Metabolites involved in plant movement and 'memory': nyctinasty of legumes and trap movement in the Venus flytrap

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The bioorganic basis of plant movement in two plant systems is described in this article: the circadian rhythmic leaf movement known as nyctinasty and trap movement in the Venus flytrap. The bioactive substances responsible for plant movement, the chemical mechanism of the rhythm, and studies on the key protein controlling nyctinasty are presented. The article also discusses the isolation of the "memory" substance from the Venus flytrap and presents a mechanism for this action.

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1 Introduction

In general, plants are rooted and are unable to demonstrate mobility, however, a variety of plants are able to move in certain ways. Some plants are known to open their leaves in the daytime and "sleep" at night with their leaves folded (Fig. 1). This circadian rhythmic leaf movement known as nyctinasty is widely observed in leguminous plants. This rhythm is regulated by a biological clock with a cycle of about 24 hours. The phenomenon has been noted by scientists for centuries, with the oldest records dating from the time of Alexander the Great, and a biological clock was

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Fig. 1 Three nyctinastic plants in the daytime (top) and at night (bottom) (from the left, *Senna obtsusifolia* L., *Phyllanthus urinaria* L., and *Mimosa pudica* L.).

discovered in 1729 from the careful observation of nyctinasty in *Mimosa pudica*.[1]

It was Charles Darwin (Fig. 2), well known for his theory of evolution, who established the science of plant movement and enthusiastically studied plant movement in his later years. In 1880, Darwin published a seminal book entitled "The Power of Movement in Plants",[2] based on experiments using more than 300 different kinds of plants, including nyctinastic species. This classic book is still cited in relevant papers today. However, despite the advances that have been made in the interim, it has proven difficult to determine the detailed molecular mechanisms of these processes. This review describes studies which have focused on the molecular mechanisms of Darwin's original observations.

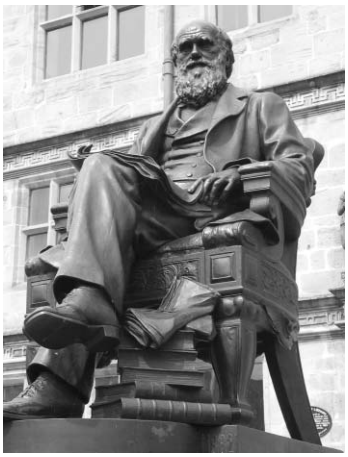

Fig. 2 Charles Darwin (1809–1892) was intrigued by nyctinasty and was the author of two books on the subject: "The Insectivorous Plants" in 1875 and "The Power of Movement in Plants" in 1880. Photo reproduced with the permission of istockphotos.

Fig. 3 shows one mechanism of leaf movement.[3,4] In this case, nyctinastic leaf movement is induced by the swelling and shrinking of motor cells in the pulvinus, an organ located in the joint of the leaf. Such motor cells play a key role in plant leaf movement. A flux of potassium ions across the plasma membranes of the motor cells is followed by a massive water flux, which results in the swelling and shrinking of these cells. At the heart of such a mechanism is the regulation of the opening and closing of the potassium channels involved in nyctinastic leaf movement, a process that is under metabolic control. Many attempts have been made to isolate the endogenous bioactive substances that control nyctinasty.[5]

Fig. 3 Mechanism of nyctinasty revealed since Darwin's early observations.

2 Endogenous bioactive substances controlling nyctinasty

Nyctinastic plants have a pair of endogenous bioactive substances that control nyctinastic leaf movement. One of these is a leaf-opening factor that "awakens" plant leaves, and the other is a leaf-closing factor that reverses this process such that the plant leaves "sleep". Five sets of leaf-closing and -opening factors in five different nyctinastic plants have been identified (Fig. 4). When the leaves of leguminous plants such as *Mimosa pudica* L. and *Cassia mimosides* L. are disconnected from the stem, they continue leaf movement according to the diurnal circadian rhythm, opening in the daytime and closing at night. Artificial application of the leaf-opening factors to the leaves can reverse these rhythms making plant leaves open at night or close during the daytime. All of these factors were effective at concentrations of 10^{-5} to 10^{-6} M. This bioactivity is very similar to that of known phytohormones such as indoleacetic acid (IAA) and the gibberellins. These studies also showed that each nyctinastic plant uses unique leaf-movement factors, but these are conserved within the same genus. None of the factors were effective in the other plants, even at a 100 000-fold concentration. Such observations clearly contradict working hypotheses, which suggest that nyctinasty is controlled by phytohormones common to all plants. The chemical mechanisms by which these individual compounds control nyctinastic leaf movement and the way in which the rhythm of nyctinasty is maintained remains to be determined in detail.

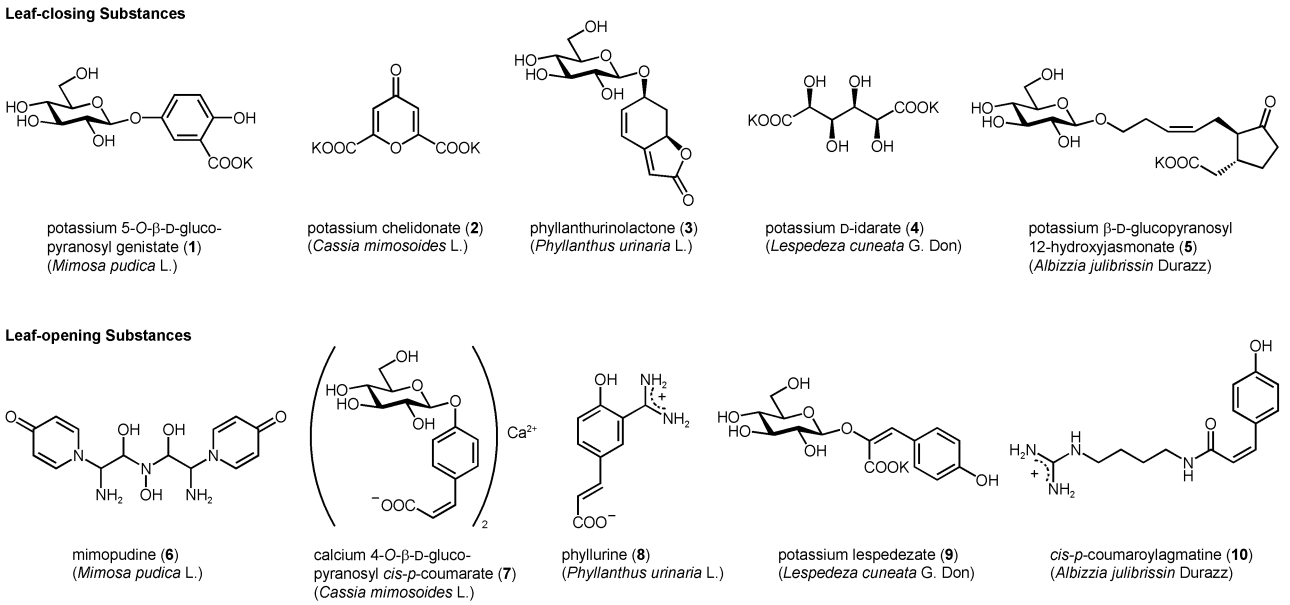

Fig. 4 Leaf-movement factors from five nyctinastic plants.

3 The chemical mechanism of rhythm in nyctinasty

Changes in the concentrations of leaf-closing and -opening factors in *Phyllanthus urinaria* over time are highlighted in Fig. 5.[7] HPLC was used to determine the levels of these factors every four hours through a daily cycle. It was found that the concentration of leaf-opening factor **8** remains nearly constant during the day, whereas that of the leaf-closing factor **3** changes by as much as 20-fold. This behaviour could be accounted for by conversion of the leaf-closing factor to its corresponding aglycon **11** in a hydrolytic reaction. It follows from this type of analysis that significant changes in the ratio of the concentrations of the leaf-closing and leaf-opening factors in the plant are responsible for leaf movement.

In *Lespedeza cuneata*,[8] the concentration of potassium lespedezate **9**, a glucoside-type leaf-opening factor, decreases in the evening whereas in this species, the concentration of the leaf-closing factor **4** remains constant during the day. Metabolite **9** is metabolized to the biologically inactive aglycon **12** in the evening (Fig. 6). These findings are consistent with the changes in β-glucosidase activity in the plant body during the day where significant activity is observed only in plants collected in the evening. This suggests that there is a temporal mechanism regulating β-glucosidase activity, which influences these factors during the diurnal cycle.

In all of the five pairs of leaf-closing and -opening factors **1–10** from the five nyctinastic plants discovered so far, one of

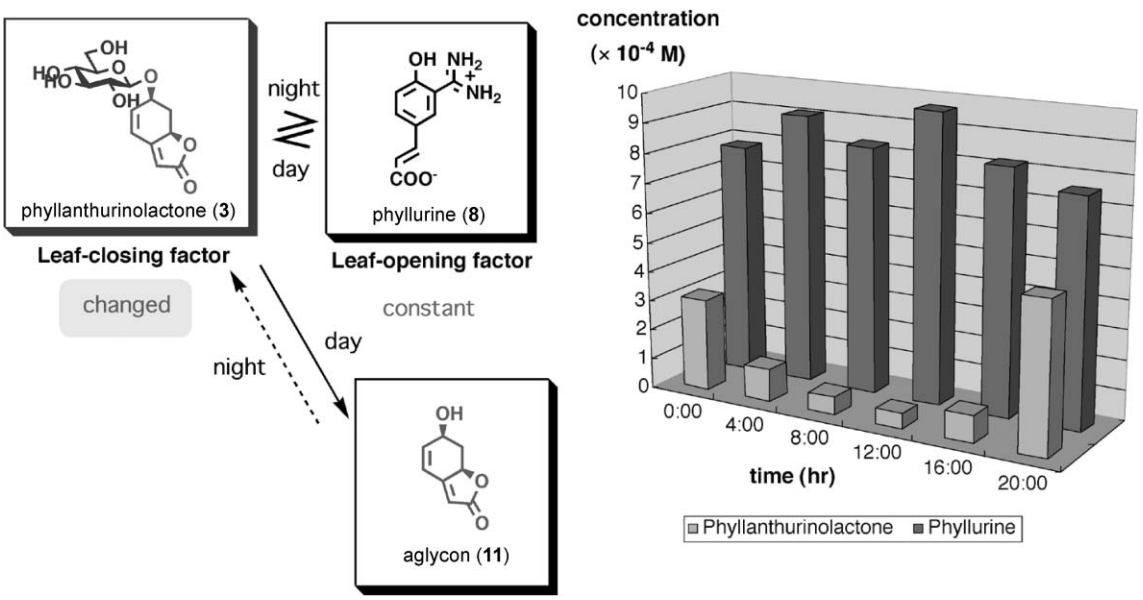

Fig. 5 Changes in the concentrations of leaf-opening and leaf-closing factors in *Phyllanthus urinaria* over time.

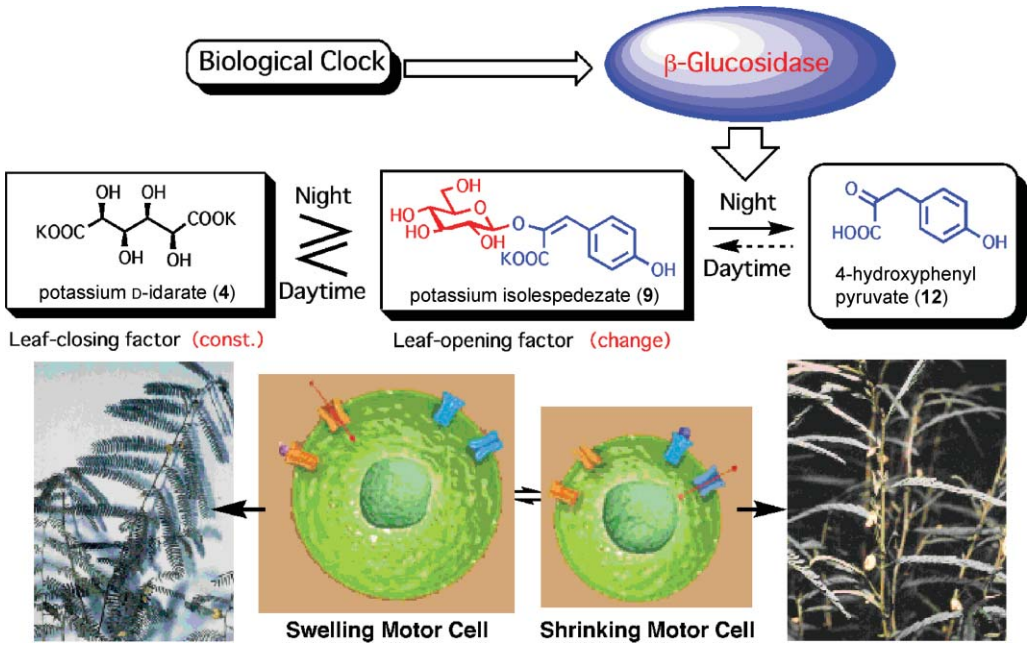

Fig. 6 Chemical mechanism of nyctinasty in *Lespedeza cuniata.*

each pair of factors is a glycoside and in all cases the concentrations of these glycoside-type leaf-movement factors change during the day in a similar manner to that described for *Lespedeza cuneata.*

This suggests that all nyctinastic leaf movement can be explained by a single mechanism as follows, involving two leaf movement factors of which one is a glucoside. β-Glucosidase activity is then regulated by some mechanism deactivating the glucoside and controlling the relative concentrations of the leaf-closing and -opening factors. Thus, nyctinastic leaf-movement is controlled by regulated β-glucosidase activity on a daily cycle.

4 Bioorganic studies of nyctinasty using functionalized leaf-movement factors as molecular probes

4.1 Fluorescence studies on nyctinasty

The mechanisms by which leaf-movement factors induce leaf movement have been examined using molecular probes consisting of chemically modified leaf-movement factors designed to identify the target cells of these factors. Structure–activity relationships for such probes were explored (Fig. 7).[9] Fluorescence-labelled

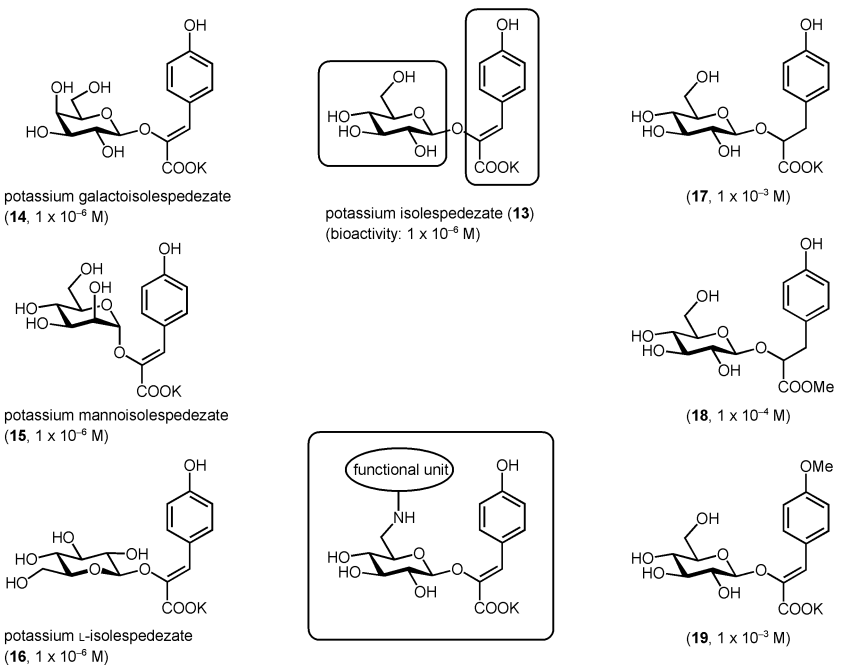

Fig. 7 Structure–activity relationships of potassium isolespedezate (**13**).

potassium isolespedezate **13** has been explored as a probe and it was found that structural modification of the glucose moiety of **13** did not diminish its bioactivity. Even the L-glucoside-**16** was as effective as the native factor. In contrast however, bioactivity was greatly diminished by modification of the aglycon moiety. For example, reduction of the double bond (as shown in **17**), protection of the carboxylate (as shown in **18**) or the phenolic hydroxy group (as shown in **19**), all lead to reduced bioactivity. The successful probes attach the fluorescent dye to the primary hydroxy group at the 6′ position of the glucose moiety. Due to the presence of esterases in the plant body, amide coupling has proven preferential over esters for connection of the fluorescent dye to the native factor and the use of galactose instead of glucose prevents β-glucosidase hydrolysis. Such design features have resulted in effective molecular probes.

The AMCA-labelled probe **20** was used to target cells containing leaf-movement factors.[10,11] Fig. 8 shows sections of *Cassia mimosoides* L. under a fluorescence microscope. It emerges that the motor cells are located in the pulvinus, the point where the leaf is attached to its stem. When sections were incubated with the molecular probe, staining was observed only in the motor cells contained within the pulvinus. No other part of the plant was stained by the probe. These observations suggest that there are specific receptors for the leaf-movement factors on the motor cells.

4.2 Photoaffinity labelling of the receptor molecules for leaf-movement factors

Photoaffinity labelling[12–18] probes based on potassium isolespedezate **13** have been developed to explore the receptor for **13**, the leaf-opening factor of *C. mimosoides* (Fig. 9). The efficiency of such a probe is a balance between achieving high binding affinity with the receptor, which is generally compromised[19] and by the size and location of the photolabile group.[20,21] Leaves of *C. mimosoides* were used to test the 2′- and 6′-modified probes **21–23** as photoaffinity labels. Compound **21**, which bears a photoaffinity group[22] and a biotin moiety[23] on the 6′-position of the galactose unit, retained leaf-opening activity at 5×10^{-5} M for *C. mimosoides* at about 2% of the natural product. The bioactivity of probe **22** (8×10^{-5} M) was one-eighteenth that of the natural

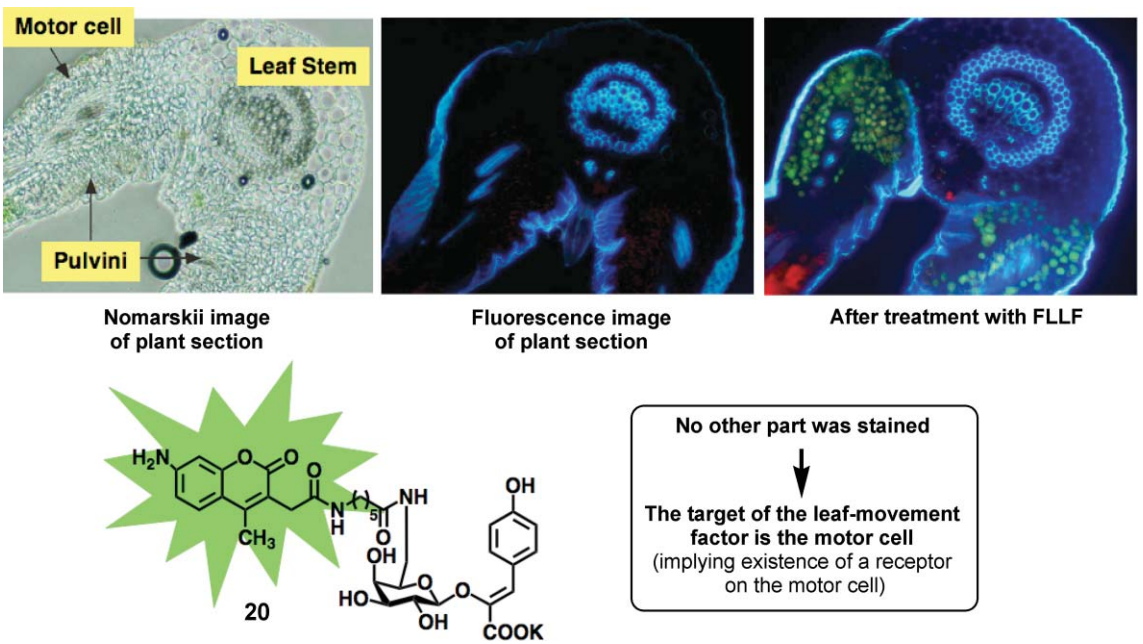

Fig. 8 Fluorescence study of the target cells of **13** using a fluorescence-labelled probe (**20**).

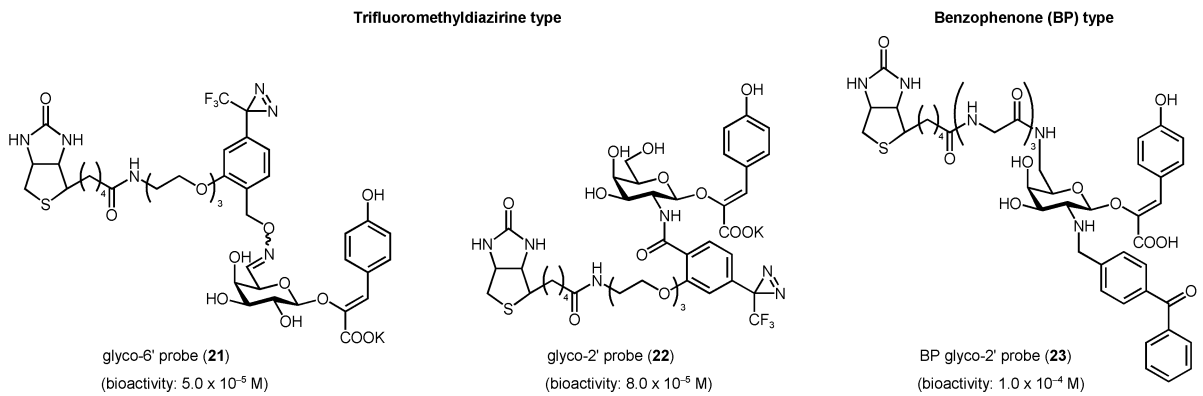

Fig. 9 Photoaffinity probes (**21–23**) based on the structure of **13**.

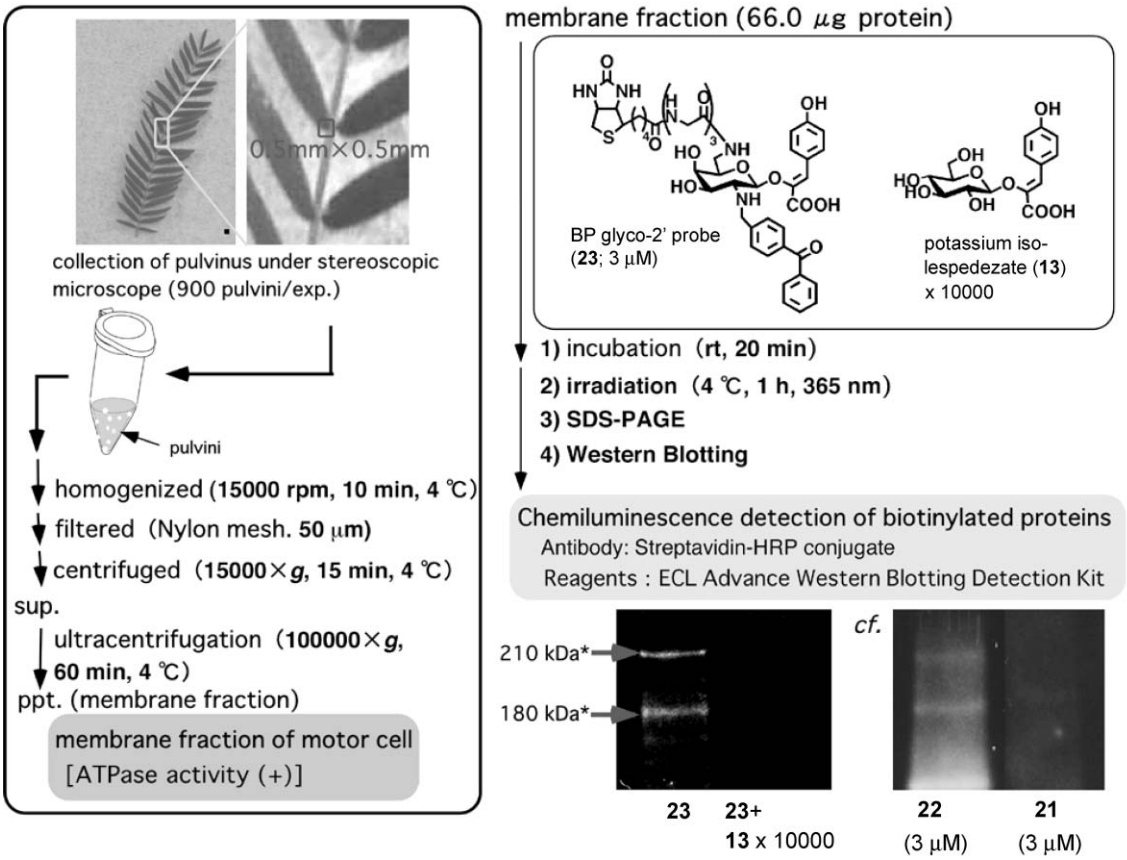

Fig. 10 Photoaffinity labelling experiment for the membrane fraction of motor cells using probes **21–23**.

product and probe **23** was effective at 1×10^{-4} M, at about 1% of the activity of the natural product, thus, all of these synthetic probes were active in this bioassay. The results tend to indicate that the nearer the photoaffinity group is to the aglycone unit, the weaker the bioactivity of the probe.

Fluorescence-labelled probe **20** has been shown to target motor cells in the pulvini of *C. mimosoides.* Plant motor cells have to be harvested by cutting off the sections of plant pulvini containing the motor cell (0.5 mm × 0.5 mm), one by one from plant leaves using a stereoscopic microscope (Fig. 10). Each experiment needs about 900 plant sections. Successive homogenization, filtration, and ultracentrifugation generates a pellet which contains the plasma membranes of the motor cells. The membranes' ATPase activity is generally checked as a measure of the purity of such membrane preparations, and then the crude membrane fractions can be used in the assays. Typically, cells are suspended and incubated with the probes (*e.g.* **22** and **23**) at 3×10^{-6} M and they are then cross-linked by irradiation with UV light (365 nm). After such experiments the membrane fractions are analyzed by SDS-PAGE. After western blotting and chemiluminescence detection, biotinylated proteins are identified with their binding. In this study two proteins, one of 210 kDa, and another of 180 kDa, were identified. Binding of the probe to these proteins was competitively inhibited by the natural ligands. On the other hand, no specific protein was detected in labelling experiments with this 6′-modified probe **21** under the same conditions. These results suggested that the close arrangement of the photolabelling group with the binding site is most important for successful photolabelling experiments. In this study, probe **23** bearing a benzophenone group near the binding site, gave the best results. It emerges that the probe with the strongest bioactivity is not always the best for photoaffinity labelling experiments.

The study also examined the localization of these binding proteins.[19] Results using fluorescent probe **20** revealed that the leaf-opening substance exclusively binds to the motor cells, and not to any other parts of the plant body. Clearly if the binding proteins described above are the genuine receptors for the leaf-movement factor, they should be localized in the plant motor cells. A control experiment revealed that photolabelling experiments, using probe **23**, with a crude membrane fraction prepared from a section of plant leaves devoid of motor cell, gave no indication of a specific band after chemiluminescence detection for biotinylated proteins. A clear conclusion from these results is that the protein receptors for the leaf-opening factors are contained in the plasma membranes of the motor cells. Moreover, a biologically inactive probe whose phenolic hydroxyl group was protected as a methyl ether gave a quite different band by SDS/western blotting from the previously identified proteins (210 kDa and 180 kDa) and clearly these previously identified proteins are strongly implicated with the biological activity of the probe, reinforcing their role as the receptors for the leaf-opening substance. These observations offer the first insights into the molecular mechanism of nyctinasty.

Nyctinastic leaf movement is induced by the opening and closing of potassium channels. It has generally been assumed that

potential receptor proteins would be closely associated with, or be a subunit of, the potassium channels of H^+-ATPase, which is involved in the regulation of channel movements. However, no such subunits have been reported with molecular masses in the range of 200 kDa. Thus, these putative receptor proteins might be previously unrecognised proteins involved in the control and regulation of potassium channels. Attempts to clone this receptor are now in progress to examine its role in more detail.

Progress to date has established a new biologically active substance which has led to the discovery of a novel membrane receptor. This type of chemical approach for identifying target proteins is increasingly reinforcing the molecular biological approach in the study of cell biology, and the tools and methods of 'chemical genetics' will clearly become increasingly important in the post-genomic era.

4.3 Are leaf-movement receptors common to the same plant genus?

The discussion so far has focused on the target cells and protein receptor for metabolite **13**, a leaf-opening substance of the *Cassia* plant. However, the majority of the physiological studies on nyctinasty have been carried out using plants belonging to the genus *Albizzia*.[23] It emerges that *Albizzia* plants utilise the leaf-movement factors **5** and **10** and these metabolites also target protein receptors in motor cells. Metabolites **5** and **10** are leaf-movement factors in at least three *Albizzia* plants, however they are not effective in plants of other genera.[24,25] The receptors for these metabolites have been explored with probes **24** and **25** (Fig. 11).[26,27] In particular these probes were used to examine whether the receptors for **5** and **10** are the same in the different *Albizzia* plants. The probes **24** and **25** bound to motor cells of *A. juribrissin* and *A. saman* as revealed in fluorescence detection studies, however they did not bind to *Cassia mimosoides* L., *Phyllanthus urinaria*, or *Leucaena leucocephara* cells showing a clear *Albizzia* genus preference, and suggesting a quite different metabolite/receptor interaction even in quite closely related plant systems. By extension, it may be the case that different plant genera have quite different leaf-opening and -closing substances interacting with unique receptors in plant motor cells. This might indicate that such molecular diversity in nyctinasty is a comparatively late process in plant evolution occurring when the leguminous plants differentiated into their various genera.

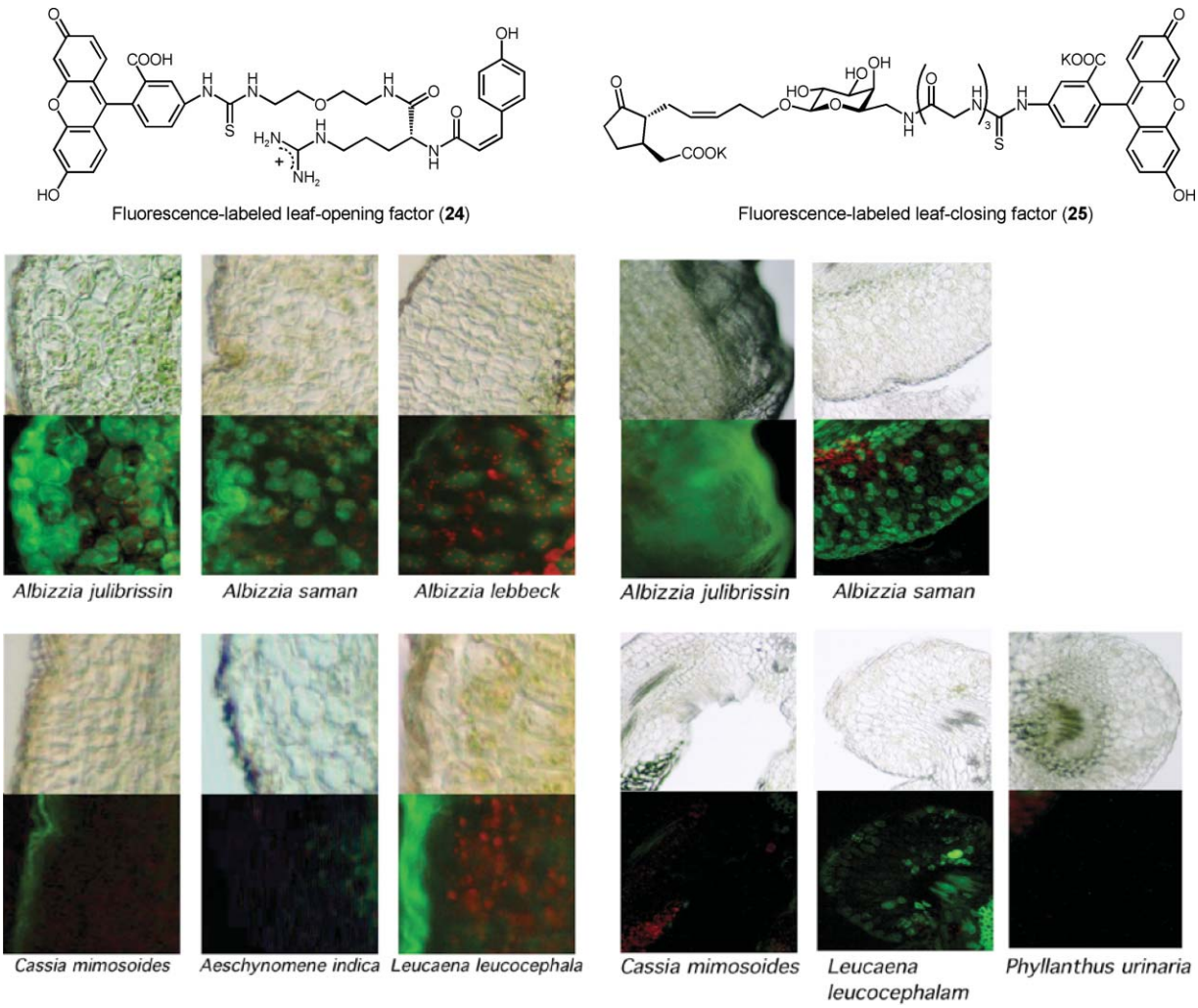

Fig. 11 Genus-specific receptors in *Albizzia* sp. using fluorescence-labelled leaf-movement factors (**24** and **25**).

5 "Why do leguminous plants sleep?"

This question has puzzled many scientists studying nyctinasty. Darwin concluded that nyctinasty provided protection from chilling or freezing.[2] Bünning, who is an authority on biological clocks, proposed that nyctinasty protected the photoperiodic timekeeping system from moonlight, because moonlight falling on leaves during the night might interfere with the accurate measurement of night length.[29] However, there has been no experimental evidence supporting these hypotheses. Research has been hindered by the lack of molecular probes to inhibit leaf movement. However, the first tools for such studies have recently emerged.

Based on the mechanism of leaf movement shown in Fig. 5, it was hypothesized that a structural analogue of the leaf-opening factor, that cannot be hydrolyzed by a β-glucosidase, should inhibit leaf closure, keeping the leaf open constantly in an "insomnia" condition (Fig. 12). Potassium isolespedezate **13** is a glucoside-type leaf-opening factor. Structure–activity relationship studies have shown that structural modification of the sugar moiety of **13** does not affect its bioactivity (Fig. 7). Based on the structure of **13**, the potential leaf-closure inhibitor **16**, which contains L-glucose instead of D-glucose, was prepared as it was anticipated that it would not be a substrate for the activating β-glucosidase.

These synthetic compounds did inhibit leaf closure. Compound **13** and the leaf-closure inhibitor **16** kept leaves open, even at night, at 1×10^{-6} mol l^{-1}. When the leaves were treated with 3×10^{-6} mol l^{-1} of **13**, the leaf-opening activity lasted for only two days, after which the leaves closed again (at night). This can be rationalised if the potassium lespedezate is gradually hydrolyzed to its corresponding aglycon within a few days in the plant body. In contrast, the leaf-opening activity of the synthetic inhibitors lasted for more than a week and they can truly induce "insomnia" in plants.

Fig. 13 shows the status of "insomniac" leaves, from 9:00 pm on days 1, 4 and 14, along with a 14 day control. The leaves became damaged as a consequence of the inhibition of leaf closure, and they withered and died within the two weeks. Thus this inhibition study suggests that nyctinastic leaf movement is essential for the health and survival of leguminous plants. These observations offer the first experimental data on the importance of leaf closure for the survival of legumes, and go some way to answering the question "Why do leguminous plants sleep?".

Fig. 13 The behaviour of "insomniac" plant leaves.

6 Chemical studies on leaf movement and ancient "memory" in carnivorous plants

The Venus flytrap (*Dionaea muscipula* Ellis) is an insectivorous plant that catches insects using a trap made of large leaves. The plant then digests the insects in the trap using a number of digestive enzymes. Charles Darwin was deeply intrigued by insectivorous plants. In his book "The Insectivorous Plants", he enthusiastically called *D. muscipula* "one of the most wonderful plants in the world".[30] Interestingly, a kind of "memory" appears to be involved in leaf closure of *D. muscipula* (Fig. 14). Rapid closure of the trap requires two stimuli within 30 seconds of each other on the sensory hairs, which are located on the internal surface of the trap leaves.

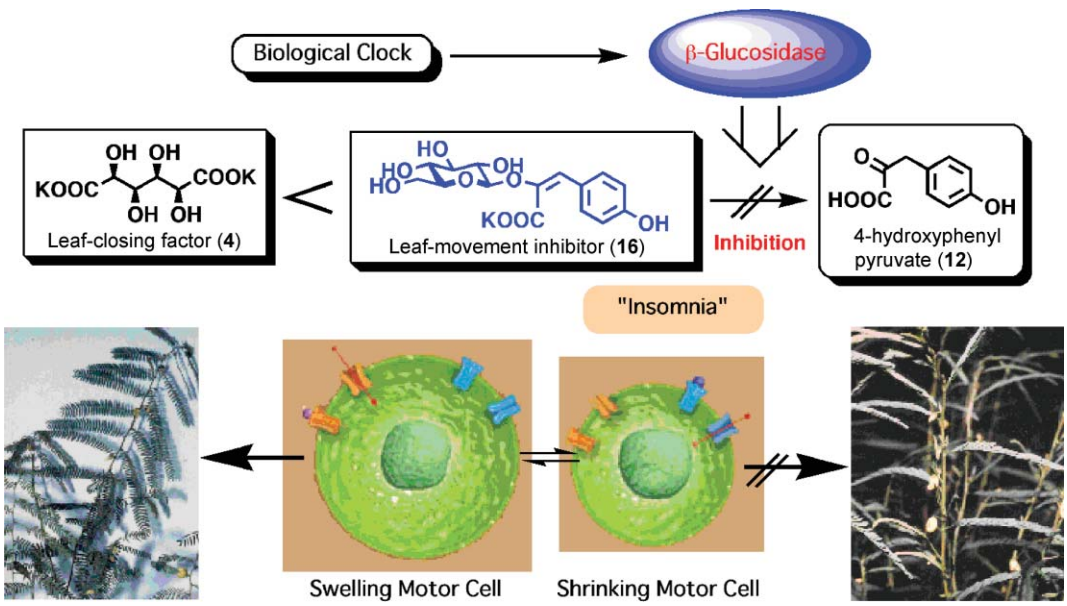

Fig. 12 Inhibition of leaf-closing by using potassium L-lespedezate (**16**).

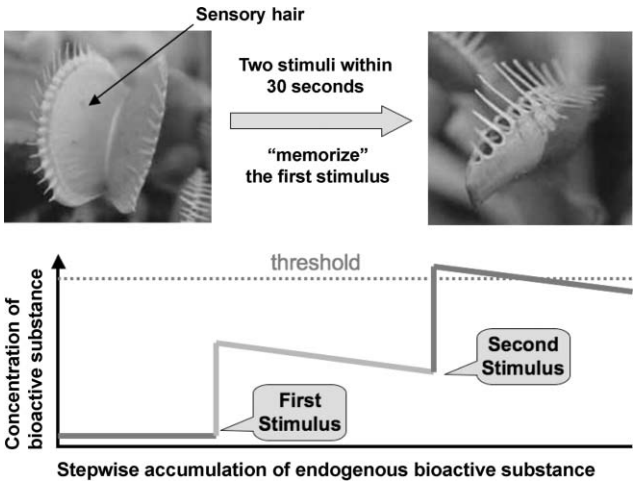

Fig. 14 "Memory" in *Dionaea muscipula* and the accumulation of bioactive substance.

Leaf closure never occurs when only a single stimulus is applied. Clearly *Dionaea muscipula* has a mechanism for "remembering" the first stimulus. We have hypothesized that a bioactive substance is involved in this "memory" process. If an appropriate metabolite is secreted stepwise in response to each stimuli, the "memory" response could be triggered by the stepwise accumulation of the secreted bioactive substance. Thus, a study was initiated to isolate an endogenous metabolite responsible for triggering closure of traps in *D. muscipula.*

The possibility that a neurotransmission mechanism similar to that in higher animals exists in the Venus flytrap was first mooted in 1873. Burden-Sanderson of London University reported that an action potential caused by the stimulation of sensory hairs is involved in the trap movement of Dionaea.[31] This observation suggested that the movement of the plant, and animal neurotransmission may have a similar general mechanism. In addition, the action potential mechanism in Dionaea was shown to be a "nothing or all" mechanism, similar to that found in higher animals. Electrophysiology has shown that both potassium and calcium ions are involved in the generation of the action potential in the Venus fly trap.[32]

If the relationship between the trigger for trap movement and the action potential generated in Dionaea is viewed from a chemist's viewpoint, then a "memory" metabolite will be gradually secreted after multiple stimulations until its concentration *in vivo* exceeds the threshold and triggers ion channel opening, which would then induce the generation of the action potential. Such a hypothesis implicates a particular metabolite in this role. A bioassay for leaf-closing activity offers an approach route to identifying and isolating such a substance. Bioassays using the leaves or plant body of Dionaea generally have a low reproducibility because of the individual differences between plants. However bioassays involving genetically uniform clones of Dionaea[33] have resulted in much more highly reproducible and useful outcomes. By this method, a bioactive fraction can be identified and isolated, which has the capacity to induce closure of Dionaea leaves without an external stimuli (Fig. 15).

Bioassay-guided separation of the extract led to the identification of an endogenous bioactive polysaccharide consisting of α-arabinofuranoside, α-galactopyranoside, and α-xylopyranoside moieties (Fig. 16). This polysaccharide has the capacity to induce the closure of traps, without an external stimuli, at very low concentrations ~2 ng/leaf. Although this compound has been isolated in very low concentrations some information on the polysaccharide nature of the compound has been obtained using 500 MHz cryoprobe NMR analysis. The mechanism: accumulation of the chemical substance → ion channel activation → action potential generation, observed in Dionaea closely resembles the stimulation transmission mechanisms found in higher order animals. Interestingly, the trap leaf movement of Dionaea was also induced by high concentrations (*ca.* 0.1–1 g L^{-1}) of the common neurotransmitters found in higher order animals, such

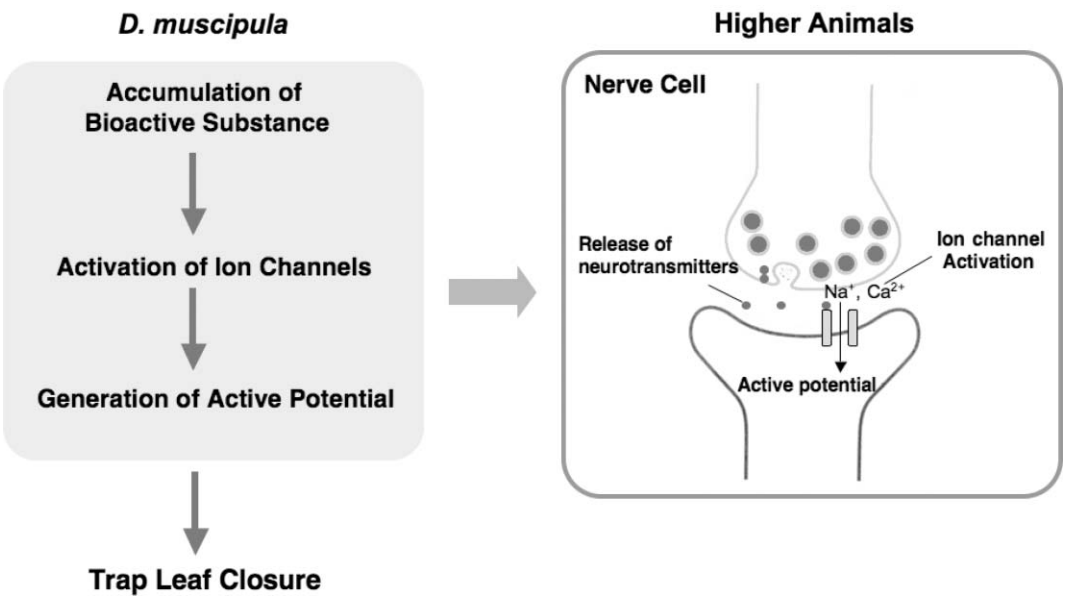

Fig. 15 A close relationship between the memories of humans and Dionaea.

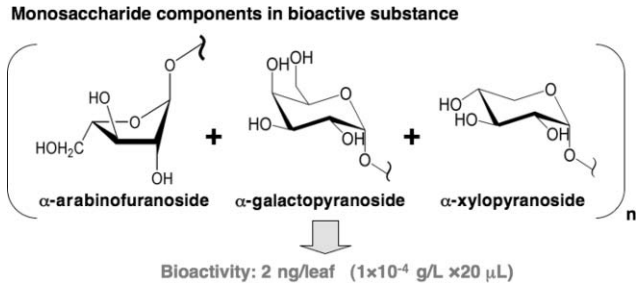

Fig. 16 The partial structure of the Venus flytrap "memory" substance.

as norepinephrine, DOPA, and glutamate. There is a clear implication that the "memory" substance in Dionaea may also possess neurotransmitter activity.

In plants, some polysaccharides, such as elicitors and Nod factors,[34] act as signal transmitters that induce cellular responses. Thus this "memory" substance is estimated to act as a direct trigger of the action potential generation.

The studies reviewed here have revealed that "memory" in the trap movement of Dionaea can be explained by the stepwise accumulation of a unique bioactive metabolite. There are real prospects now for exploring the chemical basis of this "memory" phenomenon with the full structure elucidation of the relevant bioactive agent. This would allow chemists to design appropriate molecular tools and probes to delineate the detailed mechanisms of these intriguing physiological processes.

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