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Fluorescence studies on nyctinasty using fluorescence labeled *cis*-*p*-coumaroylagmatine, a leaf-opening substance of *Albizzia* plants: existence of genus-specific receptor for leaf-movement factor

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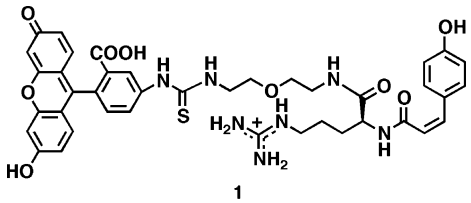
Abstract—We developed fluorescent probes (**1** and **2**) based on the structure of *cis*-*p*-coumaroylagmatine (**3**), a leaf-opening substance of *Albizzia julibrissin* Durazz. These probes were effective for the leaf-opening of *A. julibrissin*, and specifically bind to the motor cell of this plant. Moreover, binding of the fluorescent probe was specific to the plant motor cell contained in the plants belonging to the *Albizzia* genus. These results showed that the binding of a probe compound with a motor cell is specific to the plant genus and suggested that the genus-specific receptor molecule for the leaf-movement factor on a motor cell would be involved in nyctinasty.

Most leguminous plants close their leaves in the evening, as if to sleep, and open them early in the morning according to the circadian rhythm controlled by the biological clock. Nyctinastic leaf movement is induced by the swelling and shrinking of motor cells in the pulvini, an organ located in the joint of the leaf.[1] Motor cells play a key role in plant leaf-movement. A flux of potassium ions across the plasma membrane of the motor cells is followed by a massive water flux, which results in swelling and shrinking of these cells. Extensive studies on nyctinastic plants led to the isolation of a variety of leaf-closing and leaf-opening substances,[2] and we found that the biological clock regulates the balance of concentration between leaf-opening and -closing substances in the plant body during the day. Recently, we studied the mechanisms by which leaf-movement factors induce leaf movement. We developed molecular probes consisting of modified leaf-movement factors in order to identify the target cells of these factors. For example, we synthesized fluorescence-labeled potassium lespedezate, a leaf-opening substance of *Cassia* plants, and showed that some receptors for this leaf-movement factor are located on motor cells.[3–5] In this paper, we developed novel fluorescent probes (**1** and **2**) based on the structure of *cis*-*p*-coumaroylagmatine (**3**), leaf-opening substance of *Albizzia julibrissin* Durazz, and bioorganic studies using them were carried out.



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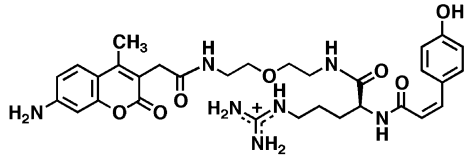

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cis-*p*-Coumaroylagmatine (**3**) was isolated as a leaf-opening substance of *A. julibrissin*.[6] We revealed that **3** operates as a leaf-opening substance among *Albizzia* genus based on the analyses of other *Albizzia* plants, such as *A. lebbeck* and *A. saman*.[7] In contrast, **3** was not effective for plants belonging to other genuses, such as *Cassia mimosoides*, *Phyllnathus urinaria*, *Mimosa pudica*. These data suggest that a common receptor for **3** would operate in the *Albizzia* genus, whereas recep-

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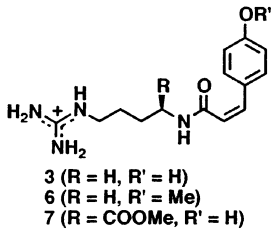
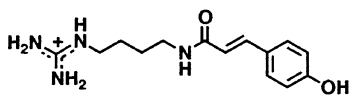
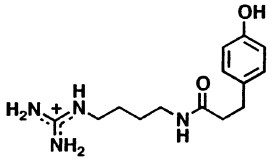
tors operating in plants belonging to other genuses would not bind to **3**. Bioactivity of the leaf-movement factor is known to be highly genus-specific.[2] Thus, it will be important to clarify whether the genus-specific bioactivity of the leaf-movement factor could be due to the difference in the specificity of the receptor on the motor cell. We carried out fluorescence studies using fluorescent probe designed on **3** to address this issue.

The molecular design of a fluorescent probe requires structure–activity relationship studies of **3**. Thus, we synthesized some analogs of **1**, such as one with a *trans*-double bond (**4**), one with a reduced double bond (**5**), one with a protected hydroxy group (**6**), and one which contains D-arginine instead of agmatine (**7**). Analogs with structure modification in the *cis*-*p*-coumaroyl moiety showed no leaf-opening activity against the leaves of *A. julibrissin*, whereas **7** was as effective as **3**. Bioassays were carried out according to the method in Ref. 7. These data suggested that the *cis*-*p*-coumaroyl moiety in **3** is indispensable for its bioactivity, and the agmatine moiety in **3** can be structurally modified with almost no decrease in bioactivity. Thus, in the molecular design of a fluorescent probe, a large fluorescence dye should be introduced in the agmatine moiety in **3**.

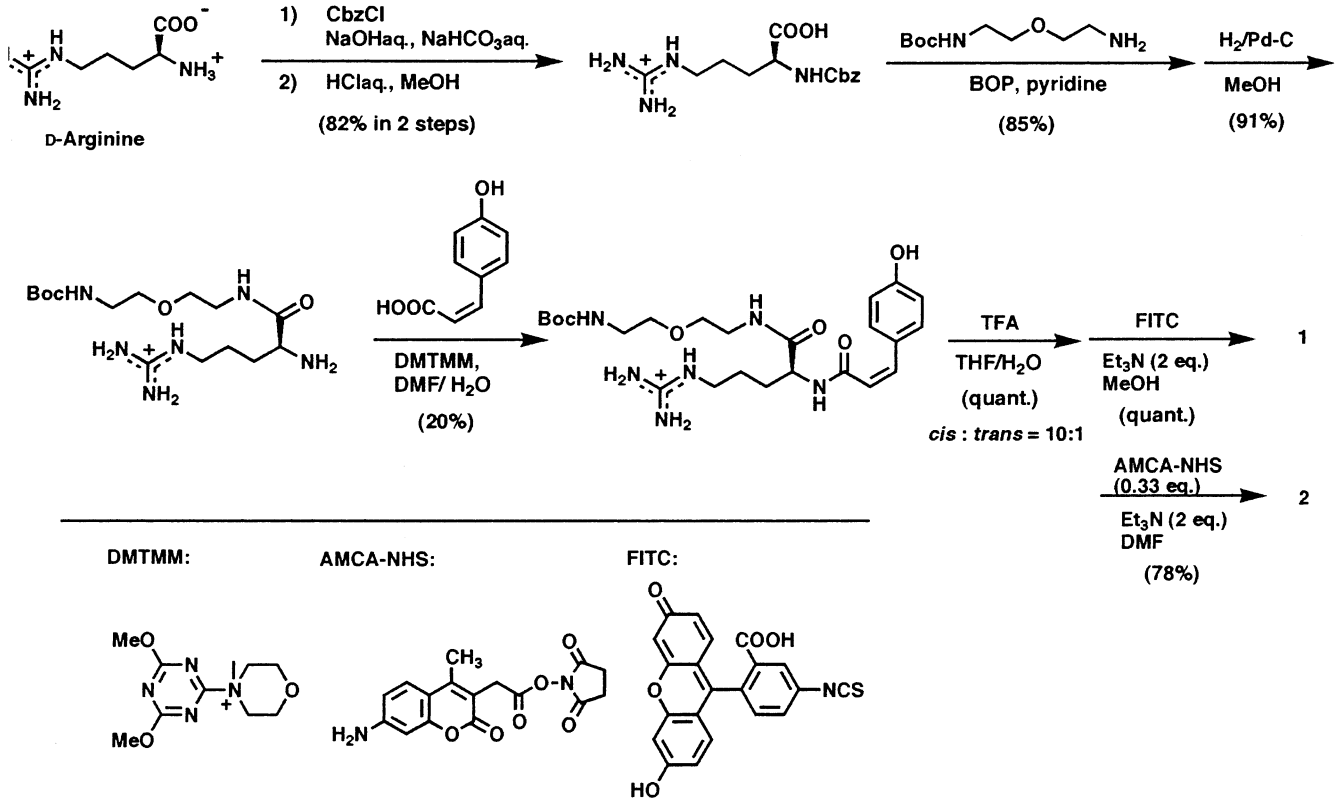
We used D-arginine, instead of agmatine, to introduce a fluorescence dye in the agmatine moiety. Use of the D-form of arginine will circumvent the hydrolysis of the amide bond by peptidase in the plant body. Also, a







fluorescence dye was connected to the carboxylate group via an amide bond to circumvent the hydrolysis by esterase in the plant body. The fluorescent probe was synthesized according to Scheme 1. D-Arginine was coupled with a diamine-ether type linker by using BOP reagent. After deprotection it was coupled with *cis*-*p*-coumaric acid by using DMTMM (dimethoxythiazolyl methylmorphorine),[8] and then with AMCA-NHS [AMCA: 6-((7-amino-4-methylcoumarin-3-acetyl) amino)-hexanoyl] or FITC [fluorescein-5-isothiocyanate] to give FITC-labeled probe (**1**)[9] or AMCA labeled probe (**2**)[10]. The bioassays using these two probes were carried out using the leaves of *A. julibrissin*.[7]



Scheme 1. Synthetic route of fluorescence labeled *cis*-*p*-coumaroylagmatine (**1** and **2**).

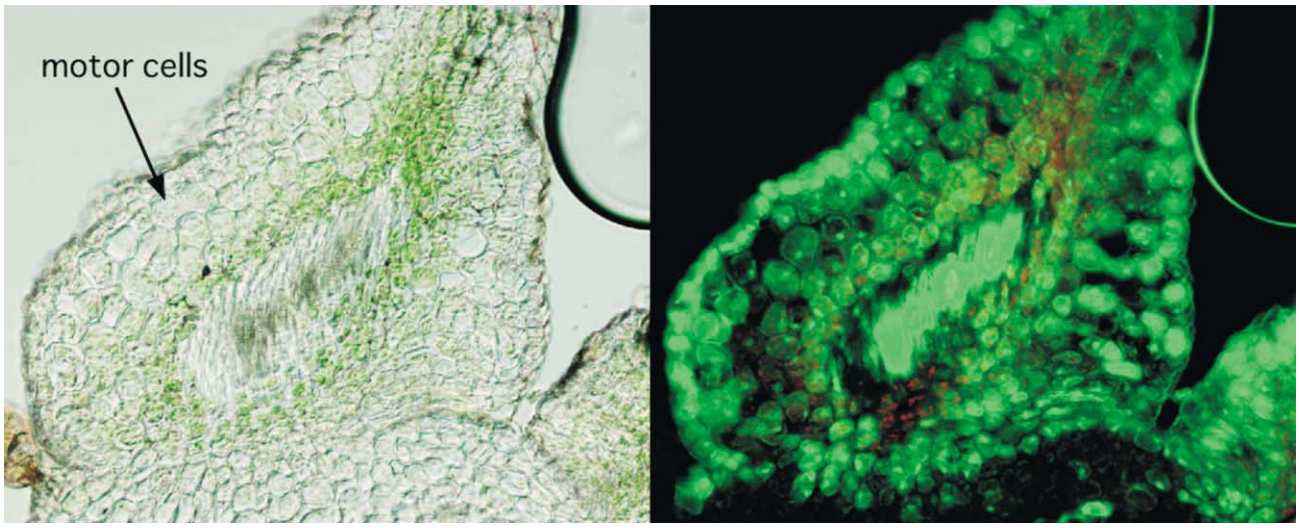


Figure 1. Binding experiment of fluorescent probe **1** with plant section of *A. julibrissin* containing motor cell (left: Nomarskii image of the plant section containing motor cell, right: fluorescence image of plant section after treatment with 1×10^{-4} M of probe **1** [excitation: 450–490 nm]).

AMCA-labeled probe (**2**) was effective at 1×10^{-5} M, which was half as strong as that of the native factor (**3**), and FITC-labeled probe (**1**) was also effective at 5×10^{-5} M. Both of the probes were of moderate bioactivity and can be used in binding experiments using plant sections containing motor cells.

We used probe **1** for a fluorescence study of the interaction between the leaf-opening substance and the plant motor cell. A leaf of *A. julibrissins* was cut by a microslicer (Dousaka EM Co., Ltd.) to a thickness of thirty micrometers. Then the section containing a motor cell was incubated for 4.5 h at 25°C in a 0.1 M citrate-phosphate buffer (pH 7.0) containing 1×10^{-4} M of **1**. After staining, the stained section was incubated for 10 min with washing buffer to remove excess fluorescent probes. Then, the stained section was monitored by using a fluorescence microscope (Nikon ECLIPSE E-800 equipped with VFM fluorescence instrument) with an appropriate filter (Nikon B-2A: excitation: 450–490 nm). The use of an antifadant reagent (Slow Fade™ Antifade Kits, Molecular Probes Inc.) was essential to prevent photobleaching (fading of fluorescence). Figure 1 shows photographs of plant pulvini (Fig. 1, left), which contains motor cells, under a fluorescence microscope. The staining pattern for the fluorescence of **1** was observed on the surface of the motor cell (Fig. 1, right). No staining was observed in other parts of the plant section treated with **1**, and the control section which was treated with a solution containing no **1**. Clear staining could be observed up to a concentration of 5×10^{-5} M of **1**. Also, binding of probe **1** was inhibited by the coexistence of 1000-fold concentration of non-labeled **3**. When the section was treated with 5×10^{-5} M of **1** together with 5×10^{-2} M of **3**, no staining was observed in the plant section. Moreover, FITC itself also gave no image. Probe **2** did not give a clear fluorescence image in the fluorescence study.[11] These results suggest that the specific binding site for **1** (or **3**) should exist on the plasma membrane of the motor cell.

From our previous studies, it was revealed that each nyctinastic plant has a different leaf-movement factor whose bioactivity is specific to the plant genus.[2,7] Thus, the fluorescent probe is expected to show specific leaf-opening activity against the leaves of plants belonging to the *Albizzia* genus, and not to be effective for the leaves of other plants. We examined the specificity of bioactivity on the probe compounds **1**. Probe compound **1** did not show leaf-opening activity against the leaves of *Cassia mimosoides* L., and *Aeschynomene indica* L. at 1×10^{-4} M, whereas it was effective at the same concentration for the leaf-opening of *Albizzia saman* and *Albizzia lebbeck*, which belong to the same *Albizzia* genus as *A. julibrissin*. From these results, the binding of a probe compound is expected to be specific to the section of plants belonging to the *Albizzia* genus, and no binding would be observed in the experiment using the section of other plants.

Then, we used probe **1** for the binding experiment with the sections of *C. mimosoides*, *Aeschynomene* indica together with that of *A. saman*, and *A. lebbeck*. The binding experiments were carried out according to the same method used in the case of *A. julibrissin*. Thus, it was revealed that the sections of *A. saman* and *A. lebbeck* gave a fluorescence image resulting from **1** and no other sections gave the image (Fig. 2). Red stains seen in the fluorescence images are due to the porphyrine in the plant tissue. These results showed that the binding of a probe compound with a motor cell is specific to the *Albizzia* genus and suggested that a genus-specific receptor molecule for the leaf-movement factor on a motor cell would be involved in nyctinasty. This result will provide a molecular basis for discussion of the following issue: why does each nyctinastic plant have a different leaf-movement factor whose bioactivity is specific to the plant genus?

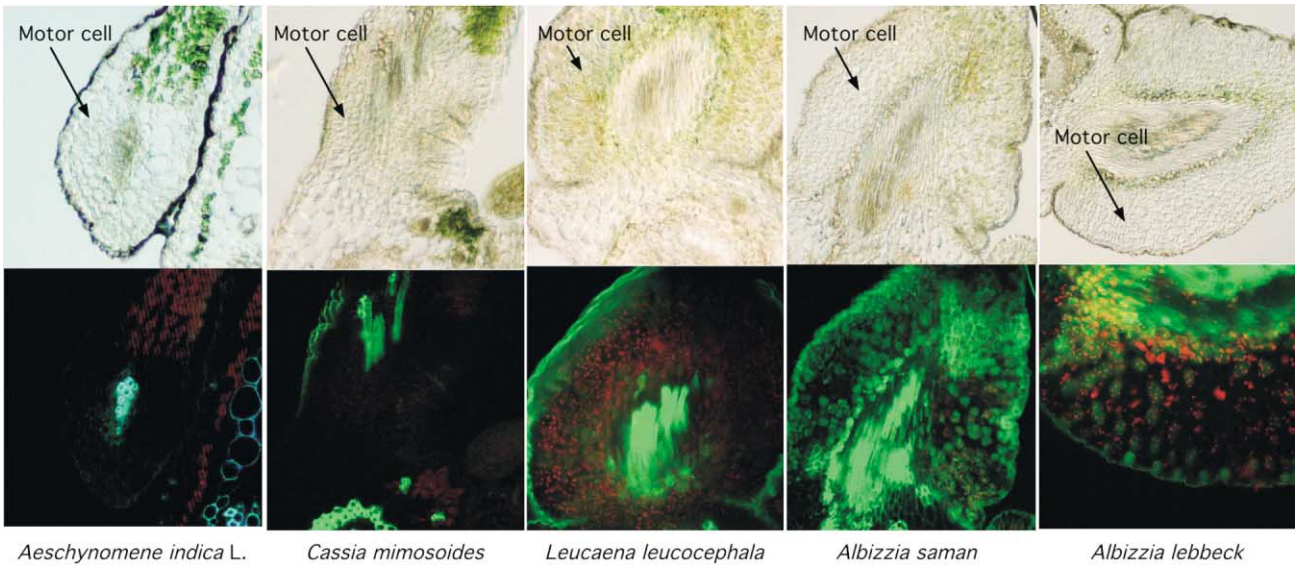


Figure 2. Photographs of plant sections in the binding experiments which shows specific binding of probe **1** with the motor cell of *Albizzia* plants (upper: Nomarskii image of plant section, lower: fluorescent image of plant section after treatment with 1×10^{-4} M of probe **1** [excitation: 450–490 nm]).

Acknowledgements

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9. **1**: ^{1}H NMR (270 MHz, CD_2OD, 18°C): 8.03 (1H, d, $J=2.0$ Hz), 7.66 (1H, brs), 7.38 (2H, d, $J=8.6$ Hz), 7.20 (1H, d, $J=8.4$ Hz), 7.13 (2H, d, $J=8.6$ Hz), 6.70–6.58 (7H, m), 5.89 (1H, d, $J=12.7$), 4.30 (1H, m), 3.94–3.37 (8H, m), 3.01 (2H, t, $J=7.0$ Hz), 1.86–1.41 (4H, m); ^{13}C NMR (100 MHz, DMSO, 18°C): 180.2, 171.8, 169.6, 166.1, 165.5, 159.1, 158.2, 156.9, 155.2, 140.2, 137.7, 132.3, 130.0, 129.3, 127.8, 126.1, 125.2, 120.8, 120.2, 118.4, 115.8, 114.8, 111.0, 102.6, 69.0, 68.2, 52.3, 45.7, 43.9, 29.1, 25.0, 11.5; HR FAB MS (positive): $[M+H]^+$ found m/z 796.2778, $C_{40}H_{42}O_9N_7S$ requires m/z 796.2765; IR (film) ν: 3273, 1589, 1466, 1387, 1265, 1209, 1111 cm^{-1}; $[\alpha]_D^{20}$ +40.2° (c 1.0, DMSO).
10. **2**: ^{1}H NMR (400 MHz, CD_3OD, 25°C): 7.49 (1H, d, $J=9$ Hz), 7.42 (2H, d, $J=9$ Hz), 6.69 (2H, d, $J=9$ Hz), 6.67 (1H, dd, $J=2$, 8 Hz), 6.64 (1H, d, $J=12$ Hz), 6.51 (1H, d, $J=2$ Hz), 5.86 (1H, d, $J=13$ Hz), 4.42 (1H, dd, $J=6$, 7 Hz), 3.54 (2H, s), 3.51 (5H, m), 3.37 (3H, m), 3.16 (2H, t, $J=7$ Hz), 2.35 (3H, s), 1.98 (6H, s), 1.82 (1H, m), 1.72–1.51 (3H, m); ^{13}C NMR (100 MHz, CD_3OD, 18°C): 175.2, 173.9, 173.3, 170.0, 164.9, 159.4, 158.6, 155.9, 153.6, 153.0, 139.5, 132.6, 127.9, 127.5, 120.7, 116.0, 114.6, 113.5, 112.0, 100.7, 70.3, 70.2, 54.3, 42.0, 40.6, 40.5, 35.2, 30.2, 26.3, 20.7, 15.4; HR FABMS (positive): $[M+H]^+$ found 622.2983 m/z, $C_{31}H_{40}O_7N_7$ requires 622.2989 m/z; IR (film) ν: 3344, 1668, 1554, 1516, 1201, 1136, 721 cm^{-1}; $[\alpha]_D^{20}$ +14.1° (c 0.5, MeOH).
11. Probe **2** gave dim image and clear image with high resolution was not obtained.