# Loading of Fura-2 into Liquid Organ Cultured Adventitious Root of Turnip (*Brassica rapa* L.) Resistant to Clubroot Pathogen *Plasmodiophora brassicae* and Determination of $[Ca^{2+}]_{cyt}$

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**Summary**

Our previous study using liquid organ cultured adventitious roots from turnip (*Brassica rapa* L.) showed that $Ca^{2+}$ is required for induction of defense responses in the cultured roots on the treatment with *Plasmodiophora brassicae* resting spores (Takahashi *et al*., 2006).

To evaluate change in $[Ca^{2+}]_{cyt}$ in cultured root cells on contact with the spores, acetoxymethyl ester derivative of Fura-2 (Fura-2/AM) was loaded into the roots. When ionophore A23187 was treated with $Ca^{2+}$ simultaneously, the Fura-2 fluorescence ratio that represents relative $[Ca^{2+}]_{cyt}$ increased promptly, showing that the Fura-2/AM system is suitable to evaluate $[Ca^{2+}]_{cyt}$ change in cultured root cells in a second range. Applicability of this method for studies on $Ca^{2+}$ fluctuation against various extracellular stimuli was supported by observations that treatment with mannitol or NaCl also immediately increased the Fura-2 ratio. When the Fura-2/AM loaded roots were treated with resting spores, no $[Ca^{2+}]_{cyt}$ change was observed during 500 second but when treated with spores pre-incubated with germination-enhancing suspension (GES), a slight but reproducible increase in $[Ca^{2+}]_{cyt}$ was observed. We conclude that although further analysis is needed, the Fura-2/AM system will contribute to revealing the $Ca^{2+}$ involvement in clubroot-resistance response.

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**Key words** : *Brassica rapa*, clubroot resistance, cytosolic $[Ca^{2+}]$, Fura-2, root culture

Plants have mechanisms for protecting themselves from unfavorable abiotic stresses and enemy attacks. After the perception of these stimuli, plants synthesize certain metabolites for the protection. In particular they respond to pathogen by accumulation of pathogen-related (PR) proteins, phytoalexin synthesis, cell death induced in hypersensitive response, etc. (Thomma *et al*., 2001; Schaller and Oecking, 1999). We previously used callus and cultured roots derived from turnip, *Brassica rapa*, and revealed the crucial $Ca^{2+}$ involvement in induction of defense responses, e.g., phenylalanine ammonia lyase (PAL) activity in *P. brassicae* resistant callus and induced cell death in resistant cultured roots (Takahashi *et al*., 2001, 2002, 2006).

Metabolic changes in plant roots by various stimuli have been well studied. On infection of *Phytophthora parasitica*, tomato roots responds by expression of several PR proteins to increase resistance toward the pathogen (Dassi *et al*., 1998). In Scots pine roots, cadmium induces antioxidative enzymes such as ascorbate peroxidase or catalase relating to plant defense, and pathogen attack and injury induce cell death, $H_2O_2$ production and lignin synthesis (Schützendübel *et al*., 2001). Roots were also used for research of signal transduction in stresses (Šamaj *et al*., 2002). Calcium signaling has been vigorously studied and became clear that $IP_3$, inositol-1,4,5-trisphosphate, is involved in cellular calcium mobilization (DeWald *et al*., 2001) and direct observations of $Ca^{2+}$ with fluorescent calcium indicators revealed the transient increase in cytosolic free $Ca^{2+}$ concentration, $[Ca^{2+}]_{cyt}$, in the response to UV, osmoticum, Nod-factor, touch, gravity, cold shock and an elicitor (Bibikova *et al*, 1997; Kitagawa and Yoshizaki, 1998; Gehring *et al*., 1997; Legué *et al*., 1997; Knight *et al*., 1991). Further, cultured adventitious roots have been used for studies such as formation of root architecture and secondary metabolism including alkaloid synthesis (Murphy *et al*., 2000; Biondi *et al*., 2002; Sugimoto *et al*., 1990; Babiker *et al*., 1999).

Here, we report our try to detect the change in $[Ca^{2+}]_{cyt}$ using Fura-2/AM loaded cultured roots in the response to *P. brassicae* spores. Although the result suggested that Fura-2/AM system is suitable to observe $[Ca^{2+}]_{cyt}$ in a second range, artificial control of germination of spores at least was shown to be necessary to reveal molecular mechanisms of signal transduction in clubroot resistance.

## MATERIALS AND METHODS

### Chemicals

Fura-2/AM, Fura-2 pentakis(acetoxymethyl)ester was purchased from Dojin Chemicals (Kumamoto, Japan). All other chemicals were biochemistry grade.

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**Culture of *Plasmodiophora brassicae* and spore preparation**

*P. brassicae* was determined as race IV as previously described (Takahashi *et al*., 2001). *Brassica rapa* L. was grown in infected sterilized soil containing spores in a growth chamber at 24℃ under 12 h dark / 12 h light at 115 μmol $m^{-2}$ $S^{-1}$. *P. brassicae* spores were isolated from the infected turnip and processed to remove contaminating microorganisms as previously described (Takahashi *et al*., 2001).

**Induction and culture of adventitious roots**

Seeds of *B. rapa* L. cv. CR Takamaru Kokabu resistant to *P. brassicae*, were placed on basal Murashige-Skoog (MS) medium (1962) solidified with 0.8% agar in 8 cm diameter-Petri dish and incubated at 24℃ under 12 h dark / 12 h light at 115 μmol $m^{-2}$ $s^{-1}$. After a week, roots of the seedlings were cut into about 1 cm-long and cultured in 20 mL MS basal medium supplemented with 25 μM 4-(3-indolyl)butyric acid, 29 nM gibberellin $A_3$ and 3% sucrose in 200 mL flask under the dark at 24℃ on a rotary shaker at 40 rpm. Adventitious roots generated were maintained as described previously (Takahashi *et al*., 2006).

**Spore treatment**

Two weeks-old cultured roots were cut and 10 mg of them were pre-cultured for 3 days in freshly made MS medium supplemented as above. The roots were transferred into 1 mL newly prepared medium in an Eppendorf tube and inoculated with $10^7$ spores in 1 μL de-ionized water (i.e., final spore concentration was $10^4$ $mL^{-1}$ medium).

**Loading of Fura-2 / AM into cultured roots**

To determine $[Ca^{2+}]_{cyt}$, Fura-2/AM was administered into cells. For loading Fura-2/AM into roots, we modified acid-loading protocol by Legué et al. (1997) and DeWald et al. (2001). Extra-cellular esterases of plant has been thought to reduce loading efficiency of the AM derivative into plant cells due to digestion of the ester bond, therefore, we added 300 μM eserine sulfate, an esterase inhibitor, to prevent Fura-2/AM from hydrolysis. Since Fura-2/AM is very insoluble in water, a nonionic detergent cremophore EL which is low toxicity to plant cells was used to dissolve the ester.

Five of 5 mm-long cultured root tips having apical meristem were prepared and incubated into 100 μL of acetate buffer, pH 4.5, containing 30 μM of Fura-2/AM, 300 μM eserine sulfate and 0.02% cremophore EL, for 3 h at room temperature under the dark. The solution was discarded and the roots were rinsed twice with 10 mM MES, pH 5.0, containing 50 mM KCl (washing buffer) for 10 min each. The segments were checked under a fluorescence microscope and only those having cells whose cytoplasm emitted fluorescence were used for experiments.

**Determination and imaging of $[Ca^{2+}]_{cyt}$ by Fura-2 fluorescence ratio**

A Fura-2 loaded root segment was placed on a cover glass covering a 10 mm $\phi$ hole on a bottom plate of 50 mm $\phi$ plastic Petri dish. The cover glass was pre-coated with poly-L-lysine to hold root specimen placed on the cover glass and prevent it from drift. The root segments were submersed in 5 μL of the washing buffer and observed with a fluorescence-inverted microscope (IX-70, Olympus Corp., Tokyo, Japan). After a few minutes of stabilization, intracellular Fura-2 was excited at 340 nm and 380 nm and the resulting fluorescence at 505 nm was recorded. Ratio of the two fluorescence intensities (Fura-2 ratio, 340 nm / 380 nm) was calculated and visualized using computer aided program MetaFluor (Universal Imaging corporation, USA) for evaluation of relative $[Ca^{2+}]_{cyt}$.

Using MetaFluor, photo bleaching of fluorescence dye can be minimized, thus excess insertion of dye, which may be harmful for the cell activity, was to be obviated.

**Treatment of spores with germination enhancing suspension (GES)**

*P. brassicae* resting spores were incubated with germination enhancing suspension (GES) prepared as Ogawa et al. (2001) and Takahashi et al. (2001) at the concentration of $10^4$ spores $mL^{-1}$ for 5 days. The spore suspension was used for inoculation of Fura-2 loaded roots segments as described above.

## RESULTS

**Localization of loaded Fura-2 in cultured roots**

Recently we found that in clubroot-resistant cultured root of *B. rapa* L., pre-treatment of the root with EGTA or verapamil inhibited induction of cell death induced by spore treatment (Takahashi *et al*., 2006). Thus, we intended to measure $Ca^{2+}$-uptake and $[Ca^{2+}]_{cyt}$ using calcium fluorescent indicator, Fura-2. First, localization of the dye loaded was checked by imaging of Fura-2 fluorescence. As shown in **Fig. 1**, Fura-2 seemed to be localized within cytoplasm of cultured root cells and those showing the fluorescence within cell were used in following experiments.

**Changes in $[Ca^{2+}]_{cyt}$ against calcium ionophore A23187 treatment**

A calcium ionophore, A23187, was used to estimate if fluorescence of Fura-2 loaded into root cells responded to change in $[Ca^{2+}]_{cyt}$. Simultaneous treatment of the roots with 25 μM A23187 and 10 mM $CaCl_2$ increased Fura-2 fluorescence (**Fig. 2**). The increase in the Fura-2 ratio, i.e., the ratio of fluorescence intensity excited at 340 nm to that excited at 380 nm, was observed immediately after simultaneous addition of the ionophore and $CaCl_2$ (**Fig. 3A**). No change in the ratio was observed in treatment with either $CaCl_2$ or the ionophore alone, whereas successive treatment with the ionophore followed by $CaCl_2$ markedly increased $[Ca^{2+}]_{cyt}$ (**Fig. 3B, C**). These results showed that in the Fura-2/AM system, the Fura-2 fluorescence ratio clearly reflected $[Ca^{2+}]_{cyt}$ and the responsiveness was kept for at least 500 sec. Additionally, the administration of $MnCl_2$ into the medium led the quench of fluorescence excited at 340 nm and 380 nm (Data not shown), indicating that detected fluorescence was resulted from intracellular dye not from self fluorescence (Dassi *et al*., 1998).

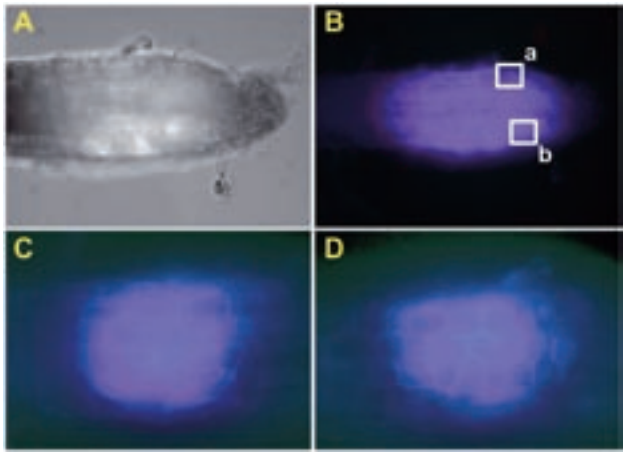


**Fig. 1**. Fluorescence imaging of Fura-2 in cultured root cells. A Fura-2/AM loaded cultured root was excited at 340 nm and 380 nm, and the Fura-2 ratio (340 nm/380 nm) was visualized as fluorescence blue color image. Since the root was not treated with any chemicals or spore, the fluorescence intensity was very faint and thus was maximized to calculate the ratio. (A) Phase contrast light micrograph of Fura-2/AM loaded root. (B) Fluorescence image of Fura-2 in the root cells shown in panel A. Two areas enclosed by two rectangular at upper (a) and lower (b) parts in Panel B were enlarged and shown in Panels C and D, respectively.

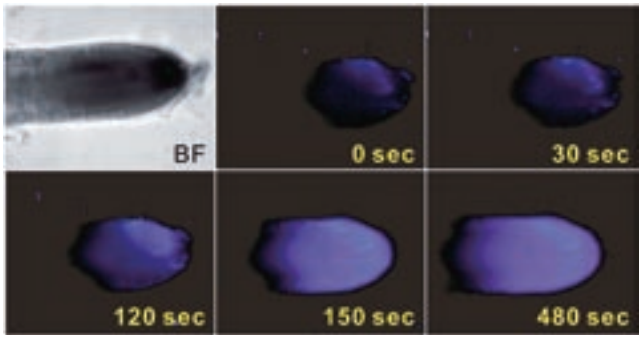


**Fig. 2.** Change in Fura-2 fluorescence image in cultured roots after treatment with A23187 and $CaCl_2$. Fura-2 fluorescence was visualized as described in **Fig. 1**. On 30 sec, right after taking a photo, A23187 and $CaCl_2$ was added to the medium. BF, bright field image at time 0.

**Evaluation of change in $[Ca^{2+}]_{cyt}$ in the response to extracellular stimuli using Fura-2/AM system**

It is known that treatment of plants with mannitol or NaCl triggers $[Ca^{2+}]_{cyt}$ increase due to osmotic and/or ionic stress (Knight *et al*., 1997), thus we then attempted to apply the Fura-2/AM system to assess their effects on $[Ca^{2+}]_{cyt}$ in cultured roots. As shown in **Fig. 4**, rapid increase in the Fura-2 ratio after addition of 0.5 M mannitol or 0.5 M NaCl was clearly observed, supporting reliability of the method in second range experiments.

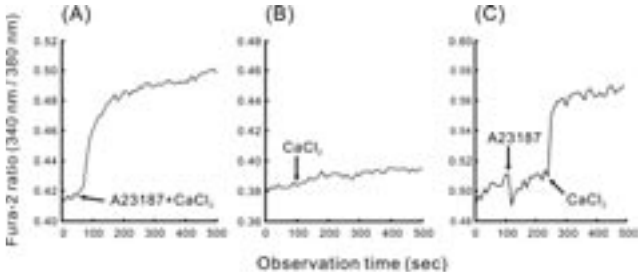


**Fig. 3.** Change in Fura-2 ratio in cultured roots after treatment with A23187 and $CaCl_2$. (A) Ten mM of $CaCl_2$ and 25 μM of A23187 were given simultaneously to the Fura-2/AM loaded roots. (B) Ten mM $CaCl_2$ alone was given to the roots. (C) Twenty-five μM of A23187 alone was given first, and after 150 second, 10 mM $CaCl_2$ was given. $[Ca^{2+}]_{cyt}$ was expressed as the Fura-2 ratio (340 nm/380 nm). Chemicals were treated at timing indicated with an arrow.

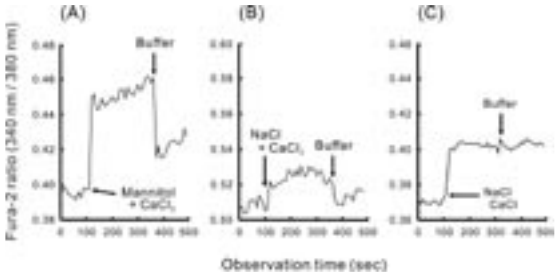


**Fig. 4.** Evaluation of change in $[Ca^{2+}]_{cyt}$ on the response to mannitol and NaCl using Fura-2/AM system. Fura-2/AM loaded roots were treated with 0.5 M mannitol alone (A), 0.5 M NaCl alone (B) or 0.5 M NaCl and 10 mM $CaCl_2$ (C), and after a few minutes of incubation, the media were diluted with washing buffer. Change in $[Ca^{2+}]_{cyt}$ was evaluated as **Fig. 3**. Timing of addition of the chemicals or buffer was indicated with an arrow.

The results shown in **Fig. 4** also indicate that there are different ways to increase $[Ca^{2+}]_{cyt}$ in the response of turnip cultured roots to mannitol and NaCl. Mannitol treatment increased $[Ca^{2+}]_{cyt}$ in the absence of extracellular $Ca^{2+}$ **(Fig. 4A)**, suggesting that $Ca^{2+}$ is supplied from intracellular $Ca^{2+}$ pools in the response to mannitol. On the other hands, the increase in $[Ca^{2+}]_{cyt}$ by NaCl treatment was significantly enhanced by the co-presence of $Ca^{2+}$, although $[Ca^{2+}]_{cyt}$ increased at a very low intensity when treated with NaCl alone **(Fig. 4B, C)**. This indicates that both intracellular and extracellular $Ca^{2+}$ contribute to the $[Ca^{2+}]_{cyt}$ increase in the response to NaCl. After addition of the buffer to the medium to reduce the stress of these substances, the Fura-2 ratio immediately decreased in the roots treated with mannitol or NaCl alone **(Fig. 4A, B)** but did not change in roots treated with NaCl plus $CaCl_2$ **(Fig. 4C)**.

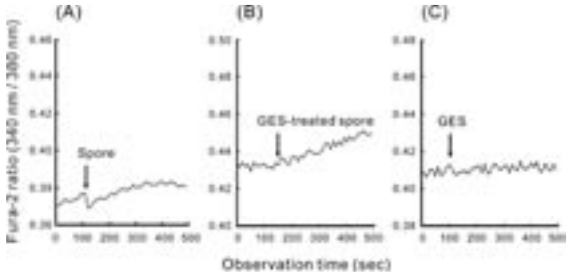


**Fig. 5.** Change in $[Ca^{2+}]_{cyt}$ in cultured roots on contact with *P. brassicae*. Fura-2/AM loaded root was treated with resting spores (A), GES-treated spores (B) or GES (C) at timing indicated with arrow. Change in $[Ca^{2+}]_{cyt}$ was evaluated as **Fig. 3**.

### Measurement of fluctuation of $[Ca^{2+}]_{cyt}$ on contact with *P. brassicae* spore

$Ca^{2+}$ is required for induction of defense responses in cultured roots from clubroot-resistant turnip (Takahashi *et al*., 2006). However, spore treatment did not affect the Fura-2 ratio over 500 sec **(Fig. 5A)**. This is most likely due to the fact that spore germination requires longer than 500 sec. Therefore, we incubated resting spores in GES, which enhances germination rate up to 30% (Ogawa *et al*., 2001) for 5 days before inoculation of Fura-2 loaded roots. As a result, $[Ca^{2+}]$ slightly increased right after the treatment **(Fig. 5B)**. This increase was much less than that by mannitol or NaCl treatment **(Fig. 4)** but was reproducible. GES itself had no such effect **(Fig. 5C)**.

## DISCUSSION

*P. brassicae* is an obligate pathogen causing clubroot disease of crucifer plants. The roots of infected plant are hypertrophied and lead to abnormal growth. Since the pathogen's life cycle occurs underground completely, it is difficult to determine initiation point of infection. Therefore, in previous report we established calluses from clubroot susceptible and resistant turnips to investigate early events in clubroot resistance response. Using the calluses, we observed crucial involvement of $Ca^{2+}$ for induction of PAL in the resistant calluses and furthermore, postulated plausible preliminary $Ca^{2+}$ movement within cell in the callus (Takahashi *et al*, 2001; 2002). However we have felt in these researches that the callus contained disaccord with real plant-parasite relationships in various aspects. Because, not only the callus has not differentiated organs such as main root or root hair but it has significant higher constitutive PAL activity or salicylic acid compared to turnip roots (Takahashi *et al*, unpublished data). Therefore, we established organ cultured roots from the resistant and susceptible turnips in MS basal medium supplemented (Takahashi *et al*., 2006). The cultured roots retain their original characteristics of susceptibility or resistance to *P. brassicae* (Takahashi *et al*., 2006). Using the cultured roots, we showed evidences that $Ca^{2+}$ was also required for induced cell death in *P. brassicae*-resistant roots on contact with the spores of the pathogen (Takahashi *et al*., 2006). These observations forced us to estimate $[Ca^{2+}]_{cyt}$. Thus, we attempted to measure $[Ca^{2+}]_{cyt}$ using fluorescent $Ca^{2+}$ indicator Fura-2 and we have succeeded to load its derivative Fura-2/AM into cells of the cultured roots. When ionophore A23187 was given simultaneously with $Ca^{2+}$, rapid increase in $[Ca^{2+}]_{cyt}$ was visualized by fluorescent microscopy **(Fig. 2)** and the change was quantified by the Fura-2 ratio **(Fig. 3)**. Moreover, mannitol and NaCl treatment substantially increased the Fura-2 ratio **(Fig. 4)**. These experiments clearly showed that fluorescence of Fura-2 loaded into the cultured roots well reflects $[Ca^{2+}]_{cyt}$ and the Fura-2/AM system is a useful tool for real-time monitoring of $[Ca^{2+}]_{cyt}$ in the response to various stimuli.

However, as predicted, the change of $[Ca^{2+}]_{cyt}$ in roots on contact with spores was not observed during 500 sec. Since spores may not germinate within 500 seconds, freshly germinated zoospores must be inoculated with roots for observation of $[Ca^{2+}]_{cyt}$ fluctuation.

In previous work, we observed that root exudates (GES) slightly enhanced germination of mature spore of *P. brassicae* (Ogawa *et al*., 2001). GES-treated resting spores induced PAL 8 h earlier compared to a timing of PAL induction by non-treated spores in the resistant callus (Takahashi *et al*., 2001). Using the GES-treated spores, a slight increase in $[Ca^{2+}]_{cyt}$ was observed **(Fig. 5B)**. However, the increase was too low to conclude that $[Ca^{2+}]_{cyt}$ increases as an intermediate in signal transduction against *P. brassicae*. Optimization of condition to further enhance spore germination rate (e.g., submerging period of spores in GES, concentration of exudates and/or salt composition) is necessary in future study using the Fura-2/AM system, which is, in this study, shown to be a useful and convenient tool for investigation of $[Ca^{2+}]_{cyt}$.

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# 根こぶ病抵抗性カブ（*Brassica rapa* L.）培養根への Fura-2の導入と細胞内 $Ca^{2+}$ 濃度の測定

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## 要　約

我々は以前の研究において、カブ（*Brassica rapa* L.）幼根から誘導した培養根を用い、根こぶ病菌 *Plasmodiophora brassicae* に対する初期抵抗反応の誘導に $Ca^{2+}$ が必要であることを示した（Takahashi *et al.,* 2006）。本研究では、根こぶ病抵抗反応におけるカブ培養根の細胞内 $Ca^{2+}$ 濃度（$[Ca^{2+}]_{cyt}$）の変動を調査するため、$Ca^{2+}$ の蛍光指示薬 Fura-2のアセトキシメチルエステル（Fura-2/AM）をカブ培養根に導入し、蛍光顕微鏡により $[Ca^{2+}]_{cyt}$ を定量的に解析することを試みた。蛍光顕微鏡下で細胞質内に Fura-2が導入されたことが確認できたカブ培養根に、カルシウムイオノフォア A23187と $Ca^{2+}$ を同時に処理すると、直ちに $[Ca^{2+}]_{cyt}$ の相対値を示す Fura-2蛍光比が上昇したことから、Fura-2/AM を用いた本手法は、培養根における $[Ca^{2+}]_{cyt}$ の変化を観察するのに適していることが示された。さらに $[Ca^{2+}]_{cyt}$ を増加させることが知られている外的刺激として、マンニトール及びNaClの高濃度処理を行ったところ、同様な Fura-2蛍光比の急激な増加が観察され、本手法の汎用性が示された。次に、我々は本来の目的である根こぶ病抵抗反応における $[Ca^{2+}]_{cyt}$ の解析に本手法を応用した。Fura-2/AM を導入した根こぶ病抵抗性カブ培養根に *P. brassicae* の休眠胞子を処理しても、500秒の測定時間内に $[Ca^{2+}]_{cyt}$ が変化することは無かった。しかし休眠胞子を発芽促進液（GES: germination enhancing suspension）で前処理し、同様の実験を行ったところ、非常にわずかであったものの、再現性ある $[Ca^{2+}]_{cyt}$ の上昇が観察された。根こぶ病に対する初期抵抗反応における $Ca^{2+}$ の重要性を立証するためには、今後さらなる解析が必要であるが、その過程において本手法は大きく貢献するものと期待される。


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