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Chemical studies on plant leaf movement controlled by a biological clock*

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Abstract: Most leguminous plants close their leaves in the evening, as if to sleep, and open them early in the morning according to the circadian rhythm controlled by a biological clock. Extensive studies on nyctinastic plants led to the isolation of a variety of leaf-closing and -opening substances. And we found that the biological clock regulates the balance of concentration between leaf-opening and -closing substances in the plant body during the day.

INTRODUCTION

In general, plants are rooted and unable to move from place to place by themselves. However, some plants are known to be able to move in certain ways. Especially, the circadian rhythmic leaf movement known as nyctinasty is widely observed in all leguminous plants. Their leaves close at night and open in the daytime, according to a circadian rhythm. This rhythm is regulated by a biological clock with a cycle of about 24 h. This phenomenon has been of great interest to scientists for centuries, with the oldest records dating from the time of Alexander the Great [1]. It was Charles Darwin, 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 an invaluable book entitled *The Power of Movement in Plants*, based on experiments using more than 300 different kinds of plants, including nyctinastic species [2]. However, despite the advances that have been made since Darwin's time [3], it can still be difficult to determine the molecular mechanisms of these processes. Our study focuses on the molecular mechanisms of Darwin's observations.

ADVANCES SINCE DARWIN'S TIME

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 [3]. Motor cells play a key role in plant leaf movement. Flux of potassium ions across the plasma membranes of the motor cells is followed by massive water flux, which results in swelling and 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. Many attempts have been made to isolate the endogenous bioactive substances that control nyctinasty. The first successful isolation of one of these bioactive substances, turgorin, did not occur until 1983 [1]. Turgorin was thought to be a new class of phytohormone, and to regulate all leaf movements by controlling the turgor of plant cells. However, our results raise important questions about the function of turgorin [4]. The turgorin molecule contains a strongly acidic free sulfuric acid group. It seems highly unlikely that

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such a strong acid could be contained in the plant body in its free form. In addition, a potassium salt of turgorin that we isolated from several plants did not induce closing of the leaves. Finally, we found that diluted sulfuric acid induced leaf closing as strongly as turgorin. Given these results, we concluded that the leaf-closing activity of turgorin is due to the strong acidity of its sulfuric acid group. Turgorin was isolated under acidic conditions, and we hypothesize that the genuine endogenous factor was lost as a result of the acidic conditions. We attempted to isolate the genuine endogenous leaf-movement factor under neutral conditions.

ENDOGENOUS BIOACTIVE FACTORS CONTROLLING NYCTINASTY

We found that nyctinastic plants have a pair of endogenous bioactive substances that control nyctinastic leaf movement: one is a leaf-opening factor that "awakens" plant leaves, and the other is a leaf-closing factor that makes plant leaves "sleep". To date, we have identified five sets of leaf-closing and -opening factors (**1**–**10**) in five nyctinastic plants (Fig. 1) [5]. All of these factors were effective at concentrations of 10^{-5} to 10^{-6} M. This strength of bioactivity is very similar to those of known phytohormones such as indole-3-acetic acid (IAA) and gibberellin. The present study 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. These findings contradict the established theory that nyctinasty is controlled by phytohormones common for all plants.

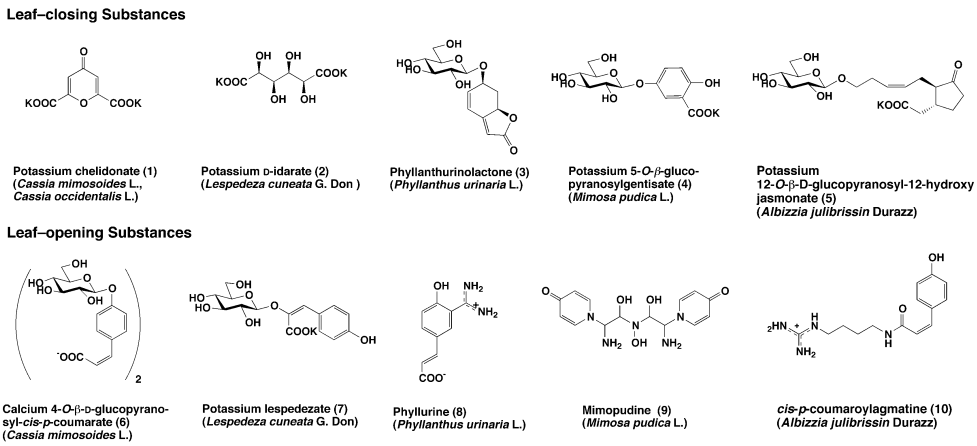

Fig. 1 Leaf-closing and -opening substances from five nyctinastic plants.

CHEMICAL MECHANISM OF THE CONTROL OF NYCTINASTY BY A BIOLOGICAL CLOCK

Other important problems to be solved regarding nyctinasty are the chemical mechanisms by which these compounds control nyctinastic leaf movement and the way in which the rhythm of nyctinasty is maintained. We found that the bioactivity of the plant extract correlated strongly with the status of the collected leaves; extract collected when leaves were closed showed leaf-closing activity, and vice versa. This suggests that the balance of concentration between leaf-closing and -opening factors is reversed during the day. We examined time-course changes in the content of leaf-closing and -opening factors in the plant body. The following results, for *Phyllanthus urinaria*, are typical [6]. High-performance liquid chromatograpy (HPLC) analyses to determine the content of these factors were performed every 4 h. We found that the content of the 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 during the day. We also found that this

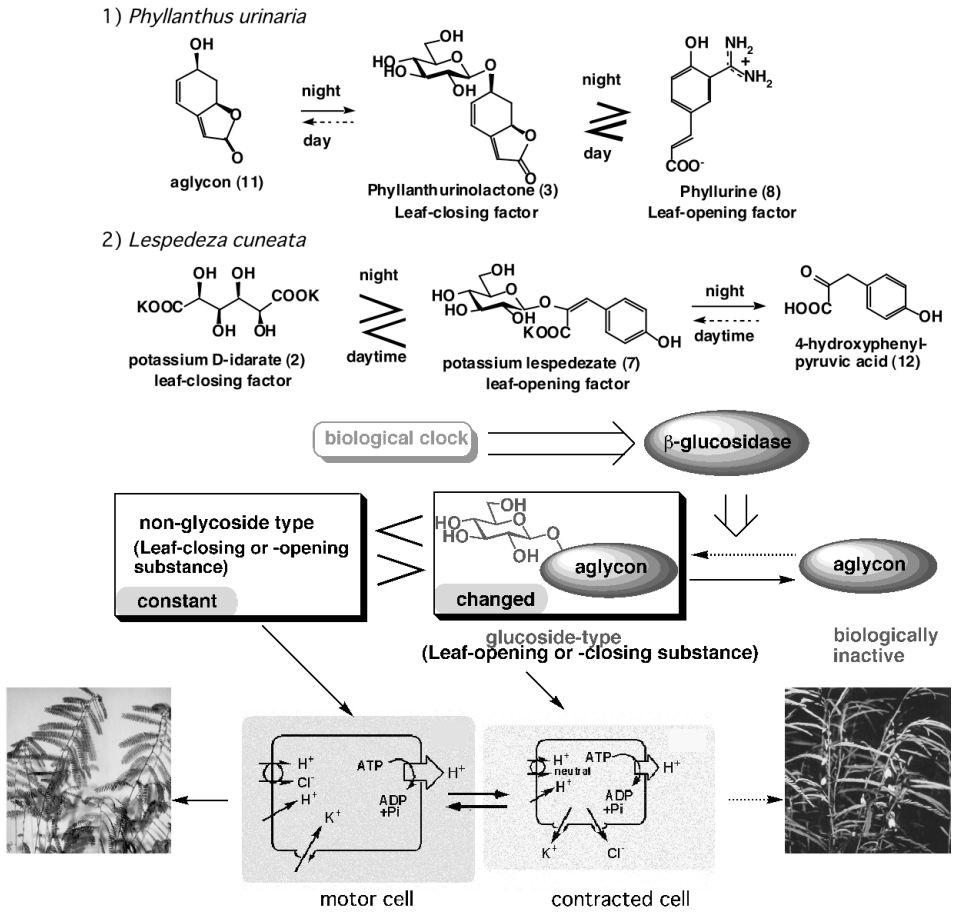

Fig. 2 Chemical mechanism of leaf movement controlled by a biological clock.

drastic change was due to hydrolysis of the leaf-closing factor into its corresponding aglycon (**11**) (Fig. 2). These results suggest that marked changes in the balance of concentration between the leaf-closing and -opening factors in the plant body during the day are responsible for leaf movement.

Similar results were obtained for *Lespedeza cuneata*, in which the relative concentration of a glucoside-type leaf-opening factor (**7**) decreases in the evening [7]. In this species, the concentration of the leaf-closing factor (**2**) remains constant during the day, whereas leaf-opening factor is metabolized to biologically inactive aglycon (**12**) in the evening (Fig. 2). These findings are consistent with the changes in β-glucosidase activity in this species during the day. β-Glucosidase activity was measured using **7** as a substrate, and activity was observed only in plants collected in the evening. This suggests that a biological clock controls β-glucosidase activity, which regulates the balance of concentration between leaf-movement factors during the day. In all pairs of bioactive substances that are identified so far, one of each pair of factors is a glycoside. HPLC analysis shows that the concentrations of these glycoside-type leaf-movement factors change during the day in all five species. These results indicate that regulation of all nyctinastic leaf movement can be explained using a single mechanism (Fig. 2) [6]. One of each pair of leaf-movement factors is a glucoside. A 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 -opening factors. Thus, nyctinastic leaf movement is controlled by a biological clock through the regulation of the activity of β-glucosidase. In other words, control of leaf move-

ment by a biological clock can be explained as a series of chemical reactions; that is, the formation and dissociation of the glycosidic bond of a leaf-movement factor. To the best of our knowledge, this is the first organic study of the mechanisms of biological phenomena that are regulated by a biological clock.

BIOORGANIC STUDIES ON NYCTINASTY USING SYNTHETIC PROBES

Next, we studied the mechanisms by which leaf-movement factors induce leaf movement. We developed molecular probes consisting of modified leaf-movement factors in order to identify the target cells of these factors. For example, we synthesized fluorescence-labeled potassium lespedezate, based on data from a structure–activity relationship study [8,9]. The structural modification of the glucose moiety of potassium isolespedezate did not diminish its bioactivity at all. Even the L-glucose type was as effective as the native factor. In contrast, bioactivity was greatly diminished by modification of the aglycon moiety. Therefore, we attached a large fluorescent dye to the primary hydroxy group at the 6′ position of the glucose moiety. Moreover, owing to the presence of esterases in the plant body, we used an amide bond instead of an ester to connect the fluorescent dye to the native factor. And also, the use of galactose instead of glucose prevents the hydrolysis by β-glucosidase. After examining the sizes of fluorescent dyes and lengths and structures of linker moiety in order to optimize bioactivity, we developed an 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-type probe (**13**), to detect the target cells of leaf-movement factors (Fig. 3) [9]. Figure 3 shows sections of *C. mimosoides* under a fluorescence microscope. The motor cells are located in the pulvivi, which is at the point where the leaf attaches to its stem. When sections were incubated with the probe, staining was observed only in the motor cells contained in the pulvini. No other part of the plant was stained by the probe. These results strongly suggest that receptors for this leaf-movement factor are located on motor cells.

To confirm the existence of receptors for leaf-movement factors, we synthesized biologically inactive probes and used them to perform binding experiments [10]. No binding was observed in sections treated with these probes. Recently, we succeeded in synthesizing biologically active photoaffinity probes based on potassium lespedezate [11]. The bioactivity of these probes was one-fiftieth of that of the native factor. We are currently using these probes to look for the native factor receptor.

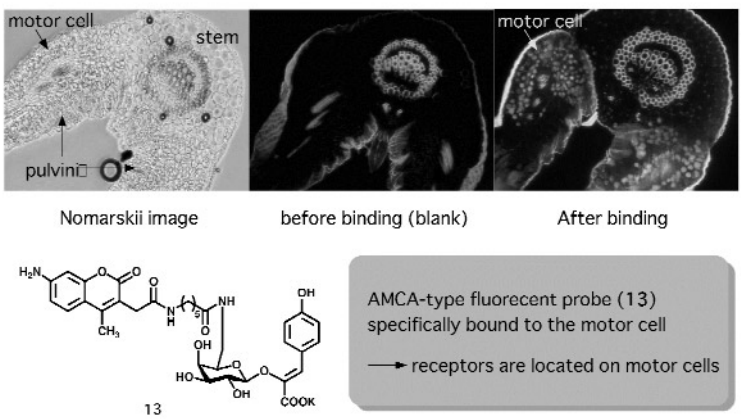

Fig. 3 Binding experiment using AMCA-labeled probe and plant section.

WHY DO LEGUMINOUS PLANTS SLEEP?

The question, "Why do leguminous plants sleep?", has puzzled many scientists studying nyctinasty. Darwin concluded that nyctinasty provided protection from chilling or freezing [2]. Büning, who is an authority on biological clocks, proposed that nyctinasty protected the photoperiodic timekeeping sys-

tem from moonlight, because moonlight falling on leaves during the night might interfere with accurate measurement of night length [12]. However, there has been no experimental evidence supporting these hypotheses. Research has been hindered by the inability to inhibit leaf movement. A genetic approach to the issue would be difficult, because there have been no reports of mutant nyctinastic plants that lack nyctinastic movement. However, we recently succeeded in inhibiting leaf closure using a synthetic substance based on a naturally occurring factor that induces leaf opening [13]. Based on the mechanism of leaf movement (Fig. 2), we hypothesized that a structurally modified leaf-opening factor that cannot be hydrolyzed by β-glucosidase by would inhibit leaf closure, keeping the leaf open constantly in a condition we call "insomnia" (Fig. 4). Potassium lespedezate (**7**) is a glucoside-type leaf-opening factor. Structure–activity relationship studies have shown that structural modification of the sugar moiety of **7** causes no decrease in bioactivity. Based on the structure of **7**, we designed and synthesized potential leaf-closure inhibitor (**14**) containing galactose instead of glucose, which are not expected to be hydrolyzed by β-glucosidase. Inhibitor **14** showed novel bioactivities in bioassays. Both **7** and **14** kept leaves open, even at night, at 1×10^{-6} mol/l. When the leaves were treated with 3×10^{-6} mol/l of **7**, the leaf-opening activity lasted for only 2 days, after which the leaves again closed at night. This is because **7** is gradually hydrolyzed into its corresponding aglycon within a few days in the plant body. In contrast, the leaf-opening activity of **14** lasted more than a week. Thus, we succeeded in inducing "insomnia" in plant leaves. Insomniac leaves were damaged by inhibition of leaf closure, and withered and died within two weeks. Using synthetic inhibitors of leaf closure to induce "insomnia" in plants, we showed that nyctinastic leaf movement is essential for the survival of leguminous plants. These results are the first experimental data of the importance of leaf closure for the survival of legumes, and provide an important clue for solving the ancient mystery, "Why do leguminous plants sleep?"

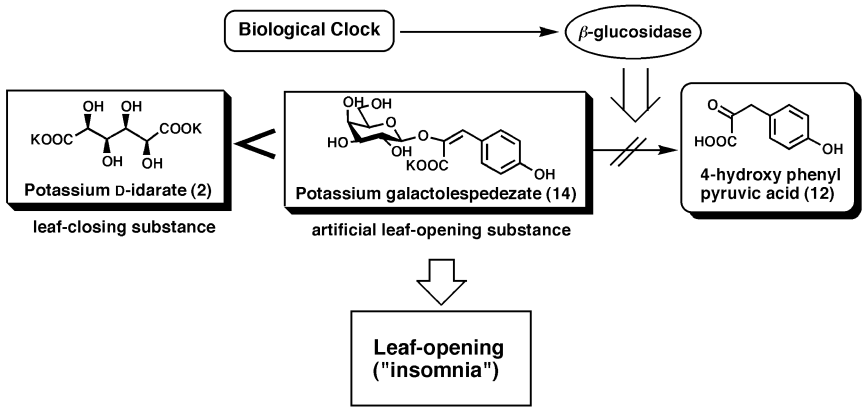

Fig. 4 Leaf-movement inhibitor-induced insomnia in plant leaves.

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