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Expression of Cell Wall Invertase from Maize Cell Suspension Culture after a Long Term Chelation of Extracellular Calcium

By

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With 4 figures

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Summary

DERMASTIA M. & CHOUREY P. S. 2001. Expression of cell wall invertase from maize cell suspension culture after a long term chelation of extracellular calcium. – *Phyton* (Horn, Austria) 41(1): 63–74, with 4 figures. – English with German summary.

The cell wall-bound form of invertase (EC 3.2.1.26) from a maize cell suspension culture was affected by removal of extracellular calcium, over time, at RNA, protein and enzyme activity level. The increase in enzyme activity was detected in cells treated with 5 mM EGTA, which preferentially chelates extracellular Ca^{2+} . Although the enzyme activity of EGTA-treated cells was low and similar to the control after 24 hours of incubation, it started to increase soon thereafter. It reached the maximum after 96 hours, when a decline of invertase activity had already been detected in the control samples. The amount of the invertase protein corresponds to its determined specific activity. Calcium depletion also induced *Incw1* mRNA accumulation, and the concentration of *Incw1* mRNA in EGTA-treated cells was 1.7 times higher than in the control after the first 24 hours. It remained high in the next 24 hours and slowly decreased throughout a 96 hour-incubation. At the end of the experiment the transcript

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level decreased and reached that of the control. The possible association of the cell wall invertase with the cell wall-bound calcium is discussed.

Zusammenfassung

DERMASTIA M. & CHOUREY P. S. 2001. Die Expression der Zellwandinvertase in Maissuspensionskulturen nach einer Langzeitkomplexierung von extrazellulärem Kalzium. – *Phyton* (Horn, Austria) 41(1): 63–74, mit 4 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die zellwandgebundene Invertase (EC 3.2.1.26) einer Maiszellsuspensionskultur wurde durch Entfernen von extrazellulärem Kalzium eine Zeit lang, auf der RNA-, Protein- und Enzymaktivitätsebene beeinflusst. Der Anstieg der Enzymaktivität konnte in Zellen, welche mit 5 mM EGTA behandelt wurden und welches vornehmlich extrazelluläres Ca^{2+} komplexiert, beobachtet werden. Obwohl die Enzymaktivität von EGTA-behandelten Zellen gering und ähnlich der von Kontrollen nach 24 h Inkubation war, begann eine Zunahme bald darauf. Sie erreichte ihr Maximum nach 96 h, als bei der Kontrollprobe bereits eine Abnahme in der Invertaseaktivität festzustellen war. Die Menge an Invertaseprotein entspricht der bestimmten spezifischen Aktivität. Der Entzug an Kalzium induzierte auch eine Incw1 mRNA Akkumulation und die Konzentration vom Incw1 mRNA war in EGTA-behandelten Zellen um $1,7 \times$ höher als in den Kontrollen nach den ersten 24 h. Die Menge blieb in den nächsten 24 h hoch und nahm im Laufe einer 96stündigen Kultur wieder ab. Am Ende des Experiments sank der Transcriptlevel und erreichte den der Kontrollen. Die mögliche Verbindung der Zellwandinvertase mit dem zellwandgebundenen Kalzium wird diskutiert.

Abbreviations: Incw1, cell wall invertase 1; EGTA, ethylene glycol – bis (β -aminoethyl ether).

Introduction

The hydrolytic enzyme invertase (β -fructofuranosidase, EC 3.2.1.26) is responsible for the irreversible cleavage of sucrose to glucose and fructose. It has been studied in many plants, including maize. At least two forms of the enzyme, soluble and particulate, are a common feature to all invertases. The soluble form is predominantly localized in vacuole and cytoplasm. The particulate form is ionically bound to the cell wall as cell wall-bound form, and is extractable with a high salt concentration (STURM & CHRISPEELS 1990, VON SCHAEWEN & al. 1990, WEIL & al. 1994). Each of the two forms has several isozymes (JAYNES & NELSON 1971). Although at the molecular level a number of invertase genes encoding both forms of the enzyme have been analyzed from a diverse group of plant species (STURM & CHRISPEELS 1990, ELLIOT & al. 1993, RAMLOCH-LORENZ & al. 1993, GREINER & al. 1995, ROITSCH & al. 1995, WEBER & al. 1995), their physiological role in various tissues is not well understood. It is believed to be in the sucrose partitioning between the source and sink regions of the plant (ESCHRICH 1980).

The possibility that higher plant genes could respond to sugar levels has been demonstrated by the effects of carbohydrates on enzymes involved in sugar metabolism in different plants and tissues (EHNESS & al. 1997, HOUSLEY & POLLOCK 1985, MITSUI & ITOH 1997, OHTO & al. 1995). Among those enzymes, the cell wall invertase in maize cell suspension culture was shown to be inducible by high levels of sucrose (CHENG & al. 1999). On the other hand, sucrose deprivation in cultured rice cells for 18 hours stimulates a marked accumulation of Ca^{2+} , whereas the addition of sucrose causes its reciprocal decrease (MITSUI & ITOH 1997).

Here we tested whether the expression of cell wall invertase from maize cell suspension cultures was affected by the calcium concentration.

Materials and Methods

Maize cell suspension culture

The Blade Mexican Sweet maize suspension-cultured cells were maintained as described (CHOUREY & ZURAWSKI 1981). Five days after subculturing, the cells were collected and aliquoted into fresh media supplemented with or without EGTA.

For sucrose starving experiment an aliquot of subcultured cells was washed three times with a medium from which sucrose had been removed and transferred to fresh corresponding medium without sucrose.

24- to 96-hour old cells were harvested, frozen in liquid nitrogen and stored at -80 °C.

Chemicals

Stock solution for EGTA (Sigma) was prepared at 1 M concentration in 10 % ammonium hydroxide. The solution was filter sterilized prior to addition to the culture media and used at the 5 mM final concentration.

Invertase isolation

Protein extracts of 24- to 96-hour-old control or treated cell suspension cultures were prepared. Cells were harvested by filtration and homogenized using 0.5 g of fresh weight to 5.0 ml of cold grinding buffer (0.05 mM Tris-maleate, 1mM DTT, pH = 7.0), then centrifuged at $14,000 \times g$ for 10 min. The pellet was washed three times with the extraction buffer and resuspended in 1 ml of 1M NaCl, vortexed in an Eppendorf mixer (model 5432; Eppendorf Corp., Madison, WI) for 30 min at 4 °C, and subsequently centrifuged at $14,000 \times g$ for 10 min. The supernatant presented a cell wall fraction was dialyzed against the extraction buffer at 4 °C for 1 hour or overnight and subsequently used in the enzyme assay.

Invertase enzyme assay

Invertase activity was measured as described (MILLER & CHOUREY 1992) as a rate of sucrose cleavage. The subsequent products of the reaction, reducing sugars, were estimated using the method described by Nelson (NELSON 1944). All measurements were performed in triplicate.

Protein concentration measurement

Protein concentrations were estimated using the Bio-Rad DC Protein Assay. BSA was used as the standard.

SDS-PAGE and protein blot analysis

SDS-polyacrylamide mini-gels (12%) were prepared (LAEMMLI 1970). Electrophoresis was performed according to the instruction manual for the Bio-Rad Mini-PROTEAN II Electrophoresis Cell.

Protein electroblotting on nitrocellulose membranes was performed as described in the instruction manual for the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell.

Antibody binding was detected via chemiluminescence using a SuperSignal CL-HRP Substrate System detection kit (Pierce) according to the instruction manual supplied. The used polyclonal antibodies were raised against maize Incw1 in mice and that against SS1 (ECHT & CHOUREY 1985) in rabbits. Primary antibodies were used at a 1 : 2000 dilution. Anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies were used at a 1: 1250 dilution.

RNA Analyses

Total RNA was isolated from 24- to 96-hour old samples as described (WADSWORTH & al. 1988) with a minor modification that increased the yield of the isolated RNA. After grinding frozen cells under liquid nitrogen in a precooled mortar, 200 mg of glass beads per gram of cells were added and then homogenized with 15 ml of extraction buffer (100 mM Tris, pH 9.0, 200 mM NaCl, 5 mM DTT, 1% sarcosyl, 20 mM EDTA, pH 8.0).

RNA samples were glyoxylated and fractionated on 1.2% agarose gels. RNA was transferred to Nytran membranes (Schleicher & Schuell) and prehybridized in 50 mM Pipes (pH 6.5), 100 mM NaCl, 50 mM sodium phosphate (pH 6.5), 1mM EDTA (pH 8.0), and 5% SDS. The blots were hybridized in the same solution with ^{32}P -labeled cDNA inserts corresponding to Incw1 (SHANKER & al. 1995) and Shl (GUPTA & al. 1988) probe (3×10^6 cpm ml⁻¹) overnight at 65 °C. Blots were washed twice for 45 min each in 6x SSC (0.5 mM EDTA, pH 8.0, 5 mM sodium phosphate, pH 6.5, and 5% SDS) and twice for 30 min each in 0.2x SSC in the same wash solution but with 1% SDS. The blots were exposed to x-ray film for 3 days at -80 °C with intensifying screens.

Results

Dry weight and protein content

Growth efficiency of cells in maize suspension culture was estimated by the dry weight of cells and the total amount of extracted protein. The treatment of cells with 5 mM EGTA had no effect on the measured parameters (Fig. 1).

Invertase activity of the cell wall fraction

Specific activity was expressed as μmol s of reducing sugars per mg per minute. That of the cell wall fraction from the control samples was stable

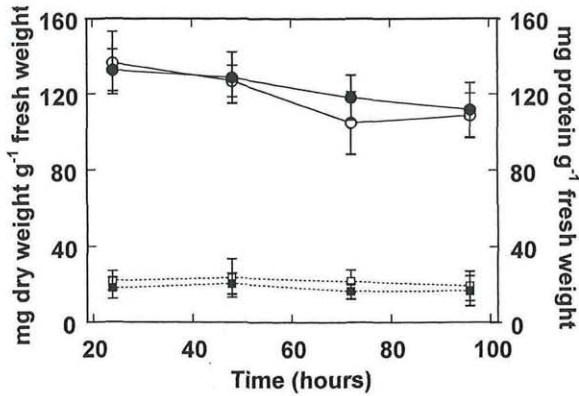


Fig. 1. Dry weight (solid line, circle) and protein content (dashed line, square) of maize suspension cultured cells expressed on fresh weight basis at various time of cultivation. Values are the means $\pm$ SEM (n=3). Open symbols, control samples; filled symbols, EGTA treated samples.

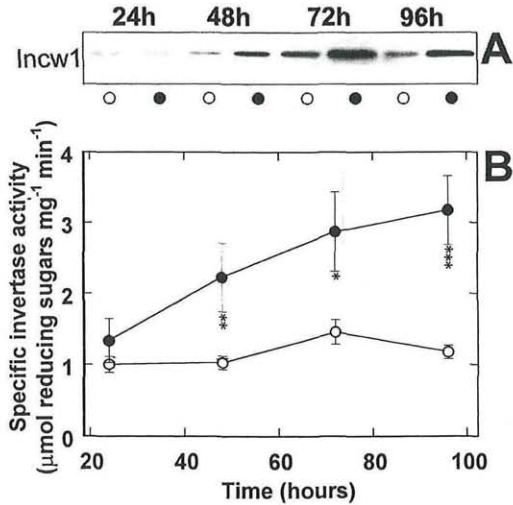


Fig. 2. Effects of treatment with 5 mM EGTA on the invertase (Incw1) protein and on the specific invertase activity of the cell wall fractions from maize cell suspension culture at various times of cultivation. Open symbols, control samples; filled symbols, EGTA treated samples. (A) Immunoblot analysis. Each lane contains 5 μ g of protein in crude extracts. (B) Specific invertase activity. Measurements represent the mean $\pm$ SEM (n is at least 5). Statistical significance is based on the Student's *t*-test. Significantly different from the control at (*) $P < 0.05$; (**) $P < 0.02$; (***) $P < 0.0001$.

from 24 to 48 hours of cultivation, after that time it only slightly increased from 1 to 1.5 μmol of reducing sugars per mg per minute and again decreased to 1.2 μmol of reducing sugars per mg per minute toward the end of cultivation (Fig. 2-B).

When the cells were grown in the presence of 5 mM EGTA, a steadily increasing invertase activity was detected after 24 hours of cultivation. The specific activity was significantly higher compared to the untreated control after 48 hours and from that time constantly increased (Fig. 2-B).

Immunodetection of Incw1

SDS-protein blot analysis of the cell wall fractions using polyclonal antibodies raised against Incw1 showed an increasing amount of the Incw1 protein in the EGTA-treated cells (Fig. 2-A) which was in correlation with the determined specific activity (Fig. 2-B).

RNA gel blot analysis

Figures 3-A(Incw1) illustrates a gel blot showing steady state levels of Incw1 RNAs from the control and EGTA treated samples at various stages of cultivation. The membrane was hybridized with the radiolabeled full-length cDNA clone of maize, Incw1. In the corresponding Fig. 3-B the mRNAs are presented as their relative levels.

In the control cells there was a steady increase of Incw1 message from 24 to 48 hours, the Incw1 mRNA concentration decreased significantly within next 24 hours and remained at the same level to the end of cultivation (Fig. 3-A,B).

However, calcium depletion induced Incw1 mRNA accumulation, and the concentration of Incw1 mRNA in EGTA-treated cells was 1.7 times higher then in the control after first 24 hours. It remained high during next 24 hours and subsequently slowly decreased. At the end of the experiment the transcript level reached that of the control (Fig. 3-A,B).

Figure 3-A(Sh1) shows the hybridization pattern with the Sh1 cDNA (gene locus Sh1 encodes sucrose synthase isozyme SS1), which was included as an internal loading control for comparison against Incw1 expression. Northern blot was made on the same membrane that was first hybridized with the Incw1 probe. There were no obvious changes in the level of Sh1 transcript in cells treated with EGTA.

Depletion of sucrose from the growth media and simultaneous addition of EGTA caused a substantial decrease in both smaller (Incw1-S) and larger (Incw1-L) Incw1 transcripts (Fig. 4).

Discussion

A cell-wall invertase from maize cell suspension cultures is encoded by the Incw1 gene, with two transcripts, Incw1-S (small) and Incw1-L

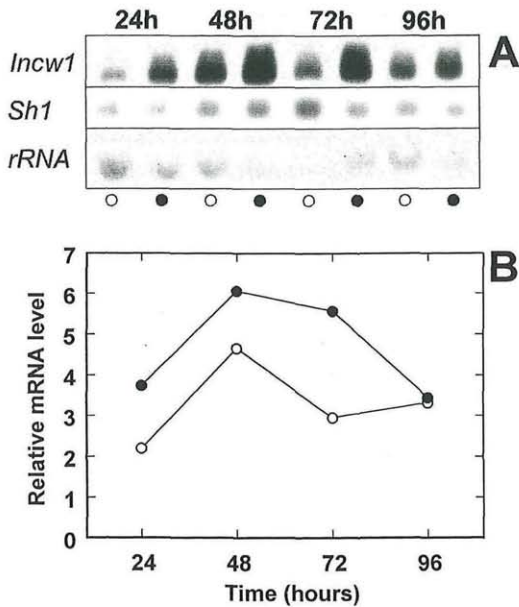


Fig. 3. Effects of treatment with 5 mM EGTA on the accumulation of invertase mRNA at different times of cultivation. Open symbols, control samples; filled symbols, EGTA treated samples. (A) 5 μ g of total RNA was loaded in each lane. RNA was subjected to Northern blot analysis using the cell wall invertase (*Incw1*) or sucrose synthase (*Sh1*) cDNA as a probe. *rRNA* transcript as a control of the equivalence of RNA loading among lanes. (B) Levels of mRNA from the same experiment as shown in A-*Incw1* were quantitated densitometrically.

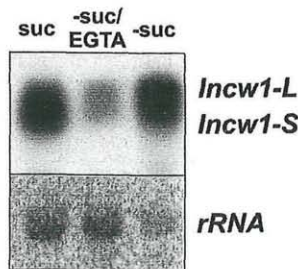


Fig. 4. Cell wall invertase mRNA accumulation after 48 hours of cultivation of maize cell suspension cultures. Cells were grown in normal medium (suc), sucrose-free medium without any treatment (-suc) or were exposed to 5 mM EGTA treatment in sucrose-free medium (-suc/EGTA). Their corresponding total RNA was isolated after 48 hours and subjected to Northern blot analysis using the cell wall invertase (*Incw1*) cDNA probe. 5 μ g of total RNA was loaded in each lane. *rRNA* transcript as a control of the equivalence of RNA loading among lanes.

(large), (CHENG & al. 1999). Both metabolizable and nonmetabolizable sugars induced Incw1-L RNA by default. However, only the metabolizable sugars, sucrose and D-glucose are associated with the steady-state abundance of Incw1-S RNA, the concomitant Incw1 protein and enzyme activity. Thus, a depletion of sucrose from the medium led to a time-dependent loss of Incw1-S RNA, protein, and enzyme activity (CHENG & al. 1999). On the other hand, it was shown that depletion of sucrose from a rice suspension culture provokes a significant influx of calcium into the cells (MITSUI & ITOH 1997). Accordingly, we hypothesized an increased invertase activity at low calcium concentrations.

In our experiment we treated maize cells with EGTA, which preferentially chelates extracellular Ca^{2+} (POOVIAH & REDDY 1987) associated with the cell wall in a substantial quantity (HIS & al. 1997). Although there are some reports of a requirement for high extracellular Ca^{2+} to sustain plant cell expansion and division (HEPLER & WAYNE 1985, JONES & al. 1998), the addition of extracellular Ca^{2+} to growing tissues inhibits cell extension, while Ca^{2+} removal can increase growth rates (LONG & al. 1997) and enhanced cell differentiation (SURI & RAMAWAT 1997). In maize cell suspension cultures there was no detectable change in the growth of cells as measured by their weight and protein content after the removal of calcium from the medium (data not shown), nor its chelation by EGTA in a 96 hour-period of cultivation (Fig. 1).

The removal of cell wall calcium, as predicted, caused an increase in cell wall invertase expression in mRNA, protein and enzyme activity levels (Fig. 2; 3-A,B).

According to the original hypothesis of increased calcium concentration in the sucrose starving cells (MITSUI & ITOH 1997) with repressed Incw1-S RNA (CHENG & al. 1999), we additionally tested a long-term effect of concurrent removals of extracellular Ca^{2+} and sucrose from the medium on the Incw1 transcripts. In contrast with the data obtained from the cells grown on the sucrose medium (Fig. 3), there was no induction of Incw1 RNA. Moreover, after 48 hours, when the effect of sucrose starvation on the repression of Incw1-S cell wall invertase transcript and derepression of Incw1-L was the most provoked (CHENG & al. 1999), (Fig. 4, -suc), both cell wall invertase transcripts were repressed in the presence of EGTA (Fig. 4, -suc/EGTA). Those observations might indicate that the long-term cell wall calcium removal did not overcome the expected influx of Ca^{2+} into the cells (MITSUI & ITOH 1997) or that cell wall invertase repression under the reduced availability of sugars was not associated with its observed high activity at low extracellular Ca^{2+} .

It has been proposed that inorganic cations, depending on whether they induce intermolecular cross-links or not, can play a significant part in the regulation of enzyme activities within the wall. The ion induced for-

mation of calcium linkages among pectic polysaccharides (HIS & al. 1997) reduces apple fruit softening by strengthening the cell wall (ROY & al. 1994) induces wall shrinkage (JARVIS 1992) makes the wall less extensible (NAKAJIMA & al. 1981) and makes cell wall substrates less accessible to hydrolytic enzymes (JAUNEAU & al. 1994). In epidermal cell walls of flax, calcium-induced cross-linking between pectin molecules reduced endopolygalacturonase mobility and activity within the wall (JAUNEAU & al. 1994). The relation between extracellular calcium and the cell wall degrading enzymes β -1,4 and β -1,3-glucanase was also demonstrated in the segments of hypocotyls from etiolated seedlings of *Phaseolus radiatus*. The activities of those enzymes were markedly decreased during the inhibition of elongation induced by the high calcium concentration (LONG & al. 1997). In the case of cell wall invertase, the high enzyme activity even at the time of slowly decreasing protein amounts (Fig. 2) might indicate higher protein mobility within the wall. The increased accessibility and motility could be a consequence of loosening of the wall induced by calcium removal as previously suggested for polygalacturonases from the tomato (RUSHING & HUBER 1990) and flax (JAUNEAU & al. 1994).

Acknowledgement

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Der Band gilt einem der bekanntesten und markantesten Berge (2139 m) in den südlichen Kalkalpen Kärntens. 29 Autoren trugen 26 Beiträge zu Geschichte und Naturgeschichte des Hochobirs bei. Den Anfang macht ein von Franz Xaver WULFEN verfaßter Bericht über eine Reise von Klagenfurt zum Obirgipfel im Jahre 1783; das Manuskript wird erstmals (größtenteils) abgedruckt und kommentiert. Wegen der durch den Bergbau bedingten, ganzjährigen Besiedlung des Berges (bis zum Gipfel) befanden sich hier die ältesten und lange Zeit auch die höchstgelegenen Wetterstationen der habsburgischen Monarchie (p. 83–94). Zur Geologie und Mineralogie (p. 97–160) sind vier Beiträge enthalten, die sich unter anderem auch mit den Hö-

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