Plant Physiology and Biochemistry 113 (2017) 20–31

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Glucose and sucrose differentially modify cell proliferation in maize during germination

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ARTICLE INFO

Article history:
Received 18 May 2016
Received in revised form
19 December 2016
Accepted 20 January 2017
Available online 21 January 2017

Keywords:
CDKs
Cell cycle
Cyclins
Germination
Glucose
Maize
Sucrose

ABSTRACT

Glucose and sucrose play a dual role: as carbon and energy sources and as signaling molecules. In order to address the impact that sugars may have on maize seeds during germination, embryo axes were incubated with or without either of the two sugars. Expression of key cell cycle markers and protein abundance, cell patterning and *de novo* DNA synthesis in root meristem zones were analyzed. Embryo axes without added sugars in imbibition medium were unable to grow after 7 days; in sucrose, embryo axes developed seminal and primary roots with numerous root hairs, whereas in glucose axes showed a twisted morphology, no root hair formation but callus-like structures on adventitious and primary seminal roots. More and smaller cells were observed with glucose treatment in root apical meristems. *de novo* DNA synthesis was stimulated more by glucose than by sucrose. At 24 h of imbibition, expression of *ZmCycD2;2a* and *ZmCycD4;2* was increased by sucrose and reduced by glucose. *CDKA1;1* and *CDKA2;1* expression was stimulated equally by both sugars. Protein abundance patterns were modified by sugars: ZmCycD2 showed peaks on glucose at 12 and 36 h of imbibition whereas sucrose promoted ZmCycD3 protein accumulation. In presence of glucose ZmCycD3, ZmCycD4 and ZmCycD6 protein abundance was reduced after 24 h. Finally, both sugars stimulated ZmCDKA protein accumulation but at different times. Overall, even though glucose appears to act as a stronger mitogen stimulator, sucrose stimulated the expression of more cell cycle markers during germination. This work provides evidence of a differential response of cell cycle markers to sucrose and glucose during maize germination that may affect the developmental program during plantlet establishment.

1. Introduction

Seed germination includes the chain of events occurring in a seed after water uptake and before root protrusion with the purpose of producing a mature plant able to reproduce and give rise to a new generation. After water uptake a plethora of metabolic events are turned on, among them, the onset of the cell cycle that has a profound impact on a successful germination completion. The cell cycle comprises four general phases, G1, S (Synthesis), G2 and M (Mitosis) phases, with three main control points along the process: the first one in the transition of G1 to S; the second in the G2 to M transition and the third one during the metaphase to anaphase transition in Mitosis. These control points require the kinase activity of a heterodimeric complex formed by a member of a family of proteins named Cyclins (Cyc) and a member of a family of proteins named Cyclin-Dependent Kinases (CDKs, Inzé and De Veylder, 2006).

Plant Cycs have been classified into different types (A, B, C, D, H, L, T, P, Q and SDS). In general, those involved in G1 and S phase (CycDs) are responsive to signals derived from nutritional status and hormones as well as growth rate or cell size (Nieuwland et al., 2007). CycDs are unstable proteins and some of them show

Abbreviations: CDK, cyclin dependent kinase; Cyc, cyclin; Glc, glucose; HAI, hours after imbibition; RAM, root apical meristem; RE, responsive element; Suc, sucrose.

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http://dx.doi.org/10.1016/j.plaphy.2017.01.018
0981-9428/

fluctuating protein levels with a typical abundance peak between G1 and S phases. They can be regulated at different levels: transcriptional, translational, posttranslational, and also by their stability, association with other proteins and cellular localization (Nieuwland et al., 2007). A bioinformatic analysis showed that in maize there are 17 CycD genes within its genome; 15 of them are expressed differentially during germination as well as under the influence of hormones, suggesting they could have non-redundant functions (Buendía-Monreal et al., 2011).

CDKs are the active partners of Cycs and are serine/threonine protein kinases able to phosphorylate several substrates (Inzé and De Veylder, 2006), among them the retinoblastoma-related protein (RBR) and Histone H1. In plants, two CDK types, A and B, have been particularly studied. A-type CDKs present in their sequence a canonical PSTAIRE motif whereas B-type CDKs, exclusive of plants, contain a divergent sequence, either PPTALRE or PPTTLRE. CDKsA have been linked to cell cycle control at the G1/S and G2/M transitions in association mainly with CycDs (Inzé and De Veylder, 2006), whereas CDKBs have been associated to G2/M transitions in partnership with A or B-type Cycs. CDK activity can be regulated either by positive or negative phosphorylation, cellular localization or interaction with other proteins (Dudits et al., 2007).

Cell cycle control plays an essential role for a successful germination and seedling establishment. Different and complex regulatory levels converging along germination are orchestrated. By instance, the transcriptional regulation of cell cycle related genes is essential to coordinate cell cycle progression with the morphogenetic program, as well as with environmental and nutritional inputs (Sablowski and Dornelas, 2013). The nutritional status plays an important role in germination. A seed cannot germinate without proper carbon and energy inputs from storage tissues. Among nutrients, sugars have been recognized as dual components: as carbon and energy donors as well as signaling molecules. Soluble sugars could have a differential impact on germination by activating or blocking key processes. It is known that hexoses like glucose (Glc) in plant tissues trigger cell division, whereas sucrose (Suc) favors differentiation and maturation (Koch, 2004; Gibson, 2004; Eveland and Jackson, 2011; Wang and Ruan, 2013).

During *Vicia faba* seed development, high Glc concentrations were found in non-differentiated cotyledon regions with high mitotic index. In contrast, high Suc concentrations were found on starch accumulating and expanding cells (Borisjuk et al., 2003; Eveland and Jackson, 2011).

Cell cycle-related markers can be regulated at different levels by sugars: in Arabidopsis suspension cultures, the expression of *CycD2* and *CycD3* was stimulated by Glc and Suc; in the presence of Suc, kinase activity in Cycs D2 and D3/CDK complexes increased up to six times from a threshold level (Riou-Khamlichi et al., 2000). Similarly, in maize embryo axes *CycD2;2* expression was stimulated by Suc (Gutiérrez et al., 2005) and in tobacco BY-2 cells the expression of *CycD2;1, D3;2, A3;2 and B1;2* increased in parallel to Glc concentration and the length of S and G2 phases could be modified by varying Glc levels (Hartig, 2005; Hartig and Beck, 2006). This regulation could be due, at least partially, to the presence of the corresponding Responsive Elements (RE) on the promoter region of these cell cycle genes.

Incidentally, recent reports indicate that Target Of Rapamycin kinase (TOR kinase) plays a critical role in Glc signaling by activating the expression of different biosynthetic pathways. It was found that Glc, via TOR, activates cell division in root meristems, being the E2F transcription factor the target for TOR kinase thus activating S-phase genes for cell cycle entry (Xiong et al., 2013). TOR is a master regulator that coordinates nutrient and energy availability as well as environmental signals with growth, development and survival. Apparently TOR senses and transduces Glc signals to control root meristem proliferation (Xiong and Sheen, 2014).

The aim of the present work is to assess the impact of adding Suc and Glc to germinating maize seeds: embryo axes morphology, cell patterning and *de novo* DNA synthesis at RAM, and on cell cycle markers at gene expression and protein abundance levels.

2. Materials and methods

2.1. Materials and treatments of maize embryo axes

Protease inhibitor cocktail tablets (Complete) were purchased from Roche (Indianapolis, IN, USA). Western chemiluminescent horseradish peroxidase substrate kit and Immobilon polyvinylidene fluoride membranes were purchased from Millipore (Billerica, MA, USA); anti-rabbit IgG-horseradish peroxidase conjugate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Caryopses of *Zea mays* cv. Chalqueño (an open pollination genotype, harvested in 2014 and 2015) were acquired at Chalco, Estado de México, México. Intact embryo axes were dissected manually from the dry caryopsis removing the scutellum (the single cotyledon embryo) with a scapel.

To follow germination (time from onset of imbibition), embryo axes (10 embryos per treatment) were disinfected by submergence in sodium hypochloride (0.5% v/v) for 15 min with occasional agitation, and rinsed four times 5 min each with sterile distilled water. Embryo axes were incubated for 6, 12, 18, 24, 30 or 36 h at 25 °C or up to 7 days in the dark on Whatman filter paper No. 1 with imbibition buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$ and 120 mM Glc or 120 mM Suc, or without a carbon source (Control axes) under sterile conditions. For *de novo* DNA synthesis analysis embryo axes were incubated as before with a slight modification: four hours prior harvesting, axes were changed to imbibition medium plus 120 μM EdU (5-ethynyl-2′-deoxyuridine), vacuum was applied 3 times 10 s each with a recovery period of 1 min and axes were put back in fresh imbibition medium without EdU (Kotogány et al., 2010).

2.2. Protein extraction

Protein extracts were produced by grinding axes at 4 °C with a polytron (Janke & Kunkel, Ultra-Turrax) on extraction buffer containing 25 mM Tris-HCl (pH 7.5), 15 mM MgCl2, 25 mM KCl, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.2% Triton X-100, 30% (v/v) glycerol, 60 mM β-glycerol phosphate, 50 mM NaF, 200 μM Na_3VO_4, 1 mM EGTA, a mini tablet of protease inhibitor cocktail/15 mL. Protein extracts were centrifuged at 16 000 g for 1 h at 4 °C. Protein concentration was determined by the BCA method (Stoscheck, 1990).

2.3. Total RNA extraction and semi quantitative RT-PCR

Total RNA was extracted from 10 dry embryo axes or imbibed for 12 and 24 h using Trizol reagent (Invitrogen, Carlsbad, CA) following supplier's instructions. Synthesis of cDNA and PCR were performed with 2.5 μg of total RNA by using a two-step kit. Reverse transcriptase (M-MLV Reverse transcriptase, Promega, Madison, WI) reaction was performed after DNA degradation utilizing DNase I (Sigma-Aldrich, St, Louis, MO).

PCR reactions were performed with specific primers (Buendía-Monreal et al., 2011) from cDNA equivalent to 100 ng of total RNA following manufacturer's instructions (JumpStart, Sigma-Aldrich). The PCR cycle number in the linear range was as reported by Buendía-Monreal et al. (2011) or empirically determined.

PCR products not reported before were sequenced to confirm