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Accounts

Bioorganic Studies on Nyctinasty of the Plant Controlled by a Biological Clock

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Most leguminous plants close their leaves in the evening, as if to sleep, and open them early in the morning. This circadian rhythm is known to be controlled by the biological clock of such plants. Extensive studies on other nyctinastic plants led to the isolation of a variety of leaf-closing and leaf-opening substances. And we found that the circadian rhythmic leaf-movement of these plants is controlled by a biological clock that regulates the balance of concentration between leaf-opening and -closing substances.

In contrast with animals, plants are rooted and unable to move from one place to another by themselves. However, they are not static but sensitively respond to a variety of environmental factors such as light, temperature, and humidity, such responses result in visually detectable movement.

This review focuses on a variety of bioactive compounds related to the leaf-movement of nyctinastic plants, whose 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 hours. This movement is called nyctinasty. Nyctinasty is observed in most leguminous plants. In any event, such an exciting biological phenomenon has attracted much attention since the fourth century B.C.

Historically, the discovery of a biological clock was based on the observation of nyctinastic movement in *Mimosa pudica* L. In the 18th century, a French scientist discovered that the rhythm involved in nyctinastic leaf-movement of this plant was maintained even under continuous darkness in a cave.[1] He hypothesized that the leaf-movement is controlled by the intrinsic rhythm of the plant. In 1880, Charles Darwin, well known for his biologically important book entitled "Origin of Species," also established the basis of this field by the publication of an invaluable book entitled "The Power of Movement in Plants" based on experiments conducted by himself and his son Francis, using more than three hundred different kinds of plants including nyctinastic ones.[2]

Leaf-Movement Factors in Nyctinastic Plants

Nyctinastic movement has been believed to be controlled by turgorins which induce leaf-closing movement of the plants.[3] Turgorin was isolated by Schildknecht as a new class of phytohormone which was said to regulate all leaf-movements by controlling the turgor of the plant cell. However, we revealed that turgorin is not a genuine factor controlling leaf-movement.[4] We isolated leaf-closing and -opening substances from several nyctinastic plants.[5–14] Nyctinastic movement is regulated by a chemical substance that differs depending on the plant. Our present study showed that every family or subfamily of plants has its own leaf-movement factor that is effective only for plants belonging to its own family. Also, we revealed that there exist leaf-opening substances together with leaf-closing ones in every nyctinastic plant. Leaf-opening substances differed in certain aspects from indole-3-acetic acid (IAA) that had been believed to be concerned with the leaf-opening of nyctinastic plants: [6] 1) the bioactivities of the leaf-opening substances (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 presence of leaf-opening substances indicates that nyctinastic movement is controlled not only by the leaf-closing factor, but also by the competitive interaction between leaf-closing and leaf-opening substances.

Nyctinastic leaf-movement is induced by the swelling and shrinking of motor cells which exist in the pulvini of the plant. Leaf-movement factors are supposed to interact directly (or indirectly) with motor cells. The cells exerts their function by swelling and shrinking in the process of leaf-opening and -closing and play a central role in the plant leaf-movement.[15–17] The ion fluxes followed by massive water fluxes across the plasma membrane of these cells produce the swelling and shrinking behavior of the motor cells.

The discovery of the co-existence of a leaf-opening substance with a leaf-closing one is very reasonable in terms of

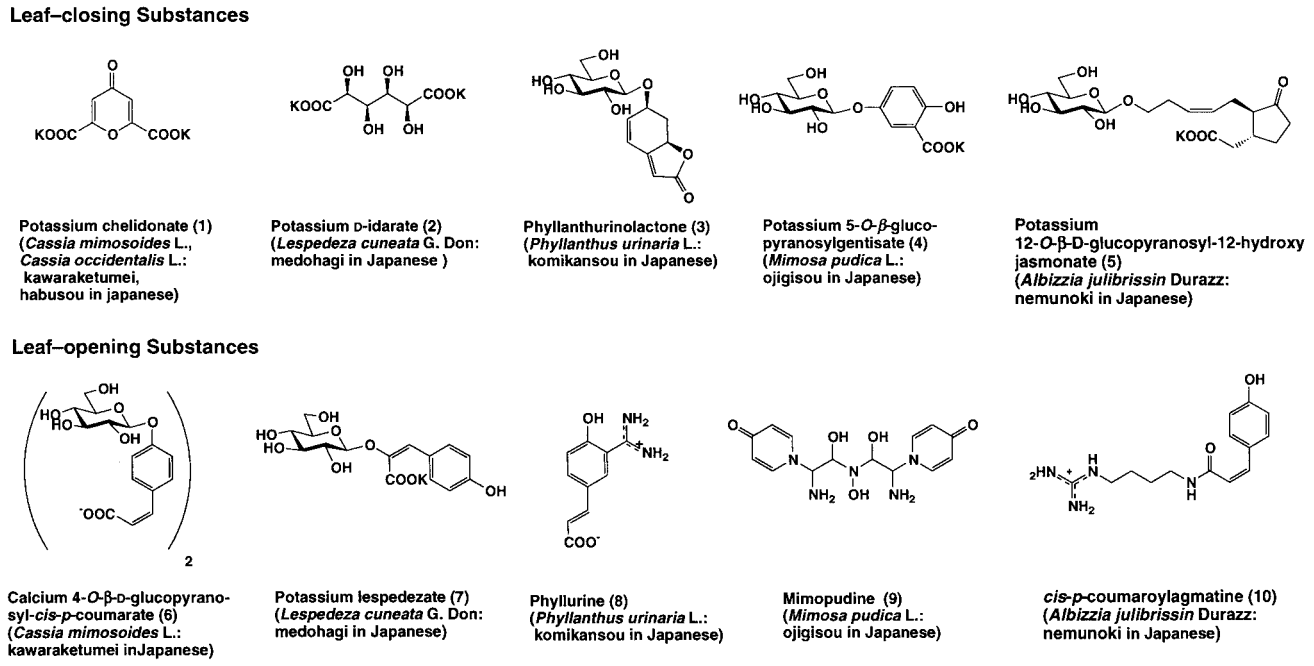


Fig. 1. Leaf-movement factors from nyctinastic plants.

the previously reported result that K^+ ions enter and leave plant motor cells via ion channels which are regulated differently to induce leaf-movement.[18]

So far, we have identified five sets of leaf-closing and -opening substances from five nyctinastic plants as shown in Fig. 1.

The leaf-closing and -opening substances of nyctinastic plants were proven to be different from each other. Therefore, every nyctinastic plant uses different chemical substances for the control of leaf-movement.

These leaf-movement factors have five properties in common;

1) At the concentration of approximately 10^{-6}–10^{-7} M, all bioactive substances exert their effects only on the plants where they originate. This strength of bioactivity is almost the same as that of known phytohormones, such as IAA and gibberellin.

2) The bioactivity of all leaf-movement factors is specific to the original plant from which it was isolated.

3) All leaf-closing substances have a common physiological property in that they compete with IAA at 10^{-6} M. However, much more IAA (ca. 10^{-4} M) is required to achieve a competitive state than the natural abundance of IAA in a plant body.

4) The bioactivity of these compounds is dependent on their stereochemistry. Optically active **5** and **7** were the only bioactive ones among their stereoisomers. This suggests that a specific receptor participates in the process of signal transduction by these substances.

5) The leaf-opening substance competes with the leaf-closing substance. When the concentration of the leaf-closing substance was higher than that of the leaf-opening substance, the leaves were closed during the day and vice versa.

Chemical Control of Leaf-Movement in Nyctinastic Plants

From the viewpoint of chemical studies, the next important problem to be solved regarding nyctinasty is how these compounds control the nyctinastic leaf-movement, and what makes the rhythm of nyctinasty.

We have isolated two leaf-movement factors of contrasting bioactivities from the same nyctinastic plants. The discovery of leaf-opening substances from nyctinastic plants prompted us to assume that nyctinastic leaf-movement is controlled by a competitive interaction between leaf-closing and leaf-opening substances. As described above, five sets of leaf-movement factors were isolated from five plants (Fig. 1). Leaf-closing and -opening substances inhibited each other competitively. Then, we assumed that the concentration of these two substances in a plant body changes through a day.

Potassium D-idarate (**2**), a leaf-closing substance of *L. cuneata*, interacts competitively with potassium lespedezate (**7**), a leaf-opening substance of the same plant. When the concentration of **2** was higher than that of **7**, the leaves were closed during the day, and vice versa. It is important that the bioactivity of the plant extract completely reflected the status of the collected leaves; the plant extract collected when the leaves were closed showed leaf-closing activity and vice versa. This phenomenon suggested that the balance of concentration between leaf-closing and -opening substances is reversed in during the day.

We demonstrate the chemical mechanism for nyctinasty of *L. cuneata*, as an example.[19,20] The extracts of *L. cuneata* collected during the day (around 10:00 a.m.) and at night (around 7:00 p.m.) showed inverse bioactivity; in other words, the former showed leaf-opening activity but the latter showed leaf-closing activity. These results suggest that the balance of concentration between **2** and **7** (or its *cis* isomer) is reversed in these two extracts. HPLC analysis revealed that the extract collected during the day contained twice as much **7** as the extract collected at night, 10:00 p.m.

This two-fold increase in the concentrations of **7** is sufficient to invert the bioactivity of the extract, as judged from our

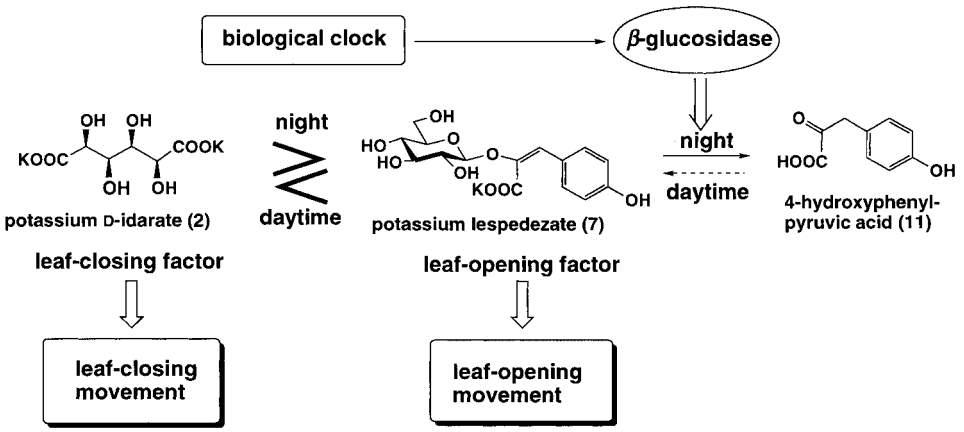


Fig. 2. Chemical control of nyctinasty in *Lespedeza cuneata* G. Don.

previous research on the competition between **2** and **7**. Thus, **7** is metabolized in the evening, and biosynthesized in the morning. Moreover, we discovered that the concentrations of **7** in the plant are inversely proportional to that of potassium 4-hydroxyphenylpyruvate (**11**) (Fig. 2).

The effectiveness of the pyruvate (**11**) (5×10^{-5} M) was one-hundredth of that of **7** (8×10^{-7} M) inducing leaf-opening movement. The extract collected at night contained fivefold as much **11** as the extract collected during the day. This result strongly suggests that **7** is biosynthesized from **11** in the morning, and is metabolized (and deactivated) to **11** by enzymatic hydrolysis in the evening, 5:00 p.m. (Fig. 2).

This new model of the regulation of leaf-movement was strongly supported by the measurement of β-glucosidase activities of the crude enzyme prepared from plants collected during the day and the evening. The crude enzyme was prepared according to the method of Watanabe et al.[21] We collected the leaves of *L. cuneata* and prepared acetone powder. We used **7** as a substrate of the enzyme, and quantitatively analyzed **11** produced by treatment of the acetone powders by HPLC. β-Glucosidase activity was observed only in the acetone powder prepared from the plant collected in the evening.[19]

We propose a new model for the regulation of nyctinastic movement in *L. cuneata*: enzymatic transformation of leaf-movement factors (**7**) inverted the balance of concentration between **2** and **7**, wherein a biological clock should control leaf-movement by activation or expression of the enzyme concerned in this step (Fig. 2). Our study on the nyctinastic movement of *L. cuneata* revealed that nyctinastic leaf-movement is controlled by a biological clock through the regulation of the activity of β-glucosidase which hydrolyzes the leaf-opening substance of this plant.

A similar model would be applicable in the cases of *P. urinaria*.[22] In the case of *P. urinaria*, the leaf-closing substance (**3**), which is a glucoside, decreases in the daytime. On the other hand, the concentration of leaf-opening substance is constant during the daytime. Thus, glycoside-type leaf-closing substance would be hydrolyzed by the β-glucosidase whose activity is regulated by a biological clock.

There is some possibility that the regulation of all nyctinastic leaf-movements can be explained by only one mechanism, namely, that either the leaf-closing or -opening substance is a glucoside in all nyctinastic plants. The biological clock regulates the activity of β-glucosidase which deactivates the glucoside-type leaf-movement factor to control the internal balance of concentration between leaf-closing and -opening substances (Fig. 3).[22]

Direct Observation of the Target Cell for the Leaf-Movement Factor by Using Synthetic Probe Compounds

We have identified several bioactive substances that regulate this leaf-movement, and as mentioned above, our recent studies revealed the mechanism for the control of nyctinasty by the biological clock. The next issue is to determine how these compounds induce leaf-movement. However, different from the studies using some cell-lines, our bioassay is carried out by using whole plant-leaf samples. Thus, we should start from determining a cell to which leaf-movement factor binds. Our leaf-movement factor is supposed to induce the swelling and shrinking of motor cells by direct or indirect interaction with motor cells. To determine the role of leaf-movement factor in plant leaf-movement, we developed molecular probes based on the leaf-movement factor for identification of its target cell in the plant body, which leads to bioorganic studies of nyctinasty. Investigation of the target cells where bioactive substances are perceived is the first step towards the bioorganic study of their receptor molecule.

Synthesis of Fluorescence-Labeled Probe Compounds with Small Fluorescence Dye

We tried to prepare the fluorescence-labeled leaf-movement factors designed on the leaf-opening substance of *C. mimosoides*. The most important problem on the design of fluorescence-labeled leaf-movement factors is the instability of **7** in the plant body; it is easily hydrolyzed by β-glucosidase.[19] Thus, we designed a probe compound based on the structure of an artificial leaf-opening substance, potassium galactolespedezate (**12**) and its geometrical isomer (potassium galactoisolespedezate (**13**)), which could not be hydrolyzed in a plant body.[23,24] Moreover, the bioactivity of **12** and **13** was as strong as the bioactivity of natural product.

The introduction of a large fluorescent dye to artificial leaf-opening substances requires careful consideration of the structure-activity relationship in iso-**7**, which is easily interconvert-

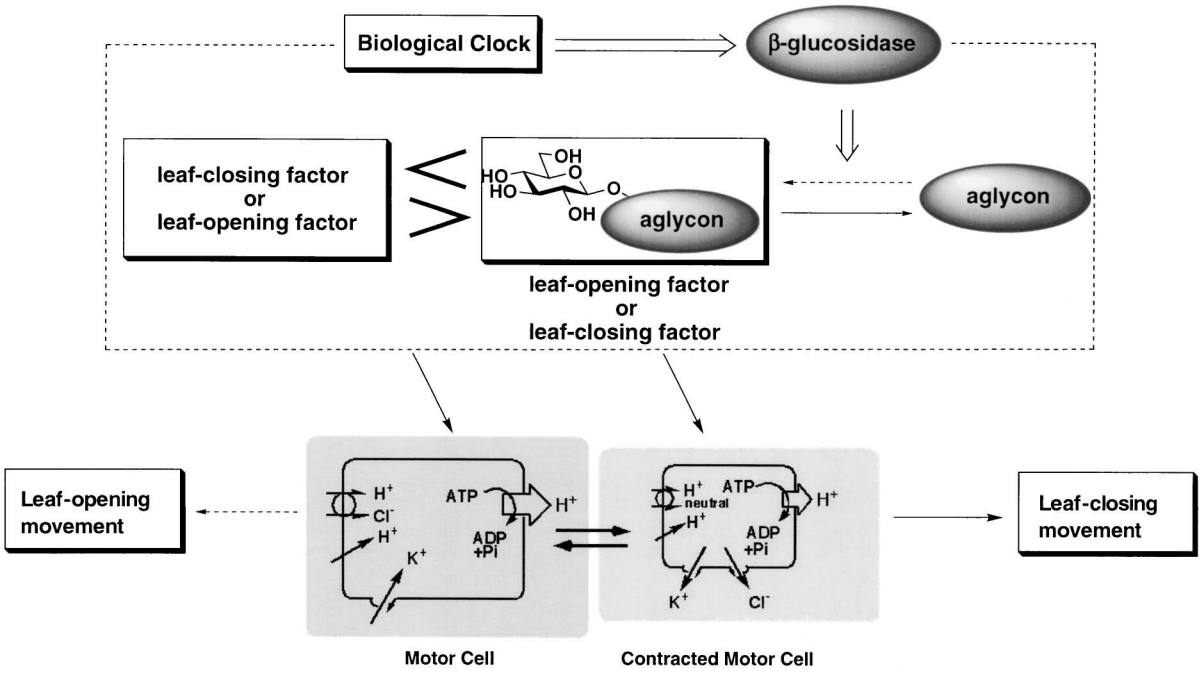


Fig. 3. Universal mechanism of nyctinasty controlled by a biological clock.

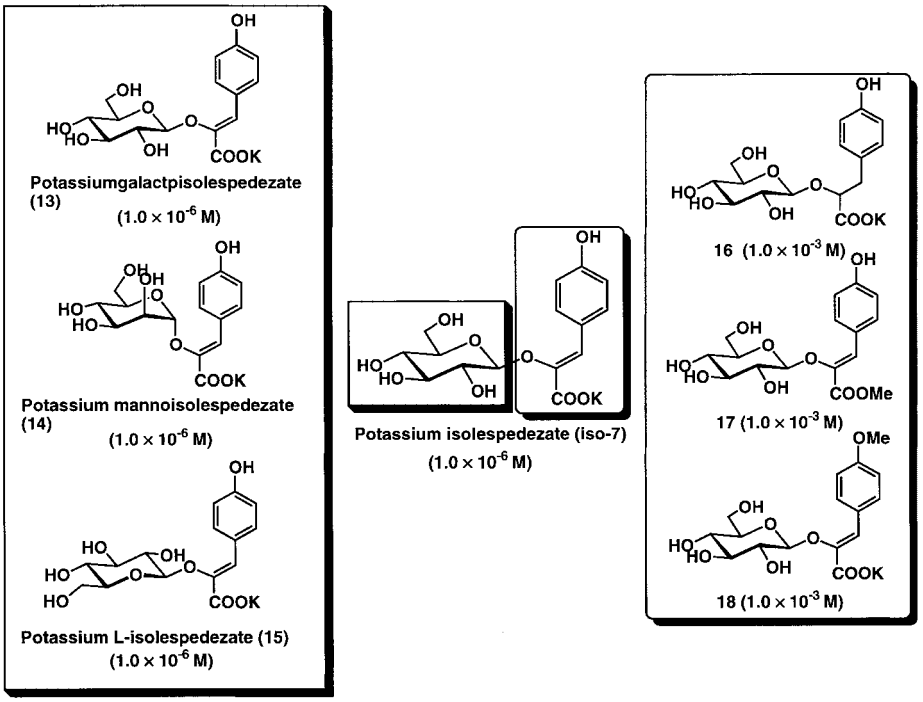
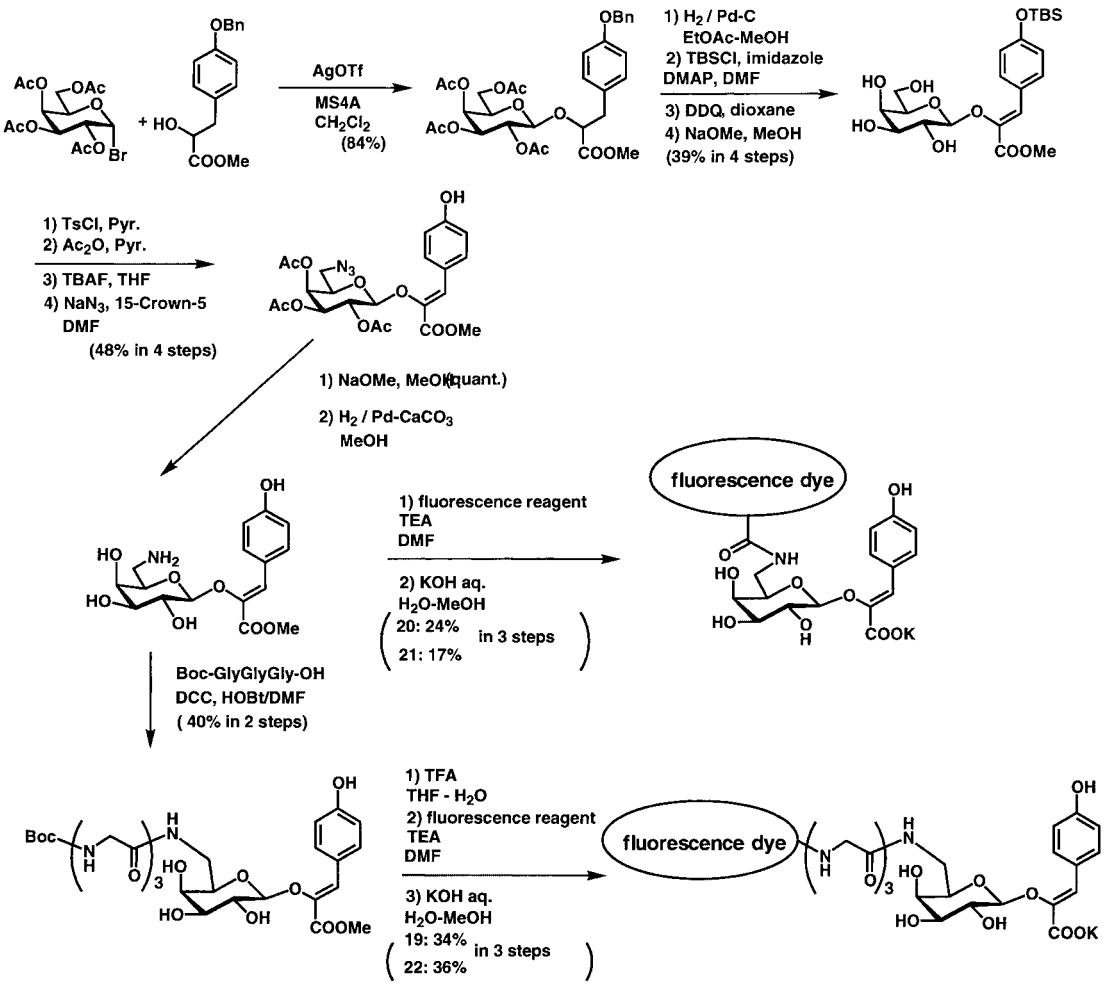


Fig. 4. The structure activity relationship in potassium isolespedezate (**7**).

ed into **7** in aqueous solution. The structure modification in the glucose moiety of iso-**7** (**14** and **15**) did not affect its bioactivity at all,[23,24] while modification in the aglycon moiety of iso-**7**, such as the reduction of the double bond (**16**), and protection of the carboxylate (**17**), or phenolic hydroxy group (**18**), greatly diminished the bioactivity (Fig. 4).[25] From these results, it is expected that the introduction of a large fluorescent functional group in the hydroxy group at the 6′ position of the galactose moiety would not weaken the bioactivity of **12** and **13** to any extent.

Moreover, because of the resistance to the esterase in a plant body, an amide bond would be better than an ester bond to connect a fluorescent dye with **12** and **13**. Thus, to introduce a fluorescent dye at the 6′ position of **12** (or **13**), we should convert the hydroxy group on the 6′ position of **12** (or **13**) into an amino group.



Scheme 1. Chemical syntheses of fluorescence-labeled probe compounds.

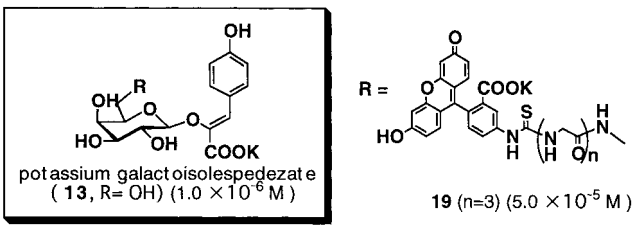


Fig. 5. Structure and bioactivity of FITC-labeled probe compound (**19**).

First, we synthesized FITC-labeled potassium galactoisolespedezate (**19**) (Fig. 5),[26,27] based on the artificial leaf-opening substance **13**, which showed leaf-opening activity against the leaf of *Cassia mimosoides* L. (Scheme 1). However, the bioactivity of **19** was one-fiftieth as strong as that of **13**; thus, a fluorescence labeled probe compound of much stronger bioactivity was required for the bioorganic study of nyctinasty. We examined the length of linker moiety in FITC-labeled probe compounds, to improve their bioactivities. However, according to this methodology, bioactivity of **19**, for example, was at most improved to one-fiftieth of iso-**7**, which is insufficient for bioorganic study. These results suggest that the weak bioactivity of **19** would be attributed to the large size of the FITC group. The bioactivity of probe compounds became higher with the increase of the length of the linker moiety. This result suggests that the large fluorescein moiety blocks the binding of the aglycon moiety to the receptor molecule to weaken the bioactivity.

Based on this idea, we examined 6-(7-amino-4-methylcoumarin-3-ylacetylamino)hexanoyl (AMCA), 6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl (NBD), and 6-(5-dimethylaminonaphthalene-1-sulfonylamino)hexanoyl (dansyl) groups as the fluorescence dyes (Fig. 6).[28] All these fluorescence dyes are much smaller than the FITC in their size.

We synthesized probe compounds bearing those fluorescent dyes (Scheme 1). An aminosugar and a hexanoic acid linker in the fluorescent reagents were connected through an amide bond, which is assumed to be stable against the hydrolysis by an esterase in the plant body.

The resulting fluorescence-labeled probe compounds (**20**-**22**) showed leaf-opening activity against the leaf of *C. mimosoides* (Fig. 6).[28] The bioactivities of all the probe compounds are shown in Fig. 6. Especially, the NBD-labeled probe compound (**21**) showed the strongest bioactivity, and was effective at 5×10^{-6} M, which was one-fifth as strong as that of the natural product (iso-**7**).

From these results, it was concluded that the size of the fluo-

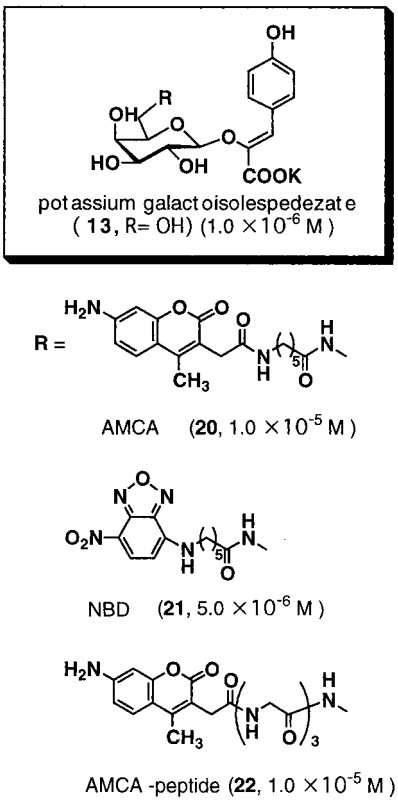


Fig. 6. Fluorescence-labeled probe compounds.

rescence dye is important for the strong bioactivity of probe compounds. Thus, fluorescence-labeled probe compounds, such as **20**–**22**, would be sufficient for the fluorescence study of nyctinasty.

Here, we have succeeded in the chemical synthesis of several probe compounds for the bioorganic study of nyctinasty. Bioorganic studies using these probe compounds are now in progress.

Direct Observation of the Target Cell for the Leaf-Movement Factor

We used probe **20** for the detection of the target cell for the leaf-movement factor. Figure 7 shows photographs of the plant sections treated with **20** under a fluorescence microscope.

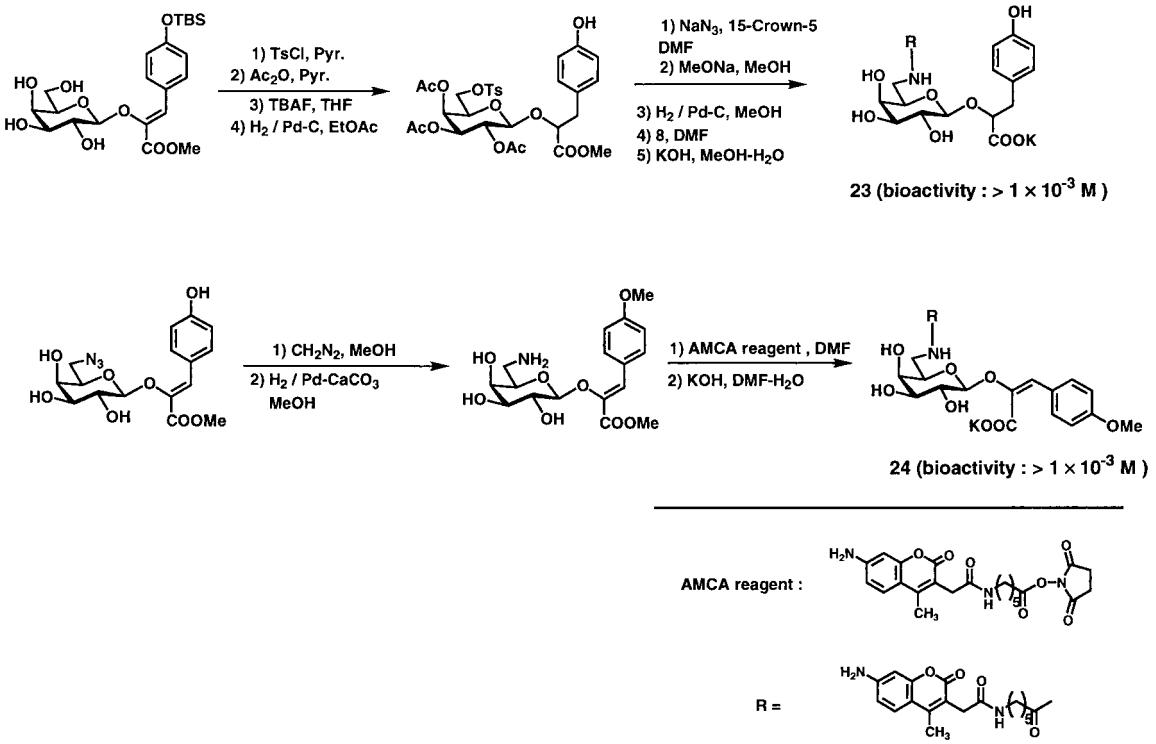
Strong autofluorescence was observed in the plant section. However, when the section was treated with probe compound (**20**), the staining pattern for **20** was observed only in the motor cells contained in pulvini (Fig. 7). No other part of the plant section showed the fluorescent stain for **20**. Also, no stain was observed in the control section, which was treated with a solution without **20**. Thus, the binding of iso-**7** and motor cell would be specific to the structure of leaf-opening substance moiety in **20**, and not non-specific resulting from AMCA moiety. These results suggest that the binding sites for **20** should exist on the the motor cell of *C. mimosoides*. Because of its high hydrophilicity, leaf-movement factors would not be able to be transmitted into the plasma membrane. Thus, it was assumed that some receptors would exist on a plasma membrane of the target cell for the leaf-movement factor.

Bioorganic Studies Using Inactive-Type Probe Compounds

In the previous section, we showed that the biologically active probe compound can bind its receptor molecule on the motor cell of *C. mimosoides*. However, to prove the existence of a receptor molecule, it is also essential to demonstrate that biologically inactive-type probe compounds cannot bind to the receptor. Thus, it was strongly desired to carry out the fluorescence study using biologically inactive fluorescence-labeled probe compounds designed on a biologically inactive analog of **7** to prove the existence of a receptor molecule and the specific binding of **20** to it.

Molecular design of the biologically inactive probe compounds was based on the structure–activity relationship of iso-**7**.[25] The structure modification in the glucose moiety of iso-**7** did not affect its bioactivity at all, while modification in the aglycon moiety of iso-**7**, such as the reduction of the double bond or protection of the carboxylate or phenolic hydroxy group, greatly diminished the bioactivity. Thus, the biologically inactive probe compounds were designed on the analogs of iso-**7** with structure modification in the aglycon moiety. The sugar part of the probe was galactose instead of glucose in iso-**7**, which prevents hydrolytic decomposition of the probe com-

Fig. 7. The binding study of fluorescence-labeled probe compounds (left: blank, right: treated with **20**).



Scheme 2. Chemical synthesis of inactive-type probe compounds.

pounds by β-glucosidase in the plant body. The introduction of a fluorescence dye was carried out at the 6-position of the galactose moiety through the amide bond. We chose 6-(7-amino-4-methylcoumarin-3-ylacetylamino)hexanoyl (AMCA) groups as a fluorescent dye which is the same as that contained in **20**.

We synthesized two probe compounds (**23** and **24**) based on the procedure reported in Ref. 27 (Scheme 2). In the case of the probe with a reduced double bond, the absolute stereochemistry of the major product was determined by the PGME method[29] to be (*R*) by using the pentaacetate.

For the synthesis of a probe compound with methyl ether (**24**), the geometry of the double bond in **24** changed to (*E*)-form. However, it was already shown that the geometry of the double bond in iso-**7**, which is a parent compound of **24**, easily isomerized in aqueous solution to give a mixture of both geometrical isomers which are equally effective against plant leaf. The resulting fluorescence-labeled probe compounds **23** and **24** showed no leaf-opening activity against the leaf of *C. mimosoides* even at 1×10^{-3} M. The bioactivities of **23** and **24** were no more than one-thousandth of that of probe **20**.

We carried out the binding studies between the biologically inactive probe (**23** and **24**) and the plant sections with motor cell. A leaf of *C. mimosoides* was cut with a microslicer to a thickness of thirty micrometers. Then the section containing a motor cell was incubated overnight in an aqueous solution containing 5×10^{-5} M of **23** (or **24**). Then, the stained section was monitored by using a fluorescence microscope with an appropriate filter. The same experiment was carried out by using **20** as a control experiment.

Figure 8 shows photographs of plant pulvini, which contains a motor cell, under a fluorescence microscope. A staining pattern for the fluorescence of probe compound (**20**) was observed on the motor cell. On the other hand, no stain was observed in the section treated with (*R*)-**23** and **24**. Thus, it was proved that biologically inactive probe compounds cannot bind to the plant motor cell at all. Results from the structure activity relationship were consistent with the results from binding experiments. Also, it was clearly shown that the binding of biologically active AMCA-labeled probe (**20**) with a motor cell is due to the specific binding of the 2-hydroxy-3-*p*-hydroxyphenylacrylate moiety which is the active site of this molecule, and is not a non-specific binding due to the hydrophobic AMCA group.

These results strongly suggested that some receptor for the leaf-opening substance exists on the motor cell. Along with the previous result, some properties were revealed on a receptor molecule of iso-**7**, that is, this receptor recognizes the aglycon moiety precisely; on the other hand, it does not recognize the sugar moiety at all.

Specific Binding Ability of Fluorescent Probe Compound to *C. Mimosoides*.

Each nyctinastic plant has a different leaf-movement factor whose bioactivity is specific to the original plant.[5–14] Thus, synthetic probe compounds are expected to show specific leaf-opening activity against the leaves of *C. mimosoides*, and not to be effective for the leaf of other plants even at higher concentrations. We examined the specificity of bioactivity on the probe compounds **20**. Probe compound **20** did not show leaf-opening activity against the leaves of *Leucaena leucocephala*, *Albizzia julibrissin* Durazz., and *Aeschynomene indica* L. at 5×10^{-5} M. On the other hand, **20** showed leaf-opening activity against the leaves of *C. mimosoides* even at 1×10^{-5} M.

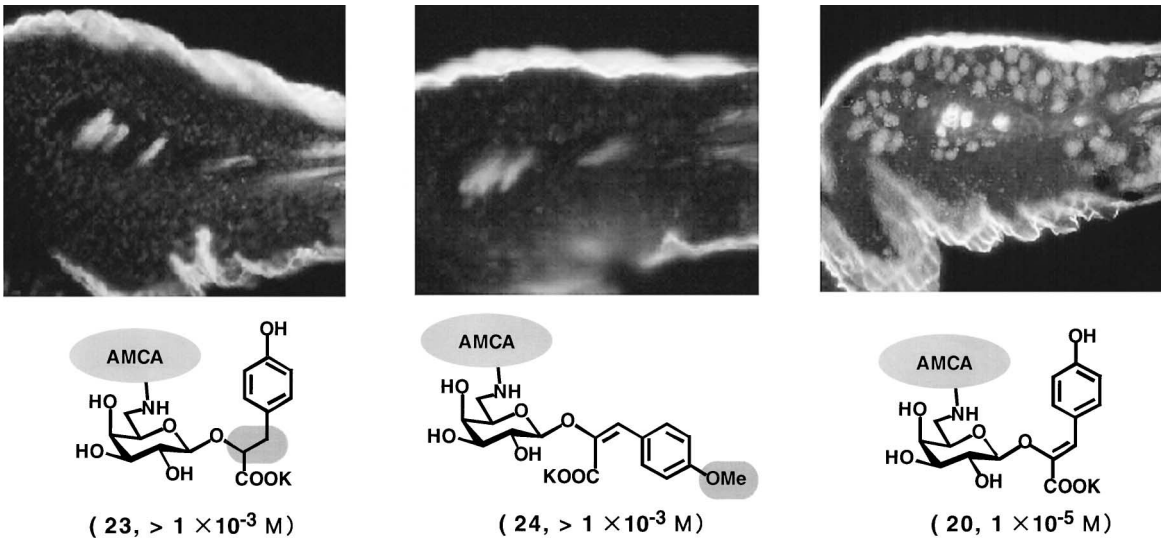


Fig. 8. The binding studies using inactive-type probe compounds.

These results showed that the bioactivity of **20** was specific to the leaves of *C. mimosoides*. From this result, the binding of the probe compound is expected to be specific against the section of *C. mimosoides* and no binding would be observed in the experiments using the section of other plants.

Then, we used probe **20** for the binding experiments with the sections of *C. mimosoides*, *L. leucocephala*, *A. julibrissin*, and *A. indica*.[30] Thus, it was revealed that only the section of *C. mimosoides* gave the fluorescence image resulting from **20** and other sections did not at all. Moderately strong red fluorescence observed on each plant section is due to the autofluorescence from chlorophylls contained in the plant section, which was also observed on the blank sample. This result showed that the binding of probe compound with a motor cell is also specific to the plant species and suggested that the specific receptor molecule would be involved in the binding of **20** with a motor cell.

In other words, this result strongly suggests that each nyctinastic plant has different receptor molecules on the motor cell to perceive external chemical signal for introduction of swelling and shrinking of motor cell. However, more examples of fluorescence study should be carried out on other nyctinastic plants, such as *L. leucocephala*, *A. julibrissin*, and *A. indica* etc., to confirm this hypothesis by using fluorescence probe compounds designed on the structure of the leaf-movement factor of each plant. The systematic studies on the identification of the receptor protein for leaf-movement factor and investigations on the difference of receptor molecule in each nyctinastic plant will give a clue for the question: Why does a different leaf-movement factor operate in each nyctinastic plant?

So far, we have shown two examples of nyctinastic plants in which the concentration of the leaf-movement factor in a plant body changes through a day according to a circadian rhythm, and this rhythmic change in concentration agrees with the nyctinastic leaf-movement. And we have shown that our leaf-movement factor binds to the motor cell. So far, the behavior of our leaf-movement factor can fully account for this physiological phenomenon in the molecular level. The next issue of our research is focused on investigation of the role of leaf-movement factors in this biological event, especially, the interaction between leaf-movement factor and its receptor molecule, which invokes the signal transduction in the motor cell, which leads to the swelling and shrinking of motor cell.

Why Does the Plant Sleep?

"Why does the plant sleep?": This fundamental question has always bothered scientists concerned with the research of nyctinasty, such as C. Darwin and E. Bünning. Darwin spoke of protection from chilling in addition to protection from actual freezing.[2] However, there also reported that protection from chilling rather than freezing gave nyctinastic plants a slight advantage.[31,32] Bünning proposed that nyctinasty protected the photoperiodic timekeeping system from moonlight, because moonlight falling on leaves during the night might prevent accurate measurement of night length.[33] Like this, two great scientists insisted on the original hypotheses which based on their original speculation.[3] So far, no experimental evidence was reported to explain the biological meaning of nyctinasty. This problem would be so difficult because we cannot inhibit the nyctinastic leaf-movement of the plants. Now, we have succeeded in the inhibition of nyctinastic leaf-movement in plants, and reported the first clue for the question, "Why does a leguminous plant sleep?", with experimental evidence.

We have shown the chemical mechanism for the control of nyctinasty. In the case of *Lespedeza cuneata* G. Don, nyctinastic leaf-movement is controlled by a change in the concentration of leaf-movement factor which is transformed into its aglycon by the action of β-glucosidase (Fig. 3).[19,20] This transformation inverted the balance of concentration between leaf-movement factors, wherein a biological clock controls leaf-movement by activation or expression of β-glucosodase concerned in this step. Thus, β-glucosidase would be a key enzyme in the control of circadian rhythm of nyctinasty. According to this mechanism, structurally modified leaf-opening substance which does not become hydrolyzed by β-glucosidase would keep the leaf open eternally, and thus, inhibit the nyctinastic leaf-closure (Fig. 9). Fortunately, it was already revealed that no decrease of bioactivity was observed by the

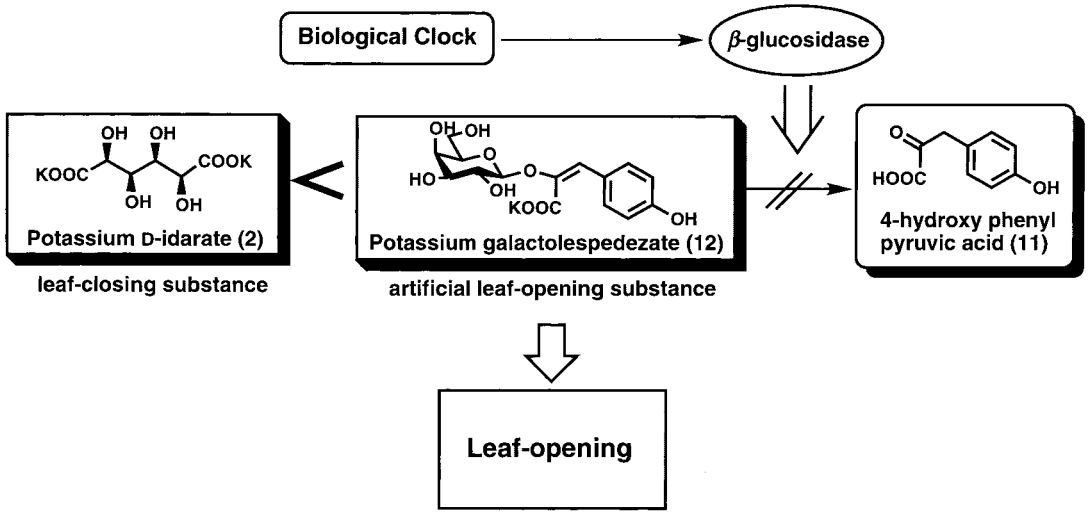
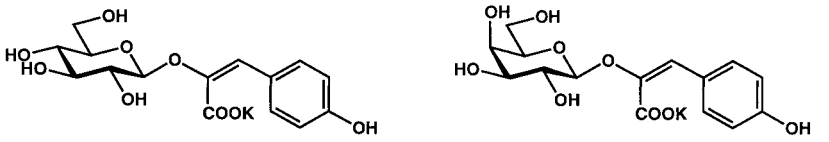


Fig. 9. Inhibition of nyctinasty by artificial leaf-opening substance (**12**).



	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
7	open	open	closed	closed	closed	closed	closed
12	open	open	open	open	open	open	open

*at 3×10^{-6} M, at 9:00 p.m.

Fig. 10. The bioactivity of artificial leaf-opening substance (**12**).

structural modification on the sugar moiety of **7** in the structure-activity relationship studies of **7**.[23,24] Then, we synthesized an artificial leaf-opening substance based on **7** which contains galactose instead of glucose in the molecule.

Our synthetic artificial leaf-opening substances showed novel bioactivity; the leaf-opening activity of **7** lasted for only two days at 3×10^{-6} M; after that, the leaves closed at night again (Fig. 10). On the other hand, that of artificial leaf-opening substances lasted even after a week (Fig. 10).

The leaves treated with 3×10^{-6} M of the artificial leaf-opening substance (**12**) kept opening until the leaves withered and died after a week. This result clearly showed that the nyctinastic leaf-closure is essential for the survival of this plant. This result would be an important clue for the historical mystery "Why does the plant sleep?".

And also, **7** would be completely hydrolyzed into **11** by β-glucosidase in a few days; on the other hand, our artificial leaf-opening substances would not be hydrolized by the enzyme in the plant body. The leaf has never closed without the deactivation of the leaf-opening substance by β-glucosidase. The artificial leaf-opening substance (**12**) was effective and specific to *C. mimosoides*, and not effective for other nyctinastic plants, such as *Mimosa pudica*, *Aeschynomene indica*, and *Phyllanthus urinaria*, at 3×10^{-5} M.

This result suggested that nyctinastic leaf-closure would greatly influence the water control of the leguminous plants. The plants mainly control their water fluence by two mechanisms; one is by stomatal transpiration, and the other by cuticle transpiration, which is a transpiration from surface of the leaf.

We examined the status of the stoma in the existence of leaf-opening substance. Thus, it was reveled that the leaf-opening substance did not affect the stomatal movement at all. Stoma usually open in the daytime and close at night, and even under the existence of leaf-opening substance, stomatal movement was not affected at all. On the other hand, the leaf-closing substance also did not affect the stomatal movement at all. Thus, the leaf-movement is supposed not to affect the stomatal transpiration at all either. From these results, nyctinastic leaf-movement would play an important role in the water-control in the plant body.

This result leads to the important application of leaf-movement factors for the development of novel herbicides with complete selectivity to the weeds. Artificial leaf-opening substance of *C. mimosoides* kept the leaf open for a week ("in-

somnia") and the leaf was observed to wither and die. This result showed that the nyctinastic leaf-movement is essential for the survival of legumes. From this finding, we envisioned that plant-specific leaf-movement factors could be useful as a herbicide. When we use such leaf-movement factor as a herbicide, it would be effective only for the legume from which it was isolated because of the highly selective bioactivity of leaf-opening substance, and have no effect on other vicinal plants, insects, birds, and animals. The isolation of leaf-opening substance of some leguminous weeds aiming at the development of this unique herbicide is now in progress. Leaf-opening substance is a potential herbicide without any environmental problem.

Conclusion

Leaf-movement in nyctinastic plants has long been believed to be controlled by a common phytohormone. However, our result contradicts this theory. Indeed, we advance a new theory of the chemical control of nyctinastic leaf-movement; nyctinastic leaf-movement is controlled by the balance of concentration between two bioactive substances, leaf-opening and -closing substances, which is inverted through the day according to the rhythm created by their biological clock. A biological clock regulates this balance through the control of the β-glucosidase activity (Fig. 3).[22]

Our leaf-movement factors, whose concentrations have been proven to change according to a circadian rhythm, should be genuine chemical signals that control leaf-movement in nyctinastic plants from a physiological viewpoint. Moreover, we have shown that leaf-movement factor directly interacts with motor cells by using fluorescence-labeled probe compounds. So far, the behavior of our leaf-movement factor can fully account for this physiological phenomenon in the molecular level. The next issue of our research is focused on investigation of the role of leaf-movement factors in this biological event, especially, the signal transduction after the perception of the leaf-movement factor by its receptor. Our present result will develop the sciencific viewpoint of this field from plant physiology into bioorganic chemistry. And we expect that these results would bring us a clue in the investigation of the nature of the biological clock in highly organized plants.

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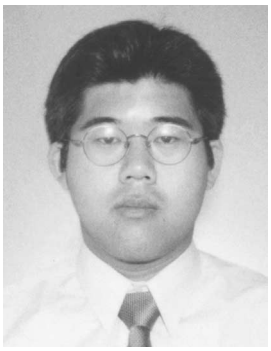

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