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Localization of Intracellular Free Calcium Ions during Adventitious Bud Initiation in *Torenia* Stem Segments

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Active meristematic divisions in *Torenia* stem segments, which were induced by application of cytokinin, a Ca^{2+} ionophore A23187 or anaerobic treatment, were suppressed by simultaneous treatment with an intracellular Ca^{2+}-binding drug, Quin II AM. Higher intracellular level of Ca^{2+} was detected by Ca^{2+}-binding fluorescent indicator in the meristematic zones during adventitious bud initiation. The increase in Ca^{2+} level could be detected at an early stage of culture. These results support the idea that adventitious bud initiation in *Torenia* stem segments may be controlled by an increase in the level of intracellular Ca^{2+}.

Introduction

Plant regeneration from somatic tissues can be attained through either adventitious bud differentiation or somatic embryogenesis. Although bud formation is mainly regulated by cytokinin and auxin[1], the controlling mechanism is still poorly understood.

We have attempted to elucidate the mechanism of adventitious bud initiation, and have previously reported on several factors which affect this process in *Torenia* stem segments cultured *in vitro*[2-13].

In higher plants, Ca^{2+} is known to play some role in physiological phenomena regulated by cytokinin. For example, cytokinin-induced retardation of senescence in corn leaf discs[14] and cytokinin-stimulated ethylene production in cucumber cotyledons[15] are significantly stimulated by increasing calcium concentration in the medium. Elliott and her co-workers reported that some cytokinin-regulated responses such as cytokinin-dependent betacyanin synthesis and cytokinin-induced soybean callus growth were inhibited by calmodulin inhibitors[16,17]. In the filamentous protonema of moss, a Ca^{2+} ionophore A23187 stimulated budding[18], and some calcium or calmodulin inhibitors blocked cytokinin-induced budding[19]. Therefore, cytokinin may control certain physiological phenomena in conjunction with the increase in the level of intracellular Ca^{2+}.

Recently, we demonstrated that bulblet differentiation could be induced by application of calcium ionophore A23187 in lily bulb-scale segments[20]. Using *Torenia* stem segments, we also reported that bud initiation induced by the Ca^{2+} ionophore A23187, or by cytokinin was inhibited by simultaneous application of ethyleneglycol-*bis*-(2-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA), an extra-cellular calcium chelator, verapamil, a calcium-channel blocker, lanthanum, a calcium antagonist, or various calmodulin inhibitors[21]. Such results support the idea that cytokinin-induced adventitious bud differentiation in *Torenia* stem segments may be mediated by cytoplasmic free Ca^{2+} and calmodulin.

Intracellular Ca^{2+} can be measured by some calcium-binding fluorescent indicators such as

chlorotetracycline (CTC)[22] and Quin II[23]. These chemicals selectively bind to calcium ions and Ca^{2+} concentration can be detected by the intensity of fluorescence. According to Tsien *et al.*[24], Quin II AM, an acetoxymethyl ester of Quin II, readily permiates the membrane and is hydrolyzed in the cytoplasm. The hydrolyzed Quin II binds to Ca^{2+} with a 1 : 1 stoichiometry[23].

With the view of elucidating the role (s) played by calcium in organogenesis, we examined intracellular Ca^{2+} concentration during adventitious bud initiation in *Torenia* stem segments.

Materials and Methods

Apical internodes of 8-week-old plants of *Torenia fournieri* Lind. were excised and surface-sterilized. Stem segments (5 mm in length) were cut from the internodes and then cultured using basal medium containing Murashige and Skoog's mineral salts and vitamines[25], 2% sucrose and 0.3% Gelrite (Merck) (hereafter referred to as MS medium). The calcium ionophore A23187 (Hoechst) was dissolved in dimethyl sulfoxide, and added to the MS medium at 10 μM. The final concentration of dimethyl sulfoxide was adjusted to 0.3% in all treatments. In another series of treatment, 0.5 μM of benzyladenine (BA) was also added to the medium. For anaerobic pre-treatment, the excised explants were treated with a stream of N_2 for 10 min. and cultured on the basal MS medium only.

For detection of intracellular calcium, epidermal strips (3×5 mm) were peeled off from the cultured explants, and incubated in 50 μM of Quin II AM (Dojin) solution for 1 hr. After incubation, the strips were washed with water and then observed under a fluorescence microscope (Optiphoto with EF, Nikon). To cope with membrane-density changes, we employed a general membrane marker, *N*-phenyl-1-naphthylamine (NPN) (Sigma). For fluorescence excitation of Quin II AM and NPN, an HBO 50-w mercury vapor lamp (Osram) was used with a U filter (Nikon) (excitation peak at 340 nm). Fluorescence of Quin II AM and NPN was monitored at the

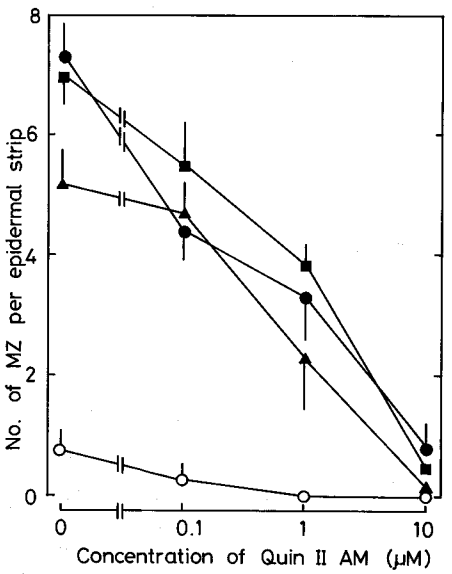

Fig. 1 Effects of Quin II AM on MZ formation in *Torenia* stem segments.
Explants were cultured on MS medium with various concentrations of Quin II AM in the absence (○) or presence of 0.5 μM BA (■) or 10 μM A23187 (●), or explants were treated with a stream of N_2 and cultured on the MS medium (▲). To calculate the average number of MZ, at least 240 epidermal strips in each treatment were observed after 7 days of culture. The experiments were repeated at least 3 times, and standard errors were then calculated.

wavelength longer than 490 nm and 400 nm, respectively. Relative intensity for Quin II AM fluorescence was measured by a microphotometer (P-1, Nikon) and expressed as percentage of maximum values of the photometer.

Bud initiation was observed as follows. Meristematic divisions induced in the epidermis of stem segments led to formation of meristematic zones (MZ) which had a potency to develop adventitious buds[3]. Therefore, the epidermal strips of stem segments cultured for 7 days were stained with aceto-carmine and observed under a bright-light microscope.

Results

When *Torenia* stem segments were cultured on the MS medium containing 0.5 μM BA or 10 μM A23187, the average number of MZ formed in an epidermal strip was about 7. The simultaneous addition of Quin II AM inhibited the MZ formation induced by both BA and A23187 (**Fig. 1**). In the case of the anaerobic pretreatment, about 5.2 MZ were formed in a strip, and Quin II AM also suppressed the MZ formation (**Fig. 1**). One μM of Quin II AM applied to the medium reduced the MZ number by about 50% and the addition of Quin II AM at 10 μM completely inhibited MZ

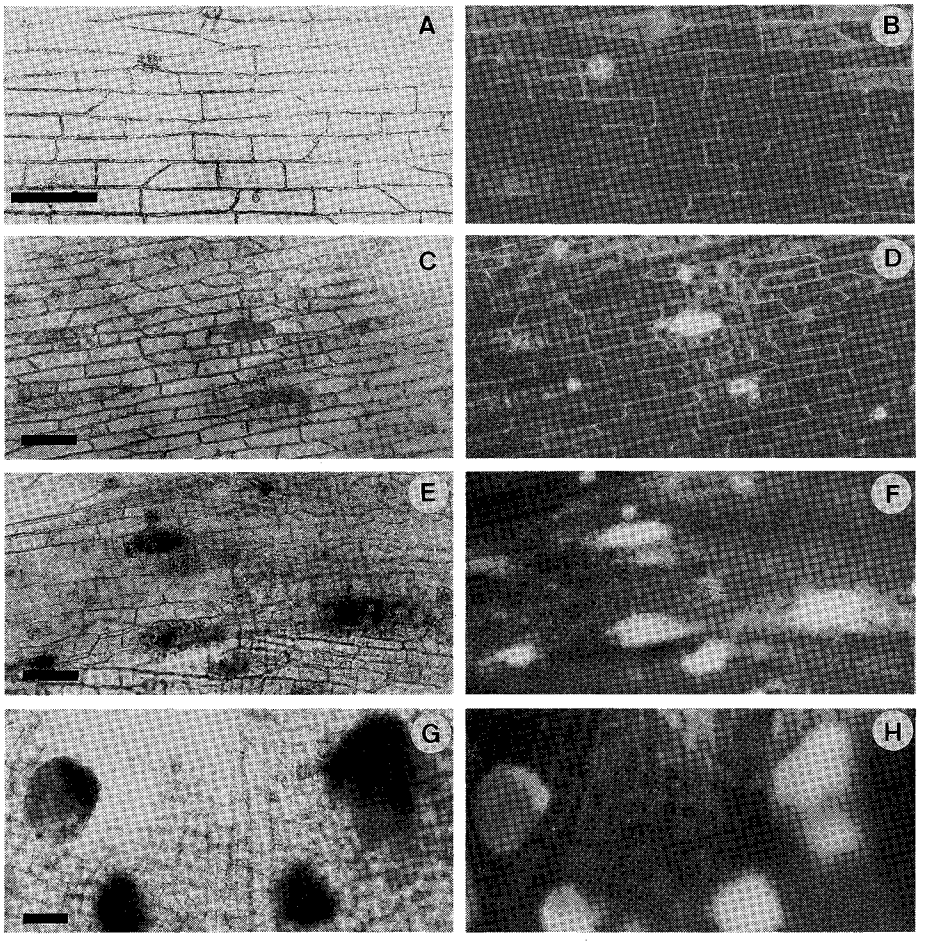

Fig. 2 Paired contrast (A, C, E and G) and Quin II AM-fluorescence (B, D, F and H) micrographs of epidermal strips of *Torenia* stem segments.
The epidermal strips of initial (A and B), 7 day-cultured with 10 μM A23187 (C and D) or with 0.5 μM BA (G and H), or N_2-treated (E and F) stem segments were peeled, incubated with Quin II AM and observed. Bars represent 100 μm.

formation.

For observation of intracellular changes of Ca^{2+}, epidermal strips from explants cultured on the medium containing 10 μM A23187 or 0.5 μM BA, or from explants treated with a stream of N_2 for 10 min. and cultured on MS basal medium, were first incubated in the Quin II AM solution, then they were observed under a fluorescence microscope. As shown in **Fig. 2**, Ca^{2+}-Quin II AM fluorescence was stronger in the MZ cells. No difference in fluorescence distribution of a general membrane marker, NPN, was observed during MZ formation (**Fig. 3**).

In the strips peeled from explants cultured on the BA- or A23187-containing medium, relative intensity of Ca^{2+}-Quin II AM fluorescence increased from the 3rd day of culture and reached its maximum level at the 5th day of culture (**Fig. 4**). In the explants treated with a stream of N_2 and then cultured on the MS medium, the intensity of fluorescence rapidly increased after 1 day of

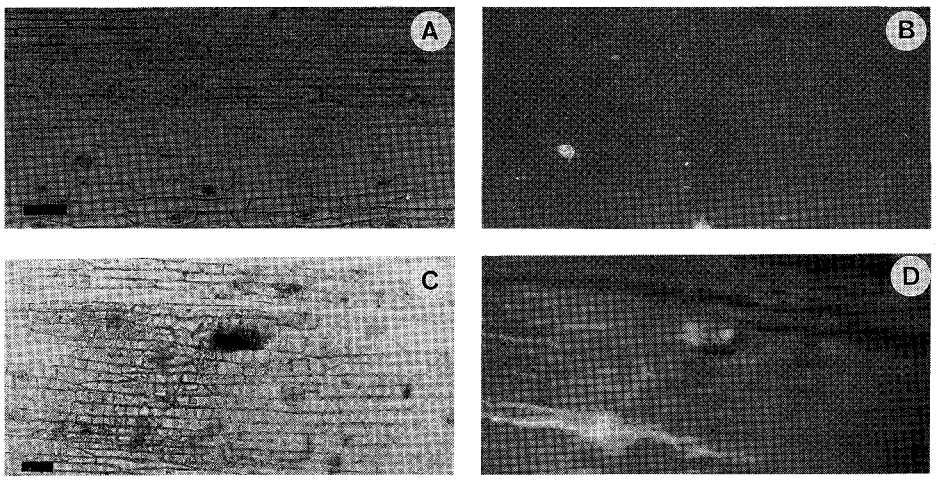

Fig. 3 Paired contrast (A and C) and NPN-fluorescence (B and D) micrographs of epidermal strips of *Torenia* stem segments.
The epidermal strips of initial (A and B) or 7 day-cultured with 10 μM A23187 (C and D) stem segments were peeled, incubated with NPN and observed. Bars represent 100 μm.

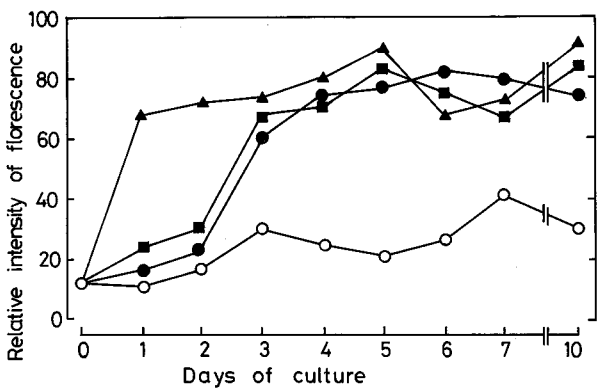

Fig. 4 Changes in relative intensity of Quin II AM-fluorescence during MZ formation.
The epidermal strips were peeled from stem segments cultured on the medium without (○) or with 0.5 μM BA (■) or 10 μM A23187 (●), or N_2 treated (▲). The strips were incubated with Quin II AM, then the fluorescence was measured by microphotometer. Measurements were made at 100 different points in each strip, and the average values of relative intensity were shown.

culture (**Fig. 4**).

Discussion

As shown in **Fig. 1**, application of Quin II AM inhibited the MZ formation induced by 0.5 μM BA, 10 μM A23187 and anaerobic pretreatment. Quin II AM binds cellular Ca^{2+} and consequently decreases the intracellular level of Ca^{2+}. A Ca^{2+}-channel inhibitor, verapamil, was also show to suppress MZ formation[21]. These results suggest that intracellular Ca^{2+} plays some important role in adventitious bud differentiation.

In moss protonema, membrane-associated Ca^{2+}, detected by CTC, was located in cells which formed buds[22]. Similar results were reported in plant cells such as lily pollen and root hairs of *Lepidium*[26]. Although these results indicate that Ca^{2+} accumulated in rapidly growing or dividing cells, it was not clear if the localization of Ca^{2+} was really required for growth and differentiation. In our experiments, however, the application of A23187 stimulated MZ formation[21] and the incorporated Ca^{2+} by A23187 or BA was localized in the cells of MZ (**Fig. 2**). Therefore, the localization of Ca^{2+} in the cells of MZ seems to be necessary for adventitious bud initiation.

We have previously reported that short term treatment with an N_2 stream promoted MZ formation and the treatment was only stimulately when applied to the explants immediately after their excision from mother plants[10]. In the explants treated by an N_2 stream, the intensity of fluorescence rapidly increased after 1 day of culture (**Fig. 4**). It is proposed that the first process of MZ formation in *Torenia* stem segments may be elicited by excising explants from mother plants, and the increase in intracellular Ca^{2+} seemed to be induced by excision itself.

In the initial period of MZ formation, some interesting biochemical changes such as new specific protein synthesis[5] and the activation of new serine-proteases[8] have been detected. These facts indicate that the protein metabolism is important in early stages of MZ formation in the process of adventitious bud differentiation. An increase in intracellular free Ca^{2+} may activate further the action of calmodulin.

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《和文要約》

トレニア茎切片における不定芽誘導時の細胞内遊離カルシウムイオンの局在

谷本靜史

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トレニアの茎切片において，サイトカイニンや Ca^{2+} イオノフォアの添加あるいは嫌気処理によって誘導される活発な不定芽原基形成にむけた分裂は，同時に添加した細胞内 Ca^{2+} 結合剤である Quin II AM の添加によって強く抑制された．Quin II AM は細胞内 Ca^{2+} 結合蛍光指示薬でもあるが，この薬剤を用いることによって検出される細胞内 Ca^{2+} レベルは，不定芽形成時に不定芽原基において有意に増加していた．この増加は培養のごく初期にみとめられた．これらの結果は，不定芽分化が細胞内 Ca^{2+} 濃度の増加によって誘導されているのではないかという考えを示唆するものである．