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The diversity of chemical substances controlling the nyctinastic leaf-movement in plants

Minoru Ueda, Hideyuki Shigemori, Noriko Sata, Shosuke Yamamura*

Department of Chemistry, Faculty of Science and Technology, Keio University, Hiyoshi, Yokohama 223-8522, Japan

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Abstract

Leaf-movement in nyctinastic plants has long been believed to be controlled by plant hormones that are common among all nyctinastic plants. We have identified several bioactive substances for nyctinasty, whose bioactivities were highly specific to the original plant, based on the bioassay using the original plant leaf, and have shown that nyctinastic leaf-movement is not regulated by plant hormones. Our present results are in accordance with Umrath et al. physiologically significant opinion. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Nyctinasty; Plant hormone; Turgorin; Leaf-closing substance; Leaf-opening substance; Biological clock

1. Introduction

The sensitive plant, *Mimosa pudica* L., is well known for the rapid movement of the leaf when it is touched. However, yet another leaf-movement is observed in this plant. It is called a nyctinastic leaf-movement; the leaf closes at night and opens again the next morning. This periodical movement of the leaf according to the biorhythm of the plant is known to be controlled by a biological clock, and its rhythm can be maintained in continuous daylight or darkness. Historically, a biological clock was discovered from the observation of plant leaf-movement in the 18th century (du Fay, 1729). Nyctinastic leaf-movement is observed in almost all leguminous plants. In any event, this exciting biological phenomenon has been attracting much attention since the fourth century B.C. In 1880, Charles Darwin and his son Francis published an important book entitled "The Power of Movement in Plants" based on his own experiments, using more than three hundred different kinds of plants, including nyctinastic ones represented by *M. pudica* (Darwin, 1882).

Many attempts were made to search for the chemical substance controlling this movement. In 1983, H. Schildknecht isolated turgorins (**1**–**4**), which induce leaf-closing movement of the plants, as chemical substances to control the nyctinastic movement of the plants (Schildknecht & Schumacher, 1982; Schildknecht, 1983). They claimed that turgorin is a new plant hormone which regulates the turgor movement of the plants. After that, nyctinastic movement has been believed to be controlled by turgorins. However, Umrath and coworkers expressed the physiologically significant opinion that every genus or subgenus of plants has its own leaf-movement factor that is effective only in plants of a given genus, although they did not identify any leaf movement factor (Bielenberg, Esterbauer, Hayn & Umrath, 1984).

In this paper, we have shown that turgorin is not a genuine leaf-movement factor, and nyctinastic movement is not regulated by common chemical substances, such as plant hormones, but rather by chemical substances that differ depending on the plant species, as suggested by Umrath.

* Corresponding author. Tel.: +81-45-563-1141; fax: +81-45-563-5967.

E-mail address: yamamura@chem.keio.ac.jp (S. Yamamura).

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Table 1
Plant-specific bioactivities of the leaf-movement factors

	Cassia mimosoides	*Cassia occidentalis*	*Phyllanthus urinaria*	*Mimosa pudica*	*Aeshyomene indica*	*Albizzia julibrissin*
K-PLMF1 (**1**)	–	–	–	1×10^{-1} M	–	–
Leaf-closing substances						
Potassium Chelidonate (**6**)	1×10^{-7} M	4×10^{-5} M	–	–	–	–
Potassium D-idarate (**7**)[a]	5×10^{-7} M	–	–	–	–	–
Phyllanthurinolactone (**8**)	–	–	1×10^{-7} M	–	–	–
Potassium 5-*O*-β-D-glucopyranosylgentisate (**9**)	–	–	–	5×10^{-5} M	–	–
Trigonelline (**10**)	–	–	–	–	1×10^{-7} M	–
Leaf-opening substances						
Calcium 4-*O*-β-D-glucopyranosyl-*cis*-*p*-coumarate (**11**)	4×10^{-6} M	1×10^{-4} M[b]	–	–	–	–
Potassium lespedezate (**12**)[a]	8×10^{-7}	–	–	–	–	–
Phyllurine (**13**)	–	–	3×10^{-5} M	–	–	–
Mimopudine (**14**)	–	–	–	2×10^{-5} M	–	–
cis-*p*-coumaroyl-agmatine (**15**)	1×10^{-2} M	–	–	–	–	1×10^{-5} M

[a] These compounds were isolated from *L. cuneata* based on the bioassay using the leaf of *C. mimosoides*.
[b] IAA was effective at as low as 1×10^{-3} M for the leaf of *C. occidentalis*.

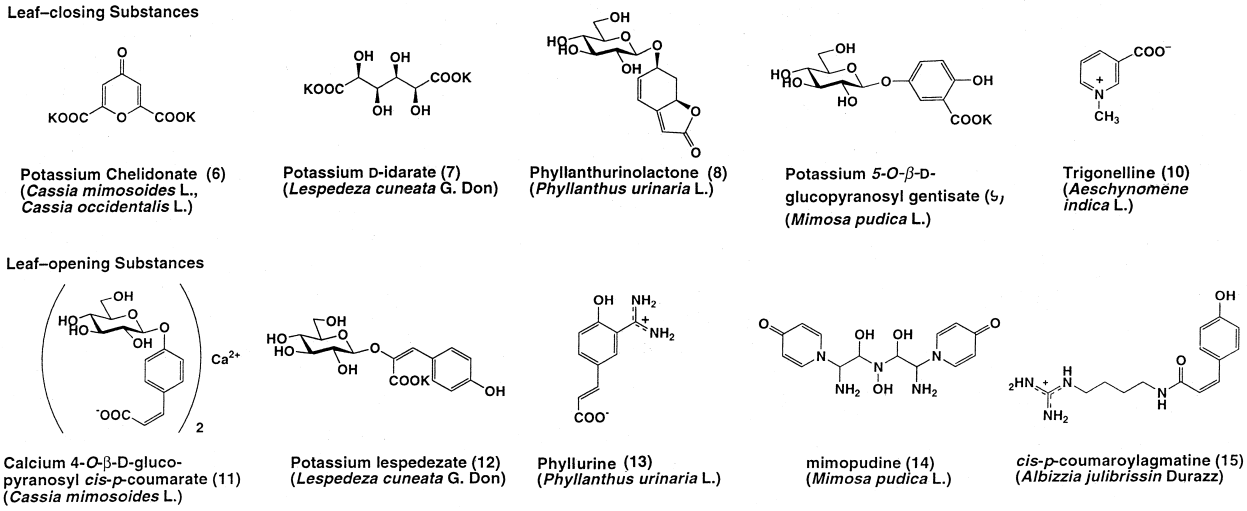


Fig. 1. Leaf-movement factors of nyctinastic plants.

2. Results and discussion

One significant issue arising from the chemical studies of nyctinasty by Schildknecht is the bioassay that is used for the detection of the nyctinastic leaf-closing activity (Schildknecht & Schumacher, 1982; Schildknecht, 1983). We have two important questions regarding this bioassay. First, if Umrath's hypothesis is true, the plant used in the bioassay must be the same as the one extracted. However, Schildknecht et al. used different plants for the bioassay and extraction. First, they observed the induction of rapid leaf-closure in the mimosa leaf for the detection of the leaf-closing activity in the extracts of all nyctinastic plants they studied. Second, they observed rapid leaf-closure of mimosa rather than the slow leaf-closure occurred in nyctinasty. Thus, they confused these two forms of leaf-movements of mimosa.

We isolated a representative turgorin K-PLMF1 (**1**) as its potassium salt from *Albizzia julibrissin* Durazz, and tested it in our bioassay. Schildknecht isolated **1** under acidic conditions; on the other hand, we carefully isolated the potassium salt of **1** (**5**) under neutral conditions. It is assumed that **1** should exist as the potassium salt in a plant; thus, **5** is the genuine chemical form of **1** when it exists in a plant. We examined the bioactivity of **5** with bioassay using the leaf of several nyctinastic plants. However, **5** was weakly effective only for *M. pudica*, and not effective for other nyctinastic plants (Table 1). Additionally, **5** induced only the rapid, but not the slow leaf-closure of *M. pudica* at 10^{-1} g/l. Thus, it was concluded that **1** is not a universal plant hormone inducing leaf-closing movement in all nyctinastic plants.

In order to detect the genuine leaf-movement factors of nyctinastic plants, we developed a bioassay using the original plant leaves. The young leaves of nyctinastic plant to be tested were immersed in distilled water and allowed to stand at room temperature overnight. The movement of the leaves, detached from each stem was also found to follow the circadian rhythm. The leaves that opened again the next morning were used for the bioassay. The bioactivity was judged by the closure of the leaves in the daytime. The bioactive substance induces leaf-closure within a few hours after the addition of the sample. We isolated several substances (**6**–**10**) active in this bioassay (Miyoshi, Shizuri & Yamamura, 1987; Ueda, Niwa & Yamamura, 1995; Ueda, Shigemori-Suzuki & Yamamura, 1995; Ueda, Ohnuki & Yamamura, 1997; Ueda & Yamamura, 1999b). Thus, different bioactive substances for nyctinasty were isolated from all the nyctinastic plants ever studied (Fig. 1) (Miyoshi et al., 1987; Shigemori, Sakai, Miyoshi, Shizuri & Yamamura, 1990; Ueda et al., 1995a, 1995b, 1997a, 1997b, 1998a, 1998b; Ueda & Yamamura, 1999a; Ueda & Yamamura, 1999b). All bioactive substances were effective at 10^{-6}–10^{-7} M, and contained in the plant body at 10^{-5}–10^{-6} M (Ohnuki, Ueda & Yamamura, 1998; Ueda, Asano, Sawai & Yamamura, 1999). It is most important that the bioactivities of all leaf-movement factors are specific for the original plant; these substances were not effective in other nyctinastic plants (Table 1). Thus, nyctinastic leaf-closure is not regulated by a universal turgorin but rather by leaf-closing substances specific to each nyctinastic plant.

On the other hand, we noted that the leaf-opening substance was contained together with the leaf-closing one in a nyctinastic plant. The leaf-opening substances (**11**–**15**) were also plant-specific (Fig. 1) (Ueda et al.,

1997b; Ueda et al., 1998a; Ueda et al., 1998b; Ueda & Yamamura, 1999a). They were effective at 10^{-5}–10^{-6} M, similar to the leaf-closing ones. These leaf-opening substances differed in certain aspects from indole-3-acetic acid (IAA) that has been considered to induce the leaf-opening of nyctinastic plants (Watanabe & Umrath, 1983; Tsurumi, Asahi & Suda, 1985; Morimoto, Schijo, Watanabe, Suda & Hashimoto, 1986) : (1) the bioactivities of the leaf-opening substances (Table 1, ca. 1×10^{-6} M) were much stronger than that of IAA ($> 1 \times 10^{-4}$ M), and (2) the bioactivity of the leaf-opening substances was specific to the genus of the plant while that of IAA was nonspecific. The concentration of IAA in the plant body is generally not sufficiently high as to make the leaf open. Thus, leaf-opening of the nyctinastic plants should be regulated by our bioactive substance that is specific to the plant species, not by IAA.

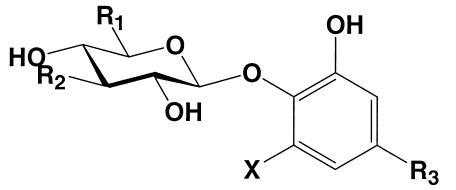
Therefore, in contradiction to Schildknecht's theory, each nyctinastic plant has its own leaf-opening and leaf-closing substances, and nyctinasty is controlled not by "plant hormones", such as turgorins and IAA, but rather by bioactive substances that differ depending on the plant species as suggested by Umrath. Each of them was effective at low concentration only for the original plant from which it had been isolated (Table 1).

However, it is assumed that the bioactive substance for nyctinasty would be common within a given plant genus. Potassium chelidonate (**6**), which was isolated as a leaf-closing substance from *Cassia mimosoides*, was also isolated from *Cassia occidentalis* belonging to the same plant genus, and was also effective in *C. occidentalis* (Table 1) (Miyoshi et al., 1987). Calcium 4-*O*-β-D-glucopyranosyl-*cis*-*p*-coumarate (**11**) isolated as a leaf-opening substance from *C. mimosoides* was also effective for *C. occidentalis* (Table 1); thus, **6** and **11** are assumed to be common chemical substances for nyctinasty of the *Cassia* genus.

Thus, we have two bioactive substances in nyctinasty: a leaf-closing substance and leaf-opening substance. It is also important that both leaf-closing and leaf-opening substances have been isolated from a nyctinastic plant, as shown in the cases of *C. mimosoides* (**6**, **11**) (Miyoshi et al., 1987; Ueda et al., 1998a), *Lespedeza cuneata* (**7**, **12**) (Shigemori et al., 1990; Ueda et al., 1997a), *Phyllanthus urinaria* (**8**, **13**) (Ueda et al., 1995b, 1998b), and *M. pudica* (**9**, **14**) (Ueda & Yamamura, 1999a, 1999b). Nyctinastic movement is controlled not only by the change in the concentration of the leaf-closing factor, but also by the competitive interaction between leaf-closing and leaf-opening substances (Ohnuki et al., 1998; Ueda et al., 1999). The idea of the co-existence of a leaf-opening substance with a leaf-closing one is very reasonable in terms of the previous report that the K^+ ion, which moves into and out of motor cells when the cell expands and shrinks, enters and leaves plant cells through ion channels which are regulated differently.

3. Conclusion

Leaf-movement in nyctinastic plants has long been believed to be controlled by plant hormones that are common among all nyctinastic plants. However, our results contradict this theory. We have identified several bioactive substances for nyctinasty based on the bioassay using the original plant leaf. Their bioactivities were highly specific to the original plant. Our present results are in accordance with Umrath et al. physiologically significant opinion.



	R_1	R_2	R_3	X
1	CH_2OSO_3H	OH	COOH	OH
2	CH_2OSO_3H	OSO_3H	COOH	OH
3	COOH	OH	COOH	OH
4	CH_2OSO_3H	OH	COOH	H
5	CH_2OSO_3K	OH	COOK	OH

4. Experimental

4.1. General procedure

Sephadex G-10 (Pharmacia Fine Chemicals) and TSKgel G3000SW (Toyo Soda) were used for gel filtration chromatography. Fuji-Davison ODS-W (Fuji-Davison Chemical) and Develosil ODS (Nomura

Chemical) were used for MPLC. HPLC experiments were carried out on Shimadzu LC-6A and LPD-6A systems, monitoring UV absorbance at 260 nm. Sizes of the columns used for HPLC were ϕ 6.0 × 250 mm (Unicil Pack 5C_{18}, Gasukuro-kogyo), ϕ 5.0 × 500 mm (Fine Pack Sil AF-102, JASCO), ϕ 8.0 × 500 mm (OH pack B-804, Shodex). ^{1}H-NMR (400 MHz) spectra were recorded on a JEOL JNM GX 400 FT NMR spectrometer in D_2O with *t*-BuOH as an internal standard (1.23 ppm for ^{1}H- NMR). IR spectra were measured on a JASCO A-202 spectrometer.

4.2. Plant materials

Plant materials that were used for bioassay were grown in a greenhouse at Keio University at 25–33°C for a few weeks. The fresh whole plant of *Cassia occidentalis* L. used for the extraction was grown in the campus of Keio University in September 1989.

4.3. Bioassays

Young leaves separated from the stems of the plants with a sharp razor blade were used for the bioassay. Two leaves were immersed in distilled water (ca. 1.0 ml) in a 20 ml glass tube in a greenhouse and allowed to stand overnight. Leaves which opened again the next morning (at around 10 a.m.) were used for the bioassay. Each test solution dissolved in water was poured into test tubes with a microsyringe at around 11 a.m., and the fraction with leaf-closing activity made the leaves completely closed within a few hours. On the other hand, the fraction with the leaf-opening activity was judged by the complete leaf opening until 9 p.m. In the bioassay of leaf-closing substance, the reaction time depended on the concentration of the bioactive substance. All bioassays were repeated several times to check their reproducibility. The lowest effective concentration of each leaf-movement factor was given in Table 1.

4.4. Isolation of the potassium salt of K-PLMF1 (**5**) from Albizzia julibrissin Durazz

The fresh whole plant of *Albizzia julibrissin* Durazz (33.4 kg) was immersed in MeOH (98 l) for one week and then concd. in vacuo. The concd aq. extract was partitioned with EtOAc (2 l × 2) and then *n*-BuOH (2 l × 2). The dried aqueous layer (1.17 kg) was carefully separated by Sephadex G-10 (800 g) column chromatography eluted with H_2O. The fractions containing **5** were collected and separated by Toyopearl HW-40S (800 g) column chromatography with H_2O, and then further purified with MPLC using a Fuji Davison ODS-W glass column (700 cm^3) with H_2O–MeOH = 9:1 to give the fraction containing **5** (316 mg). The resulting fraction was separated with MPLC using a Fuji Davison ODS-W glass column (400 cm^3) with H_2O–MeOH = 9:1 to give a fraction (224 mg), which was further purified using a Toyopearl HW-40S glass column (200 cm^3) with H_2O–MeOH = 95 : 5 to give the fraction containing **5** (27.0 mg). Final purification was carried out by MPLC using a Fuji Davison ODS-W column (100 cm^3) with H_2O–MeOH = 99:1 to give the potassium salt of K-PLMF1 (**5**) (11.3 mg). Potassium salt of K-PLMF1 (**5**) IR (Nujor): ν_{max} 1650 (sh), 1635 cm^{-1}; ^{1}H-NMR (400 MHz, D_2O): δ 6.85 (2H, *s*), 4.81 (1H, *d*, *J* = 7.3 Hz), 3.47 (1H, *dd*, *J* = 10.2, 7.3 Hz), 3.46 (1H, *m*), 3.45 (1H, *dd*, *J* = 10.2, 7.8 Hz), 3.44 (1H, *m*).; ^{13}C-NMR (100 MHz, D_2O): δ 151.7, 137.5, 112.1, 107.0, 78.1, 76.5, 71.6, 69.4.

4.5. Isolation of potassium chelidonate (**6**) from Cassia occidentalis L.

The fresh whole plant of *C. occidentalis* (1.2 kg) was immersed in hot water (9 l) for 15 min and concd. in vacuo. The concd aq. extract was partitioned with *n*-BuOH (500 ml × 2). The bioactive aqueous layer (30 g) was carefully separated by Sephadex G-10 (800 g) column chromatography eluted with H_2O. The bioactive fraction (9.0 g) was further separated by TSK gel G3000S column chromatography with H_2O. The bioactive fraction (3.4 g) was separated with MPLC using Fuji Davison ODS-W column (400 cm^3) to give bioactive fraction (490 mg), which was further purified with Develosil ODS glass column (400 cm^3) to give the fraction containing potassium chelidonate (160 mg). This fraction was separated by HPLC using a combination of three columns (Unicil pack 5C_{18} + Fine Pack Sil AF-102 + OH pack B-804) with H_2O–^{i}PrOH = 19:1 to give 20 mg of bioactive fraction. Final purification was carried out by HPLC using a combination of four columns (Unicil pack 5C_{18} × 4) with H_2O–MeOH = 9:1 to give potassium chelidonate (**6**) (3.1 mg): IR (Nujor): ν_{max} 1650 (sh), 1635 cm^{-1}; ^{1}H-NMR (400 MHz, D_2O): $\underline{\delta}$ 7.08 (*s*); ^{13}C-NMR (100 MHz, D_2O): δ 119.0, 162.4, 167.8, 187.0.

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References

Bielenberg, W., Esterbauer, H., Hayn, M., & Umrath, K. (1984). *Phyton*, *24*, 1.

Darwin, C. (1882). The power of movement in plants. In *Third thousand*. London: John Murray.

du Fay (1729). *Historie de L'Academie Royale des Science Paris*, p. 35.

Miyoshi, E., Shizuri, Y. & Yamamura, S. (1987). *Chem. Lett*., 511.

Morimoto, N., Schijo, C., Watanabe, S., Suda, S., & Hashimoto, T. (1986). *Physiol. Plantarum*, *68*, 196.

Ohnuki, T., Ueda, M., & Yamamura, S. (1998). *Tetrahedron*, *54*, 12173.

Schildknecht, H., & Schumacher, K. (1982). *Pure Appl. Chem*, *54*, 2501.

Schildknecht, H. (1983). *Angew. Chem. Int. Ed. Engl*., *22*, 695 (references cited therein).

Shigemori, H., Sakai, N., Miyoshi, E., Shizuri, Y., & Yamamura, S. (1990). *Tetrahedron*, *46*, 383.

Tsurumi, S., Asahi, Y., & Suda, S. (1985). *Bot. Mag. Tokyo*, *98*, 89.

Ueda, M., Niwa, M., & Yamamura, S. (1995a). *Phytochemistry*, *39*, 817.

Ueda, M., Shigemori-Suzuki, T., & Yamamura, S. (1995b). *Tetrahedron Lett*, *36*, 6267.

Ueda, M., Ohnuki, T., & Yamamura, S. (1997a). *Tetrahedron Lett*, *38*, 2497.

Ueda, M., Tashiro, C., & Yamamura, S. (1997b). *Tetrahedron Lett*, *38*, 3253.

Ueda, M., Asano, M., & Yamamura, S. (1998a). *Tetrahedron Lett*, *39*, 9731.

Ueda, M., Ohnuki, T., & Yamamura, S. (1998b). *Phytochemistry*, *49*, 633.

Ueda, M., & Yamamura, S. (1999a). *Tetrahedron Lett*, *40*, 353.

Ueda, M., & Yamamura, S. (1999b). *Tetrahedron Lett*, *40*, 2981.

Ueda, M., Asano, M., Sawai, Y., & Yamamura, S. (1999). *Tetrahedron*, *55*, 5781.

Watanabe, S., & Umrath, K. (1983). *Phyton*, *23*, 49.