centrifugation, the precleared supernatants were incubated overnight at 4 °C in the presence of the LeKRP1 (1:500) antibody. 20 μl of 25% (v/v) protein A-agarose beads were subsequently added, and the mixture was incubated for 2 h at 4 °C on a rotating wheel. After centrifugation, the supernatants were collected and used as the immunodepleted extracts. The pelleted beads were washed twice with phosphate-buffered saline buffer. After a final centrifugation, the immunoprecipitated material was resuspended in 30 μl of Laemmli loading buffer (20). Samples were separated in a 15% SDS-PAGE and transferred to Immobilon-P membrane prior to immunological detection by Western blot analyses using the LeKRP1 (1:600) antibody. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated IgG diluted 1/10,000 (Chemicon, Temecula, CA) with the BM Chemiluminescence blotting substrate (POD) system from Roche Applied Science.

The same procedure was applied for demonstrating the in vivo interaction between LeKRP1 and CDKA, except that 800 μg of total protein from gel tissue were used. A negative control was performed using the preimmune antiserum to precipitate the proteins. Samples were analyzed by Western blot using an anti-PSTAIR (1/1000) antibody (Santa Cruz Biotechnology).

**In Vitro Kinase Assay**—p9(Cd3ε) was purified from an overproducing strain of E. coli and linked to CNBr-Sepharose 4B (Amersham Biosciences) according to Azzi et al. (21). A 50-μl aliquot of packed p9(Cd3ε)
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% Nonidet P40, and anti-protease mixture) and was mixed with the protein extract. The tubes were rotated constantly 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 (50 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol), and then used for kinase assay. The histone H1 kinase reaction was initiated by resuspending the pellet of p9CksHs1-Sepharose beads with 30 μl of the reaction mixture containing 1 mg ml⁻¹ histone H1 as the substrate and 2.5 μCi of [γ-³²P]ATP (22). Assays were terminated by placing the tube on ice. After a brief centrifugation at 10,000 × g, 10 μl of 4X Laemmli sample buffer was added to the supernatant. Samples were analyzed in a 15% SDS-PAGE, followed by Coomassie Blue staining of the gel to visualize the histone H1 and subsequent autoradiography to detect histone H1 phosphorylation. 30 μl of Laemmli sample buffer was added to the bead pellet, and Western blot analysis was performed to detect CDKA using the anti-PSTAIR (1/1000). Detection of CDKA was performed by standard nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining.

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**FIGURE 2.** Comparison of amino acid sequences of LeKRP1 and LeKRP2 with plant KRPs. A, unrooted neighbor-joining tree of the plant KRPs with the Poisson correction for evolutionary distance calculation. Bootstrap values of 500 bootstrap iterations are shown. Numbers indicate evolutionary distance. The phylogenetic analysis was performed over the last 43 C-terminal residues of the KRPs. Accession numbers are indicated in parentheses. B, sequence alignments of LeKRP1 with Arath;KRP4 and LeKRP2 with Arath;KRP3. Gaps (—) were introduced to maximize the alignments. Conserved motifs in plant KRPs are boxed.
For assaying the retinoblastoma-related protein (RBR) kinase activity, tobacco NtRb1 was expressed using the pGEX-5X-2 plasmid (23) as a glutathione S-transferase-NtRb1 fusion protein and purified by affinity chromatography on glutathione-Sepharose 4B column. Protein extracts were mixed with glutathione S-transferase-NtRb1 immobilized on glutathione-Sepharose 4B beads. Kinase reactions were initiated at 30 °C by adding 5 μCi of [γ-32P]ATP and unlabeled ATP to a final concentration of 30 μM. After 30 min of incubation, the reactions were stopped by adding 30 μl of 2X Laemmli sample buffer and heating at 95 °C for 5 min. The visualization of phosphorylated NtRb1 was as described above. Signals obtained from autoradiography, Western blot, and gel staining were quantified by image scanning using the Quantity One software from Bio-Rad Laboratories (Hercules, CA).

Detection of Protein-Protein Interactions—Protein-protein interactions between LeKRP1 and the different CDK and cyclins were detected by a two-hybrid assay as described previously (19).

Phylogenetic Analysis of Plant KRPs—The sequence analysis of plant KRPs was performed using BioEdit (24), and the phylogenetic tree was constructed with the neighbor-joining algorithm using the software packages TREECON (25). Bootstrap analysis with 500 replicates was performed to test the significance of the nodes.

RESULTS

Molecular Characterization of Two cDNAs Encoding p27Kip1-related Proteins from Tomato—Exploiting the genomic resources of The Institute for Genomic Research (Rockville, MD) Tomato Gene Index (LeGI), we were able to identify two Tentative Consensus (TC) sequences corresponding to cDNAs encoding CDK-specific inhibitors of the plant KRP family (13). TC89204 was 1014 bp and contained a complete open reading frame coding for a 212-amino acid translation product of MW 24.0. TC89097 was a partial sequence of 458 bp harboring a truncated open reading frame. We obtained the corresponding full-length cDNA by screening a 20-DPA tomato gel tissue cDNA library. The full-length cDNA corresponding to TC89097 was 1061 bp and encoded a 190-amino acid product (MW 19.9).

The sequence similarities of plant KRPs are mostly in the C-terminal part of the proteins (13, 26). Thus the 43-amino acid C termini of the two predicted proteins were used for phylogenetic analysis (Fig. 2A).

FIGURE 3. Differential expression analysis by semiquantitative RT-PCR of tomato KRP genes in various organs of tomato and during fruit development. A, expression analysis in various organs from tomato plants: young leaves (YL), differentiated leaves (DL), roots (Ro), stems (St), mature seeds (Sd), and inflorescences (In). C1 and C2 correspond to the negative control (absence of cDNA matrix) and positive control (presence of cDNA of interest as a matrix) of PCR, respectively. The LeKRP1 and LeKRP2 mRNA abundances were normalized toward that of Actin1 and expressed as a ratio of arbitrary units for pixel intensities. B, expression analysis in whole fruits harvested at the following developmental stages: A, 3, 5, 8, 10, 15, and 20 DPA and MG. C, expression analysis in the dissected fruit tissues (epidermis, pericarp, and gel) at the following developmental stages: 10, 15, and 20 DPA, MG, and RR.
The translation products corresponding to TC89204 and TC89097 displayed the highest sequence homology with Arath;KRP4 and Arath;KRP3, respectively, rather than their closest evolutionary counterparts from tobacco, Nicta;KRP (26) and Niccy;KRP (GenBank™ accession numbers KRP3, respectively, rather than their closest evolutionary counterparts, over the C-terminal 43 residues which encompass domains I, II, and III as defined by De Veylder et al. (13). Based on this phylogenetic and sequence analysis, the two cDNAs were identified as putatively encoding tomato KRP s and thus named LeKRP1 (GenBank™/EBI accession number AJ441249 for TC89204) and LeKRP2 (GenBank™/EBI accession number AJ441250 for TC89097).

**Differential Expression of LeKRP1 and LeKRP2 Genes in Tomato**—The transcripts for LeKRP1 accumulated in all plant organs, especially those displaying meristematic activity such as young leaves (YL), roots (Ro), and inflorescences (In) (Fig. 3A). In fully differentiated leaves (DL), the level of LeKRP1 transcripts was much lower. The LeKRP2 transcripts were most abundant in stems (St), and roots and weakly expressed in seeds (Sd) and leaves (YL and DL).

During fruit development, the LeKRP1 and LeKRP2 transcripts were highly expressed very early in the cell division phase (Fig. 3B). After 3 DPA, their respective expression profiles differed. The abundance of LeKRP1 mRNA decreased, reaching its lowest level during the cell expansion phase (8–20 DPA), whereas the abundance of LeKRP2 transcripts decreased gradually, not reaching their lowest level until the mature green (MG) stage.

The different tissues that compose the fruit were dissected to analyze the gene expression from a spatial and developmental perspective. As shown in Fig. 1C, the epidermis tissue per se is composed of a single cell layer. During the tissue preparation fruits were peeled to obtain the epidermis that consists of a few external layers made of small cells. The pericarp corresponds to the fleshy part of the fruit and the gel to the jelly-like locular tissue that surrounds the seeds. The above mentioned difference in the expression profiles of the two tomato KRP genes was also observed during the course of fruit development (Fig. 3C). The maximum expression of LeKRP1 was observed at 20 DPA in all the dissected tissues, especially in the gel tissue where mitosis is impaired and endoreduplication occurs (2) as evidenced by the disappearance of LeCYCA2;1 transcripts. In all three tissues, the expression of LeKRP2 gradually increased to reach its maximum level at the red ripe (RR) stage.

To further characterize LeKRP1 and LeKRP2 gene expression, in situ hybridizations were performed using longitudinal sections of vegetative shoot meristems and developing floral organs (Fig. 4). In vegetative shoot meristems, the in situ hybridization signals using LeKRP1 as a probe were detected in the regions where cell divisions predominantly occur, such as the central and peripheral zones of the meristem and the leaf primordia (Fig. 4, A and B). In 5-mm floral buds, a strong signal was observed in the developing ovules and the carpel wall (Fig. 4D). A higher magnification of the flower bud and an ovule hybridized with LeKRP1 revealed a patchy pattern of expression (Fig. 4, E and F). The hybridization signal obtained with LeKRP2 as a probe was uniformly distributed within the different developing organs of a 7-mm flower bud (Fig. 4G). This uniform distribution was confirmed at the level of the ovules (Fig. 4J) and also observed in every developmental stage of flower buds we analyzed (data not shown). A stronger expression was detected in the epidermis and the 2–3 subepidermal cell layers of the carpel wall (Fig. 4H). These data not only show a differential expression of tomato KRP genes during fruit and plant development but also sug-
gest the involvement of tomato KRPs in endoreduplication during tomato fruit development (2).

LeKRP1 Is a Functional CDK Inhibitor in Vitro —We then focused on the functional analysis of LeKRP1, as its transcriptional expression was maximal at 20 DPA in the developing gel tissue when only endoreduplication occurs (2). LeKRP1 was produced in E. coli as an N-terminal His_6-tagged recombinant protein (Fig. 5A, left panel). After purification by Ni-NTA affinity chromatography, the identification of the recombinant His_6-tagged LeKRP1 was first ascertained by mass spectrometry analysis after two-dimensional gel separation (data not shown). The specificity of a polyclonal antibody raised against LeKRP1 was then analyzed by immunoblotting of E. coli BL21 (DE3) crude extracts (Fig. 5A, right panel). The antibody reacted clearly with the induced and Ni-NTA-purified recombinant LeKRP1. In addition, an antibody raised against the His tag was used as a positive control and gave similar results, confirming the specificity of the induced LeKRP1 protein (data not shown). As shown below in immunoprecipitation experiments (Fig. 7A), the anti-LeKRP1 antibody was able to react with a single species of protein of the expected molecular mass (23.8 kDa), thus indicating the specificity of the antisera.

The purified LeKRP1 was then tested for its ability to inhibit the CDK-cyclin kinase activity in _in vitro_ phosphorylation assays using histone H1 as a substrate. Protein extracts were prepared from dissected epidermis tissue, referred to as Epidermis extract, of 20-DPA fruits and used as the source of CDK-cyclin complexes that were then purified by p9<sub>CksHs1</sub>-affinity chromatography. As shown in Fig. 5B, the addition of 100 ng of purified LeKRP1 prior to p9<sub>CksHs1</sub>-affinity chromatography (+) resulted in a 57% inhibition of the kinase activity present in the untreated Epidermis extract (−). The effect mediated by LeKRP1 appeared to be specific as the levels of CDKA and histone H1 used as the substrate in the phosphorylation reaction were equally detected in both samples in the absence or in the presence of LeKRP1. Increasing amounts of purified LeKRP1 were then mixed with the Epidermis extract prior to purification of the CDK-cyclin complexes (Fig. 5C). In the presence of added LeKRP1, the kinase activity decreased in a dose-dependent manner. The addition of 100 ng of LeKRP1 resulted in a 60% inhibition of kinase activity. Furthermore, a progressive heat denaturation of the purified LeKRP1 restored the kinase activity. It is noteworthy that treatment at 100°C for as long as 30 min was necessary to inactivate LeKRP1 totally and to recover the initial level of kinase activity.

LeKRP1 Protein Accumulates in the Gel Tissue during Endoreduplication —In the gel tissue of tomato fruits, the CDK-cyclin kinase activity is strongly regulated at the post-translational level (2). This suggests the presence of an inhibitory mechanism. We therefore investigated whether LeKRP1 could be involved in this inhibition.

As a first step, protein extracts from 20-DPA Epidermis (dividing tissue) were used as a source of CDK-cyclin kinase activity. These extracts were mixed with extracts from 20-DPA gel referred to as Gel extract (endoreduplicating tissue lacking CDK-cyclin histone H1 kinase activity) (2) (Fig. 6). The Epidermis extract displayed a high level of kinase activity, whereas the activity from the Gel extract was insignificant even though CDKA was present. When both extracts were mixed, the kinase activity present in Epidermis extract decreased in a dose-dependent manner. Adding equal amounts of Gel extract and Epidermis extract resulted in 67% inhibition of the kinase activity. By progressively increasing the duration of protein denaturation, the kinase activity of the Epidermis extract was similar to that measured in the absence of Gel extract or after the addition of an equal amount (100 μg) of bovine serum albumin. A treatment of 30 min at 100°C was necessary to recover 100% of the kinase activity, as previously observed with the exogenously added LeKRP1. Thus the effect of the Gel extract mimicked the effect of purified LeKRP1 on the activity of the CDK-cyclin complex present in epidermis. This indicates that a putative CDK inhib-
itory protein is expressed in the gel tissue after 20 DPA during endoreduplication (2).

The accumulation of LeKRP1 in gel protein extracts of developing fruits was then analyzed by immunoprecipitation experiments using the specific antibody raised against LeKRP1 (Fig. 7A, top panel). The level of LeKRP1 in the gel increased throughout fruit development to reach a maximum at 20 DPA. At the onset of ripening and in fully matured fruits, the level of LeKRP1 then decreased but still remained elevated compared with that at 10 DPA. As a control, we performed a second round of immunoprecipitation using the LeKRP1-immunodepleted extracts and demonstrated that they lacked LeKRP1 (Fig. 7A, bottom panel). Thus the accumulation profile of LeKRP1 in the developing gel tissue of tomato fruit paralleled that of LeKRP1 transcripts (Fig. 3C).

The Presence of LeKRP1 Accounts for the Inhibition of CDK-Cyclin Kinase Activity Mediated by Gel Protein Extracts—To demonstrate that LeKRP1 was involved in the CDK-cyclin complex inhibition observed in the Gel extract, the LeKRP1 immunoprecipitates (Fig. 7A) were used in phosphorylation assays. The Epidermis extract was used as the source of CDK-cyclin kinase activity. As shown in Fig. 7B, the addition of LeKRP1 immunoprecipitates obtained from 10- to 15-DPA gel extracts resulted in a 75% inhibition of the activity. When compared with the histone H1 autophosphorylation control (in the absence of any added protein extracts), the addition of the LeKRP1 immunoprecipitates from gel extracts of 20-DPA, MG, and RR fruits resulted in the total inhibition of the activity. In the presence of the LeKRP1-immunodepleted extracts, the level of the CDK-cyclin kinase activity remained almost constant up to 20 DPA, representing a mean value of 80% of the activity present in the Epidermis extract. After 20 DPA and during maturation, the level of the kinase activity decreased to 60% of the initial value. This inhibition may be due to the increased production of other KRP proteins in the gel during ripening, such as LeKRP2 as observed in Fig. 3C. We conclude that LeKRP1 expression in the gel tissue of developing tomato fruits contributes to the inhibition of CDK-cyclin kinase activity during endoreduplication.

LeKRP1 Only Interacts with Tomato CDKA and CYCD3;1—A yeast two-hybrid approach was used to investigate putative protein-protein interactions between LeKRP1 and the tomato CDKA and CYCD3;1 proteins. The results of these interactions are shown in Fig. 7B. The data indicate that LeKRP1 interacts specifically with the CDKA and CYCD3;1 proteins, but not with any other proteins tested.

FIGURE 6. Inhibition of CDK-cyclin kinase activity by protein extracts from endoreduplicating gel tissue of developing tomato fruits. Equal amounts (100 μg) of extracted proteins from 20-DPA Epidermis were preincubated at room temperature for 10 min with 20-DPA Gel protein extract using various amounts (indicated in μg) of heat-treated extract. The phosphorylation assay, controls, and quantification of kinase activity were performed as in Fig. 5B.

FIGURE 7. Inhibition of the CDK-cyclin complex activities by LeKRP1 in the endoreduplicating gel tissue of developing tomato fruits. A, 100 μg of Gel protein extracts prepared from fruits harvested at 10-, 15-, and 20-DPA, MG, and RR stages were used to prepare immunoprecipitated (top panel) and immunodepleted (bottom panel) LeKRP1 extracts as described under “Experimental Procedures.” B, the LeKRP1 immunoprecipitates and LeKRP1-immunodepleted extracts were mixed to equal amounts (100 μg) of 20-DPA Epidermis extracts, and the mixtures were subsequently preincubated at room temperature for 10 min prior to binding to p9CksHs1-Sepharose beads and kinase activity assay. The phosphorylation assay, controls, and quantification of kinase activity were performed as in Fig. 5B.
interactions between LeKRP1 and tomato CDKs and cyclins (2, 18, 19). As shown in Table 1, LeKRP1 interacted strongly with CDKA and CYCD3;1. No interaction was detected between LeKRP1 and the other CDKs and cyclins tested. To demonstrate an in vivo interaction between CDKA and LeKRP1, we performed an immunoprecipitation assay, trapping the KRP1-interacting CDK-cyclin complexes with the anti-KRP1 antibody. The immunoprecipitated proteins were subsequently detected using an anti-PSTAIRE antibody. As shown in Fig. 8, the incubation of total protein extracts from 15-DPA gel with the anti-KRP1 antibody resulted in the specific immunoprecipitation of CDKA (lane 1), thus demonstrating the in vivo KRP1-CDKA interaction occurring in tomato fruit cells.

LeKRP1 Is Able to Inhibit a CDK-Cyclin Kinase Activity Targeting RBR—Increasing evidence indicates that CDKA and D-type cyclins associate to form complexes controlling the G1/S transition (27). Plant CDKA-CYCD complexes can phosphorylate the RBR protein both in vitro and in vivo (29, 34). Therefore we investigated the inhibitory effect of LeKRP1 on the CDK-cyclin kinase activity isolated from endoreduplicating cells of the gel tissue, using the tobacco RBR (NtRb1) protein (23) as a substrate.

First the kinase activity of the Epidermis and Gel protein extracts (both prepared from fruits at 20 DPA) were assayed (Fig. 9A). A basal kinase activity was present in the Epidermis extract. The Gel extract showed a 1.4-fold greater kinase activity than the epidermis. We then tested the kinase activity of the gel tissue during fruit development (Fig. 9B). The maximum RBR phosphorylation was observed during the earliest developmental stages, i.e. at 10 and 15 DPA. The kinase activity decreased at 20 DPA and then increased again until the RR stage. The pattern of kinase activity was therefore inversely proportional to the transcripational (Fig. 3C) and translational (Fig. 7A) profile of LeKRP1, suggesting that LeKRP1 may affect the activity of this kinase complex during the development of the gel tissue. When added exogenously to the Gel extracts, LeKRP1 was able to significantly inhibit the kinase activity. These results suggest that a kinase activity targeting RBR is putatively regulated by LeKRP1 binding.

### DISCUSSION

The tight binding of specific inhibitors is essential for the control of CDK activities governing cell cycle progression. In mammals CDK inhibitors belong to two classes of proteins, the INK4 family and the Cip/Kip family (28). Their classification is based on their structural features and CDK targets. Both types of CDK inhibitors are involved in regulating the progression of the G1 phase. In plants, all CDK inhibitors isolated so far belong to a single family with weak homology to the mammalian Cip/Kip family (13, 26, 29, 30). In planta functional analyses of plant KRP s (13, 31–33) indicate that, unlike their mammalian counterparts, they may not be solely involved in regulating the timing of exit from the cell cycle and differentiation (13, 34). Cell proliferation is controlled by stimulatory signals such as sugars and hormones through the activation of D-type cyclins (35). Therefore, inhibitory signals may alter the mitotic activity of plant cells in response to unfavorable environmental conditions. Plant KRP s may integrate these negative signals, as their expression, at least at the transcriptional level, is regulated by growth conditions. For instance, Himanen et al. (36) demonstrated that the expression of ArathKRP2, which is involved in maintaining the arrest of root pericycle cells, is rapidly repressed by auxin. However, the function of plant KRP s in the regulation of the cell cycle progression as well as their putative involvement in endoreduplication remains poorly understood.

We isolated cDNAs of the tomato CDK inhibitors, LeKRP1 and LeKRP2. Their amino acid sequence is typical of plant KRP s (Fig. 2), with a conserved C-terminal region and a highly variable N-terminal region (13). The C-terminal region of KRP s plays a major role in protein-protein interactions with both CDKA and CYCD3;1 (31). The N-terminal domain contains a sequence determining protein stability in vivo that could drive the temporal selective degradation of KRP proteins at different time points of the cell cycle (37). This sequence variation may also reflect the specificity of KRP gene expression at a developmental and spatial level. Indeed, like Arabidopsis KRP s (13, 30), LeKRP1 and LeKRP2 display differential patterns of expression in the various organs of tomato plants (Fig. 3A). The expression of LeKRP1 is associated with dividing tissues and restricted to a limited interval of the cell cycle as seen by its patchy expression pattern during in situ hybridization (Fig. 4). The increased expression of LeKRP2 in flower buds compared with that of LeKRP1 (Fig. 3A) may be due to the uniform distribution of the transcripts as observed in Fig. 4, C–I. This suggests that the regulation of LeKRP2 expression is independent of the cell cycle. The first data dealing with the differential expression of plant KRP genes during cell cycle progression were produced in highly synchronous Arabidopsis suspension cultures (38). Unfortunately, the lack of a synchronizable cell suspension culture for tomato prevents us from investigating the expression of LeKRP1 and LeKRP2 during cell cycle progression. During the course of fruit development, LeKRP1 and LeKRP2 display

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**TABLE 1**

| Protein fused to | DBD | AD | His<sup>a</sup> growth<sup>b</sup> | His<sup>c</sup> growth<sup>c</sup> | β-Galactosidase<sup>e</sup> |
|------------------|-----|----|----------------|----------------|-----------------|
| LeKRP1           | CDKA|    | +             | +             | 33.11 ± 0.36    |
| LeKRP1           | CDKB|    | +             | +             | 101.7 ± 14      |
| LeKRP1           | CDKB2|    | +             | +             | 71.7 ± 9.2      |
| LeKRP1           | CYCA1;1|   | +             | +             | 274.6 ± 54      |
| LeKRP1           | CYCA2;1|   | +             | +             | 274.6 ± 54      |
| LeKRP1           | CYCA3;1|   | +             | +             | 274.6 ± 54      |
| LeKRP1           | CYCB1;1|   | +             | +             | 274.6 ± 54      |
| LeKRP1           | CYCB2;1|   | +             | +             | 274.6 ± 54      |
| LeKRP1           | CYCD3;1|   | +             | +             | 274.6 ± 54      |
| LeKRP1           | LeKRP1|    | +             | +             | 50.2 ± 1.31     |
| CDKA1           | LeKRP1|    | +             | +             | 33.11 ± 0.36    |
| CDKB1           | LeKRP1|    | +             | +             | 101.7 ± 14      |
| CDKB2           | LeKRP1|    | +             | +             | 71.7 ± 9.2      |
| CDKC           | LeKRP1|    | +             | +             | 274.6 ± 54      |
| CYCA1;1         | LeKRP1|    | +             | +             | 274.6 ± 54      |
| CYCA2;1         | LeKRP1|    | +             | +             | 274.6 ± 54      |
| CYCA3;1         | LeKRP1|    | +             | +             | 274.6 ± 54      |
| CYCB1;1         | LeKRP1|    | +             | +             | 274.6 ± 54      |
| CYCB2;1         | LeKRP1|    | +             | +             | 274.6 ± 54      |
| CYCD3;1         | LeKRP1|    | +             | +             | 274.6 ± 54      |
| Laminin y1      | LeKRP1|    | +             | +             | 50.2 ± 1.31     |
| RAS             | RAF|    | +             | +             | 50.2 ± 1.31     |

<sup>a</sup> Ability of transformed yeast cells to grow on selection plates containing His (His<sup>a</sup>).

<sup>b</sup> Ability of transformed yeast cells to grow on selection plates lacking His (His<sup>c</sup>).

<sup>c</sup> β-Galactosidase activity was measured only for positive interactions (indicated as Miller units).
Tomato KRP1 and Endoreduplication

De Veylder et al. (39) suggested that the inhibition of M-phase CDK-cyclin kinase activity triggers endoreduplication, thus preventing mitosis. The plant-specific CDKB1;1, whose activity peaks at the G2/M boundary, is likely to prevent the transition from the mitotic cycle to the endocycle. Indeed, the impairment of CDKB1;1 activity resulted in enhanced endoreduplication (7). Recently it was shown in Arabidopsis that CDKB1;1 phosphorylates KRP2, triggering its proteolytic degradation, and in turn maintains a high CDKA;1 activity required for the mitotic cell cycle (15). When the CDKB1;1 activity declines, KRP2 is able to inactivate CDKA;1, thus driving the cell toward endoreduplication.

Transgenic plants that strongly overexpress KRP1 show not only reduced growth due to the alteration of cell proliferation through the reduction of CDK activity but also reduced ploidy levels as endoreduplication is suppressed (13, 26, 31). These data suggest that KRP1s can inhibit both types of CDK-cyclin complexes, mitotic cell cycle-specific ones and endoreduplication cycle-specific ones. Interestingly, the generation of weak KRP2 overexpressors in Arabidopsis resulted in the inhibition of only the mitosis CDK-cyclin complex activity and not that of the endocycle-specific CDK-cyclin complex. Data obtained from the misexpression of ICK1/KRP1 in the single cell Arabidopsis trichomes also supported the idea that plant CDK inhibitors act in a concentration-dependent manner (34).

Endoreduplication (and consequently the absence of mitosis) in the gel tissue of tomato fruit coincides with the lack of transcription of mitosis-associated genes (e.g. CDKBs and A- and B-type cyclins) (18, 19). Therefore, it is likely that M-phase CDK-cyclin complexes are not synthesized and assembled in endoreduplicating tissues. The production of tomato KRP1s in the gel (Figs. 3C and 7), in the absence of cell division (2), suggests a role for CDK inhibitors in addition to the inhibitory binding of M-phase CDK-cyclin complexes for the control of the transition toward endoreduplication.

The RBR pathway controls the G1/S transition in plant cells (40). The CDK-cyclin complexes that drive the G1/S transition are formed from the association of a D-type cyclin with CDKA. They act by phosphorylating RBR (23, 27, 41, 42). Compelling evidence for the involvement of RBR pathway components in the control of endoreduplication arises from in planta functional analyses (39, 43). We (Table 1; Fig. 8) and others (13, 31, 37) have shown that plant KRP1s only interact with CDKA and Cyclin D. During endoreduplication, the transcription of LeCDKB1;1 and LeCYCD3;1 is sustained in tomato (18), arguing for a role of CDKA and CYCD3;1 in the endocycle. Here, we have shown that LeKRP1 can inhibit a putative kinase activity present in the endoreduplicating gel tissue of the fruit that targets RBR (Fig. 9). It remains to be demonstrated whether the CDK-cyclin complex containing this kinase activity, that is targeted by LeKRP1, is indeed composed of CDKA and CYCD3;1.

As demonstrated in transgenic lines overproducing plant KRP1s (13, 32), cell growth can be uncoupled from cell cycle progression and DNA content. However, in most plant cells, endoreduplication correlates with cell size increase to maintain the nucleus-to-cell volume ratio (3, 44). The effects of a mis-expression of ICK1/KRP1 in Arabidopsis trichomes indicated that cell growth may be controlled by two distinct
mechanisms, a DNA amount-independent mechanism and a DNA-de-
pendent mechanism (14). For instance, the cell may need to reach a
minimal size to pass the Start/restriction point in the G-phase of the
endocycle prior to DNA replication. This was demonstrated in yeast
where the control of cell size involves the action of the CDK inhibitor
p25rum1, which delays the passage through Start until the required cell
mass is reached (45). As a working hypothesis, we propose that plant
KRPs regulate the phase transitions of the endocycle by modulating the
CDKA-CYCD kinase activity on RBR in late G-phase of the endocycle
to prevent a premature passage into S-phase. This control of the CDKA-
CYCD kinase activity could be exerted at two distinct levels: first, by
slowing down the assembly of the CDKA-CYCD kinase complex,
assuming that KRP may interact with the CYCD subunit alone (as shown in
plants and Refs. 14, 32, 37); and second, by inhibitory binding to
the CDKA-CYCD complex itself. An additional level of control by KRP
may also be during re-entry into the successive G-phase of the endo-
cycle. At this point it may act by inhibiting the CDKA-CYCD kinase
activity when the DNA replication phase is completed. Whether plant
KRPs participate in these different mechanisms is an intriguing matter
for investigation, and some recent findings seem to support these puta-
tive roles of plant KRPs (34).

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REFERENCES
1. Gillaspy, G., Ben-David, H., and Gruissem, W. (1993) Plant Cell 5, 1439–1451
2. Joubé, J., Phan, T.-H., Just, D., Rothan, C., Bergouin, C., Raymond, P., and Chev-
alier, C. (1999) Plant Physiol. 121, 857–869
3. Joubé, J., and Chevalier, C. (2000) Plant Mol. Biol. 43, 735–745
4. Grafi, G. (1998) Exp. Cell Res. 244, 372–378
5. Dewitte, W., and Murray, J. A. H. (2003) Annu. Rev. Plant Biol. 54, 235–264
6. Leiva-Neto, J. T., Grafi, G., Sabelli, P. A., Rousseau, Y.-M., Maddock, S.,
Gordon-Kamm, W. J., and Larkina, B. A. (2004) Plant 16, 1854–1869
7. Boudolf, V., Vlieghe, K., Beemster, G. T. S., Magyar, Z., Torres Acosta, J. A., Maes, S., Van Der Schueren, E., Inzé, D., and De Veylder, L. (2004) Plant Cell 16, 2683–2692
8. Grafi, G., and Larkins, B. A. (1995) Science 269, 1262–1264
9. Sun, Y., Dilkis, B. P., Zhang, C., Génard, R. A., Carneiro, N. P., Lowe, K. S., Jung, R.,
Gordon-Kamm, W. J., and Larkins, B. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4180–4185
10. Gonzalez, N., Hernould, M., Delmas, F., Gevaudant, F., Dufle, P., Causse, M., Mouras, A., and Chevalier, C. (2004) Plant Mol. Biol. 56, 849–861
11. Cebolla, A., Vinardell, J. M., Kiss, E., Olah, B., Roudier, F., Kondorosi, A., and Kondo-
rozi, E. (1999) EMBO J. 18, 4476–4484
12. Vinardell, J. M., Fedorova, E., Cebolla, A., Kevei, Z., Horvath, G., Koelmen, Z., Tarayre,
S., Roudier, F., Mergaert, P., Kondorosi, A., and Kondorosi, E. (2003) Plant Cell 15, 2093–2105