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Nitrate and Ammonium-induced Ca^{2+} Influx in *Arabidopsis* Leaf Cells

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In plants, nitrogen is utilized as amino acids and other nitrogen compounds for plant growth and development. Interestingly, nitrate and ammonium nitrate (NO_3^-) and ammonium (NH_4^+) play not only as nitrogen source but as signal molecules. To investigate the signaling pathway, the changes in cytosolic free Ca_2^+ concentration ($[Ca^{2+}]_c$), one of the major second messenger, was monitored. The nitrate-induced increase in $[Ca^{2+}]_c$ was not observed when plants were grown on the synthetic medium deficient of nitrate, hence ammonium-induced increase in $[Ca^{2+}]_c$ was comparably observed when applied in high concentration. According to the presented results, the role of $[Ca^{2+}]_c$ increase in nitrogen metabolism is discussed.

Key words: Ca^{2+} signaling, Nitrate, Ammonium, H^+-coupled transporter, Bio-imaging

Introduction

In plants, nitrate is assimilated via its reduction by nitrate reductase and metabolized to produce amino acids and other nitrogen compounds for plant growth and development. Recent studies revealed that nitrate is not only an important nitrogen source but also as signal molecule to control plant development and metabolism (Wang *et al.*, 2003). Nitrate has been known to stimulate seed germination in various plant species including *A. thaliana*, and recently Alberesi *et al.* (2005) revealed that *NRT1. 1*, H^+-coupled nitrate transporter (NRT), may be involved in conveying the nitrate into the seeds as signal molecule. With a great similarity to nitrate, the signaling function of sugars also has been focused on (Gibson, 2000; Koch, 1996; Sheen *et al.*, 1999) and the importance of Ca^{2+}-signaling in sugar-signaling was indicated (Ohto *et al.*, 1995). Calcium ion (Ca^{2+}), one of the major necessary elements in living cells, plays an essential role as an intracellular second messenger in plants. Basically, the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) is strictly lowered by continuous export of Ca^{2+} by Ca^{2+}-ATPase and H^+/Ca^{2+} antiporter. When Ca^{2+}-permeable channel(s) are activated in response to a variety of external and internal stimuli, the entrance of only a small amount of Ca^{2+} causes a drastic increase in the $[Ca^{2+}]_c$. Then the entered Ca^{2+} may bind to Ca^{2+}-regulated proteins such as calmodulin (CaM), calcium dependent protein kinase (CDPK) and modify these activities or affinities of these proteins in binding to their specific targets. These signal transduction pathways mediated by transient or spontaneous changes in the $[Ca^{2+}]_c$, termed Ca^{2+} signaling, are mainly initiated by the activation of Ca^{2+}-permeable channels. To investigate the in-

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crease in $[Ca^{2+}]_c$, bioluminescence-based assay is a powerful tool because it is extremely sensitive and free of background based on the cytosol specific expression of apoaequorin. Aequorin, a photoprotein originally isolated from jellyfish comprises an apoaequorin protein and the luminophore, coelenterazine. For the aequorin-based Ca^{2+}-monitoring, the transgenic plants and cultured cells which expressing apoaequorin were established and then chemically synthesized coelenterazine were applied to reconstitute the aequorin complex. When Ca^{2+} bind to the aequorin, coelenterazine is oxidized to coelenteramide, with a concomitant release of carbon dioxide and blue light (Takahashi *et al.*, 1997). Using the *Arabidopsis* and tobacco plants or suspension cultured cells expressing apoaequorin, $[Ca^{2+}]_c$ increases coupled with a wide range of biotic and abiotic stimuli has been reported (Kawano *et al.*, 1998; Knight *et al.*, 1991). In leaves of *A. thaliana* expressing apoaequorin, the sugar-induced transient increase in $[Ca^{2+}]_c$ was monitored (Furuichi *et al.*, 2001a and b). Choiu and Bush (1998) reported that the transcript level of a sucrose-H^+ symporter is specifically regulated by sucrose. Thus, sucrose-induced $[Ca^{2+}]_c$ increase in *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 $[Ca^{2+}]_c$ increase. Furthermore, the intensity of sucrose-induced increase in $[Ca^{2+}]_c$ and the expression level of sucrose-H^+ symporters (*NtSUT1A*) were nicely synchronized in tobacco BY-2 suspension cells (Furuichi and Muto, 2005).

When the plant NRT transport one molecule of nitrate, two H^+ are co-transported into the cells, same as in sucrose-H^+ symporters which co-transport one sucrose and one H^+. In addition, the expression levels of nitrate transporter family genes were reported that to be nitrate inducible and nitrate starvation causes the reduction of NRT expression (Tsay *et al.* 1993). In the present study, we monitored the image of aequorin luminescence in *Arabidopsis* excised leaves using an ultra-sensitive photo-imaging system and showed that nitrate generates a transient increase in $[Ca^{2+}]_c$, and the relation to the NRT are discussed to clarify the signaling and pathway for nitrate metabolism.

Materials and Methods

Chemicals

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

Arabidopsis thaliana ecotype Wassilewskija transformed with apoaequorin cDNA by binary vector system (Furuichi *et al.*, 2001b) was used. The binary vector pHAQ, containing apoaequorin cDNA on the downstream of cauliflower mosaic virus 35S promoter was transformed to root-derived calli with *Agrobacterium tumefacience* EHA101. pHAQ possesses hygromycin phoshpotransferase and apoaequorin genes independently driven by CaMV 35S promoter. The regenerated plants were selected for hygromycin B resistance. Seeds of homozygous luminous plant were sown on 0.3% gellan gum plate containing GM medium (Valvekens *et al.*, 1988) with 1% sucrose and grown at 22℃ under continuous light at 4,000 lux with day-light fluorescent lamps. Two-week-old intact plants were transplanted onto fresh plates containing GM medium (GM plants), GM-nitrate medium deficient of nitrate (GM-NO_3 plants). Transplanted plants were grown further at least for 1 week under the same condition. In order to reconstitute aequorin, excised leaves were floated overnight on liquid grown medium con-

taining 2.5 μM coelenterazine in the dark.

Monitoring of $[Ca^{2+}]_c$

The $[Ca^{2+}]_c$-dependent aequorin luminescence intensity of excised leaves were measured with with a Lumicounter 2500 luminometer (Microtech Nition, Funabashi, Japan) or a ultra-sensitive VIM camera system (a charge coupled device camera equipped with an intensifier, Model C-1400-47; Hamamatsu Photonics Co., Hamamatsu, Japan). Excised leaves were put into cylindrical plastic cuvettes (diameter, 1 cm) or wells of 24-well titer plate respectively, both containing 500 μl of appropriate liquid media to immerse the leaves. One-fifth volume of media containing potassium nitrate (KNO_3, ammonium chloride (NH_4Cl) was injected and the luminescence reflecting the increase in $[Ca^{2+}]_c$ was measured. The intensity of aequorin luminescence was recorded real time using luminometer and accumulated for 5 min to record the total luminescence counts in response to nitrate and ammonium.

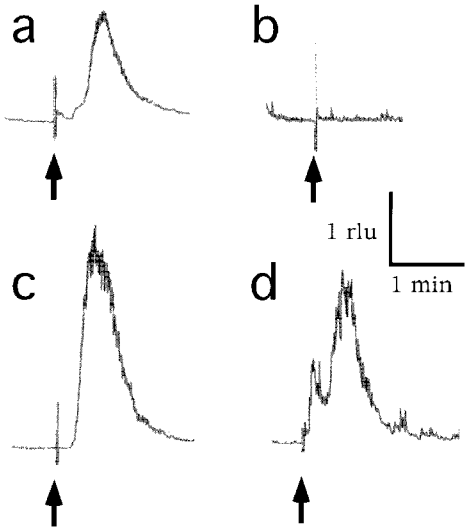

Fig. 1. Nitrate and ammonium induced the transient and immediate increase in $[Ca^{2+}]_c$ in Arabidopsis leaf cells.
Aequorin luminescence indicating $[Ca^{2+}]_c$ in excised leaves of GM plants (a and c) and GM-NO_3 plants (b and d) are recorded by luminometer. Arrows indicate the time for the application of nitrate (a and b) and ammonium (c and d). rlu: relative luminescence intensity.

Results

Nitrate-induced transient $[Ca^{2+}]_c$ increases in excised leaves of *Arabidopsis*

To investigate the signaling effect of nitrate, the changes in $[Ca^{2+}]_c$, one of the major phenomena in subcellular signal transduction, were monitored in *Arabidopsis* excised leaves expressing apoaequorin with the stimulation with nitrate (Fig. 1). The intensity of aequorin luminescence, reporting $[Ca^{2+}]_c$ is immediately and gradually increased after the application of 0.1 M and reached its maximum value within 1–2 min and gradually decayed within 5 min (Fig. 1a). The accumulated counts of intensity of nitrate induced changes in $[Ca^{2+}]_c$ were reduced in dose dependently (Fig. 2a). Remarkably, nitrate-induced increase in aequorin luminescence didn't observed in leaves excised from GM-NO_3 plants (Figs. 1b and 2a). There is no differences in aequorin luminescence between GM and GM-NO_3 plants in response to the application of same concentration (0.1 M) of sugars and salts (data not shown), indicate that above phenomena is a nitrate specific effect and not due to the effect of the changes in osmolarity or other cationic or anionic concentration. In general, nitrate is uptaken by nitrate transporters (NRTs), sorts of H^+-coupled transporters. Because of the positive charge of $2H^+$ for 1 nitrate (NO_3^-), nitrate uptake *via* NRTs causes the transient depolarization in plasma membrane of the cells (Huang *et al.*, 1999). The expression levels of NRTs were reduced by long term (around 1 week) nitrate starvation as reported previously (Tsay *et al.*, 1993), indicating that NRTs were not expressing in GM-NO_3 plants and may play important role(s) in nitrate-induced $[Ca^{2+}]_c$.

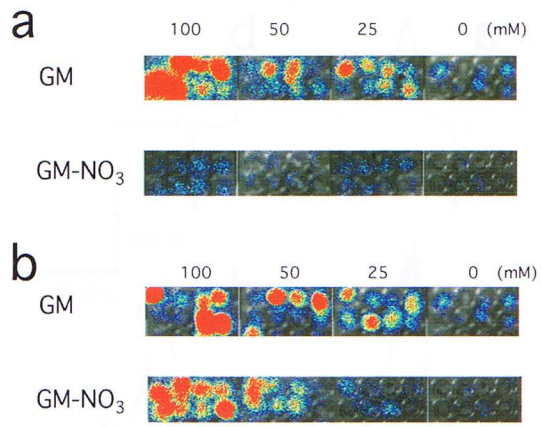

Fig. 2. Dose dependency of nitrate and ammonium induced increase in $[Ca^{2+}]_c$.
Aequorin luminescence indicating $[Ca^{2+}]_c$ in excised leaves of GM plants and GM-NO_3 plants are recorded by ultrasensitive CCD camera equipped with VIM intensifier. Nitrate (a) and ammonium (b) were applied to the excised leaves putted in each well of 24-well culture dishes. Aequorin luminescence was accumulated for 5 min since the stimulation. Luminescence images were superimposed on its blight images. Luminescence intensity was indicated by pseudo color (strength order: red, yellow, green, blue).

Ammonium-induced transient $[Ca^{2+}]_c$ increases in excised leaves of *Arabidopsis*

The ammonium-induced luminescence was also observed in leaves excised from both GM plants and GM-NO_3 plants (Figs. 1c and d) to investigate its signaling effect and define the differences with nitrate-signaling. Although the luminescence patterns are similar to nitrate-induced one and these were also dependent of dose (Fig. 2b), the application of ammonium induced the $[Ca^{2+}]_c$ increase both in GM and GM-NO_3 plants, hence the application of nitrate didn't induced the $[Ca^{2+}]_c$ increase in leaves of GM-NO_3 plants (Fig. 1b). There is no mean difference in the intensity of nitrate and ammonium induced aequorin luminescence in leaves of GM plants (Fig. 2). Both ammonium and nitrate are supposed to be the principal source of nitrogen for plant, although, when the two compounds are provided to plants at similar concentrations, ammonium is generally taken up more rapidly than nitrate (Howitt and Udavardi, 1999 and references therein). The preference for ammonium over nitrate is explained, at least in part, by the extra energy the plant must expend in reducing nitrate to ammonium before it can be incorporated into organic compounds. In addition, it is revealed that the expression of ammonium transporter in *Arabidopsis*, *AtAMT1; 1* is rapidly increased during nitrogen deprivation, and decrease rapidly in response to nitrogen supply (5 mM NH_4NO_3), both in parallel to the expression of NRTs, decreased in nitrogen starvation and increased in nitrogen supply (Rawat *et al.*, 1999). In the presented works, Arabidopsis plants were starved only in nitrate, and the comparable intensity of ammonium-induced $[Ca^{2+}]_c$ increase was observed against the existence of high ammonium (approximately; 20 mM) in culture medium. Interestingly, the intensities of low concentration of ammonium-induced $[Ca^{2+}]_c$ increase in GM-NO_3 plants were lower than that in GM plants (Fig. 2b). These results indicate that the expression of high affinity ammonium transporter(s) in leaves may down regulated by high ammonium, but the expression of low affinity ammonium transporter(s) may regulated by nitrate rather than ammonium.

Discussion

Nitrogen-induced Ca^{2+} signaling and following events

In the presented study, we reported that nitrate induces an transient increase in $[Ca^{2+}]_c$ in excised leaves of *A. thaliana* and it wasn't observed in leaves of nitrate-starved plants. As an analogy of the molecular mechanism for sugar-induced $[Ca^{2+}]_c$ increase and the synchronized expression levels, NRTs are supposed to be one of the major factor for the nitrate-induced $[Ca^{2+}]_c$ increase. Although what kinds of

Ca^{2+}-permeable channels are involved in nitrate and ammonium-induced $[Ca^{2+}]_c$ increase, the molecular mechanism for sugar-induced $[Ca^{2+}]_c$ increase, with great similarity, is well documented. In rat pancreatic β-cells and human sperm, sugar-stimulation cause transient changes in plasmamembrane voltage and following activation of voltage-dependent Ca^{2+} channel (Best, 2002, Guzmán-Grenfell *et al.*, 2000). In case of the plants, the sucrose-induced increase in $[Ca^{2+}]_c$ in aequorin-expressing *Arabidopsis* leaves, was enhanced by overexpression and suppressed by antisense expression of *AtTPC1*, a voltage-dependent Ca^{2+} channel in *A. thaliana* (Furuichi *et al.*, 2001a), implying that the sugar-induced $[Ca^{2+}]_c$ increase in plant also regulated by voltage-dependent Ca^{2+} channel. Further analysis using overexpressing and knockout lines of *AtTPC1* and other cloned Ca^{2+}-permeable channels such as cyclic nucleotide gated channels may elucidate that *AtTPC1* is the Ca^{2+}-permeable channel for nitrate and ammonium induced $[Ca^{2+}]_c$ increases or not. Using DNA microarray technique and further analysis on RNA blot, Wang *et al.* (1999) identified that *Arabidopsis* genes responsible to nitrate and revealed that some of Ca^{2+}-signaling related genes such as *CAX1*, Ca^{2+}/H^+ antiporter and some of Ca^{2+}-regulated enzymes are included in nitrate-inducible genes to adopt high nitrate condition. Further analysis on the molecular mechanisms of Ca^{2+} signaling triggered by nitrate and ammonium, especially voltage-dependent Ca^{2+} channels and relationship for nitrate metabolism especially on its reduction might be necessary to elucidate the mechanism of nitrogen metabolism in plants.

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