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Fluorescence studies on nyctinasty which suggest the existence of genus-specific receptors for leaf-movement factor

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Periodic leaf-movement of legumes is called nyctinasty and has been known since the age of Alexander the Great. We found that nyctinasty is controlled by a periodic change of the internal concentration of leaf-opening and leaf-closing substances in the plant body. Now, we have developed novel fluorescent probes (**1**) based on the structure of *cis-p*-coumaroylagmatine (**3**), which was isolated as a leaf-opening substance of *Albizzia juribrissin* Durazz. Binding experiments using probe **1** showed that *Albizza* plants have receptors for a leaf-opening substance in their motor cells. By using probes **1** we then found that genus-specific receptors are involved in nyctinasty.

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Introduction

Plants are unable to move from one place to another. However, the folding and opening movement of the leaves according to the circadian rhythm have been widely observed in leguminous plants (Fig. 1). This periodic leaf-movement is called nyctinasty and has been known since the age of Alexander the Great.[1] It is widely known that Charles Darwin carried out the pioneering work in this field, and Since Darwin, many scientists have worked on research in this field. It was revealed that 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] We found that the biological clock regulates the balance of concentration between leaf-opening and -closing substances in the plant body during the day.[2] Recently, we studied the mechanisms by which leaf-movement factors induce leaf movement and 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 (**2**), a leaf-opening substance of *Cassia* plants, and showed that some receptors for this leaf-movement factor are located on motor cells.[3–5]

Fig. 1 Nyctinasty of *Albizzia julibrissin* Durazz. (left: leaf in the daytime, right: leaf at night).

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

In this paper, we report the development of a novel fluorescent probe (**1**) based on the structure of *cis-p*-coumaroylagmatine (**3**), a leaf-opening substance of *Albizzia julibrissin* Durazz. We found that genus-specific receptors are involved in nyctinasty by using probe **1**.

Results and discussion

The molecular design of a fluorescent probe required structure–activity relationship studies of **3**. Thus, we synthesized some analogs of **3**, such as one with a *trans*-double bond (**4**),[8] one with a reduced double bond (**5**), one with a protected hydroxy group (**6**), and one which contains D-arginine instead of agmatine (**7**) (Schemes 1 and 2). Commercially available *trans-p*-coumaric acid was reduced by hydrogen gas using Pd–C and resulting **13** was coupled with agmatine to give **5** (Scheme 1). An analog **6** was synthesized from **16** which was prepared from *trans-p*-coumaric acid with methyl iodide (Scheme 2). Analog **7** was synthesized from *N*-hydroxysuccinimide ester of *cis-p*-coumaric acid which was prepared according to the method in reference 8.

Analogs with structure modification in the *cis-p*-coumaroyl moiety, such as **4**, **5**, and **6**, showed no leaf-opening activity against the leaves of *A. juribrissin*, whereas **7** was as effective as **3** that is effective at 5×10^{-5} M. 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

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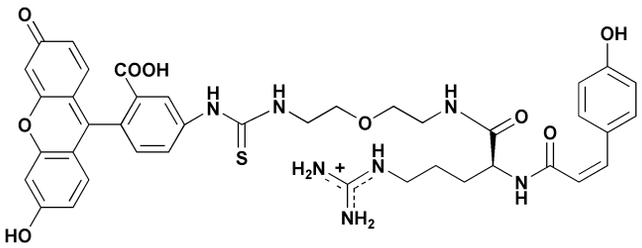

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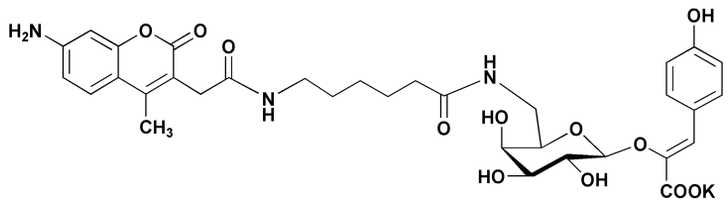

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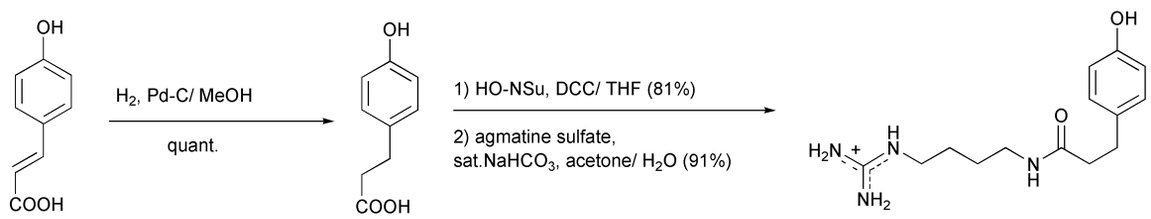

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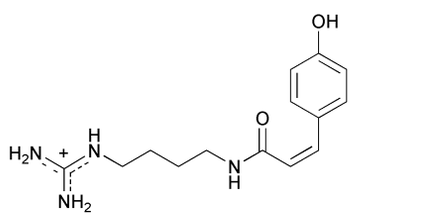
Scheme 1 Synthetic route of analog of *cis-p*-coumaroylagmatine (**5**).



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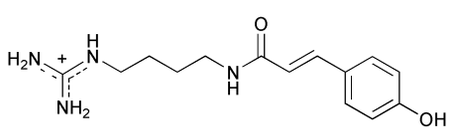

4

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 circumvented 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 3. D-Arginine was coupled with a diamine-ether type linker by using BOP (benzotriazole-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate) reagent. After deprotection it was coupled with *cis-p*-coumaric acid by using DMTMM (dimethoxythiazolyl methylmorphorine),[9] 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**) or AMCA labeled probe (**24**).

The bioassays using these two probes were carried out with the leaves of *A. julibrissin*. AMCA-labeled probe (**24**) 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. Then the section containing a motor cell was incubated for 4.5 hours 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 minutes with washing buffer to remove excess fluorescent probes. Then, the stained section was monitored by using a fluorescence microscope. The use of an antifadant reagent was essential to prevent photobleaching (fading of fluorescence). Fig. 2 shows photographs of plant pulvini (Fig. 2, left), which contains motor cells. The staining pattern for the fluorescence of **1** was observed on both the plasma membrane and nuclei (Fig. 2, center and right) of the motor cell. No staining was observed in the cells contained 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 a 1000-fold concentration of non-labeled **3**. When the section was treated

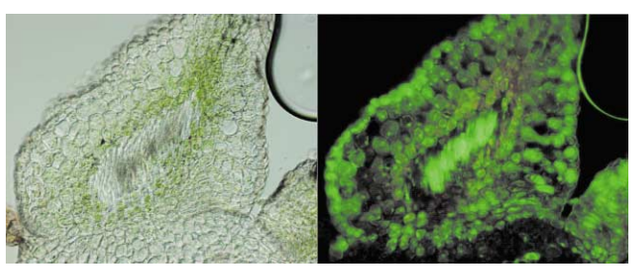

Fig. 2 Binding experiment of fluorescent probe **1** with a plant section of *A. juribrissin* containing a motor cell (left: Nomarskii image of the plant section containing motor cell, right: fluorescence image of plant section after treatment with 1×10^{-4} M of probe **1**; [Excitation: 450–490 nm]. Right: expansion of the fluorescence image of motor cell).

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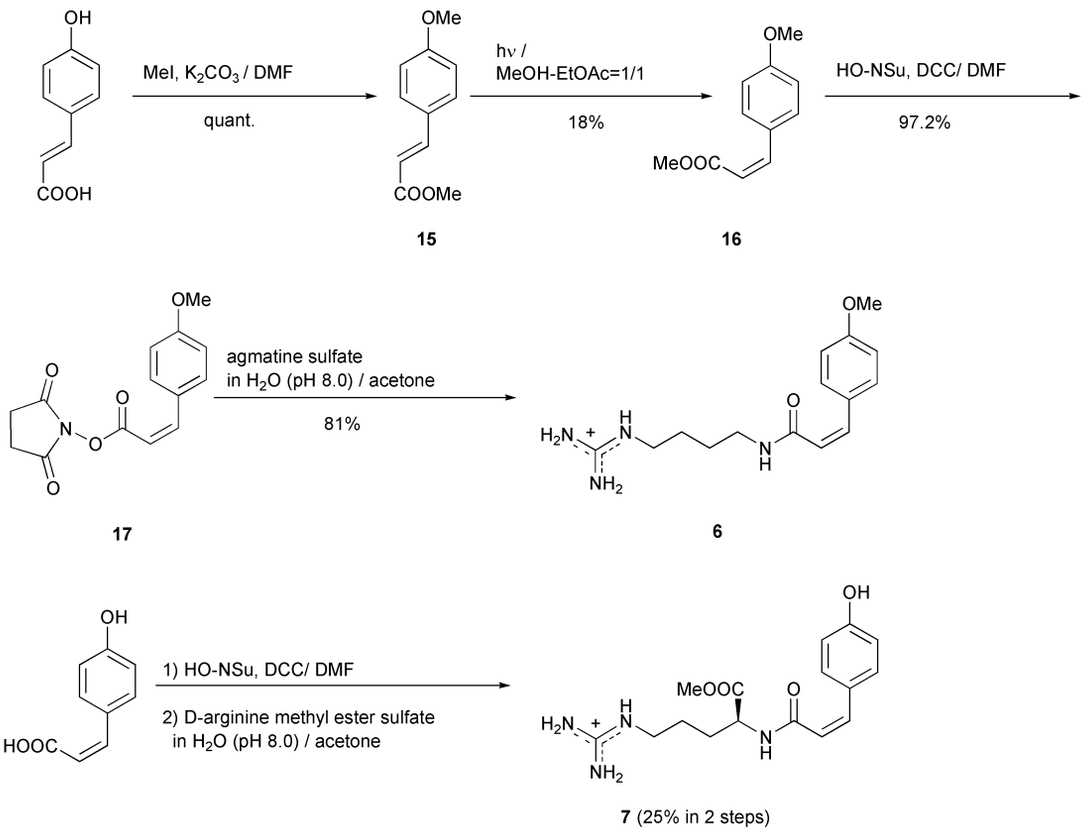

Scheme 2 Synthetic route of analogs of *cis-p*-coumaroylagmatine (**6** and **7**).

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with 5×10^{-5} M of **1** together with 5×10^{-2} M of **3**, no staining was observed in the plant section on both membrane and nuclei. These results suggest that the specific binding site for **1** (or **3**) should exist in the plasma membrane and nuclei of the motor cell.

Interestingly, we found that the binding of **1** with nuclei was inhibited in the presence of *cis-p*-coumaric acid (**25**). We carried out the binding examination of probe **1** at 1×10^{-4} M in the presence of 1000-fold molar excess of **25**, that was not effective for the leaf-opening of *A. juribrissin* even at 1×10^{-4} M. Binding of **1** to the nuclei was strongly inhibited by the coexistence of **25**, whereas binding to the plasma membrane was not (Fig. 3). Together with the result using **3**, the competitive inhibition of the binding of **1** to the plasma membrane was strongly correlated to the leaf-opening activity of the coexisting molecule. These results strongly suggest that the binding of **1** to the nuclei is attributed to *cis-p*-coumaric acid which has no leaf-opening activity on the bioassay using the whole leaf. Thus, the genuine receptor of leaf-opening substance **3** would be located in the plasma membrane of the motor cell.

On the other hand, this result showed that some receptors for **25** could be located in the nuclei of the motor cell as well as leaf-opening substances that have so far been isolated from several genuses contained some analogs of *cis-p*-coumaric acid in the molecule.[2] The structural variation of the leaf-opening substances is due to the variety of the other parts. Thus, it would be estimated that **25** would be a genuine bioactive substance for leaf-opening, and an agmatine moiety would be important for transport in the plant body and recognition by a membrane receptor. After recognition by a receptor molecule on the membrane, **1** would be transferred into the cytosol and transported to the nuclei to develop the leaf-opening activity.

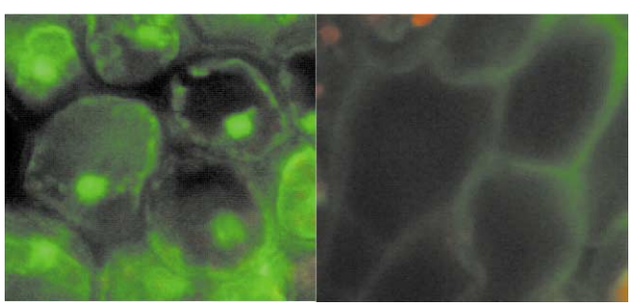

Fig. 3 Expansion of the fluorescence image of an *Albizzia* section treated with **1** (left), and of the section treated with **1** in the presence of 1000 molar exess of *cis-p*-coumaric acid (**25**) (right).

From the result of the fluorescence studies mentioned above, the specific recognition of **1** by the membrane receptor could be due to the whole structure of the molecule containing a *cis-p*-coumaroyl moiety and agmatine part. *A. juribrissin* contains two types of receptor: one is the membrane receptor that recognizes the whole structure of the molecule, and the other is the nucleus receptor that binds to the *cis-p*-coumaric acid. This result suggests that the leaf-opening substance **3** is perceived by the membrane receptor, and then the *cis-p*-coumaric acid is released, transported into the cytosol, and bound to a nucleus receptor to induce leaf-opening.

This model 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? The genus specificity on the recognition of the leaf-movement factor can be attributed to the ligand specificity of the membrane receptor, and the nucleus receptor which binds to *cis-p*-coumaric acid would be common among leguminous plants because almost all leaf-opening substances contain the *cis-p*-coumaroyl moiety in the molecule.

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] We revealed that **3** is commonly contained in three *Albizzia* plants, such as *A. julibrissin*, *A. lebbeck*, and *A. saman*.[7] Compound **3** was effective for these *Albizzia* plants, whereas it was not effective for the plants belonging to other plant genera. This result strongly suggested the existence of some specific receptor for **3** that is common among genus *Albizzia*. First, we examined the

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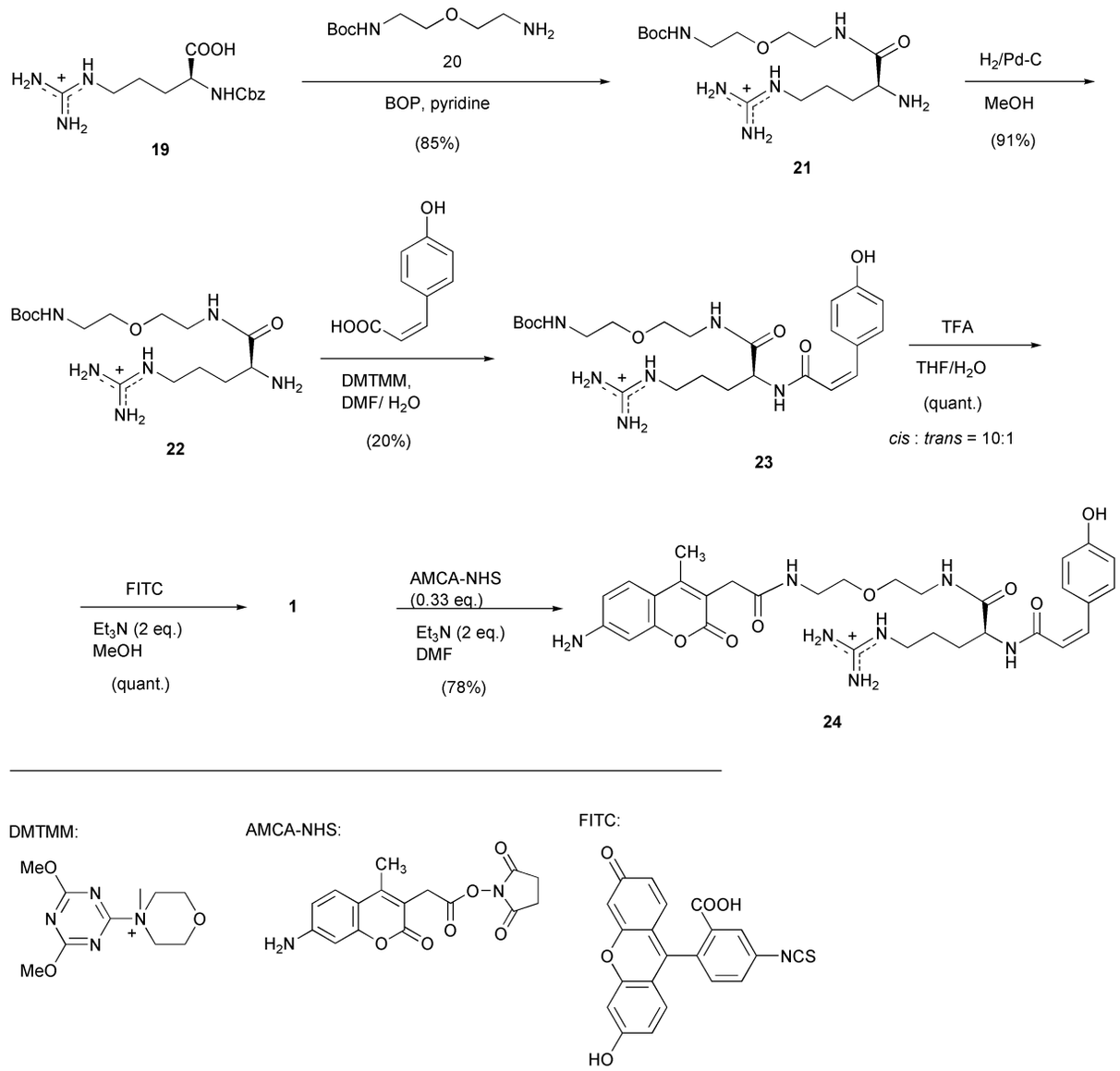

Scheme 3 Synthetic route of fluorescence labeled *cis-p*-coumaroylagmatines (**1** and **24**).

specificity of the bioactivity on the probe compound **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 **1** is expected to be specific to the section of plants belonging to genus *Albizzia*, 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. juribrissin*. 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. 4). Red stains seen in the fluorescence images are due to the porphyrin in the plant tissue. These results showed that the binding of a probe **1** with a motor cell is specific to genus *Albizzia* and suggested that a genus-specific receptor molecule for the genus-specific leaf-movement factor on a motor cell would be involved in nyctinasty.

From these results, we have shown that *Albizza* plants have receptors for leaf-opening substances in their motor cells. The specificity of the receptors for the ligands is common among the plant genus and this result strongly suggests that the genus specific receptor would be involved in the binding between the leaf-movement factor and a motor cell. It was estimated that each plant genus would have different receptor molecules on the motor cells which are specific to each genus-specific leaf-movement factor. Genus specific recognition of the ligand by a receptor of a leaf-movement factor strongly suggests that membrane receptors concerning nyctinasty would be differentiated in the comparatively latter process of evolution in the plant kingdom.

Experimental

General

NMR spectra were recorded on a Jeol JNM-A400 spectrometer [^{1}H (400 MHz) and ^{13}C (100 MHz)] or a Jeol JNM-EX 270 spectrometer [^{1}H (270 MHz) and ^{13}C (67.5 MHz)] using TMS in $CDCl_3$, CD_2HOD in CD_3OH (^{1}H; 3.33 ppm, ^{13}C; 49.8 ppm), or *t*-BuOH (^{1}H; 1.23 ppm, ^{13}C; 32.1 ppm) in D_2O as internal standards at various temperatures. The FAB-MS and HR FAB-MS spectra were recorded on a Jeol JMS-700 spectrometer, using glycerol or *m*-nitrobenzylalcohol as a matrix. The IR spectra were recorded on a JASCO FT/IR-410. The specific rotations were measured by JASCO DIP-360 polarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. The HPLC purification was carried out with a Shimadzu LC-6A pump equipped with

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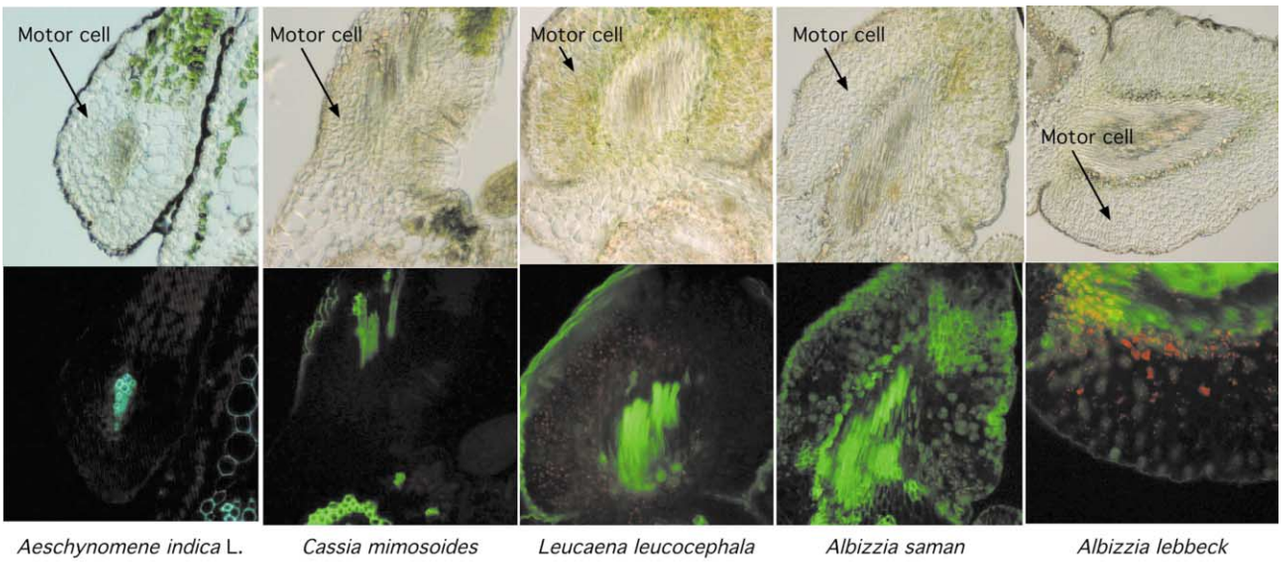

Fig. 4 Photographs of plant sections in the binding experiments which show specific binding of probe **1** with the motor cells of *Albizzia* plants (upper: Nomarskii image of the plant section, lower: fluorescent image of the plant section after treatment with 1×10^{-4} M of probe **1** [excitation: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

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a SPD-6A detector using a COSMOCIL $5C_{18}$-AR column (ϕ 20 × 250 mm) (Nakalai Tesque Co. Ltd.). The solvents used for HPLC were available from Kanto Chemical Co. and were filtered through a Toyo Roshi membrane filter (cellulose acetate of 0.45 μm pore size, 47 mm diameter) before use. Silica gel column chromatography was performed on silica gel 60 K070 (Katayama Chemical Co. Ltd.) or silica gel 60N (Kanto Chemical Co. Ltd.). Reversed-phase open-column chromatography was performed on Cosmosil 75C18-OPN (Nakalai Tesque Co. Ltd.). TLC was performed on Silica gel F_{254} (0.25 mm or 0.5 mm, MERCK) or RP-$18F_{254S}$ (0.25 mm, MERCK). Photochemical reactions were performed with a high-pressure Hg-lump system (UM-452 equipped with UM-453B-A, Ushio Electronics Co. Ltd.)

cis-p-Coumaroyl-D-arginine methyl ester (7)

To a solution of **10** (16.2 mg, 98.7 μmol) in DMF (0.2 mL) was added HO–NSu (17.3 mg, 150 μmol) and DCC (23.2 mg, 112 μmol) at 0 °C and then the solution was stirred for 24 hours. The reaction mixture was concentrated *in vacuo*, and the residue was mixed with ethyl acetate, and then filtered with Hyflo super-cell (Wako Pure Chemical Industries). The filtrate was washed with saturated cold $NaHCO_3$aq. and then cold brine. The organic layer was concentrated *in vacuo*, and the residue was separated by silica gel column chromatography ($CHCl_3$–MeOH = 10 : 1) to give **11**. To the solution of **11** in acetone (0.7 mL), D-arginine methyl ester sulfate (11.1 mg, 49.4 μmol) in H_2O (1.0 mL), which was adjusted to pH 8.0 with saturated $NaHCO_3$ aq., was added at 0 °C. This reaction mixture was stirred for 24 hours, adjusted to pH 3 with acetic acid, and then evaporated to remove acetone. This solution was extracted by ethyl acetate, and the aqueous layer was concentrated to dryness. The residue was purified by HPLC Cosmosil 5C18AR (30% MeOH aq. containing 1% AcOH) to give **7** (5.6 mg, 25%).

7: ^{1}H-NMR (270 MHz, CD_3OD, 19 °C): 7.46 (2H, d, J = 8.9 Hz), 6.71 (2H, d, J = 8.9 Hz), 6.69 (1H, d, J = 12.5 Hz), 5.86 (1H, d, J = 12.5 Hz), 4.49 (1H, dd, J = 8.6, 4.9 Hz), 3.73 (3H, s), 3.18 (2H, dd, J = 12.9, 6.3 Hz), 1.92–1.87 (1H, m), 1.75–1.57 (3H, m) ppm; ^{13}C-NMR (100 MHz, CD_3OD, 20 °C): 176.1, 173.4, 159.3, 158.4, 139.5, 132.5, 127.8, 120.2, 115.8, 53.2, 52.8, 41.9, 29.6, 26.4 ppm.; HR FAB MS (positive): $[M + H]^+$ Found m/z 335.1749, $C_{16}H_{23}O_4N_4$ requires m/z 335.1719; IR (film) ν: 3197, 1736, 1653, 1608, 1514, 1219, 1173 cm^{-1}; $[\alpha]_D^{20}$ 48.9 (c 0.17, MeOH).

3-(4-Hydroxyphenyl) propionyl agmatine (5)

To a solution of **13** (30.6 mg, 0.184 mmol) in THF (0.5 mL), HO–NSu (36.2 mg, 0.315 mmol) and then DCC (58.2 mg, 0.282 mmol) was added at 0 °C. After stirring for 2 hours under an argon atmosphere, the solution was filtered through Celite, and was diluted with ethyl acetate. The filtrate was washed with saturated $NaHCO_3$ aq., dried over abs. Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel column chromatography and TLC ($CHCl_3$: MeOH = 9 : 1) to give activated ester **14** (39.2 mg, 81%). Compound **14** (32.1 mg) was dissolved in acetone (1 mL) at 0 °C and was then mixed with agamatine sulfate (26.3 mg, 0.115 mmol) in 1 mL of H_2O, which was adjusted to pH 8 by saturated $NaHCO_3$aq. After stirring for 19 hours, the reaction mixture was adjusted to pH 3 by acetic acid and was washed with ethyl acetate. The aqueous layer was evaporated to dryness, and the residue was dissolved in 30% MeOH aq. The residue was purified by HPLC Cosmosil 5C18AR (30% MeOH aq.) to give **5** (29.2 mg, 91%).

5:^{1}H-NMR (270 MHz, CD_3OD, 18 °C): 6.93 (2H, d, J = 8.3 Hz), 6.63 (2H, d, J = 8.3 Hz), 2.66 (2H, t, J = 6.9 Hz), 2.32 (2H, t, J = 6.9 Hz), 1.13–1.05 (2H, m), 1.01–0.95 (2H, m) ppm; ^{13}C-NMR (100 MHz, CD_3OD, 20 °C): 175.6, 156.9, 132.8, 130.4, 116.2, 42.1, 39.5, 39.3, 32.2, 27.7, 27.0 ppm; HR FAB MS (positive): $[M + H]^+$ Found m/z 279.1796, $C_{14}H_{23}O_2N_4$ requires m/z 279.1821; IR (film) ν: 3172, 1641, 1552, 1514, 1406, 1248 cm^{-1}.

4-*O*-Methyl-*cis-p*-coumaryl succinimide ester (18)

To a solution of **17** (25.1 mg, 0.14 mmol) in DMF (0.3 mL) was added *N*-hydroxysuccinimide (HO–NSu, 32.9 mg, 0.29 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI, 54.0 mg, 0.28 mmol) at 0 °C under an argon atmosphere. After stirring for 4 hours, additional EDCI (39.2 mg, 0.20 mmol) was added to this solution. After stirring overnight, the reaction mixture was concentrated *in vacuo*, and the residue was purified with silica gel column chromatography and preparative TLC ($CHCl_3$–MeOH = 10 : 1) to give **18** (37.7 mg, 97.2%).

18:^{1}H-NMR (270 MHz, $CDCl_3$, 18 °C): 7.77 (2H, d, J = 8.9 Hz), 7.18 (1H, d, J = 12.5 Hz), 6.89 (2H, d, J = 8.9 Hz), 6.00 (1H, d, J = 12.5 Hz), 3.83 (3H, s), 2.84 (4H, s) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$, 19 °C): 169.3, 161.3, 160.8, 150.3, 133.0, 126.1, 113.5, 108.8, 55.3, 25.6 ppm; HR FAB MS (positive): $[M + Na]^+$ Found m/z 298.0680, $C_{14}H_{13}O5N_1Na_1$ requires m/z 298.0691; IR (film) ν: 1767, 1736, 1597, 1512, 1208, 1071 cm^{-1}

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cis-p-Methoxycinnamoylagmatine (6)

To a solution of Agmatin sulfate (26.8 mg, 0.12 mmol) in H_2O (0.8 mL) was added saturated $NaHCO_3$ aq. in order to adjust the pH to 8. To this solution was added compound **18** (36.1 mg, 0.13 mmol) in acetone (1 mL) at 0 °C. After stirring for 3 days, the reaction mixture was evaporated to remove acetone, extracted with EtOAc, and the resulting aqueous layer was evaporated to dryness. The residue was purified with HPLC using a Cosmosil 5C18AR column (1% AcOH–30% MeOH aq.) to give **6** (31.0 mg, 81%).

6: ^{1}H-NMR (270 MHz, CD_3OD, 20 °C): 7.49 (2H, d, J = 8.9 Hz), 6.86 (2H, d, J = 8.9 Hz), 6.66 (1H, d, J = 12.5 Hz), 5.90 (1H, d, J = 12.5 Hz), 3.78 (3H, s), 3.24 (2H, br s), 3.15 (2H, br s), 1.57 (4H, br s) ppm; ^{13}C-NMR (100 MHz, CD_3OD, 20 °C): 175.5, 161.4, 158.6, 138.0, 132.2, 129.2, 122.3, 114.5, 55.7, 42.0, 39.7, 27.4, 27.2 ppm; HR FAB MS (positive): [M + H]$^+$Found m/z 291.1823, $C_{15}H_{23}O_2N_4$ requires m/z 291.1821; IR (film) v: 3163, 1643, 1512, 1255, 1176, 1093, 613 cm^{-1}.

N-(2-(*tert*-Butoxycarbonylamino)ethoxyethyl) N_α-benzyloxycarbonyl-D-arginine amide (21)

Compound **19** (60.7 mg, 0.18 mmol, Fluka Co., Ltd.) was dissolved in pyridine (1.3 mL), and to this solution was added **20** (27.0 mg, 0.13 mmol) followed by benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (83.2 mg, 0.19 mmol) at 0 °C under an argon atmosphere. After stirring for 15 hours, the reaction mixture was evaporated with the addition of toluene to remove pyridine. The resulting residue was purified with silica gel column chromatography and preparative TLC ($CHCl_3$–MeOH–AcOH = 10 : 2 : 1) to give **21** (54.2 mg, 85%).

21: ^{1}H-NMR (270 MHz, CD_3OD, 17 °C): 7.37–7.29 (5H, m), 5.11 (1H, d, J = 12.8 Hz), 5.07 (1H, d, J = 12.8 Hz), 4.10 (1H, m), 3.49 (2H, t, J = 5.2 Hz), 3.45 (2H, t, J = 5.2 Hz), 3.36 (2H, m), 3.20–3.16 (4H, m), 1.84–1.58 (4H, m), 1.42 (9H, s) ppm; ^{13}C-NMR (67.5 MHz, CD_3OD, 19 °C): 174.3, 158.3, 158.2, 137.9, 129.3, 128.9, 128.7, 80.1, 70.8, 70.2, 67.7, 56.0, 41.9, 41.3, 40.3, 30.4, 28.8, 26.3 ppm.; [M + H]$^+$ Found m/z 495.2912, $C_{23}H_{39}O_6N_6$ requires m/z 495.2931; IR (film) v: 3411, 1668, 1523, 1252, 1169, 845 cm^{-1}; $[a]_D^{20}$ −5.7 (c 0.5, MeOH).

N-(2-(*tert*-Butoxycarbonylamino)ethoxyethyl) D-arginine amide (22)

Compound **21** (146.6 mg, 0.27 mmol) was dissolved in methanol (3 mL) and 10% Pd–C powder was added to this solution. This solution was stirred for one hour under a hydrogen atmosphere, filtered with Celite, and evaporated to dryness to give **22** (100.8 mg, 91%).

22: ^{1}H-NMR (270 MHz, CD_3OD, 25 °C): 3.61 (1H, t, J = 6.3 Hz), 3.54 (2H, t, J = 4.9 Hz), 3.47 (2H, t, J = 5.6 Hz), 3.42 (2H, m), 3.21 (4H, t, J = 5.9 Hz), 1.94 (3H, s), 1.81–1.72 (2H, m), 1.67–1.62 (2H, m), 1.43 (9H, s) ppm; ^{13}C-NMR (67.5 MHz, CD_3OD, 19 °C): 181.8, 170.2, 158.8, 157.3, 81.7, 70.0, 69.2, 53.7, 41.2, 40.5, 39.9, 28.9, 28.5, 24.5, 24.1 ppm; HR FAB MS (positive): [M + H]$^+$ Found 361.2550 m/z, $C_{15}H_{33}O_4N_6$ requires 361.2563 m/z; IR (film) v: 3263, 1676, 1547, 1406, 1173, 1124 cm^{-1}; $[a]_D^{20}$ −4.4 (c 1.0, MeOH)

N-(2-(*tert*-Butoxycarbonylamino)ethoxyethyl) N_α-*cis-p*-coumaroyl-D-arginine amide (23)

Compound **22** (78.9 mg, 0.164 mmol) was dissolved in DMF (1.5 mL) with a trace amount of water. To this solution was added **10** (40.4 mg, 0.246 mmol) and then 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (75.2 mg, 0.272 mmol) at 0 °C. This solution was stirred under an argon atmosphere. After 12 hours, additional DMTMM (68.7 mg, 0.25 mmol) was added. After 3 hours, the reaction mixture was evaporated, and the residue was purified with silica gel column chromatography and preparative TLC ($CHCl_3$–MeOH–H_2O = 24 : 8 : 1) to give **23** (22.6 mg, 27%).

23: ^{1}H-NMR (270 MHz, CD_3OD, 18 °C): 7.45 (2H, d, J = 8.6 Hz), 6.73 (2H, d, J = 8.6 Hz), 6.69 (1H, d, J = 12.5 Hz), 5.89 (1H, d, J = 12.5 Hz), 4.40 (1H, m), 3.53–3.44 (4H, m), 3.39–3.34 (2H, m), 3.19 (4H, t, J = 5.6 Hz), 1.87–1.79 (1H, m), 1.71–1.52 (3H, m), 1.43 (9H, s) ppm; ^{13}C-NMR (67.5 MHz, CD_3OD, 19 °C): 181.7, 172.4, 169.8, 167.8, 159.2, 139.4, 132.5, 127.8, 120.6, 115.8, 82.2, 70.9, 70.2, 54.2, 42.0, 41.3, 40.4, 30.2, 28.8, 26.4 ppm; HR FAB MS (positive): [M + H]$^+$ Found m/z 507.2932, $C_{24}H_{39}O_6N_6$ requires m/z 507.2931; IR (film) v: 3273, 1655, 1514, 1252, 1173, 843 cm^{-1}; $[a]_D^{20}$ +31.7 (c 0.5, MeOH).

N-{2-[(Fluorescein-4-isothiocyanato)amino]ethoxyethyl} N_α-*cis-p*-coumaroyl-D-arginine amide (1)

Compound **23** (22.6 mg, 44.7 μmol) was dissolved in THF–H_2O (1 : 1) (0.6 mL) and TFA (1.5 mL) was added to this solution at 0 °C. After stirring for 2 hours, this solution was evaporated to dryness to give a TFA salt of the resulting amine. The TFA salt of the resulting amine (10.7 mg) was dissolved in methanol (1 mL). To this solution, triethylamine (4.8 μL, 34.5 μmol) and then fluorescein isothiocyanate (FITC) (isomer I, 6.6 mg, 17.0 μmol) were added at 0 °C. After stirring overnight at rt, the reaction mixture was evaporated to dryness and the residue was purified with preparative TLC ($CHCl_3$–MeOH–H_2O = 24 : 8 : 1) to give **1** (21.0 mg, quant.).

1: ^{1}H-NMR (270 MHz, CD_3OD, 18 °C): 8.03 (1H, d, J = 2.0 Hz), 7.66 (1H, br s), 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) ppm; ^{13}C-NMR (100 MHz, DMSO-d_6, 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 ppm; 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) v: 3273, 1589, 1466, 1387, 1265, 1209, 1111 cm^{-1}; $[a]_D^{20}$ +40.2 (c 1.0, DMSO).

N-{[2-(7-Amino-4-methylcoumarin-3-acetyl)amino]ethoxyethyl} N_α-*cis-p*-coumaroyl-D-arginine amide (24)

Compound **23** (31.1 mg, 61 μmol) was dissolved in THF–H_2O (1 : 1) (0.6 mL) and TFA (1.0 mL) was added to this solution at 0 °C. After stirring for one hour, the reaction mixture was evaporated to give a TFA salt of the resulting amine. The TFA salt of the resulting amine was dissolved in DMF (0.5 mL) and triethylamine (17.7 μL, 127.8 μmol) and then 7-amino-4-methylcoumarin-3-acetic acid–NHS (AMCA–NHS) (8.4 mg, 25.5 μmol) in DMF (0.5 mL) was added to this solution at 0 °C. After stirred overnight, the reaction mixture was adjusted to pH 5 with acetic acid and evaporated to dryness. The residue was then purified by HPLC using a Cosmosil 5C18AR column (1% AcOH–30% MeOH aq.) to give **24** (14.8 mg, 79%).

24: ^{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), 1.82 (1H, m), 1.72–1.51 (3H, m) ppm; ^{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 ppm; HR FAB MS (positive): [M + H]$^+$ Found 622.2983 m/z, $C_{31}H_{40}O_7N_7$ requires 622.2989 m/z; IR (film) v: 3344, 1668, 1554, 1516, 1201, 1136, 721 cm^{-1}; $[a]_D^{20}$ +14.1 (c 0.5, MeOH).

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Bioassay

Albizzia julibrissin Durazz, *Albizzia saman, Albizzia lebbeck, Cassia mimosoides*, *Aeschynomene indica* L, and *Leucaena leucocephala*, which were used for bioassay, were grown in the greenhouse of Keio University. The young leaves detached from the stem of the plant with a sharp razor blade were used for the bioassay. One leaf was placed in H_2O (*ca.* 1.0 mL) using a 5 mL glass tube in the greenhouse kept at 25–30 °C and was allowed to stand overnight. The leaves which opened again the next morning (around 10:00 am) were used for the bioassay. Each test solution was carefully poured into test tubes around 10:00 am. The bioactive fraction was judged by the leaf–opening at 21:00 after the leaf-closing of the plant leaf in the blank solution containing no sample.

Fluorescence study using a fluorescence microscope

The leaf of *A. julibrissin* opening in the daytime was cut into an appropriate size and fixed in agar. The agar was sliced perpendicular to the petiole by a microslicer (DSK-1000, DOUSAKA EM Co. Ltd.) by 20–30 micrometers, and the sections containing the pulvini were floated on a 0.1 M citrate–0.2 M phosphate buffer (pH 7.0). The sections were immersed in a solution containing various concentrations of fluorescent-labelled probe compounds, and were allowed to stand for 4.5 hours under shielded conditions at room temperature for staining. After staining, the sections were washed and incubated with equilibration buffer (SlowFade™ Light Antifadant Kit, Molecular Probes Inc.) for 10 min. This section was placed on a slide glass and was covered by a cover glass after adding a drop of antifade reagent (SlowFade™ Light Antifadant Kit, Molecular Probes Inc.). The observation of these sections was carried out by using an ECLIPSE E-800 microscope (Nikon Co. Ltd.) equipped with VFM fluorescence instrument. B-2A (Nikon Co. Ltd.) filter (λ_{em}: 450–490 nm) was used on the observation with microscope. The plant sections of other nyctinastic plants, *C. mimosoides*, *L. leucocephalam*, *A. saman, A. lebbeck* and *Aeschnomene. indica*, were prepared and treated with a fluorescent-labelled probe compound in the same procedure.

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