Nyctinastic plants open and close leaves with a circadian rhythm. Here we discuss chemical aspects of the mechanism of nyctinastic leaf movement. Nyctinastic plants from five different genera are known to contain species-specific leaf-opening and leaf-closing factors. The relative concentrations of leaf-closing and leaf-opening factors of the nyctinastic plant *Phyllanthus urinaria* change circadianly, suggesting that nyctinastic movement is regulated by two classes of circadianly regulated factors with opposing functions. A closing and an opening factor of *Albizia*, when linked to a fluorescent dye, both specifically labeled motor cells of pluvini. A membrane fraction of pluvini contains proteins of 210 and 180 kDa that bind to a leaf-opening factor of *Cassia mimosoides*. The molecular identification of these proteins is underway.

Keywords: Endogenous bioactive substance — Legumes — Motor cell — Nyctinasty — Receptor.

Abbreviation: FITC, fluorescein isothiocyanate; SAR, structure–activity relationship.

Introduction

In general, plants are rooted and unable to move from place to place by themselves. However, some plants are able to move in certain ways. For example, some plants open their leaves in the daytime and 'sleep' at night with their leaves folded (Fig. 1). This leaf movement, known as nyctinasty, is regulated by a circadian clock with a cycle of about 24 h. Nyctinasty is widely observed in leguminous plants, and has been of great interest to scientists for centuries, with the oldest records dating from the time of Alexander the Great.

It was Charles Darwin, well known for his theory of evolution, who established the science of plant movement in his later years. His invaluable book, *The Power of Movement in Plants* (Darwin 1880), was based on experiments using >300 different plant species, including some that are nyctinastic. However, despite the great advances in science that have been made since Darwin's time, it is still difficult to establish the molecular basis of these processes. Our study focuses on the endogenous chemical mechanisms underlying Darwin's observations.

The physiological mechanisms of leaf movement have been extensively studied (for reviews, see Satter et al. 1981, Satter and Moran 1988, Satter et al. 1990). Nyctinastic leaf movement is induced by volume changes in motor cells in the pulvinus, a joint-like thickening located at the base of the petiole. Motor cells play a key role in leaf movement. A flux of potassium ions across the plasma membrane of the motor cells is followed by massive water flux, which results in swelling or shrinking of these cells. An issue of great interest is the regulation of the opening and closing of the potassium channels involved in nyctinastic leaf movement. The channels involved in the light-induced control of leaf movement have been studied in various ways (Moshelion and Moran 2000, Suh et al. 2000, Moshelion et al. 2002). However, there is no information on the circadian rhythmic regulation of the channels involved in nyctinasty. Many attempts have been made to isolate the endogenous chemical factors that are expected to play a role (for a review, see Schildknecht et al. 1983), but no known phytohormones are effective under physiological conditions.

Leaf-opening and leaf-closing substances in nyctinastic plants

Two types of bioactive substances were isolated from several nyctinastic plants: leaf-opening and leaf-closing factors, which possibly mediate nyctinastic leaf movement (for a review, see Ueda and Yamamura 2000). When the leaves of a leguminous plant are disconnected from the stem, their leaflets continue to open in the day and close at night according to a circadian rhythm. Artificial application of the leaf-closing factor causes the leaflets to fold even in the daytime, whereas the leaf-opening factor induces leaflet opening even at night. Five sets of leaf movement factors have been isolated from five nyctinastic plant species (Miyoshi et al. 1987, Shigemori et al. 1989, Ueda et al. 1995a, Ueda et al. 1995b, Ueda et al. 1997a,
Fig. 1  Three nyctinastic plants in daytime (left) and at night (right) (from the left, *Senna obtusifolia* L., *Phyllanthus urinaria* L. and *Mimosa pudica* L.).

**Leaf-closing Substances**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium 5-O-β-D-glucopyranosyl gentisate (1)</td>
<td>(\text{KOCO}_2)</td>
</tr>
<tr>
<td>Potassium Cheilidonate (2) (Cassia minosoides L.)</td>
<td>(\text{KOCO}_2)</td>
</tr>
<tr>
<td>Phyllanthurinolactone (3) (Phyllanthus urinaria L.)</td>
<td>(\text{KOCO}_2)</td>
</tr>
<tr>
<td>Potassium D-idarate (4) (Lespedeza cuneata G. Don)</td>
<td>(\text{KOCO}_2)</td>
</tr>
<tr>
<td>Potassium β-D-glucopyranosyl 12-hydroxyjasmonate (5) (Albizia julibrissin Durazz)</td>
<td>(\text{KOCO}_2)</td>
</tr>
</tbody>
</table>

**Leaf-opening Substances**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mimopudine (6) (Mimosa pudica L.)</td>
<td>(\text{NH}_2\text{OH} \text{NH}_2)</td>
</tr>
<tr>
<td>Calcium 4-O-β-D-glucopyranosylcyclop-coumarate (7) (Cassia minosoides L.)</td>
<td>(\text{Ca}^{2+})</td>
</tr>
<tr>
<td>Phyllurine (8) (Phyllanthus urinaria L.)</td>
<td>(\text{KOCO}_2)</td>
</tr>
<tr>
<td>Potassium lespedezate (6) (Lespedeza cuneata G. Don)</td>
<td>(\text{KOCO}_2)</td>
</tr>
<tr>
<td>cia-p-coumaroylajugmatine (10) (Albizia julibrissin Durazz)</td>
<td>(\text{NH}_2\text{OH} \text{NH}_2)</td>
</tr>
</tbody>
</table>

Fig. 2  Leaf movement factors of five nyctinastic plants.

Ueda et al. 1997b, Ueda et al. 1998a, Ueda et al. 1998c, Ueda and Yamamura 1999, Ueda et al. 1999b, Ueda et al. 2000a). For convenience, we refer to each factor by a number (1–10); the factors along with their numbers are shown in Fig. 2. All of the factors are effective in the plant species from which they were isolated at concentrations of \(10^{-5}–10^{-6}\) M when applied exogenously. The concentration of these factors in the plant body is estimated to be \(10^{-5}–10^{-6}\) M by HPLC analyses (Ueda et al. 1998c, Ueda et al. 1999a), which shows that they were effective at physiological concentration. Each nyctinastic plant has a specific set of leaf movement factors (Ueda et al. 2000a, Ueda et al. 2000b). None of the factors is effective in plants belonging to other genera, even at a 10,000- to 100,000-fold concentration (Miyoshi et al. 1987, Shigemori et al. 1989, Ueda et al. 1995a, Ueda et al. 1995b, Ueda et al. 1997a, Ueda et al. 1997b, Ueda et al. 1998a, Ueda et al. 1998c, Ueda et al. 1999b, Ueda and Yamamura 1999a, Ueda et al. 2000a, Ueda et al. 2000b). For example, 1 is effective as leaf-opening factor for *Albizia julibrissin* Durazz. at \(10^{-5}\) M, but it is not effective for other genera, such as *Cassia*, *Phyllanthus*, or *Mimosa*, even at \(10^{-1}\) M. These findings support the idea that nyctinasty is controlled by genus-specific leaf-closing and leaf-opening factors (Ueda and Yamamura 2000, Ueda et al. 2000b). The work presented below demonstrates that these leaf movement factors operate as endogenous chemical factors controlling nyctinasty in the plant body.

**Bioorganic studies of nyctinasty using functional leaf movement factors as molecular probes: leaf movement factors bind specifically to motor cells in a genus-specific manner**

Most of the physiological studies on nyctinasty have been carried out in plants belonging to the genus *Albizia*...
The motor cells in the pulvinus of nyctinastic plants are of two types: extensors, located at the upper (adaxial) side of a leaflet; and flexors, at the lower (abaxial) side (Fig. 3). Leaflets move upward during closure and downward during opening. Extensor cells increase their turgor pressure during opening and decrease their turgor during closing, while flexor cells decrease their turgor pressure during opening and increase their turgor pressure during closing.

Compounds 5 and 10 were isolated as a leaf-closing factor and leaf-opening factor, respectively (Ueda et al. 1997b, Ueda et al. 2000a). These factors were effective in three Albizzia species, A. julibrissin, A. saman, and A. lebbeck (Ueda et al. 2000a), but were ineffective in plants belonging to other genera. In the work described below, probes based on factors 5 and 10 were used as tools for tracing their molecular dynamics in vivo.

To identify target cells of leaf-closing and leaf-opening factors, fluorescein isothiocyanate (FITC)-labeled 5 (11) and rhodamine-labeled 10 (12) were synthesized based on the results of structure–activity relationship (SAR) studies (Fig. 4) (Nagano et al. 2003, Nakamura et al. 2006a, Nakamura et al. 2006c). FITC-labeled 5 (11) and rhodamine-labeled 10 (12) retain their activity to close and open Albizzia leaves, respectively, but they have no activity for other genera, similar to the non-labeled natural products (5 and 10). A double fluorescence labeling experiment using FITC-labeled 5 (11) and rhodamine-labeled 10 (12) was conducted to identify their target cells in the plant body (Nakamura et al. 2006c). Fig. 4 shows fluorescence images of sections of pulvini of A. saman. Somewhat unexpectedly, both of the probes bound to the extensor cells, motor cells located on one side of the pulvinus (see Nakamura et al. 2006b). Strong fluorescence signals observed in the xylem and epidermis were due to non-specific binding of probe 11, which we propose does not reflect the bioactivity of the leaf-closing factor 5 (discussed below). The labeled closing and opening factors 11 and 12 localize to the extensor cells but not the flexor cells (Fig. 4). This suggests that induction of volume change in the extensor cells is the trigger for leaf movement. It is likely that binding of 5 and 10 to the extensor cell induces changes in K⁺ channel activity in the cell, which result in changes in K⁺ levels in the extensor cell, in turn inducing leaf closing and opening. Therefore, an important issue yet to be examined is whether these factors affect K⁺ channel activity.

An enantio-differential approach clearly demonstrated the involvement of some specific binding protein for 5 in the extensor cell. We synthesized biologically inactive probe 11', which is an enantiomeric aglycon of 11, as a negative control for probe 11 because an SAR study of 5 showed that a structural modification in the stereochemistry in the aglycon part greatly diminished its leaf-closing activity (Nakamura et al. 2006b). Comparison of the localization of 11 and 11' enables the differentiation between specific binding of the probe, which is associated with bioactivity, and non-specific binding due to chemical adsorption. Bioactive probe 11 stained the vessel, epidermis and extensor cells in pulvini sections (Fig. 5). In contrast, probe 11' stained the vessel and epidermis of the pulvini, but did not stain motor cells at all (Fig. 5). Thus, it was clearly shown that the extensor cells contain some specific binding protein that can differentiate the stereochemistry of the ligands. It was also shown that the strong fluorescence
observed in the vessel and epidermis of the pulvini is due to non-specific binding of the probes, since it occurs regardless of the stereochemistry of the ligand. Also, the specificity of the binding of rhodamine-labeled $10^{12}$ was confirmed by repelling with an excess amount of non-labeled $10^{10}$. Thus, these results suggested that receptors for the closing factor $5^{5}$ and opening factor $10^{10}$ which are involved in changes in turgor in extensor cells are both present in the extensor cells in $A. saman$.

So far we have found that leaf-closing and leaf-opening factors act in a genus-specific manner. Therefore, we examined whether the labeled factors bind to the target cells genus specifically. As expected, FITC-labeled $5^{11}$ and FITC-labeled $10^{10}$ bound to motor cells of $A. saman$ and $A. julibrissin$, whereas they did not bind to the cells of $Cassia mimosoides$ L., $Phyllanthus urinaria$ L. or $Leucaena leucocephara$ (Nagano et al. 2003, Nakanura et al. 2006b) (Fig. 6).

The target cell of leaf movement factors was also confirmed to be a motor cell in other nyctinastic species, $C. mimosoides$ L. and $P. urinaria$ L., by using fluorescence-labeled leaf movement factors of the corresponding plant (Sugimoto et al. 2001, Kato et al. 2006). A leaf-opening factor of $C. mimosoides$, potassium isolespedezate ($14^{14}$), was also labeled to give biologically active probe $15^{15}$ (Shigemori et al. 1990, Ueda et al. 1999b). Probe $15^{15}$ labeled motor cells of $Cassia$ (Fig. 7), but not of $P. urinaria$, $A. julibrissin$ or $Mimosa pudica$, again revealing genus specificity (Sugimoto et al. 2001, Sugimoto et al. 2002). Similar results were obtained in the case of $3^{5}$ (Kato et al. 2006). It was concluded that genus-specific binding of fluorescently labeled leaf movement factors to motor cells can account for the genus-specific bioactivity of the factors.

**Photoaffinity labeling of the receptor molecule for leaf movement factor**

Next, the identification of receptors for leaf movement factors was examined using photoaffinity labeling,
Fig. 5  An enantio-differential fluorescence study using a pair of probes, 11 and 11′, which were prepared from an enantiomeric pair of 5, respectively. Stereospecific recognition of the probes was observed only in the extensor motor cells of the left image.

Fig. 6  Fluorescence labeling of Albizzia saman using probes 11 and 13. (a) Sections containing motor cells were prepared by cutting the pulvini of Albizzia saman perpendicular to the vessel. A fluorescence images of the extensor side of section is shown in (b).
a powerful method to detect the receptors of small ligands (Kotzyba-Hilbert et al. 1995, Matsubayashi et al. 2000, Yamaguchi et al. 2006). We synthesized a biologically active biotinylated photoaffinity probe 16, which is a derivative of 14 (potassium isolespedezate), a leaf-opening factor of C. mimosoides whose target cells were identified to be motor cells by a fluorescence study using 15 (Sugimoto et al. 2001). The photoaffinity probe 16 (for molecular design, see Fujii et al 2005) was effective at 1 \times 10^{-4} \text{M} in a bioassay with leaves of C. mimosoides, and the activity is 100-fold less strong than that of the natural product 14. A membrane fraction containing plasma membrane and microsomal membrane was prepared from a large number of leaflet pulvini which are composed largely of motor cells, and was incubated with 3 \times 10^{-6} \text{M} of probe 16. After cross-linking by irradiation with UV light (365 nm) at 4°C, the membrane fraction was analyzed by SDS–PAGE and Western blotting, which identified two probe-coupled target proteins of 210a and 180 kDa (Fig. 8) (Sugimoto et al. 2004). Specific binding of the probe was confirmed by the disappearance of the corresponding bands after photolabeling in the presence of a 5,000-fold molar excess of non-labeled leaf-opening substance 14. Binding of the probe was inhibited competitively under these conditions. Photolabeling experiments using probe 16 with a crude membrane fraction prepared from sections of plant leaves that contain no motor cells gave no specific bands on chemiluminescence detection of biotinylated proteins (Fujii et al. 2005). Moreover, a biologically inactive probe in which the phenolic hydroxyl group of 16 was protected as a methyl ether gave a band of 200 kDa, instead of 210 and 180 kDa. The above results indicate that the Cassia opening factor 14 specifically binds to two proteins of 210 and 180 kDa, which are localized in the membrane fraction of motor cells of leaflet pulvini, but not in the membrane fraction of other cells. Thus, it is highly likely that the two proteins are the specific receptors of the Cassia opening factor. The identification of receptors for other leaf movement factors is now under way.

### Chemical basis of the rhythm in nyctinasty

If a pair of leaf movement factors regulate nyctinasty, their levels in plants should be regulated by the circadian clock. Fig. 9 shows time course changes in the concentration of leaf-closing and leaf-opening factors in P. urinaria, Euphorbiaceae, grown in the greenhouse under sunlight (Ueda et al. 1999). HPLC analyses to determine the content of these factors in entire stems and leaves were performed every 4 h. It was found that the concentration of the leaf-opening factor 8 remained nearly constant during the day, whereas that of the leaf-closing factor 3 decreased during the day. The concentration of 3 began to decrease from 4:00 h, and began to increase from 16:00 h. The experiment was conducted in the summer in Japan. During this season, the sun rises at around 5:30 h and sets around 18:30 h; thus it was still fully dark at 4:00 h and still fully light at 16:00 h. These results suggest that changes in the concentration of 3 in the plant body are not regulated by environmental light conditions, but by the circadian clock. The level of 3 in the daytime was as little as one-twentieth that at night. This drastic change appeared to result from hydrolysis of the leaf-closing factor into its corresponding aglycon (17), which is inactive as a leaf-closing factor (Ueda et al. 1999a). These results suggest that marked changes in the balance of concentration between the leaf-closing factor and the leaf-opening factor in the plant body during the day are responsible for leaf movement. In contrast, in Lespedeza cuneata (Ohnuki et al. 1998), the leaf-opening factor is a glucoside potassium isolespedezate (9), and its relative concentration decreases in the evening, but the leaf-closing factor 4 remains constant during the day. The decrease of 9 is due to the change of 9 into the biologically inactive aglycon in the evening. We measured β-glucosidase activity of acetone-powder samples prepared from the the leaves of L. cuneata in the daytime and in the evening, using 9 as a substrate. The activity was detectable only in plants collected in the evening (Ohnuki et al. 1998, Kato et al. 2005, Kato et al. 2007). These results suggest that a biological clock controls β-glucosidase activity,
Fig. 8  Photoaffinity labeling experiment for the membrane fraction of *Cassia pulvini* with probe 16. The crude membrane fraction was prepared from pulvini of *Cassia* plants, which was then photo-cross-linked by probe 16 to give bands corresponding to the potential receptor for 14. Specific binding of 16 was confirmed by the disappearance of the bands in the presence of an excess amount of non-labeled 14.

**Fig. 9**  Time course changes in concentrations of the leaf-closing factor 3 and the leaf-opening factor 8 in *Phyllanthus urinaria*. 
which regulates the balance of concentration between leaf movement factors during the day.

In *P. urinaria* and *L. cuneata*, the concentrations of the non-glucoside movement factors are maintained nearly constant during the day and night, whereas those of the glucoside closing factor in *P. urinaria* and of the glucoside opening factor in *L. cuneata* drastically decrease during the day and night, respectively. Therefore, the ratio of the closing and opening factors is the key element of nyctinastic leaf movement, and this ratio is changed by the hydrolysis of glucoside-type factors. To the best of our knowledge, this is the first chemical study of the mechanism of a biological phenomenon that is regulated by a biological clock. Although there is evidence that β-glucosidase activity measured with a glucoside-type factor as substrate appears to change according to the decrease of glucoside factors, whether or not the change is regulated by the circadian clock awaits further studies. However, it is a tempting hypothesis that the biological clock regulates the activity of β-glucosidase, which deactivates the glucoside-type leaf movement factor, controlling the balance of concentration between leaf-closing and leaf-opening factors.

**Future perspectives**

Chemical factors inducing leaf movement were isolated from several nyctinastic plants. Diurnal change in the levels of these factors was observed in some plants. Target cells and binding proteins of these chemical factors in the plant body were also revealed. To confirm these chemical factors as genuine factors controlling nyctinasty, it is important to clone genes for proteins that bind leaf-opening and leaf-closing factors and to clone genes for proteins that bind leaf-opening and leaf-closing factors. These factors were observed in some plants. Target cells and binding proteins of these chemical factors in the plant body were also revealed. To confirm these chemical factors as genuine factors controlling nyctinast, it is important to clone genes for proteins that bind leaf-opening and leaf-closing factors.

**References**


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