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Nanometer-scale direct observation of the receptor for the leaf-movement factor in plant cell by a novel TEM probe

Yoshiyuki Manabe,[a] Takanori Sugimoto,[a] Tomoyuki Kawasaki[b] and Minoru Ueda[a,*]

[a]*Department of Chemistry, Faculty of Science, Tohoku University, Aoba-ku, Sendai 980-8578, Japan*
[b]*Faculty of Science, Tohoku University, Aoba-ku, Sendai 980-8578, Japan*

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Abstract—Electron microscopy is a useful method for observing localization of some proteins in a cell. In this Letter, we report a nanometer-scale direct observation of the receptor for a bioactive substance of small molecular weight by using TEM (transmission electron microscopy). We developed a novel TEM probe compound (**1**) that is a modified leaf-movement factor with benzophenone as a photoaffinity group and FITC as an antigen that is recognized by a nano-gold bound anti-FITC antibody. By using probe **1**, we revealed that the receptor for the leaf-movement factor of *Cassia mimosoides* is localized in the plasma membrane of the plant motor cell.

Most leguminous plants close their leaves in the evening, as if to sleep, and open them in the morning according to the circadian rhythm controlled by a biological clock. Charles Darwin, well-known for his theory of evolution, carried out the pioneering work in this field.[1] And in the 1970s, physiological studies revealed that nyctinastic leaf movement is induced by the swelling and shrinking of motor cells in the pulvini, a small organ located in the joint of the leaf to the stem.[2] Flux of potassium ions across the plasma membranes of the motor cells is followed by massive water flux, which results in swelling and shrinking of these cells. We revealed that nyctinasty is controlled by a pair of leaf-movement factors: leaf-opening and leaf-closing substances.[3,4] Recently, we have revealed that a receptor for the leaf-movement factor is involved in the membrane fraction of the motor cell by using a photoaffinity-labeling method.[5] Photoaffinity experiment using a crude membrane fraction of *Cassia* plant gave two potential receptors of 210 and 180 kDa.[5] However, the crude membrane fraction that was used in the experiment contained both a vacuolar membrane and a plasma membrane. It was extremely difficult to separate them in such a small scale. Thus, it was impossible to determine which membrane contains the receptor for the leaf-movement factor.

Determination of the localization of the receptor is very important in the bioorganic study of nyctinasty (Fig. 1): If the receptor is involved in the plasma membrane, the leaf-movement factor is an inter-cellular chemical messenger and controls the activity of the potassium channel in a plasma membrane (Fig. 1a). On the other hand, if the receptor is involved in the vacuolar

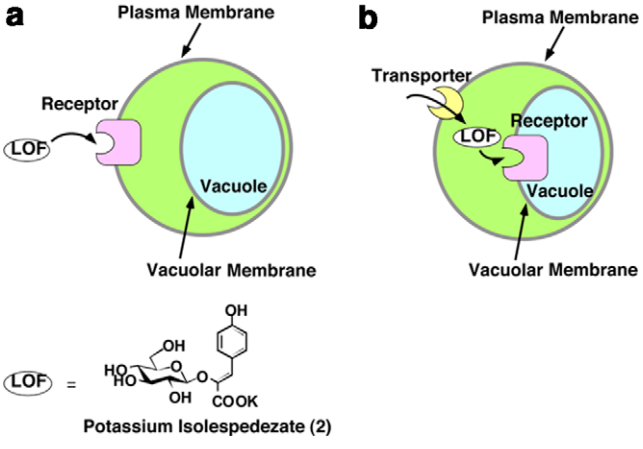

Figure 1. Two possible mechanisms in the turgor control of the motor cell by potassium isolespedezate (**2**).

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* Corresponding author. Tel./fax: +81 22 795 6553; e-mail: ueda@mail.tains.tohoku.ac.jp

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membrane, the leaf-movement factor is an intra-cellular chemical messenger that activates the potassium channel in the vacuolar membrane which triggered the flux of potassium ions across plasma membranes (Fig. 1b). To examine these two possibilities, we tried to identify the direct observation of the exact localization of the receptor in the motor cell. However, it required the observation with nanometer-scale resolution. Thus, we thought that a transmission electron microscopy (TEM) which has a resolution of 0.10 nm would be suitable for this purpose.

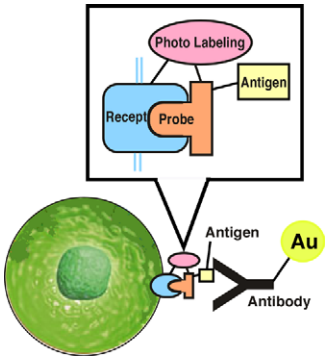
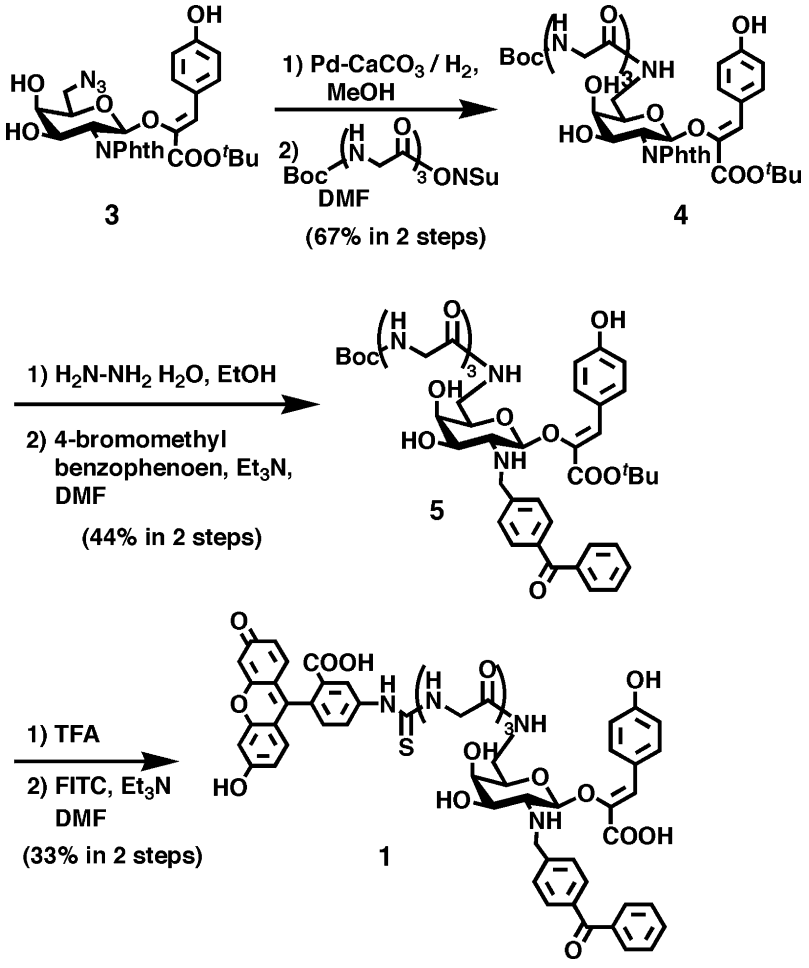


Figure 2. Concept of TEM probe: photoaffinity antigen-tagging of receptor on cell membrane.

In this Letter, we report the design and synthesis of a novel TEM probe compound (**1**) that enables the nanometer-scale direct observation of the localization of the receptor for **1**.

The TEM probe has a photoaffinity labeling group and antigen for immunoelectronmicroscopy (Fig. 2). After cross-link formation to the receptor by photoaffinity labeling, the TEM probe will act as an antigen which can be recognized by a nano-gold[5] bound corresponding antibody. Then, we can label the receptor for **2** by a nano-gold which can be monitored by using TEM.

Based on this concept, TEM probe (**1**) was designed according to the results of structure–activity relationship studies on **2**.[6,7] Structure modification in the sugar moiety of **2**, such as conversion into galactoside, α-mannoside, and even L-glucoside, did not cause any decrease in bioactivity. These results suggested that there would be little structure recognition by the receptor on the sugar moiety of **2**. Thus, retention of bioactivity can be expected after structural modification in the sugar moiety of **2**. Benzophenone was introduced in the 2′-po-



Scheme 1. Synthesis of TEM probe (**1**).

sition of the sugar moiety as a photoaffinity group and FITC was introduced in the 6′-position as an antigen.

The combination of benzophenone and FITC is very important. No photobleaching of the photoaffinity labeling group which is induced by an intramolecular-electron transfer was observed among them. It was supposed that other photolabeling groups, such as trifluoromethyldiazirine, whose absorption spectrum has an overlap with the fluorescence spectrum of FITC, cannot form a cross-link with a receptor efficiently because of the photobleaching due to the intramolecular electron transfer from the photoaffinity group to FITC.

We synthesized probe **2** according to the synthesis of the photoaffinity probe.[8] Synthetic intermediate **3**[5] was reduced and the resulting amine was coupled with Boc-protected glycylglycylglycine. After deprotection, stepwise introduction of benzophenone and then FITC was carried out to give TEM probe (**1**)[9] (Scheme 1).

We prepared a section of the plant pulvini which contains a motor cell in 30 μm-thickness. After being incubated with 1×10^{-4} M aqueous solution of TEM probe (**2**) at 4 °C for 4 h, the section was irradiated by UV (365 nm) to form a cross-linkage between probe **1** and its receptor. Then the section was washed by Milli Q thoroughly to remove the unreacted **1**. After washing thoroughly, the cross-link formation was examined by the observation of fluorescence on the plant section by using a fluorescence microscope (Fig. 3). Cross-linked **1** cannot be removed from the plant section by the washing and gave fluorescence due to the fluorescein moiety in **1**. Without UV irradiation, all of **1** was removed by washing and no fluorescence was observed on the plant section. Moreover, the specific binding of **1** was confirmed by the competitive binding experiment in the presence of 1000-fold amount (1×10^{-1} M) of non-labeled natural product (**2**). As shown in Figure 3, decrease in the strength of fluorescence around the motor cell was observed under this condition.

The cross-linked section was treated with the nano-gold bound Fab′ fragment of the anti-FITC antibody. To improve the penetration of the antibody into the plant section of 30-μm thickness, Fab′ fragment was used as an antibody which was prepared from commercially available anti-FITC antibody $F(ab')_2$ (from Goat, Rockland Inc.) (Fig. 4).[5,10] Anti-FITC antibody $F(ab')_2$ was reduced by cysteamine. The resulting Fab′ fragment was purified by FPLC, and coupled with monomaleimido nano-gold, and used in labeling of the plant section.

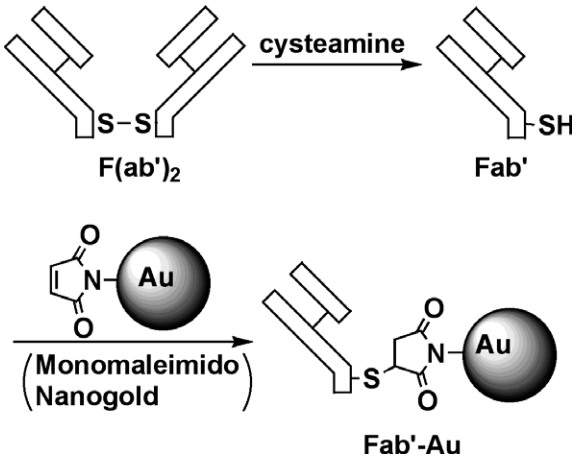


Figure 4. Preparation of nano-gold bound Fab′ fragment of anti-FITC antibody.

The section was then fixed with 4% glutaraldehyde and 0.5% formaldehyde. The HQ Silver Enhancement-Kit (Nanoprobe Inc.) for silver enhancement was also used to improve the sensitivity in TEM observation.[5] After dehydration with a series of EtOHaq, the section was embedded with Spurr resin. Finally, the embedded section was cut to a thickness of about 90 nm, stained by $UO_2(OAc)_2$, and used for the observation by TEM (Hitachi H-8100).

Figure 5 shows the TEM image of the plasma membrane of the motor cell. FITC-tagged receptors were labeled by nano-gold and can be observed as black stain in the plasma membrane. From this result, the receptor for **1** is confirmed to be localized in the plasma membrane of the motor cell; thus the proposed mechanism in Figure 1a is correct: the receptor of **2** is involved in the plasma membrane, **2** is the inter-cellular chemical messenger and controls the activity of the potassium channel in the plasma membrane of the motor cell.

This concept can be widely applicable for the nanometer scale-direct observation of the receptor for any small molecules.

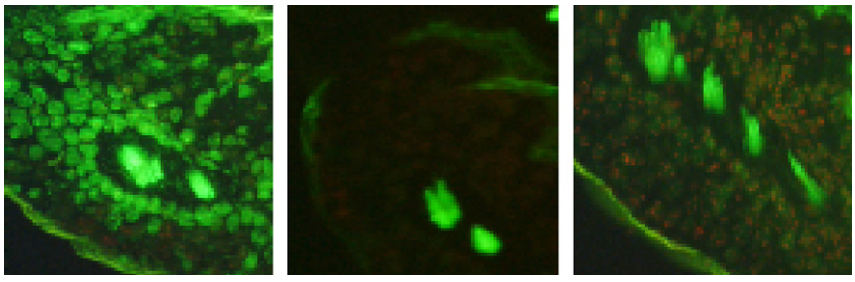


Figure 3. Fluorescence image of plant pulvini containing motor cell. Cross-link formation was examined by fluorescence of **1** on the motor cell. Strong fluorescence observed in the center of each photograph is due to the non-specific binding of **1** to the plant vessel (left: cross-linked **1**, center: without irradiation, right: under competitive binding with **2**).

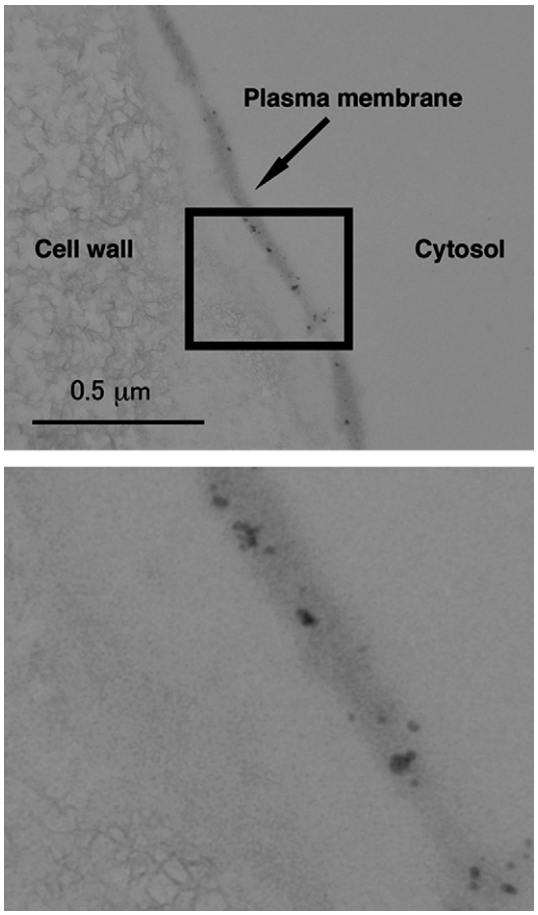


Figure 5. TEM image of plant motor cell (upper: TEM image of plant motor cell, under: expansion of square part in upper photograph).

Acknowledgments

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9. Compound **1**: ^{1}H NMR (300 MHz, CD_3OD, rt): 8.33 (1H, br s), 7.80–7.61 (10H, m), 7.48 (2H, t, $J = 7.7$ Hz), 7.22 (1H, s), 7.15 (1H, d, $J = 8.3$ Hz), 6.76–6.66 (6H, m), 6.57 (2H, m), 5.24 (1H, d, $J = 8.4$ Hz), 4.73 (1H, d, $J = 12.8$ Hz), 4.51 (1H, d, $J = 12.8$ Hz), 4.30 (2H, s), 4.06 (1H, dd, $J = 3.3$, 10.8 Hz), 3.88 (3H, s), 3.74 (2H, s), 3.69 (1H, t, $J = 7.0$ Hz), 3.60 (1H, dd, $J = 8.4$, 10.8 Hz) ppm; ^{13}C NMR (75 MHz, CD_3OD, rt): 197.7, 184.2, 173.4, 172.4, 170.7, 169.2, 168.0, 162.4, 160.8, 154.8, 142.1, 139.7, 139.1, 138.4, 137.1, 134.5, 134.1, 131.9, 131.5, 131.4, 130.1, 130.8, 130.1, 129.6, 126.5, 124.4, 112.1, 103.5, 101.1, 89.2, 74.6, 70.5, 68.9, 61.6, 52.2, 44.1, 43.3, 40.2, 30.8, 22.9 ppm. HR ESI MS (positive): $[M+H]^+$ found *m/z* 1095.3083, $C_{56}H_{50}N_6O_{16}S$ requires *m/z* 1095.3082; IR (film) *v*: 3294, 1654, 1605, 1541, 1512 cm^{-1}; $[\alpha]_D^{19}$ −7.1 (*c* 0.50, MeOH).
10. Anti-FITC $F(ab')_2$ fragment (0.2 mg in 167 μL, Goat-Poly, Rockland) was diluted by 5 mM EDTA—0.1 M phosphate buffer (pH 6.0) to 1 mL, and then cysteamine hydrochloride (6.0 mg, Aldrich) was added to this solution. After being allowed to stand at rt for 1 h, the reaction mixture was separated by using gel filtration using Fast Desalting Column HR 10/10 (Amersham) with 0.02 M sodium phosphate (pH 6.5) containing 1 mM EDTA and 0.15 M NaCl, and then concentrated with Centricon YM-10 (Millipore). Aqueous solution of monomaleimido nano-gold (250 μL, Nanoprobe) was added to the resulting Fab′ fragment (0.1 mg), and then allowed to stand at 4 °C overnight. The solution was concentrated with Centricon YM-30 (Millipore), and then separated by Superdex 75 HR 10/30 with 0.02 M sodium phosphate (pH 7.4) containing 0.15 M NaCl to give nano-gold-bound anti-FITC Fab′ fragment.