Trends in Cell & Molecular Biology
Vol. 6, 2011

Original Communication

Lipid bodies in lipotubuloids of *Ornithogalum umbellatum* ovary epidermis contain diacylglycerol acyltransferase 2 (DGAT2) and lipase, incorporate ^{3}H-palmitic acid and are connected with cuticle synthesis

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ABSTRACT

Lipid bodies of *O. umbellatum* ovary epidermis are surrounded by a network of microtubules and assembled in lipotubuloids i.e. cytoplasm domains containing ribosomes, ER cisternae and vesicles as well as some mitochondria, microbodies, Golgi structures and autolytic vacuoles. Lipid bodies *in statu nascendi* grow from 0.04 up to about 0.1 µm. In maturity they are about 0.1-0.4 µm in diameter and maintain this dimension during epidermis development, accompanied by lipotubuloid enlargement resulting in the higher number of lipid bodies. Using the immunogold technique with anti-diacylglycerol acyltransferase 2 and anti-lipase antibodies it was shown that mature lipid bodies were labeled with gold grains near a phospholipid monolayer. Autoradiography with ^{3}H-palmitic acid demonstrated that lipid body surface is the site of this precursor incorporation into lipids. Selective labeling of lipotubuloid lipid bodies with silver grains observed after 2-h incubation of the ovary disappeared after 6-h postincubation in the non-radioactive medium. It can be suggested that the size of mature lipid bodies does not change because of the dynamic balance between synthesis and degradation of lipids inside them. It was shown that lipotubuloid growth dynamics was closely correlated with the growth of ovary epidermis cells. Lipids of lipotubuloids are probably building block of cuticle.

KEYWORDS: DGAT2, lipase, lipid bodies *in statu nascendi*, mature lipid bodies, ^{3}H-palmitic acid, autoradiography, ovary epidermis, immunogold, cuticle synthesis

INTRODUCTION

Plant and animal cell lipid bodies are spherical and have simple construction. They are surrounded by a unique phospholipid monolayer (half lipid membrane; [1]), with adjacent structural proteins [2] among which oleosins are most common in plants while those of perilipin family (PAT family; [3]) in animals. Moreover, different functional proteins involved in lipid metabolism were also identified in the lipid body fraction [4]. The hydrophobic core of lipid bodies is mostly filled with triacylglycerols and free fatty acids that is why they used to be treated only as the source of energetic substances. However, during the last decade they were shown to be active organelles involved in lipid homeostasis, in intracellular signaling, in transient protein storage and their degradation, in protein lipidation, in membrane biogenesis and in eicosanoid synthesis [5-9]. Disturbances in their functioning cause numerous diseases both in animals and humans e.g. obesity, diabetes, arteriosclerosis, cardiovascular disease,

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Annals of Botany **110**: 1581–1591, 2012
doi:10.1093/aob/mcs215, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
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DNA replication stress induces deregulation of the cell cycle events in root meristems of *Allium cepa*

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Received: 1 June 2012 Returned for revision: 23 July 2012 Accepted: 22 August 2012 Published electronically: 18 October 2012

• *Background and Aims* Prolonged treatment of *Allium cepa* root meristems with changing concentrations of hydroxyurea (HU) results in either premature chromosome condensation or cell nuclei with an uncommon form of biphasic chromatin organization. The aim of the current study was to assess conditions that compromise cell cycle checkpoints and convert DNA replication stress into an abnormal course of mitosis.
• *Methods* Interphase-mitotic (IM) cells showing gradual changes of chromatin condensation were obtained following continuous 72 h treatment of seedlings with 0·75 mM HU (without renewal of the medium). HU-treated root meristems were analysed using histochemical stainings (DNA-DAPI/Feulgen; starch-iodide and DAB staining for H_2O_2 production), Western blotting [cyclin B-like (CBL) proteins] and immunochemistry (BrdU incorporation, detection of γ-H2AX and H3S10 phosphorylation).
• *Key Results* Continuous treatment of onion seedlings with a low concentration of HU results in shorter root meristems, enhanced production of H_2O_2, γ-phosphorylation of H2AX histones and accumulation of CBL proteins. HU-induced replication stress gives rise to axially elongated cells with half interphase/half mitotic structures (IM-cells) having both decondensed and condensed domains of chromatin. Long-term HU treatment results in cell nuclei resuming S phase with gradients of BrdU labelling. This suggests a polarized distribution of factors needed to re-initiate stalled replication forks. Furthermore, prolonged HU treatment extends both the relative time span and the spatial scale of H3S10 phosphorylation known in plants.
• *Conclusions* The minimum cell length and a threshold level of accumulated CBL proteins are both determining factors by which the nucleus attains commitment to induce an asynchronous course of chromosome condensation. Replication stress-induced alterations in an orderly route of the cell cycle events probably reflect a considerable reprogramming of metabolic functions of chromatin combined with gradients of morphological changes spread along the nucleus.

Key words: Chromatin, cyclin B-like proteins, DNA damage, DNA replication, γ-H2AX, histone H3 phosphorylation, hydrogen peroxide, hydroxyurea, mitosis, premature chromosome condensation.

INTRODUCTION

The large-scale changes in chromatin packaging associated with cell cycle progression produce two opposite states of nuclear DNA, one adapted to transcribe and to precisely replicate genomic sequences during interphase, and the other adjusted to distribute chromosomes into daughter cells at mitosis (Morales *et al.*, 2001). A series of transitions between up- and downstream events involved in these processes depends on checkpoint mechanisms that preserve causal ordering of specific activities and prevent cells from entering M phase before the completion of DNA synthesis and G2 functions (Hartwell and Weinert, 1989; Cools and De Veylder, 2009). Molecular components which contribute to this system create regulatory circuits keeping track of hierarchies and dependencies for making the cell cycle a one-way route composed of successive stages, each demonstrating a discrete level of nuclear organization capable of facilitating emergent tasks. As a rule, induction of signalling pathways in response to unfavourable external conditions or damaging factors combined with subsequent checkpoint activities slows down or blocks cell cycle progression, thus providing additional time needed either to supply indispensable nutrients (Van't Hof, 1985; Polit, 2009), to correct genetic lesions or to direct irreparably damaged cells towards apoptosis (Abraham, 2001; Stevens *et al.*, 2010).

Perhaps the most remarkable cell cycle control pathway conserved across all eukaryotic forms of life, the S-M checkpoint (referred to also as the DNA replication checkpoint), assembles similar biochemical elements into a three-level molecular response system composed of: (1) sensors that detect stalled replication forks or double-strand DNA breaks (DSBs), (2) specific transducers that convey appropriate signals and (3) effectors, which reply to these signals so that the cell triggers a suitable chain of events to make defence (or, alternatively, apoptotic destruction) more effective (Lukas *et al.*, 2004). An essential role for the S-M mechanism is to perceive distorted, abnormally or partially replicated DNA molecules and to prevent the assembly of active M-phase cyclin-dependent kinases (CDKs) which, together with their specific regulatory subunits (cyclins), provide the driving force for an abrupt G2-to-M phase transition. Another type of function

FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 50, No. 3, 2012
pp. 427–431

ORIGINAL STUDY

VIA MEDICA

DGAT2 revealed by the immunogold technique in *Arabidopsis thaliana* lipid bodies associated with microtubules

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Abstract: The immunogold technique with anti-diacylglycerol acyltransferase 2 (DGAT2) antibody revealed in *A. thaliana* embryo and root meristematic cells gold particles manifesting the presence of DGAT2 in ER as well as in lipid bodies. This being so, lipid synthesis could take place both in ER and in the lipid bodies. The presence of microtubules around the lipid bodies was evidenced under transmission EM. Detection of tubulin around the lipid bodies using the immunogold technique with anti-α-tubulin is in agreement with the above observations. Connection of lipid bodies with microtubules was also detected by us in other plants where they probably participated in lipid synthesis. A similar phenomenon may take place in *A. thaliana*. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 3, 427–431)*

Key words: DGAT2, lipid bodies, lipid synthesis, microtubules, ultrastructural immunogold technique

Introduction

Lipid bodies (lipid droplets, oleosomes) are the focus of interest for many scientists, as demonstrated by the large number of reviews concerning them [1–7]. They have become an important, and fascinating, object of research because they take part in many intracellular processes. The lipid bodies which are surrounded by a phospholipid monolayer are significantly different from other cell structures covered with a phospholipid bi-layer. This monolayer covers a triacylglycerol core.

Recent studies have suggested that the lipid bodies are not only lipid reservoirs, as previously thought, but are organelles which contain numerous proteins participating in signal transduction, regulation of synthesis, degradation and accumulation of lipids [1]. Disturbance of normal lipid homeostasis results in numerous human diseases [8]. Understanding lipid body functions is also important because of plant productivity and biotechnology [9].

One problem concerning lipid bodies which needs explanation is the cellular site of lipid synthesis and accumulation. It is accepted that ER containing appropriate enzymes is such a site [10]. However, the presence of diacylglycerol acyltransferase 2 (DGAT2), an enzyme which, according to biochemical data [10] is able to transform diacylglycerol into triacylglycerol, indicates that lipid synthesis could still proceed in lipid bodies formed with contribution of ER [11]. An earlier paper also reached similar conclusions concerning palm [12]. Localization of this enzyme in plant cells with the immunogold technique has not been demonstrated to date. However, Shockey et al. [13], using the immunofluorescence technique, demonstrated that in tung tree DGAT1 and DGAT2 were located in distinct dynamic regions of ER. They concluded that these enzymes had non-redundant functions in the production of storage oils in plants.

The aim of the present study was to localise DGAT2 in *A. thaliana* cells. It has been demonstrated that there is a relationship between lipid bodies

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Plant Cell Rep (2010) 29:845–856
DOI 10.1007/s00299-010-0869-x

ORIGINAL PAPER

Inter- and intrachromosomal asynchrony of cell division cycle events in root meristem cells of *Allium cepa*: possible connection with gradient of cyclin B-like proteins

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Received: 19 March 2010 / Revised: 17 April 2010 / Accepted: 5 May 2010 / Published online: 21 May 2010

Abstract Alternate treatments of *Allium cepa* root meristems with hydroxyurea (HU) and caffeine give rise to extremely large and highly elongated cells with atypical images of mitotic divisions, including internuclear asynchrony and an unknown type of interchromosomal asynchrony observed during metaphase-to-anaphase transition. Another type of asynchrony that cannot depend solely on the increased length of cells was observed following long-term incubation of roots with HU. This kind of treatment revealed both cell nuclei entering premature mitosis and, for the first time, an uncommon form of mitotic abnormality manifested in a gradual condensation of chromatin (spanning from interphase to prometaphase). Immunocytochemical study of polykaryotic cells using anti-β tubulin antibodies revealed severe perturbations in the microtubular organization of preprophase bands. Quantitative immunofluorescence measurements of the control cells indicate that the level of cyclin B-like proteins reaches the maximum at the G2 to metaphase transition and then becomes reduced during later stages of mitosis. After long-term incubation with low doses of HU, the amount of cyclin B-like proteins considerably increases, and a significant number of elongated cells show gradients of these proteins spread along successive regions of the perinuclear cytoplasm. It is suggested that there may be a direct link between the effects of HU-mediated deceleration of S- and G2-phases and an enhanced concentration of cyclin B-like proteins. In consequence, the activation of cyclin B-CDK complexes gives rise to an abnormal pattern of premature mitotic chromosome condensation with biphasic nuclear structures having one part of chromatin decondensed, and the other part condensed.

Keywords *Allium cepa* · Biphasic nuclei · Cell cycle · Chromatin · Cyclin B-like proteins · Hydroxyurea · Nuclear asynchrony · Preprophase band

Communicated by A. Feher.

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Introduction

Although most of the cells in Eukaryotes are mononucleate, a vast number of fungi, plants, and animals are known to produce bi-, poly-, or multinucleate forms (termed "syncytia") that appear occasionally at various stages of morphogenesis (Baluška et al. 2004 and references therein). Examples of these include, but are not limited to, plasmodia in the slime mold *Physarum polycephalum*, endosperm in seeds of flowering plants, embryos of *Drosophila melanogaster* at early stages of development, and giant osteoclasts, myoblasts, or placental trophoblasts in mammals, which all create syncytia by cell fusion events. As a general rule, the emergence of a multinucleate state is correlated with synchronous mitotic divisions or mitotic waves. A number of cases relating to plants include tip cells of the thallus in coenocytic algae (e.g. *Cladophora*), milk tubes (laticiferous tissue) in the seedlings of *Euphorbia marginata*, secretory tapetum characterized by nuclear morphologies changing with respect to developmental stage, and common areas of polarized cytoplasm in cereal endosperm alveoli (Kapraun 2005 and references therein). Synchronization of mitotic divisions is also observed in groups of symplasmically interconnected

Lipotubuloids – Structure and Function

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1. Introduction

In the 19th and 20th centuries these structures were called „elaioplasts" according to Wakker (1888) who introduced this term with regard to nucleus-size lipid bodies present in *Vanilla planifolia* leaf epidermis which strongly refracted light. Since they are clearly visible under light microscope (Fig. 1A,B) they became the focus of interest in those days and were described in about 120 mono- and dicotyledonous plant species. Also in our laboratory "elaioplasts" were for the first time observed in 12 *Gentiana* species (Kwiatkowska, 1959, 1961) and in *Dahlia variabilis* (Kwiatkowska, 1963).

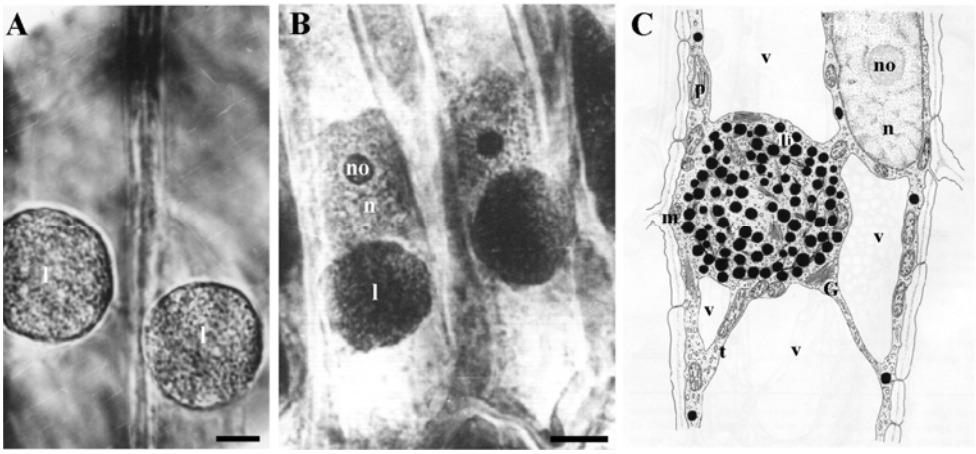

Fig. 1. *Ornithogalum umbellatum* "elaioplast" (lipotubuloid); A – in a living cell; B - after OsO_4 fixation; C – a scheme of epidermis cell with lipotubuloid; G – Golgi apparatus, l – lipotubuloid, lb – lipid bodies, m – mitochondrion, n – nucleus, no – nucleolus, p – plastid, t – tonoplast, v - vacuole; bars: 10 µm.

In 1883 Schimper introduced the term plastids which has been widely accepted since then. Among plastids, which are cell organelles containing double phospholipid bilayer, there are those producing lipids, i.e. elaioplasts which this term actually means. However, they are totally different from Wakker's "elaioplasts", the latter not being plastids in the contemporary meaning of this term. This was unequivocally proved by EM observations of

Protoplasma (2013) 250:251–259
DOI 10.1007/s00709-012-0402-x

ORIGINAL ARTICLE

Increased transcription in hydroxyurea-treated root meristem cells of *Vicia faba*

Konrad Winnicki · Justyna Teresa Polit · Janusz Maszewski

Received: 19 December 2011 / Accepted: 21 March 2012 / Published online: 15 April 2012

Abstract Hydroxyurea (HU), an inhibitor of ribonucleotide reductase, prevents cells from progressing through S phase by depletion of deoxyribonucleoside triphosphates. Concurrently, disruption of DNA replication leads to double-strand DNA breaks. In root meristems of *Vicia faba*, HU triggers cell cycle arrest (preferentially in G1/S phase) and changes an overall metabolism by global activation of transcription both in the nucleoplasmic and nucleolar regions. High level of transcription is accompanied by an increase in the content of RNA polymerase II large subunit (POLR2A). Changes in transcription activation and POLR2A content correlate with posttranslational modifications of histones that play a role in opening up chromatin for transcription. Increase in the level of H4 Lys5 acetylation indicates that global activation of transcription following HU treatment depends on histone modifications.

Keywords 5-Ethynyl uridine · Transcription · Acetylation · RNA polymerase · RPB1 · *Vicia faba*

Introduction

Cell cycle transitions throughout interphase and mitosis are regulated by sophisticated metabolic pathways comprising diverse proteins. To ensure DNA integrity and correct functioning of important cellular processes, such as replication or mitotic division, cells take advantage of their evolutionary developed mechanisms called cell cycle checkpoints. Genotoxic stress caused by a variety of potential stressors (e.g., ultraviolet light, ionizing radiation, chemicals that affect DNA integrity) combined with disruption of checkpoint control functions may have a destructive impact on cellular functioning (Shackelford et. al. 1999; Bartek and Lukas 2001; Rybaczek and Kowalewicz-Kulbat 2011). Adequate response to DNA damage is possible owing to the presence of specific sensor kinases that constitute upstream factors to their effectors. Multidimensional character of cell cycle checkpoints allows not only to block phase-to-phase transitions but also, if necessary, to activate diverse genes and to trigger DNA repair processes (Jackson 2002; Yang et al. 2004). Replication fork stalling or DNA lesions are detected by two sensor kinases (PIKK family members), ATM (ataxia telangiectasia-mutated) and ATR (ATM and Rad 3-related) and their downstream factors—Chk1 and Chk2 kinases. Consequently, the activity of the latter proteins drives inhibitory phosphorylation of Cdc25 phosphatases, which become unable to activate cyclin and Cdk complexes, ultimately resulting in cell cycle arrest (Abraham 2001; Ricaud et al. 2007; McNeely et al. 2010). In turn, repair factors involved in homologous recombination or non-homologous end-joining are recruited to DNA lesions, owing to gamma phosphorylation of H2AX histones by sensor kinases (Rogakou et al. 1998; Hanakahi et al. 2000; Bassing et al. 2002). Molecular components of cell cycle checkpoints are well known, yet mutual relationships between these factors and other cellular proteins are still not clear.

Hydroxyurea (HU) is a well-known inhibitor of ribonucleotide reductase (RNR) which, by transformation of ribonucleotides to deoxyribonucleoside triphosphates, plays a fundamental role in establishing balanced quantities of precursors required for DNA synthesis and DNA repair systems (Roa et al. 2009; Koç et al. 2004; Alvino et al. 2007).

Handling Editor: Pavla Binarova

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Acta Physiol Plant (2010) 32:1189–1196
DOI 10.1007/s11738-010-0514-x

ORIGINAL PAPER

"Elaioplasts" of *Haemanthus albiflos* are true lipotubuloids: cytoplasmic domains rich in lipid bodies entwined by microtubules

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Received: 22 July 2009 / Revised: 13 March 2010 / Accepted: 22 April 2010 / Published online: 11 May 2010

Abstract In the cells of *Haemanthus albiflos* leaf epidermis there are structures containing lipids analogous to "elaioplasts" (Wakker in Jahrb F Wiss Bot 19:423–496, 1888). Ultrastructural analysis has shown that they are cytoplasmic domains—lipotubuloids, since they exhibit all the features of *Ornithogalum umbellatum* lipotubuloids. They are composed of numerous lipid bodies surrounded by microtubules, ER cisternae and vesicles, some mitochondria, Golgi structures, and microbodies. In the center of some lipotubuloids there are also autolytic vacuoles. Microtubules adjacent to *H. albiflos* lipid bodies were revealed only when taxol preincubation was used before fixing the epidermis in the mixture of glutaraldehyde and OsO_4. The presence of tubulin in *H. albiflos* and *O. umbellatum* lipotubuloids was confirmed with use of the immunogold method involving antibodies against tubulin α. It is possible that the association of microtubules with lipid bodies may be more common than originally thought, but it is difficult to reveal due to the methodological problems.

Keywords "Elaioplasts" · *Haemanthus albiflos* · Lipid bodies · Lipotubuloids · Microtubules

Communicated by A. Kononowicz.

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Introduction

Lipid bodies (lipid droplets, oil bodies, oleosomes) (Athenstaedt and Daum 2006) were not in the focus of research studies for many years because they were treated as passive lipid droplets, present in the cytoplasm of both animal and plant cells. However, the pioneer works concerning lipid bodies conducted for the past ten years have proved that they are active structures playing an important role in lipid homeostasis and involved in intracellular signaling (Robenek et al. 2006). Disturbance of their functioning in animals is correlated with many health problems, e.g., obesity, type 2 diabetes, liver dysfunctions, and arteriosclerosis (Welte 2007). On the other hand, the production of plant oil which is used for consumption and for industry is of economic importance. Thus, the lipid bodies have become the focus of interest in many laboratories worldwide.

The structure of a lipid body is simple: a hydrofobic core made of neutral lipids (triacylglycerols) is surrounded by a monolayer of polar lipids with proteins bound to them by hydrofobic or electrostatic forces. These proteins are not well known, especially in plants, since during their isolation and purification contamination with different organelles producing artifacts is possible. Damage of lipid bodies during isolation exposing the hydrofobic core may cause non-specific adhesion of proteins from the lisate. That is why identification of proteins and examination of the functions of lipid bodies directly in the cells are very important (Welte 2007); however rather rare.

One of the controversies is the site of triacylglycerols synthesis. It is generally assumed that it takes place through the lipid accumulation inside a bilayer of ER membranes (Lung and Weselake 2006). The accumulated lipids bulge an ER external monolayer and form a bud

Journal of Plant Physiology 169 (2012) 1597–1606

Contents lists available at SciVerse ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Sucrose synthase activity and carbohydrates content in relation to phosphorylation status of *Vicia faba* root meristems during reactivation from sugar depletion

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ARTICLE INFO

Article history:
Received 7 March 2012
Received in revised form 25 April 2012
Accepted 27 April 2012

Keywords:
Glucose localization
Invertase
Meristem cell ultrastructure
Plant cell cycle
Sugar starvation
Sucrose metabolism *in situ*

SUMMARY

Carbohydrate starvation of *Vicia faba* root meristems leads to readjustment of carbohydrate metabolism and blocks the cell cycle in two principal control points (PCP1/2). The cell cycle reactivation is possible after sucrose provision, although with a delay of about 12 h. During this period, the cells are sensitive to 6-dimethylaminopurine (6-DMAP) and okadaic acid (OA), inhibitors of protein kinases and phosphatases, respectively. The aim of the present study was to investigate whether those inhibitors are involved in inhibition of cell cycle revival through interference with the activities of two sucrose-cleaving enzymes: sucrose synthase (SuSy; EC 2.4.1.13) and invertase (INV; EC 3.2.1.26).

In sugar-starved cells, the *in situ* activity of both enzymes decreased significantly. Following supplementation of root meristems with sugar, INV remained inactive, but SuSy activity increased. Despite the lack of INV activity, glucose was present in meristem cells, but its content was low in cells treated with OA. In the latter case, the size of plastids was reduced, they had less starch, and Golgi structures were affected. In sugar-starved cells, SuSy activity was induced more by exogenous sucrose than by glucose. The sucrose-induced activity was strongly inhibited by OA (less by 6-DMAP) at early stages of regeneration, but not at the stages preceding DNA replication or mitotic activities. The results indicate that prolongation of regeneration and a marked decrease in the number of cells resuming proliferation (observed in previous studies) and resulting from the action of inhibitors, are correlated with the process of SuSy activation at the beginning of regeneration from sugar starvation.

Introduction

Cell divisions in root meristems strongly rely on a constant supply of nutrients, including sucrose. Depending on physiological activities of heterotrophic root tips, sucrose is stored in vacuoles or utilized for respiration, changed into structural and storage polysaccharides or sugar derivatives in different subcellular compartments. Sucrose can also act as a signaling and regulatory molecule causing alterations in gene expression or enzymatic activities, and can consequently turn cell cycle progression on or off (Riou-Khamlichi et al., 2000; Ciereszko and Kleczkowski, 2002a; Koch, 2004; Rolland et al., 2006; Ciereszko, 2009). Plant cells are equipped with two principal control points (PCP1 and PCP2), which block cell cycle progression in response to carbohydrate starvation at G1 and G2 phases (Van't Hof, 1985; Polit et al., 2003). Since this blockade is reversible, application of sucrose switches the cell cycle on again, yet with a delay (about 12 h) defined as metabolic regeneration period (Polit and Maszewski, 2004; Polit et al., 2004), during which numerous phosphorylation and dephosphorylation processes occur, especially just after provision of sucrose. Meristematic cells are extremely sensitive to inhibitors of protein kinases (cyclin-dependent kinases, CDK) and protein phosphatases (PP1/PP2A), which give rise to the prolonged cell cycle block (Polit and Maszewski, 2004, 2005). However, the pathway(s) and messengers passing information about the abundance of nutrients to main regulators that trigger DNA replication and mitosis are still unknown.

In plants, sucrose cleavage into hexoses is catalyzed by invertases (INVs; EC 3.2.1.26; β-D-fructofuranoside fructohydrolases) and sucrose synthase (SuSy; EC 2.4.1.13; UDP-glucose: D-fructose-2-α-D-glycosyl transferase). INV catalyzes irreversible hydrolysis of sucrose to glucose and fructose. SuSy, in the presence of UDP,

Abbreviations: 6-DMAP, 6-dimethylaminopurine; INV, invertase; MPF, M-phase promoting factor; NBT, nitroblue tetrazolium salt; OA, okadaic acid; PCP1-2, principal control points; PP1/PP2A, specific protein phosphatases; SPF, S-phase promoting factor; SuSy, sucrose synthase; WM, White's nutrient medium.

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0176-1617/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.jplph.2012.04.017

BIOLOGY OF REPRODUCTION **80,** 572–580 (2009)
Published online before print 10 December 2008.
DOI 10.1095/biolreprod.108.067918

Immunogold Evidence Suggests That Endoplasmic Reticulum Is the Site of Protamine-Type Protein Synthesis and Participates in Translocation of These Proteins into the Nucleus During *Chara vulgaris* Spermiogenesis[1]

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ABSTRACT

During spermiogenesis of an alga *Chara vulgaris*, which in many aspects resembles that of animals, histones are replaced by protamine-type proteins. Our earlier immunocytochemical studies showed that this replacement started during the short stage V of spermiogenesis, when electronograms revealed an extensive system of cisternae and vesicles of endoplasmic reticulum (ER). The present studies revealed at stage V intensive incorporation of labeled ^{3}H-arginine and ^{3}H-lysine quickly translocating into a nucleus visualized with pulse-chase autoradiography of semithin sections. The immunogold technique with the use of the antibodies to protamine-type proteins isolated from *Chara tomentosa* show that both ER cisternae and vesicles are labeled with gold grains, which are absent from the spermatids not treated with the antibodies; thus, the ER is probably the site of the protamine-type protein synthesis. These proteins then are translocated to a nucleus through ER channels connected with the nuclear envelope, as suggested by gold labeling of an inner membrane of the nuclear envelope adjacent to condensed chromatin. The above results correspond with those of other authors showing that in animals, protamines bind with lamin B receptors localized in the inner membrane of the nuclear envelope. A hypothesis has been put forward that during *Chara* spermiogenesis the inner membrane of the nuclear envelope invaginates into a nucleus together with protamine-type proteins, which become separated from the membrane and penetrate into chromatin.

Chara vulgaris, *endoplasmic reticulum, ER cisternae and vesicles, gamete biology, immunogold technique, nucleohistones, protamine-type proteins, spermatid, spermiogenesis*

INTRODUCTION

Spermiogenesis, the process of spermatid differentiation leading to the formation of mobile spermatozoids, shares many common features in animal and plant organisms, among the most important being extremely strong chromatin condensation connected with the replacement of histones by protamines or other strongly basic proteins that protect the male genome against harmful external factors [1, 2]. This process is very common and well studied in animals; it has been observed, among others, in fish and mammals, including humans [2], as well as in *Drosophila melanogaster* [3]. In plants, spermiogenesis takes place only in the organisms producing free-moving spermatozoids, which reach ovary cells in water. The process of nuclear protein exchange was described in *Marchantia polymorpha* [4] and *Marsilea vestitia* [5], as well as in higher algae *Chara corallina* [5], *Chara vulgaris* [6–8], and *Chara tomentosa* [8, 9]. Unlike in mammals, where spermatids result from meiosis, in the haplobionts from *Chara* species, spermiogenesis is only preceded by some synchronous mitotic divisions of antheridial filament cells (cell cycle S + G_2 + M) [10–13].

The replacement of nucleohistones by nucleoprotamines during spermiogenesis is the key morphogenetic process whose correct course is a prerequisite for proper development and functioning of spermatozoids [14–17]. However, further studies are required for both medical and reproduction purposes. For example, it is not clear whether the synthesis of protamines in the cytoplasm occurs in free ribosomes or in those bound with the endoplasmic reticulum (ER). There is also no information concerning the way by which the protamines are transported to a nucleus. Contrary to histone H1, for which importin 5 (IPO5, also called importin beta 3) was identified [18], and to transition protein 2, for which importin 4 (IPO4) is known to be responsible for its importation into a nucleus [19], no factors facilitating the replacement of histones by protamines have been discovered [20].

The results concerning animal cells indicate that protamines are translocated to a nucleus very shortly after their synthesis (half-time approximately 1–2 min) [21]. Moreover, synthesized protamines are soon phosphorylated [21, 22], and this process is mediated by SR protein-specific kinase 1 [23]. Protamine phosphorylation is a rapid process facilitating their correct binding with DNA [24]. It is also indispensable for protamine translocation to the nucleus by transient association with the receptor of lamin B (present in the inner membrane of a nuclear envelope), whose phosphorylation enables binding with phosphorylated protamines after the receptor is unblocked because of p32 removal [25].

The mechanism of nuclear protein exchange during spermiogenesis is much less known in plant cells. No information is available concerning the site of synthesis of protamines and their way of transport into a nucleus in *M. polymorpha* and *M. vestitia* [4, 5]. The only suggestions concerning this problem come from our ultrastructural, cytochemical, autoradiographic, immunocytochemical, and electrophoretic studies of *C. vulgaris* and *C. tomentosa* spermiogenesis. The first hypothesis—that ER was the site of protamine synthesis and its translocation to a nucleus via endocytosis of an inner membrane of the nuclear envelope—was based only on very suggestive ultrastructural pictures of *C. vulgaris* spermiogenesis [26]. Then, this hypothesis was supported by cytochemical studies of *C. vulgaris* showing that

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Received: 24 January 2008.
First decision: 10 March 2008.
Accepted: 20 November 2008.

eISSN: 1259-7268 http://www.biolreprod.org
ISSN: 0006-3363

FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 49, No. 2, 2011
pp. 285–290

ORIGINAL STUDY

VIA MEDICA

Microtubule heterogeneity of *Ornithogalum umbellatum* ovary epidermal cells: non-stable cortical microtubules and stable lipotubuloid microtubules

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Abstract: Lipotubuloids, structures containing lipid bodies and microtubules, are described in ovary epidermal cells of *Ornithogalum umbellatum.* Microtubules of lipotubuloids can be fixed in electron microscope fixative containing only buffered OsO_4 or in glutaraldehyde with OsO_4 post-fixation, or in a mixture of OsO_4 and glutaraldehyde [1]. None of these substances fixes cortical microtubules of ovary epidermis of this plant which is characterized by dynamic longitudinal growth. However, cortical microtubules can be fixed with cold methanol according immunocytological methods with the use of β-tubulin antibodies and fluorescein. The existence of cortical microtubules has also been evidenced by EM observations solely after the use of taxol, microtubule stabilizer, and fixation in a glutaraldehyde/OsO_4 mixture. These microtubules mostly lie transversely, sometimes obliquely, and rarely parallel to the cell axis. Staining, using Ruthenium Red and silver hexamine, has revealed that lipotubuloid microtubules surface is covered with polysaccharides. The presumption has been made that the presence of a polysaccharide layer enhances the stability of lipotubuloid microtubules. *(Folia Histochemica et Cytobiologica 2011, Vol. 49, No. 2, 285–290)*

Key words: immunocytochemistry, electron microscopy, taxol, Ruthenium Red, hexamine

Introduction

Microtubules are polar filaments of a cytoskeleton composed of α/β tubulin heterodimers. The cytoskeleton is a complex, dynamic fibrillar net which plays a major role in the generation and regulation of cell architecture and its mechanical features. Moreover, microtubules constitute a scaffold for different biochemical processes. Recent studies have shown that the cytoskeleton is also involved in the spatial organization and regulation of translation [2].

Tubulin may undergo post-translational modulations (PTMs) which result in a functional variety of microtubules. There are many types of PTMs: detyronisation/tyronisation, glutamylation, glycylation, phosphorylation, acetylation, palmitoylation (s-acylation), Δ2-modification [3–5]. Moreover, microtubule features are modified by microtubule-associated proteins (MAPs). These can be microtubule-stabilizing proteins, microtubule-polymerizing proteins, microtubule-depolymerizing kinesins or microtubule-severing proteins [6].

In a single cell there can be microtubules with different features and stability. In mitotic cells, kinetochor and interpolar microtubules are strongly modified by polyglutamylation, acetylation and detyronisation while astral microtubules are unmodified [7]. Because of this, they exhibit different stability depending on various factors [8]. Neuronal microtubules are the most differentiated; they play an important role in neuronal differentiation-regulation by controlling tubulin availability and modulating microtubule dy-

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Plant Cell Rep (2009) 28:165–173
DOI 10.1007/s00299-008-0642-6

CELL BIOLOGY AND MORPHOGENESIS

Protein phosphorylation in *Vicia faba* root meristem cells during the first steps of leaving principal control points after sucrose application

Justyna Teresa Polit

Received: 28 August 2008 / Accepted: 2 November 2008 / Published online: 21 November 2008

Abstract Before *Vicia faba* root meristem cells stopped by carbohydrate starvation in principal control points (PCP1 and PCP2) start sucrose induced replication and division they go through a phase of metabolic regeneration. This interval is characterised st great sensitivity to the inhibitors of cyclin-dependent protein kinases and protein phosphatases (PPs). In the present research, changes of phosphoprotein levels in the nucleolus, nucleus and cytoplasm were analysed using okadaic acid and 6-dimethylaminopurine (6-DMAP) during the first period of cell regeneration in sucrose (0–3 h). It was established that when the cells start to leave checkpoints, the balance between protein phosphorylation and dephosphorylation shifts towards the intensified activity of PPs. Furthermore, it was also established that the structures appearing during cell regeneration, which were located around cell nuclei and which contained large amounts of phosphorylated proteins, were plastids. The reactions of protein phosphorylation which took place in the plastids were directly correlated with starch synthesis and were stopped by inactivation of protein phosphatases (PP1 and/or PP2A).

Keywords Carbohydrate starvation · Plant cell cycle · Principal control points · Protein phosphorylation · Sucrose recovery

Communicated by F. Brandizzi.

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Abbreviations

CDK	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitor
6-DMAP	6-Dimethylaminopurine
MPF	M-phase promoting factor
OA	Okadaic acid
PCP	Principal control point
PP1/PP2A	Protein phosphatases 1/2A
SPF	S-phase promoting factor

Introduction

In plant cell cycle, the checkpoints termed as principal control points: PCP1, located near the end of G1 phase; and PCP2, located near the end of G2 phase, are the basis of cell cycle progression mechanism (Van't Hof and Kovacs 1972; Van't Hof 1985). Countless proteins which, in the presence of other molecules, act at this time and area ensure the successful completion of one step of the cycle prior to the initiation of the next. Amongst these are S-phase promoting factor and M-phase promoting factor—complexes of cyclin-dependent kinases (CDK) with cyclins. These complexes are highly specific, and their expression and activity are tightly controlled by endogenous cyclin-dependent kinase inhibitors, protein phosphatases (PPs), and other kinases. In addition, they are self regulated by a feedback reaction to different extracellular and intracellular signals (Menges et al. 2002; Stals and Inzé 2001; Vandepoele et al. 2002; De Veylder et al. 2007; Polit and Kaźmierczak 2007). Molecular mechanisms that control cell cycle progression are largely based on countless reactions of phosphorylation and dephosphorylation of enzymatic, structural and regulatory proteins (Inzé 2005; Polit and Kaźmierczak 2007;

Protoplasma (2012) 249:131–137
DOI 10.1007/s00709-011-0272-7

ORIGINAL ARTICLE

Cell cycle-dependent phosphorylation of pRb-like protein in root meristem cells of *Vicia faba*

Justyna Teresa Polit · Andrzej Kaźmierczak · Aurelia Walczak-Drzewiecka

Received: 24 January 2011 / Accepted: 9 March 2011 / Published online: 29 March 2011

Abstract The retinoblastoma tumor suppressor protein (pRb) regulates cell cycle progression by controlling the G1-to-S phase transition. As evidenced in mammals, pRb has three functionally distinct binding domains and interacts with a number of proteins including the E2F family of transcription factors, proteins with a conserved LxCxE motif (D-type cyclin), and c-Abl tyrosine kinase. CDK-mediated phosphorylation of pRb inhibits its ability to bind target proteins, thus enabling further progression of the cell cycle. As yet, the roles of pRb and pRb-binding factors have not been well characterized in plants. By using antibody which specifically recognizes phosphorylated serines (S807/811) in the c-Abl tyrosine kinase binding C-domain of human pRb, we provide evidence for the cell cycle-dependent changes in pRb-like proteins in root meristems cells of *Vicia faba*. An increased phosphorylation of this protein has been found correlated with the G1-to-S phase transition.

Keywords Phosphorylation · Plant cell cycle · Retinoblastoma-like protein

Handling Editor: Heiti Paves

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Introduction

A vast number of similarities and differences have been shown to interlace in structure, function, and mutual relationships between the principal regulators of the cell division cycles in yeast, mammals, and plants (de Jager et al. 2005). The entry into S phase in mammalian cells, but not in yeasts, is controlled by key regulators of the retinoblastoma protein (pRb) pathway (Weinberg 1995). Recently, Rb-related genes and their products called retinoblastoma-related proteins (RBRs) have been discovered in various plant species (Ach et al. 1997; Grafi et al. 1996; Miskolczi et al. 2007; Vandepoele et al. 2002; Xie et al. 1996). In mammals, pRb is a tumor suppressor protein that prevents cells from progressing through the cell cycle and mitotic divisions. Oncogenic proteins, such as those produced by virus-infected cells, can bind and inactivate pRb, eventually leading to tumorogenesis. Accordingly, in a number of cancer types, pRb was found to be dysfunctional (Das et al. 2005; Funk et al. 1997; Ludlow and Skuse 1995; Nevins 2001; Sellers and Kaelin 1997). RBRs are also involved in DNA repair, DNA damage checkpoint control, differentiation, cellular senescence, and apoptosis.

pRb is a member of the pocket protein family which has three distinct binding domains: the first, which binds proteins with the LxCxE motif (e.g., D-type cyclins and oncoproteins; Dowdy et al. 1993; Zamanian and LaThangue 1992); the second, large A/B pocket, which binds the E2F family of transcription factors and the third, C (more divergent in plant RBRs), which binds the c-Abl tyrosine kinase in mammals (Knudsen and Wang 1996; Korenjak and Brehm 2005; Münger and Howley 2002).

Acta Physiol Plant (2008) 30:315–324
DOI 10.1007/s11738-007-0124-4

ORIGINAL PAPER

An improved method for the cell cycle synchronization of *Vicia faba* root meristem cells

Justyna Teresa Polit

Received: 23 October 2006 / Revised: 18 November 2007 / Accepted: 20 November 2007 / Published online: 7 December 2007

Abstract Plant root meristem cells divide asynchronously which makes biochemical analysis of cell cycle regulation particularly difficult. In the present article a high level of cell cycle synchronization in *Vicia faba* root meristems was obtained by using a rich medium (HNS), special culture conditions and a double-block method with replication inhibitor—hydroxyurea (HU). Two HU concentrations were tested and different periods of the first and the second synchronization, and of cycle recommencement between the first and the second blockage. The level of synchronization was estimated on the basis of ^{3}H-thymidine labeling indices, mitotic, and phase indices and indices determining the percentage of G1 and G2 cells, which were identified by cytophotometric measurements of DNA content in individual nuclei. The highest level of cell cycle synchronization was obtained after double treatment of meristems with 1.25 mM HU (18 and 12 h) separated by 6-h incubation in HNS without HU. During the second postincubation in HNS in subsequent hours: 4, 7, 10, 11, over 90% of cells in the S phase, nearly 70% in G2 phase, 86% in mitosis, and nearly 70% in G1 phase were received, respectively. The use of 2.5 mM HU in a similar experimental procedure caused disturbed divisions.

Keywords Cell cycle synchronization · Double block · Hydroxyurea · *Vicia faba*

Communicated by H. Gabrys.

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Introduction

The eukaryotic cell division cycle normally consists of four phases, the most important of which in respect of exactness are the S phase, when DNA replication occurs and the M phase, when chromosomes are precisely separated on a mitotic spindle into two daughter cells and cytoplasmic division occurs. In the majority of organisms in somatic cells, S and M phases are separated by two gap phases: the first gap—G1, when a cell grows, duplicates its organelles and prepares for DNA replication, and the second gap—G2, when a cell after replication prepares for mitosis. In cell cycle, there are several checkpoints that coordinate subsequent phase-specific events in space and time. They can stop the cell cycle in response to some irregularities in the progress of particular phases of the cycle (Ciliberto et al. 2003; Novák and Tyson 2004; Van't Hof 1985).

Extensive cell cycle research has revealed that the basic mechanism of cell division may be universal for all eukaryotic systems. Great number of genes relevant to cell cycle regulation has equivalents in yeast, animal as well as plant cells. The discovery of their products: protein kinases, cyclins, protein phosphatases, endogenic inhibitors, regulatory proteins, and transcription factors are landmark events in cell cycle research (Ciliberto et al. 2003; Dyson 1998; Magyar et al. 1997, 2005; Novák and Tyson 2004; Stals and Inzé 2001; Verkest et al. 2005). However, biochemical analysis of metabolism and molecular studies of gene expression in a given phase of cell division *in planta* are often difficult, because cell cycle progression in somatic tissues is asynchronous and only some cells are cycling in a given moment. Perfect synchronization would permit precise biochemical and molecular studies of regulatory mechanisms, which determine cell cycle regulation at the level of gene expression and protein phosphorylation

Cell Biology International

Cell Biol. Int. (2012) 36, 455–462 (Printed in Great Britain)

Research Article

Lipid body biogenesis and the role of microtubules in lipid synthesis in *Ornithogalum umbellatum* lipotubuloids

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Abstract

Lipid bodies present in lipotubuloids of *Ornithogalum umbellatum* ovary epidermis take the form of a lens between leaflets of ER (endoplasmic reticulum) membrane filled with a highly osmiophilic substance. The two enzymes, DGAT1 [DAG (diacylglycerol) acyltransferase 1] and DGAT2 (DAG acyltransferase 2), involved in this process are synthesized on rough ER and localized in the ER near a monolayer surrounding entities like lipid bodies. After reaching the appropriate size, newly formed lipid bodies transform into mature spherical lipid bodies filled with less osmiophilic content. They appear to be surrounded by a half-unit membrane, with numerous microtubules running adjacently in different directions. The ER, no longer continuous with lipid bodies, makes contact with them through microtubules. At this stage, lipid synthesis takes place at the periphery of lipid bodies. This presumption, and a hypothesis that microtubules are involved in lipid synthesis delivering necessary components to lipid bodies, is based on strong arguments: (i) silver grains first appear over microtubules after a short [^{3}H]palmitic acid incubation and before they are observed over lipid bodies; (ii) blockade of [^{3}H]palmitic acid incorporation into lipotubuloids by propyzamide, an inhibitor of microtubule function; and (iii) the presence of gold grains above the microtubules after DGAT1 and DGAT2 reactions, as also near microtubules after an immunogold method that identifies phospholipase D1.

Keywords: DGAT1; DGAT2; electron microscopy autoradiography; immunogold technique; phospholipase D1; ultrastructure

1. Introduction

Lipid bodies (lipid droplets, i.e. oil bodies) are common organelles found in yeast, plants and animals, including some prokaryota (Murphy, 2001; Martin and Parton, 2005). In comparison with other organelles, the structure of lipid bodies is unique. The core consisting of neutral lipids [TAG (triacylglycerol) and sterol esters] is surrounded by a monolayer of polar lipids (cholesterol and phospholipids) with a specific fatty acid composition (Tauchi-Sato et al., 2002). These separate the aqueous and hydrophobic phases.

Lipid bodies are a pool of lipids in a harmless form of esters that is not only an energy source, but is involved in membrane biogenesis, protein lipidation and eicosanoid synthesis. Oil bodies contain numerous proteins taking part in signal transductions and those transiently stored or degraded in them (Martin and Parton, 2005; Cermelli et al., 2006; Welte, 2007; Fujimoto et al., 2008).

Although there are numerous reports concerning lipid bodies, their biogenesis and functioning requires elucidation. Moreover, research on lipid bodies is important because disturbances in lipid homoeostasis are the basis of many animal and human diseases (Bozza et al., 2009). It may also contribute to plant productivity and biotechnology (Shockey et al., 2006; Lardizabal et al., 2008).

One of the difficulties in lipid bodies analysis is the surrounding phospholipid monolayer that can be easily damaged during biochemical procedures and could contaminate fractions obtained with cytoplasm components (Welte, 2007). This explains why light microscopy and EM (electron microscopy) of different cell components combined with autoradiography and immunocytochemistry should be helpful in understanding lipid body biogenesis and function, and their interactions with other organelles.

The ER (endoplasmic reticulum) is involved in lipid body formation resulting from lipid accumulation between the phospholipid monolayer leaflets. The growing number of lipids form a bud that enlarges and finally separates from the ER (Ducharme and Bickel, 2008; Fujimoto et al., 2008; Guo et al., 2009; Ohsaki et al., 2009); simultaneously a phospholipid monolayer is formed. Robenek et al. (2009), using freeze–fracture replica immunolabelling, claimed that lipid body formation did not occur in this way. Instead, lipid bodies appear to develop externally to both membranes at specialized sites, in which the ER enwraps the droplet thereby 'enclosing it rather like an egg-cup'.

Some authors believe that a phospholipid monolayer covering a bud contain anchored enzymes of lipid synthesis allowing lipid body enlargement (Bouvier-Navé et al., 2000; Lung and Weselake, 2006; Czabany et al., 2007). However, Boström et al. (2005) and Olofsson et al. (2009) suggest that small lipid bodies merge into bigger ones, a process that is mediated by microtubules and dynein. Another hypothesis assumes that formation of lipid bodies is independent of the ER; lipids are secreted into the cytoplasm as naked droplets that become surrounded by phospholipid monolayers (Ichihara, 1982; Murphy, 1988; Murphy et al., 1989; Lung and Weselake, 2006).

Most authors believe that after lipid body formation, further lipid synthesis in a cell takes place in the ER, since it contains

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Abbreviations: DAG, diacylglycerol; DGAT, DAG acyltransferase; ER, endoplasmic reticulum; EM, electron microscopy; TAG, triacylglycerol.

Physiologia Plantarum
An International Journal for Plant Biology

Physiologia Plantarum 135: 342–350. 2009

In situ activities of hexokinase and fructokinase in relation to phosphorylation status of root meristem cells of *Vicia faba* during reactivation from sugar starvation

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Received 26 September 2008; revised 17 December 2008

doi: 10.1111/j.1399-3054.2008.01201.x

The plant cell cycle is equipped with two principal control points: PCP1 in G1 and PCP2 in G2 phase. These checkpoints can arrest the cell cycle in response to carbohydrate starvation, while sugar presence can revive the replication and mitotic activity. The process of cell cycle revival is strongly repressed by okadaic acid (OA) or 6-dimethylaminopurine (6-DMAP), inhibitors of specific protein phosphatases 1 or 2A or kinases (cyclin-dependent kinases), respec tively. In the present study, it was investigated whether inhibition of cell cycle revival is performed through interference of the above-mentioned inhibitors with the metabolic pathway of sucrose applied to the cells. Changes of hexokinase (HK) and fructokinase (FK) activities, key enzymes of hexose metabolism, were analyzed in *Vicia faba* root meristem cells arrested in G1 and G2 phase by carbohydrate starvation as well as in those recovered with glucose or sucrose in the presence of OA or 6-DMAP. It was shown that in the sugar-starved cells, the activity of both enzymes decreased significantly. During cell regeneration with carbohydrates, the activity of HK was induced more by sucrose than by glucose, while FK remained inactive after glucose addition. Moreover, in situ investigation of the activities of HK and FK showed that OA-induced and 6-DMAP-induced repression of the cell cycle revival is connected with the interference of these drugs in the metabolic pathway of sucrose. It was also indicated that stronger OA-induced and 6-DMAP-induced inhibition of the replication and mitosis revival, at the early stages of sucrose regeneration, was correlated with the stronger influence of these inhibitors on HK and FK activities.

Introduction

Plant meristematic cells are equipped with a complicated network of mechanisms that coordinate metabolic processes with dividing function of meristematic tissue in response to external conditions. One possible mediator of these events is sucrose, the major transported product of photosynthesis, which in non-photosynthetic tissues is stored in vacuoles and is used up in the processes of cell respiration or changed into structural and storage polysaccharides or sugar derivatives (ap Rees 1988, Ciereszko 2006, Koch 2004, Quick and Schaffer 1996). Sucrose is also a signaling and regulatory molecule because it may

Abbreviations – 6-DMAP, 6-dimethylaminopurine; CDK, cyclin-dependent kinases; FITC, fluorescein isothiocyanate; FK, fructokinase; G6PDH, glucose-6-phosphate dehydrogenase; HK, hexokinase; NBT, nitroblue tetrazolium salt; OA, okadaic acid; PCP, principal control points; PGI, phosphoglucose isomerase; PP1/PP2A, protein phosphatases 1 or 2A; SuSy, sucrose synthase; WM, White's medium; WMG, White's medium containing 1% glucose; WMS, White's medium containing 2% sucrose.