H^+-Coupled Sugar Transporter, an Initiator of Sugar-induced Ca^{2+}-signaling in Plant Cells

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Z. Naturforsch. **60 c**, 764–768 (2005); received February 1/March 9, 2005

Using Ca^{2+}-dependent photoprotein aequorin-transformed tobacco BY-2 cell suspensions, the sugar-induced increase in cytosolic free Ca^{2+} concentration ($[Ca2+]_{cyt}$) was investigated by measuring the luminescence intensity. When 0.5 M sucrose or some other sugars were fed to the cells, strong and transient luminescence was observed. Salts or sugar analogues didn't show this effect. In addition, the intensity of sucrose-induced aequorin luminescence was gradually enhanced when cells were exposed to sugar-starvation. This was observed with the concurrent expression of the sucrose/H^+ co-transporter, *NtSUT1A*. The $[Ca^{2+}]_{cyt}$ increase may initiate Ca^{2+}-signaling leading to the expression of genes related to biosynthesis of storage carbohydrates in a sink organ. The sugar-signaling may play an important role in the conversion on nutritional stage of plant tissue, source organ to sink organ.

Key words: Sugar Transporter, Sugar-signaling, Ca^{2+}-signaling

Introduction

In plants, photosynthesized sugars are utilized not only as carbon sources but also as signal molecules for growth and development. In addition to the widely studied functions in metabolic processes, the signaling function of sugars has recently been focused on (Gibson, 2000; Koch, 1996; Sheen *et al.*, 1999). Using luminous *Arabidopsis* and tobacco plants or suspension cultured cells, a cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) increase coupled with a wide range of biotic and abiotic stimuli has been reported (Kawano *et al.*, 1998; Knight *et al.*, 1991). Treatment of tobacco leaf discs expressing aequorin with sucrose significantly reduced the level of cold-induced luminescence reflecting the total cellular level of aequorin (Ohto *et al.*, 1995). In leaves of *A. thaliana* expressing aequorin, the level of $[Ca^{2+}]_{cyt}$ was raised in response to sugar but not to unmetabolizable analogues of it (Furuichi *et al.*, 2001b). This response is observed in autotrophically grown plants, but not in heterotrophically grown plants, and the mRNA level of sucrose-H^+ symporters in the former is clearly higher than that in the latter. Choiu and Bush (1998) reported that the transcript level of a sucrose-H^+ symporter is specifically regulated by sucrose. The relationship between sucrose-H^+ symporters and the increase in $[Ca^{2+}]_{cyt}$ should be investigated to clarify the molecular mechanism and physiological role of sugar-signaling. Previously, we have reported that sucrose-induced luminescence, which reflects an increase in $[Ca^{2+}]_{cyt}$ in aequorin-expressing *Arabidopsis* leaves, was suppressed by antisense expression of sucrose-H^+ symporters AtSUC1 and 2 (Furuichi *et al.*, 2001a), implying that sucrose-H^+ symporters are the initiator of sugar-induced $[Ca^{2+}]_{cyt}$ increase. Although concentrations of sugars in leaf extracts from autotrophically grown plants were higher than that from heterotrophically grown plants (data not shown), we could not measure the exact concentrations of sugars in the phloem sap. Because of this, the sugar-dependent expression of sucrose-H^+ symporters and the relation to $[Ca^{2+}]_{cyt}$ increase is still unclear. In the present study, this relationship is further confirmed using tobacco BY-2 suspension cells. We could accurately measure the concentrations of sugars in the culture media, instead in the phloem sap, as

0939–5075/2005/0900–0764 $ 06.00

well as the sucrose-induced increase in $[Ca^{2+}]_{cyt}$, and the expression level of sucrose-H^+ symporters.

Materials and Methods

Chemicals

The chemically synthesized luminophore coelenterazine (Isobe *et al.*, 1994) was a generous gift from Prof. M. Isobe, Nagoya University. Murashige-Skoog salt mixture, myo-inositol and other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Plant material

Tobacco suspension culture cells (*Nicotiana tabacum* L. cv. Bright Yellow-2, cell line BY-2), which express apoaequorin specifically in the cytosol (Takahashi *et al.*, 1997), were used at the indicated days after subculturing. Cells were propagated as described (Kawano *et al.*, 1998).

Monitoring of $[Ca^{2+}]_{cyt}$

To monitor $[Ca^{2+}]_{cyt}$ in the transgenic BY-2 cells, the apoaequorin-expressing cells were incubated with 1 μM coelenterazine in the darkness for 8 h to reconstitute the Ca^{2+}-responsive luminescence protein aequorin. Luminescence reflecting the increase in $[Ca^{2+}]_{cyt}$ induced by sugars was measured with a Lumicounter 2500 luminometer and the evaluated by luminescence-curve analyzing program Ver. 1.01 (Microtech Nition, Funabashi, Japan).

Reverse transcriptase (RT)-PCR analysis of NtSUT1A expression in Arabidopsis

The total RNA was extracted from BY-2 cells after the indicated days of subculturing, with a FastRNA® Kit-GREEN, using a FastPREP machine (Bio 101., Carlsbad, CA, US.), according to the manufacturer's protocol and quantified spectrophotometrically. First-strand cDNA was synthesized from 5 μg of total RNA with a RNA PCR Kit (AMV) Ver.2.1 (Takara Co., Otsu, Japan), diluted 1:5, and used as the template for PCRs (30 cycles at 94 °C for 30 s, at 52 °C for 2 s, and at 72 °C for 80 s). The obtained cDNAs were used as the template for the PCR using 2 sets of synthetic primers to amplify the partial cDNAs of *NtSUT1A* and *NtACT1*, as internal control of cDNA libraries. The KOD dash DNA polymerase from Toyobo (Osaka, Japan) was used according to the manufacturer's protocol. PCR products were analyzed by agarose gel electrophoresis. To confirm the results, DNA sequencing was done using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster, CA, USA) and an ABI PRISM 310 DNA Sequencer (PE Applied Biosystems).

Results and Discussion

Sugar-induced increase in $[Ca^{2+}]_{cyt}$ in tobacco suspension cultured cells

To investigate the increase in $[Ca^{2+}]_{cyt}$, a bioluminescence-based assay is a powerful tool because it is extremely sensitive and free of background. Aequorin, a photoprotein originally isolated from jellyfish, comprises an apoaequorin protein and the luminophore coelenterazine. When Ca^{2+} binds to aequorin, coelenterazine is oxidized to coelenteramide, with a concomitant release of carbon dioxide and blue light (Takahashi *et al.*, 1997). Application of various kinds of sugars, NaCl and KCl (0.5 M) induced an immediate and transient increase in aequorin luminescence in the 7-day-cultured cells (Fig. 1). The luminescence was scarcely induced by 2-deoxyglucose, NaCl and KCl although the increases in osmolarity are equal or relatively higher than that by sugars. These results indicate that the increase in luminescence is spe-

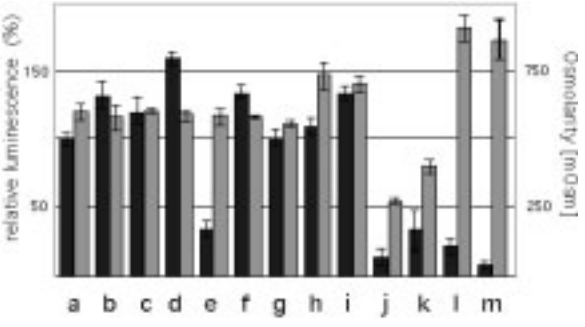

Fig. 1. Sugar-induced $[Ca^{2+}]_{cyt}$ increase in aequorin-expressing BY-2 cells. Relative intensities of luminescence are shown as solid bars. Comparison was made by expressing the peak height of glucose-induced aequorin luminescence as 100%. Increases in osmolarity after the application of each solution are shown as gray bars. Sugars and salts (0.5 M each) used are: a, glucose; b, galactose; c, mannose; d, fructose; e, 2-deoxyglucose; f, sorbitol; g, mannitol; h, sucrose; i, maltose; j, glycerol; k, dehydroxyacetone; l, NaCl; m, KCl. Bars represent standard deviations (n = 5).

cific for sugar species, as reported previously using *A. thaliana* intact plants (Furuichi *et al.*, 2001b). The sucrose-induced transient increase in aequorin luminescence reflecting $[Ca^{2+}]_{cyt}$ was caused dose-dependently (data not shown), indicating that the amount of sugar or H^+ influx may affect the response in $[Ca^{2+}]_{cyt}$.

Sugar-starvation-dependent increase of NtSUT1A transcripts and the intensity of $[Ca^{2+}]_{cyt}$ increase

Although we tried to amplify a couple of tobacco sucrose-H^+ symporters registered and available in the DNA data bases (http://www.ncbi.nlm.nih.gov/), only one of those predicted genes, *NtSUT1A* (Accession No. X82276), was expressed in BY-2 suspension cultured cells (data not shown). The intensity of a transient increase in aequorin luminescence reflecting $[Ca^{2+}]_{cyt}$ in response to sugars became gradually stronger for up to 14 d (Fig. 2A). Therefore the resulting sugar contents in the medium and the expression level of *NtSUT1A* were investigated in 3-, 7- and 14-day-cultured cells. When cells were cultured for 3 d, aequorin luminescence was scarcely induced by 0.1 M sucrose. In these cells, any *NtSUT1A* transcript wasn't detected by RT-PCR analysis, and sucrose in the culture medium remained although the contents of glucose was increased by the secreted or cell wall-bound invertase (von Schaewen *et al.*, 1990). After the 7-day-culturing, sucrose and glucose in the medium were already exhausted and the transcription of *NtSUT1A* was activated, corresponding to the enhancement of sugar-induced aequorin luminescence (Fig. 2). These results imply that the transcriptional activity of *NtSUT1A* and the intensity of sugar-induced increase in $[Ca^{2+}]_{cyt}$ are synchronized. Tökés-Füzesi *et al.* (2002) found that both transport and phosphorylation of hexose are necessary for the extracellular Ca^{2+} influx in the sugar-induced

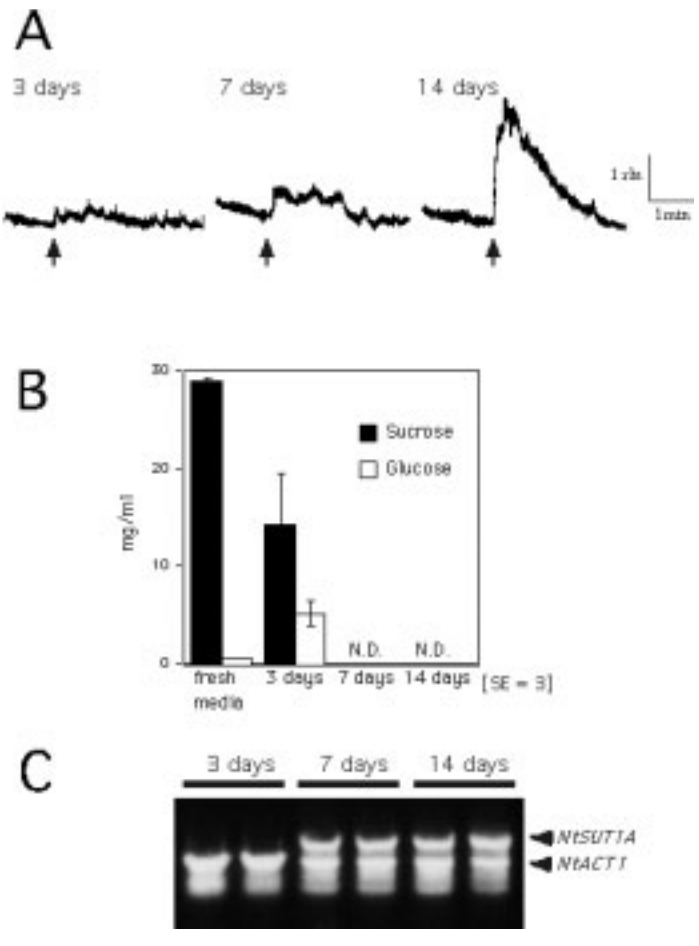

Fig. 2. Sugar-induced aequorin luminescence, levels of mRNA for *NtSUT1A* and sugar contents in tobacco suspension culture. Days after subculturing are noted. (A) Typical records of sucrose-induced aequorin luminescence in BY-2 cells. Arrows indicate the addition of 0.1 M sucrose as final concentration. rlu; relative luminescence unit. (B) Sugar contents in medium after culturing. Concentration of sucrose (solid bars) and glucose (open bars) were obtained with a commercial kit from Boehringer Mannheim Biochemicals GmbH (Mannheim, Germany) and used. (C) Effect of sucrose on levels of mRNA for *NtSUT1A* in tobacco BY-2 suspension cells. Products of RT-PCR amplification were electrophoresed and visualized by ethidium bromide-staining.

$[Ca^{2+}]_{cyt}$ increase in yeast cells. The activation mechanism of a Ca^{2+} channel for Ca^{2+} influx was also well discussed. In rat pancreatic β-cells, a volume-sensitive anion channel is activated by glucose, and following depolarization of plasma membrane was observed (Best, 2002). In these cells, Ca^{2+} influx through a depolarization-activated Ca^{2+} channel is caused. In comparison, Guzmán-Grenfell *et al.* (2000) reported that glucose induces a Na^+,K^+-ATPase-dependent transient hyperpolarization in human sperm, implying that both depolarization and hyperpolarization and following activation of voltage-gated Ca^{2+} channels may occur in response to sugars. *In planta*, a putative voltage-gated channel of *Arabidopsis* (AtTPC1; Furuichi *et al.*, 2001a) and homologous proteins in BY-2 (NtTPC1A and B; Kadota *et al.*, 2004) and rice (OsTPC1; Hashimoto *et al.*, 2004) were identified. Although both depolarization-activated and hyperpolarization-activated Ca^{2+} currents were recorded by electrophysiological analysis (Piñeros and Tester, 1997 and referenced therein), the activation mechanism of the TPC1 family is still unknown. Overexpression of AtTPC1 promoted a sucrose-induced increase in $[Ca^{2+}]_{cyt}$ in *Arabidopsis* and tobacco BY-2 cells (Furuichi *et al.*, 2001a; Kawano *et al.*, 2004), implying that further electrophysiological studies on TPC1 channels may give the evidence for the molecular mechanism of sugar-signaling in detail.

Photosynthesized sugars are transported from mature leaves (source organ) to the sink organ, such as fruits. Although premature leaves also percept sugars from mature leaves like sink organ, it may change to source organ according to the growing and development of photosynthetic activity. This is a sink to source conversion. In some species without fruit or specific storage organs such as *Arabidopsis*, photosynthesized sugars are converted to starch and stored in elder leaves, with lowering photosynthetic activity. As a general character, sucrose-H^+ symporters promote sugar influx and efflux under control of sucrose and H^+ gradients across the plasma membrane (Carpaneto *et al.*, 2004).

We conclude that sugar-deprivation induced the expression of sugar-H^+ symporters, and it is a trigger of sugar-induced $[Ca^{2+}]_{cyt}$ increase by following discussion. When the photosynthetic activity in a leaf is lowered by aging, the concentration of sucrose in the cytosol also reduced. As a following event, the expression of sugar-H^+ symporters in the leaf is activated. In contrast, the concentration of sucrose in phloem sap is maintained constant, so the gradient of sugar concentration across the plasma membrane is reversed and the direction of the sugar flux too. Then the reversed sugar-influx promotes the $[Ca^{2+}]_{cyt}$ increase, and induces the expression of sink-specific gene products. Further analysis of down-stream components of Ca^{2+} signaling triggered by sugars is necessary to elucidate the mechanism of sugar-signaling in plants.

Acknowledgement

The authors acknowledge Prof. A. J. Trewavas for permitting the use of aequorin-expressing plant material. The authors also thank Prof. M. Isobe for chemically synthesized coelenterazine. This work is supported by JSPS Research Fellowship for Young Scientists (15000639) to T. F. from the Japanese Society for the Promotion of Science.

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