Double Fluorescence-labeling Study on Genus *Albizzia* Using a Set of Fluorecence-labeled Leaf-movement Factors to Identify the Spatial Distribution of Their Receptors

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We synthesized rhodamine-labeled leaf-opening factor of genus *Albizzia*. We carried out double fluorescence-labeling study on genus *Albizzia* using rhodamine-labeled leaf-opening factor and fluorescein-labeled leaf-closing factor. This study showed that both probes bound the same motor cell. These results indicated that each motor cell had both receptors for leaf-opening and leaf-closing factors.

Circadian rhythmic leaf-closing and leaf-opening movements called nyctinasty are widely observed in legumes. It is well known that Charles Darwin is a pioneer in this field.[1] Since then, numerous studies were carried out and revealed that motor cells located in the pulvini, an organ located in the joint of the leaf, play the key role in plant leaf-movement.[2] A flux of potassium ions across a plasma membrane on motor cells is followed by a massive water flux, which results in swelling or shrinking of these cells.

Potassium β-D-glucopyranosyl-12-hydroxyjasmonate (**1**)[3] and *cis*-*p*-coumaroylagmatine (**2**) (Chart 1)[4] were isolated as leaf-closing factors and leaf-opening factors of leguminous plants belonging to genus *Albizzia*, respectively.[3,4] Recently, we revealed that the target cells for these two leaf-movement factors are motor cells, by using fluorescein (FITC)-labeled probes.[5,6] These results indicated that a pair of leaf-movement factors stimulated nyctinasty, as an endogenous signaling factor to interact specific receptors that would be located on motor cells.

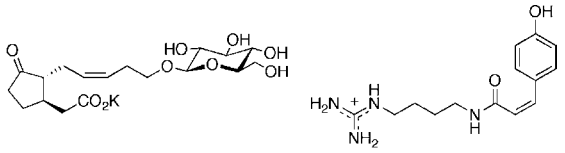


Chart 1.

However, in macroscopic view, leaf-movement requires the adverse actions of motor cells that are located in the inside part and outside part of pulvini; when the leaf closes the motor cells in the inside part of pulvini should shrink and the motor cells in the outside part should swell, and vice versa.[2] There would be three hypothetical mechanisms to explain this phenomenon. The first is the unequal distribution of receptors in pulvini; the motor cells in the inside part of pulvini have only receptors for leaf-closing factor and the motor cells in the outside part have only receptors for leaf-opening factor (Figure 1a). The second is the unequal distribution of leaf-movement factors in pulvini; in this case the receptors for each leaf-movement factor is equally distributed (Figure 1b). The third is that the motor cell with a set of receptors for each leaf-movement factor is located

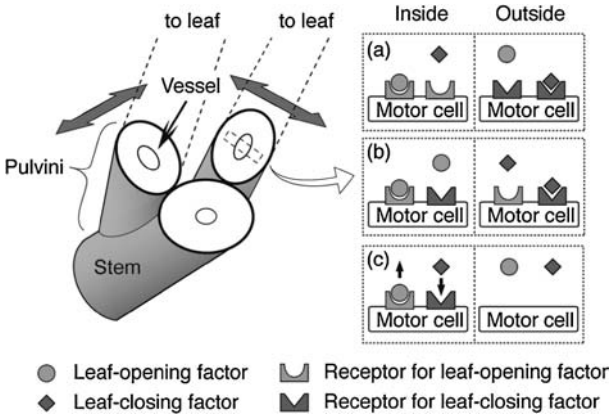


Figure 1. The cross sectional view of pulvini and three hypothetical mechanisms of leaf-movement. a) The unequal distribution of receptors. b) The equal distribution of receptors. c) The unequal distribution of motor cells with a set of receptors for each leaf-movement factor.

in the one side, outside or inside part of pulvini; in this case the changes in the balance of concentration between two leaf-movement factors induce nyctinasty (Figure 1c).

Thus, next important issue to understand molecular mechanism of the nyctinasty is a spatial distribution of the receptors corresponding to leaf-closing and leaf-opening factors. For this purpose, we examined the double fluorescence-labeling study[7] using fluorescence-labeled leaf-closing and leaf-opening factors. By coupling leaf-closing factor with FITC and leaf-opening factor with rhodamine, the distribution of the receptors corresponding to different molecules can be compared by the sequential detections of each probe; the two molecular probes, binding to its receptor, are visualized separately in the microscope by switching back and forth between two sets of filter, each specific for one dye. We have already synthesized the FITC-labeled leaf-closing factor **3** (Chart 2),[5] then we synthesized novel rhodamine-labeled leaf-opening factor **7** as a probe for double fluorescence-labeling study.

The combination of FITC/rhodamine-labeled antibodies is usually used in the immunofluorescence experiment because these dyes can be separately excited by the different wave length.[8] Probe **7** was designed according to the molecular design

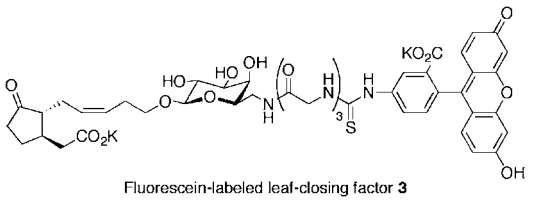
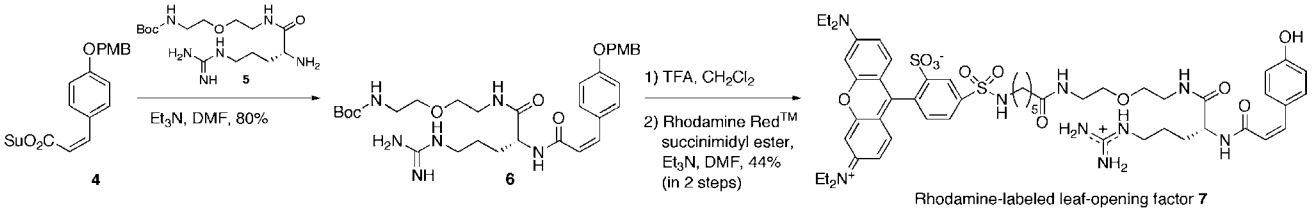


Chart 2.



Scheme 1. Synthesis of rhodamine-labeled leaf-opening factor **7**.

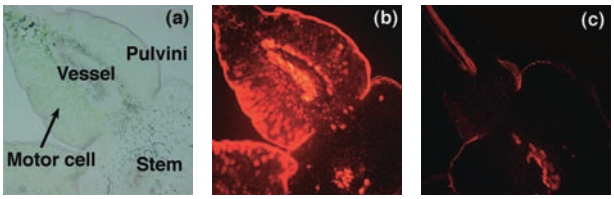


Figure 2. The binding experiments of **7**. a) Nomarskii image and fluorescence images excited at 510–560 nm after b) incubation with 1×10^{-5} M solution of **7** and c) co-incubation with 1×10^{-5} M solution of **7** and 5×10^{-2} M solution of **2**.

of previously reported FITC-labeled **2**.[5] Scheme 1 showed the improved synthetic route of a novel rhodamine-labeled probe **7** based on the previously reported route of the FITC-labeled one.[5] *p*-Methoxymethylbenzoyl (PMB)-protected *cis*-*p*-coumaric acid succinimidyl ester (**4**) was coupled with agmatine derivative **5**. Resulting **6** was deprotected and coupled with Rhodamine Red™ succinimidyl ester (Molecular probes Inc.) to give the desired rhodamine-labeled leaf-opening factor **7**.[9]

The bioactivity of **7** was confirmed by the bioassay using a leaf of *A. saman* at 5×10^{-4} M. Then, we carried out binding experiment of **7** using a plant section of *A. saman* containing motor cell according to the previous method.[5,6] A section of pulvini of *A. saman* containing motor cells was incubated with 1×10^{-5} M solution of **7**. After staining, the section was washed to remove excess amount of the probe and monitored by a fluorescent microscope. The red staining pattern due to probe **7** was observed in the motor cell, whereas the binding of **7** was competitively inhibited by the co-existence of excess amount (5×10^{-2} M) of **2** (Figure 2). These results showed that the specific binding of rhodamine-labeled probe **7** to the motor cell could be observed similar to that of FITC-labeled one.

Subsequently, we performed the double fluorescence-labeling experiment of the pulvini of *A. saman* using a combination of **3** and **7**. The plant section that was cut perpendicular to the vessel was incubated with a mixed solution containing 1×10^{-4} M of **3** and 1×10^{-5} M of **7**. After washing, the localization of each probe, binding to corresponding receptor, was visualized separately in the microscope by switching two sets of filter, each specific for one dye. The results in Figure 3 showed that the staining by each probe was monitored in the same cell, which is located in precisely the same position of the pulvini. Therefore, the motor cell with a set of receptors for leaf-movement factors is located in the one side. This result strongly suggested that our third hypothetical mechanism, which is the case (c) in Figure 1, would be the genuine location of the receptors for leaf-movement factors.

The macroscopic view such as distribution of some proteins or bioactive substances in a living body should be combined with the microscopic view such as interaction between endogenous

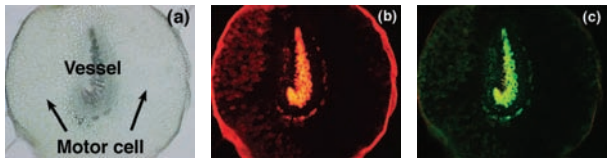


Figure 3. The double fluorescence-labeling experiment of **7**. Fluorescence images excited at a) 510–560 nm and b) at 450–490 nm after co-incubation with 1×10^{-5} M solution of **7** and 1×10^{-4} M solution of **2**.

bioactive substances and receptor proteins for the basic understanding of a biological event in the molecular level.

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9 **7**: ^{1}H NMR (300 MHz, CD_3OD): δ 8.66 (1H, d, $J = 1.8$ Hz), 8.12 (1H, dd, $J = 7.8$, 1.8 Hz), 7.52 (1H, d, $J = 7.8$ Hz), 7.44 (2H, d, $J = 8.4$ Hz), 7.09 (2H, m), 6.93 (4H, m), 6.70 (2H, d, $J = 8.4$ Hz), 6.63 (1H, d, $J = 12.3$ Hz), 5.86 (1H, d, $J = 12.3$ Hz), 4.33 (1H, dd, $J = 8.7$, 5.4 Hz), 3.65 (8H, q, $J = 6.9$ Hz), 3.51–3.45 (4H, m), 3.11 (2H, m), 3.04 (2H, m), 2.16 (2H, t, $J = 7.5$ Hz), 1.84 (1H, m), 1.71–1.43 (7H, m), 1.28 (14H, m); ^{13}C NMR (75 MHz, CD_3OD): δ 176.3, 174.0, 170.0, 159.5, 158.5, 157.5, 157.2, 152.9, 146.9, 144.4, 139.6, 135.3, 133.7, 133.5, 132.6, 129.4, 127.9, 127.7, 120.8, 116.0, 115.2, 115.1, 97.0, 70.4, 70.2, 54.5, 46.8, 44.1, 42.0, 40.4, 36.8, 30.8, 30.3, 30.1, 27.2, 26.5, 26.2, 12.8; HR–ESI–MS (positive): $[M + H]^+$ (m/z) Found 1060.4616, calcd. for $C_{52}H_{70}O_{11}N_9S_2$, 1060.4631. IR (film) ν: 3382, 1647, 1204, 1148 cm^{-1}.

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