INDIRECT SEMIQUANTITATIVE DETERMINATION OF p34^{cdc2} LEVELS IN G₁ AND G₂ CELLS OF THE CARBOHYDRATE-STARVED ROOT MERISTEMS IN *VICIA FABA* VAR. *MINOR*

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ABSTRACT

In eukaryotes, the 34kDa kinase (p34) encoded by the *cdc2* gene is a key regulator of both the onset of DNA synthesis (G₁ to S phase transition) and the onset of mitosis (G₂ to M phase transition). Using mouse anti-human PSTAIRE and FITC-labelled goat antibodies, indirect semiquantitative determination of p34^{cdc2} levels was performed in meristematic cells from the control (intact) and excised, carbohydrate-starved main roots of *Vicia faba* var. *minor*. No evident differences in the intensity of fluorescence was found either between the G₁ and G₂ cells or between the control cells and the cells arrested at both Principal Control Points by carbohydrate starvation. It seems thus, that the cell cycle block induced in meristematic cells of *V. faba* var. *minor* is not correlated with the absolute level of the key cell cycle enzyme responsible for phosphorylation of cellular proteins, but primarily with the altered activity of p34^{cdc2}.

KEY WORDS: cell cycle, p34^{cdc2}, Principal Control Point hypothesis, protein kinases, root meristems, *Vicia faba* var. *minor*.

INTRODUCTION

The most prominent events during the cell division cycle, i.e. DNA replication and mitosis, must be coupled in the correct order to ensure that each daughter cell receives a full complement of the hereditary material. Cell cycle progression is principally regulated before the onset of S phase and before the onset of mitosis by one or more protein kinases (e.g. Jacobs 1992; Francis and Halford 1994). Activation of these enzymes requires the formation of a complex with a family of other regulatory proteins, named cyclins. Specific cyclin dependent kinase (CDK) – cyclin complexes operate during the transitions from G₁ to S and from G₂ to mitosis. In addition, the controls termed as the cell cycle checkpoints maintain the temporal order of S phase and mitosis and ensure that: (1) DNA replication takes place only once per each cell division cycle (Li 1995; Maszewski and Polit 1998), (2) mitosis is not initiated until S phase is completed, and (3) the next cell cycle does not begin until the chromosomes are properly segregated at mitosis (Jacobs 1992; Martin-Castellanos and Moreno 1996).

The basic mechanisms of the plant cell cycle are likely to be the same as those of the yeast and animal cell cycles, and indeed plant homologs of both CDKs and cyclins have been identified (Deckert et al. 1994; Chasan 1995). In plant cells, the principal controls are manifested at two stages during the lifetime of a root: once when the cells are proliferative but cease dividing temporarily because of stress or other causes and again when they cease dividing and differentiate to form mature tissue (Evans and Van't Hof 1974). Cytological data

show that the phase in which cells of higher plants arrest is prescribed, suggesting that the phenomenon is genetically controlled. The ratio of cells arrested in G₁:G₂ in nutritionally starved root meristems is species-specific and similar ratios are seen in mature root tissue of unstressed seedlings. This finding demonstrates that the phase in which cells become blocked (arrested cell phenotype) is consistent and independent, regardless of the cause of arrest.

The controls of G₁ and G₂ phases in plant cells need to be defined in molecular terms and more effort must be directed toward the question of how the regulatory processes responsible for triggering the key events of the cell cycle correlate with protein phosphorylations and dephosphorylations. The present work was aimed at determining the levels of p34^{cdc2} in the G₁ and G₂ cells of intact and excised, carbohydrate-starved root meristems in *Vicia faba* var. *minor*, the latter representing populations of G₁ cells blocked at the Principal Control Point I (PCP-I; inhibited prior to the onset of S phase) and the G₂ cells blocked at the PCP-II (inhibited prior to the onset of mitosis). The semiquantitative assays have been made adopting an indirect immunocytochemical method with the use of mouse anti-human PSTAIRE motif and goat anti-mouse FITC-labelled antibodies.

MATERIALS AND METHODS

Plant material

Seedlings of *Vicia faba* var. *minor* were grown on moist blotting paper at 20-22°C in the dark. For all experiments

main roots of about 3.0 cm in length were selected and fixed either for cytophotometry or immunocytochemical staining.

Experimental procedures for excised, nutrient-starved roots

Apical parts of roots (5–10 mm long) excised from seedlings were surface sterilized with acetone-chlorophorm according to methods described earlier (Maszewski et al. 1998), washed several times with distilled water and transferred to Erlenmeyer flasks (100 ml) filled with sterile White's (1943) medium without sucrose (15 ml/5–10 root tips/flask). Using this medium, excised roots were cultivated at 22°C in a water-bath shaker (100 rpm) for 3 days, fixed and stained.

Fixation and staining for cytophotometry

Intact, control roots (*in planta*) and excised, carbohydrate-starved root tips were fixed in cold Carnoy's mixture of absolute ethanol and glacial acetic acid (3:1) for 2 h, washed with ethanol and kept in 70% ethanol. For Feulgen staining, root meristems were cut off, rehydrated, hydrolysed in 4N HCl and stained with Schiff's reagent (pararosaniline; Sigma) according to standard methods. After washing in SO₂-water (3 times) and distilled water, root tips were placed in a drop of 45% acetic acid and squashed onto gelatinized microscope slides. Following freezing with dry ice, cover slips were removed and the dehydrated dry slides were embedded in Canada balsam.

Cytophotometry

Cytophotometry was made using Jena-Med-2 microscope with the computer-aided IMAL-512 system for image analysis (Maszewski et al. 1998). To estimate the absolute DNA content per nucleus, absorbance in Feulgen-stained samples was measured at 565 nm.

Immunocytochemistry

Immunocytochemical methods have been performed according to Colasanti et al. (1993), with considerable modifications. Root tips from both control (intact) and excised, carbohydrate-starved meristems were immediately immersed in fixative containing 4% paraformaldehyde, 10 mM Tris, 10 mM EDTA-Na₂, 100 mM NaCl (pH 7.2) with 0.1% Triton X-100, at 0°C for 20 min. After fixation, the root tips were washed in several changes of water, Tris-Triton X-100 buffer and dried. Then, root tips were placed in digestion solution (5% cellulysin/2% macerace in 0.4 M mannitol/5 mM EGTA) and incubated at room temperature for 30 min. After the digestion solution was removed, the root tips were washed as before. Following the last wash, root cells were released by squashing onto poly-L-lysine-coated slides. Large pieces of tissue were removed and the slides were quickly immersed in 100% methanol at -20°C for 7 to 8 min and then washed with PBS. Excess PBS was blotted from the slides and antibody solutions were overlayed on the cells in the wells prepared using plasticine rings. Mouse monoclonal anti-human p34^{cdc2} antibody, raised against PSTAIRE motif (Novocastra, NCL-p34^{cdc2}), was diluted 1/100 in PBS. Slides were incubated overnight in a humidified incubator at 37°C, then washed for 20 min with several changes of Tris-Triton X-100 buffer and incubated with normal goat serum. Following saturation of non-specific binding sites, FITC conjugated goat anti-mouse IgG (Fab specific; Sigma BioSciences) was applied, and the slides were washed as before, embedded in PBS/glycerol/DABCO mixture (9:1:2.4%). Negative controls, made in order to examine non-specific labeling consisted of slides incubated with the 2nd antibody, solely.

Semiquantitative determination of p34^{cdc2} levels in G₁ and G₂ cells

Slides prepared using the immunocytochemical methods were viewed under Nikon Optiphot-2 microscope and photographed using Kodak Elite 400 film. To allow for a semi-quantitative determination of p34^{cdc2} levels, all micrographs were taken at exactly the same time of exposure. Following development, the relative intensity of fluorescence was examined on films, using Jena-Med-2 microscope with the computer-aided IMAL-512 system for image analysis. G₁ and G₂ cells were selected according to phenotypic parameters and morphometric examination of nuclear sizes.

RESULTS AND DISCUSSION

In recent years, much progress has been made in identifying and characterizing components of the fundamental molecular machinery that drives the cell cycle in apparently all eukaryotes. The 34kDa kinase (p34) encoded by the *cdc2* gene is central to the molecular machinery that controls key steps at both the onset of DNA synthesis (G₁ to S phase transition) and the onset of mitosis (G₂ to M phase transition). At each of these checkpoints, p34^{cdc2} functions in complex with phase-specific sets of proteins known as cyclins, which are thought to regulate its catalytic function during specific phosphorylations (McKinney and Heintz 1991). Such association allows for a progression of CDK activities that modify changing populations of proteins, initiate transcription, DNA biosynthesis and structural changes in nucleus and cytoplasm (e.g. Krek and Nigg 1991; Jacobs 1992; Francis and Halford 1994).

Fig. 1. Frequency distribution (%) for nuclear DNA content (a.u., arbitrary units) in root cells of *Vicia faba* var. *minor*.

Fig. 1A – control plants

Fig. 1B – carbohydrate-starved meristems

Fig. 2. Immunofluorescence of root meristem cells labeled with mouse anti-PSTAIRE (p34^{cdc2}) monoclonal antibodies and goat anti-mouse FITC-conjugated secondary antibodies. Magnification: 1200 $\times$.

Fig. 2A – control meristem

Fig. 2B – carbohydrate-starved meristem

Fig. 2C – negative control (FITC-conjugated antibody, solely).

Plants conform to this model in revealing cell size dependent control, the presence of p34^{cdc2}-like protein and cell cycle dependent changes in phosphorylation (Hunter 1995; John 1996). However, two aspects of the developmental regulation of cell division in plants seem of uttermost importance. First, adequate cdc2 protein level is needed for cell division; experiments with lateral root formation clearly indicate that p34 is restored prior to mitotic reactivation in tissues that have fully ceased cellular proliferation and attained low p34^{cdc2} level, as in pith (Zhang et al. 1996), cotyledon (Gorst et al. 1991), and root cortex (Miao et al. 1993). Second, the presence of p34^{cdc2} enzyme is not in itself sufficient to drive cell proliferation, indicating that the activity of the enzyme is controlled (John 1996). Equivalent data, concerning the amount of p34^{cdc2} and of other proteins per cell available for differentiating animal cells indicate that low levels of p34^{cdc2} correlate with the exit from the cell cycle. Restorative increases in p34^{cdc2} level accompany resumption of division when stimulated by serum (Lee et al. 1988) or by adenovirus infection (Draetta et al. 1987).

The Principal Control Point hypothesis raised primarily by Van't Hof and Kovacs (1972) states that cell division in complex plant tissue is regulated by factors that operate during interphase and that under non-permissive conditions these factors become limiting, causing cell cycle block at either G₁ or G₂. Induction of stationary meristems in carbohydrate-starved roots provides invitation to check out whether the levels of p34^{cdc2} in "arrested cell phenotypes" change and, if it is the case, how are these alterations manifested in cells inhibited before the onset of S phase (PCP-I) and before the G₂ $\rightarrow$ M transition (PCP-II).

Frequency distributions of the nuclear DNA contents in meristematic zones from the control (Fig. 1A) and excised roots in *Vicia faba* var. *minor* (Fig. 1B) indicate evident differences. Increased resolution between the peaks representing 2C and 4C DNA levels in carbohydrate-starved meristems corresponds well with the typical biphasic separation of stationary G₁ and G₂ subpopulations of cells arrested at PCP-I and PCP-II, respectively (comp. Van't Hof and Kovacs 1972). Moreover, this result is in good agreement with complete cessation of DNA replication and mitosis, evidenced using ³H-thymidine autoradiography, BrdUrd labeling and Feulgen staining (Politi; in prep). Basing on morphometric estimations, the G₁ and G₂ cells from the control and excised roots have been further tested immunocytochemically in order to determine the levels of p34^{cdc2}.

The p34^{cdc2} proteins from various organisms are similar, especially in a conserved stretch of 16 amino acids (EGV-PSTAIREISLLKE) around the core sequence PSTAIR. Antibodies against yeast p34^{cdc2} and p36^{CDC28} were used by Draetta et al. (1987) to detect the human equivalent of p34^{cdc2}. On the other hand, antibodies raised against animal cdc2 kinase have been employed in search for p34 in plant cells (Colasanti et al. 1993).

Irrespective of the experimental series, fluorescence of FITC-conjugated secondary antibodies recognizing the presence of PSTAIRE in root meristem cells of *Vicia faba* is confined basically to the cytoplasm (comp. Fig. 2 and 3). This result is consistent with the data presented by Colasanti et al. (1993); although in plant interphase and early prophase cells cdc2 protein is localized mainly within the nucleus, antibodies raised against the PSTAIRE motif bring about predominantly cytoplasmic staining. However, some specific labeling of cell nuclei could also be observed, with the fluorescence being even more prominent in the central regions corresponding to nucleoli (Fig. 2A, B and Fig. 3).

Fig. 3. Indirect semiquantitative determination of p34^{cdc2} levels in G₁ and G₂ cells of the control and carbohydrate-starved root meristems in *Vicia faba* var. *minor*. Immunofluorescence of root meristem cells labeled with mouse anti-PSTAIRE monoclonal antibodies and goat anti-mouse FITC-conjugated measured cytophotometrically.

N – nucleus
nuc – nucleolus
C – cytoplasm

Direct microscopic observations and semiquantitative measurements revealed no evident differences of fluorescence either between the G₁ and G₂ cells or between the control cells (obtained from the intact roots) and the cells arrested at PCPs by excision and carbohydrate starvation of root meristems. It seems thus, that according to some more general rules operating among the eukaryotes, the cell cycle arrest induced in carbohydrate-starved meristematic cells of *Vicia faba* var. *minor* is correlated primarily with altered activity of p34^{cdc2} and not with the absolute level of the key cell cycle enzyme responsible for phosphorylation of cellular proteins.

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PÓLILOŚCIOWA POŚREDNIA ANALIZA ZAWARTOŚCI p34^{cdc2}
W MERYSTEMATYCZNYCH KOMÓRKACH KORZENI *VICIA FABA* VAR. *MINOR*
ZABLOKOWANYCH W FAZIE G₁ I G₂ GŁODZENIEM WĘGLOWODANOWYM

STRESZCZENIE

Podstawowym regulatorem cyklu komórkowego u Eukaryota, odpowiedzialnym za inicjację replikacji DNA (przejście z fazy G₁ do fazy S) oraz za inicjację mitozy (przejście G₂ → M) jest kinaza białkowa o masie cząsteczkowej 34 kDa (p34), kodowana przez gen *cdc2*. Wykorzystując mysie przeciwciała skierowane przeciw motywowi PSTAIRE oraz kozie przeciwciała sprzężone z FITC, przeprowadzono pośrednią, póliłościową analizę zawartości p34^{cdc2} w komórkach kontrolnych (pochodzących z całych siewek; *in planta*) i komórkach z odciętych (głodzonych węglowodanowo) merystemów korzeni *Vicia faba* var. *minor*. Wykazano brak istotnych różnic w intensywności fluorescencji komórek będących w fazie G₁ i fazie G₂ zarówno w merystemach korzeni kontrolnych, jak też po zablokowaniu proliferacji komórek w efekcie odcięcia korzeni i ich hodowli w warunkach głodu węglowodanowego. Wydaje się więc, że zatrzymanie cyklu komórkowego w merystemach *Vicia faba* var. *minor* nie jest związane ze zmianami bezwzględnej zawartości kluczowego regulatora cyklu komórkowego (odpowiedzialnego za fosforylację wielu białek komórkowych), lecz ze zmianami jego aktywności enzymatycznej.

SŁOWA KLUCZOWE: cykl komórkowy, p34^{cdc2}, hipoteza głównych punktów kontrolnych, kinazy białkowe, merystemy korzeniowe, *Vicia faba* var. *minor*.