Endogenous factors involved in the regulation of movement and "memory" in plants*

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Abstract: 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 nyctinastic leaf movement is induced by a pair of leaf-movement factors, and one of each pair is a glucoside. There are two key proteins that are involved in the control of nyctinasty. One is β-glucosidase: a biological clock regulates the activity of β-glucosidase, which deactivates the glucoside-type leaf-movement factor, controlling the balance in the concentrations of the leaf-closing and -opening factors. The other is the specific receptor for each leaf-movement factor: the genuine target cell for each leaf-movement factor is confirmed to be a motor cell from leaflet pulvini, and the specific receptors that regulate the turgor of motor cells are localized in the membrane fraction.

The article also discusses the isolation of the "memory" substance from the Venus flytrap and presents a mechanism for this action.

Keywords: plant movement; circadian; nyctinasty; glucosidase; motor cells; leaflet pulvini.

INTRODUCTION

In general, plants are rooted and do not exhibit 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. This circadian rhythm with respect to leaf movement is known as nyctinasty and is widely observed in leguminous plants. This rhythm is regulated by a biological clock with a cycle of about 24 h. The phenomenon has been observed by scientists for centuries, with the oldest records dating back to the time of Alexander the Great. The biological clock itself was discovered in the 18th century from careful observations of nyctinasty in *Mimosa pudica*.

Charles Darwin established the science of plant movement and studied the topic enthusiastically in his later years. In 1880, Darwin published a seminal book titled *The Power of Movement in Plants*, which was based on experiments using more than 300 different kinds of plants, including nyctinastic species [1]. 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 that are involved in these processes.

Physiological studies revealed that nyctinastic leaf movement is induced by the swelling and shrinking of motor cells in the pulvinus, an organ located at the joint of the leaf [2]. These motor cells

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play a key role in plant leaf movement. A flux of potassium ions across the plasma membranes of the motor cells is followed by massive water flux, which results in the swelling and shrinking of these cells. At the heart of this 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 [3–7]. Many attempts have been made to isolate the endogenous bioactive substances that control nyctinasty [8].

ENDOGENOUS FACTORS CONTROLLING NYCTINASTY

Nyctinastic plants have a pair of endogenous bioactive substances that control nyctinastic leaf movement [9]. 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-opening and -closing factors have been identified in five different nyctinastic plants (Fig. 1) [10–21].
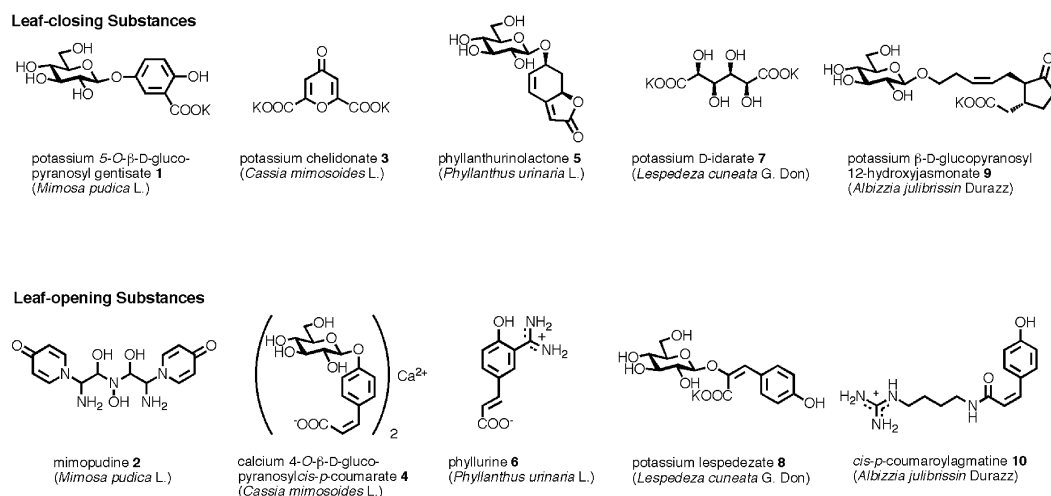

Fig. 1 Leaf-movement factors from five leguminous plants.

When the leaves of a leguminous plant are disconnected from the stem, they continue these leaf movements according to the circadian rhythm, opening in the daytime and closing at night. Artificial application of the leaf-movement factors to the leaves makes plant leaves open at night or close during the daytime [22]. All of these factors are effective at concentrations of 10^{-5} to 10^{-6} M. This bioactivity is very similar to that of known phytohormones, such as IAA and the gibberellins. These studies also showed that nyctinastic plants use unique leaf-movement factors, and that these are conserved within the same genus. None of the factors were effective in other plants, even at concentrations of 100 000-fold [23].

Leaf-movement factors are involved in the control of nyctinasty. Consequently, it was assumed that the rhythm of nyctinasty would be affected by the metabolism of leaf-movement factors in the plant body. We therefore investigated the time-course changes of leaf-movement factors within the body of the plants [24–26].

The time-course changes in the concentration of leaf-closing and -opening factors in the plant *Phyllanthus urinaria* are shown in Fig. 2 [24]. HPLC was used to determine the levels of these factors every four hours of a daily cycle. It was found that the content of leaf-opening factor **6** remains nearly constant during the day, whereas that of leaf-closing factor **5** changes by as much as 20-fold. This behavior could be accounted for by conversion of the leaf-closing factor **5** to its corresponding aglycon **11** in a hydrolytic reaction. Thus, it was revealed that significant changes in the concentration of the ratio
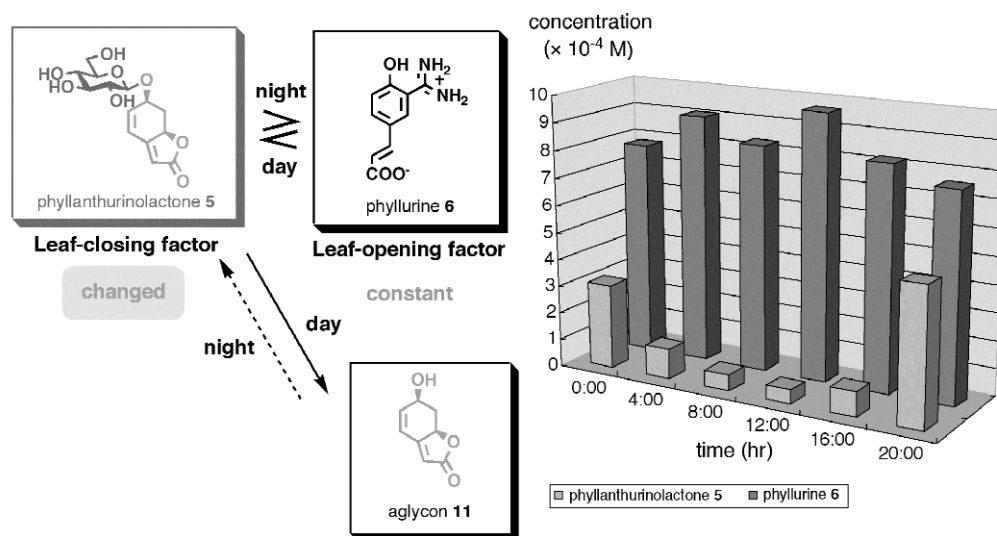
Fig. 2 Changes in the concentration of leaf-movement factors in *P. urinaria* in nyctinasty.

between leaf-closing and -opening factors in the plant are responsible for leaf movement. And this is a universal mechanism in five nyctinastic plants. These results indicate that regulation of all nyctinastic leaf movement can be explained using a single mechanism, as follows.

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 in the concentrations of the leaf-closing and -opening factors. Thus, nyctinastic leaf-movement is controlled by a biological clock through the regulation of β-glucosidase activity. In other words, control of leaf movement by a biological clock can be explained as a series of chemical reactions, specifically, the formation and dissociation of the glycosidic bond of a leaf-movement factor [9].

MOLECULAR PROBES FOR MOLECULAR MECHANISM OF NYCTINASTY

Most of the physiological studies on nyctinasty have been carried out in plants belonging to the *Albizzia* genus. Considering that each nyctinastic plant has a pair of leaf-movement factors whose bioactivities are specific to a particular plant genus, bioorganic studies of nyctinasty using *Albizzia* plants are important. We revealed that these compounds are common leaf-movement factors **9,10** among *Albizzia* plants [16,21]. We developed molecular probes consisting of modified leaf-movement factors of *Albizzia* plants in order to identify their target cells [27–30]. We conducted a double fluorescence-labeling study using FITC-labeled leaf-closing factor **13** and rhodamine-labeled leaf-opening factor **12** (Fig. 3) [30]. The experiment was performed using the pulvini (pulvinus) of *A. saman*. Figure 4 illustrates the fluorescence image of plant sections that were cut perpendicular to the vessel.

The motor cells in the pulvini of nyctinastic plants consist of two types: extensors and flexors. Leaflets move upward during closure and downward during opening due to the actions of the extensors located on the upper side of a leaf and the flexors on the lower side.

Interestingly, both of the probes bound to the same extensor cell in the pulvini. Therefore, the motor cell with a set of receptors for leaf-movement factors is located on the extensor side of pulvini in *A. saman*. Since extensor cells are defined as cells that increase their turgor during opening, and decrease their turgor during closing, the leaf-movement factors must facilitate a decrease or increase in the turgor of extensor cells of *A. saman*. In *A. saman*, the trigger for leaf-closure or -opening might be related to the change in the turgor of the extensor cells.
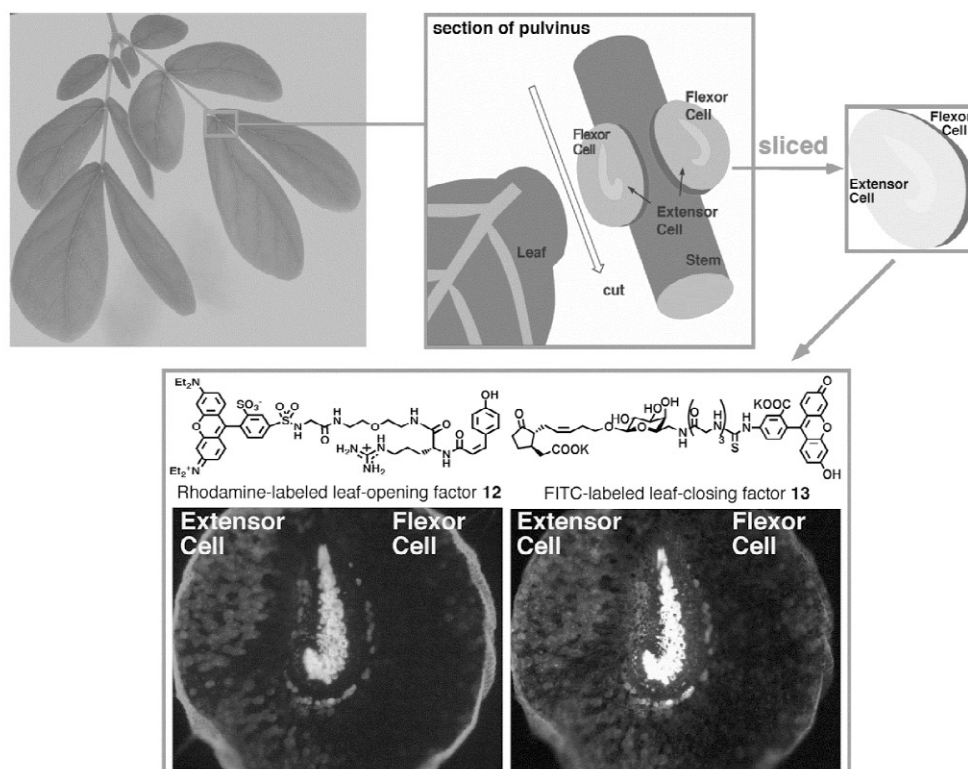
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Fig. 3 Schematic presentation of the double fluorescence-labeling study using the pulvini from *A. saman*.
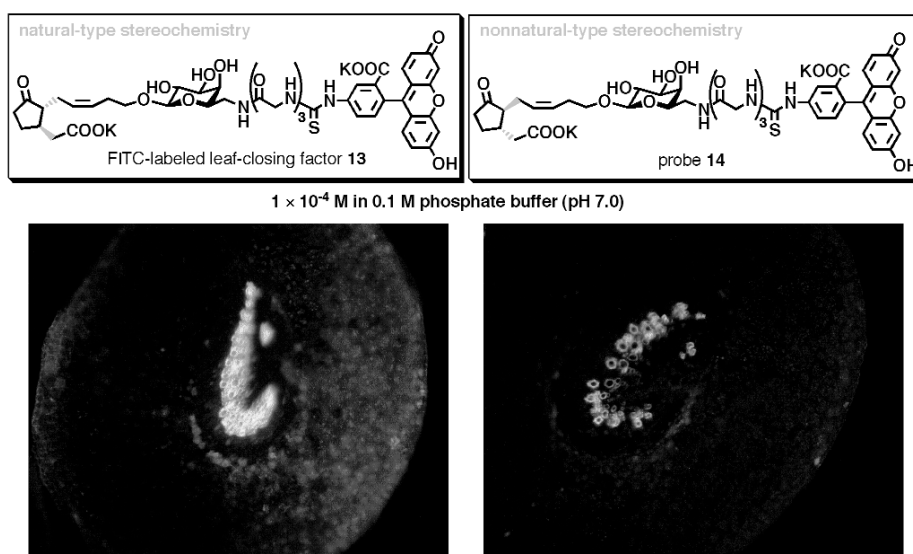

Fig. 4 Fluorescent study on specific receptor for leaf-closing factor in *A. saman* using enantio-differential approach.

Moreover, the "enantio-differential approach" clearly demonstrated the involvement of a receptor in the extensor cell, which recognizes the stereochemistry of jasmonate-type leaf-closing factor (Fig. 4) [29]. Comparing the results by leaf-closing factor probe **13** and its enantiomer **14**, it was clearly shown that fluorescence occurring in the extensor cell is due to the specific binding which is affected by natural-type stereochemistry. In addition, the strong fluorescence observed in the xylem for both enantiomers was attributed to nonspecific binding of the probes. Thus, the genuine target cell for leaf-closing factor was confirmed to be a motor cell. These results strongly suggested the involvement of some specific receptor for leaf-movement factor.

In other nyctinastic plants, the target cells of leaf-movement factors have also been found to be motor cells. Fluorescence-labeled leaf-movement factors, such as potassium isolespedezate **16** and phyllanthurinolactone **5** were also synthesized to identify their target cells [31,32]. It was revealed that target cells for these chemicals were the motor cells in the pulvini, similar to the case of *Albizzia*.

In the following sections, we will discuss the findings of studies on the receptors of leaf-movement factor. We focused on the receptor of potassium isolespedezate **16**, a leaf-opening factor of *Cassia* plants in this next section.

We then tried to identify the receptor for the leaf-movement factors. We synthesized a biologically active biotinylated photoaffinity [33–36] probe **15** based on potassium isolespedezate **16**, a leaf-opening factor of *Cassia mimosoides* [37,38]. The photoaffinity probe **15** was effective at concentrations of 1×10^{-4} M in a bioassay with leaves of *C. mimosoides*, and had an activity that was one-hundredth as effective as the natural product. The crude membrane fraction that was prepared according to Fig. 5 was incubated with 3×10^{-6} M of probe. After cross-linking, the membrane fraction was analyzed by SDS-PAGE and Western blotting was performed. Two probe-coupled target proteins of 210 and 180 kDa were detected by chemiluminescence [37,38]. Specific binding of the probe was confirmed by the disappearance of the corresponding bands during photolabeling in the presence of an excess amount of nonlabeled leaf-opening substance. The binding of probe to these binding proteins was competitively inhibited under these conditions. And these proteins could not be detected in the leaf cell which contains no motor cell.

These results indicate that the *Cassia* opening factor binds specifically to the 180 and 210 kDa proteins that are localized in the membrane fraction of motor cells from leaflet pulvini, but that the specific binding does not occur in the membrane fraction of other cells. It is, therefore, highly likely that the two proteins are the specific receptor of the *Cassia* opening factor.

We have found that two-key proteins are involved in the control of nyctinasty: one is β-glucosidase, which is responsible for controlling the rhythm of nyctinasty [39], and the other is the membrane receptor for leaf-movement factor which regulates the turgor of motor cells. Nyctinastic leaf movement is induced by opening and closing of potassium channels.

So these findings represent an important advance in the bioorganic study of nyctinasty and provide important clues regarding the molecular mechanism of nyctinasty, which has been a historical mystery since the era of Darwin.
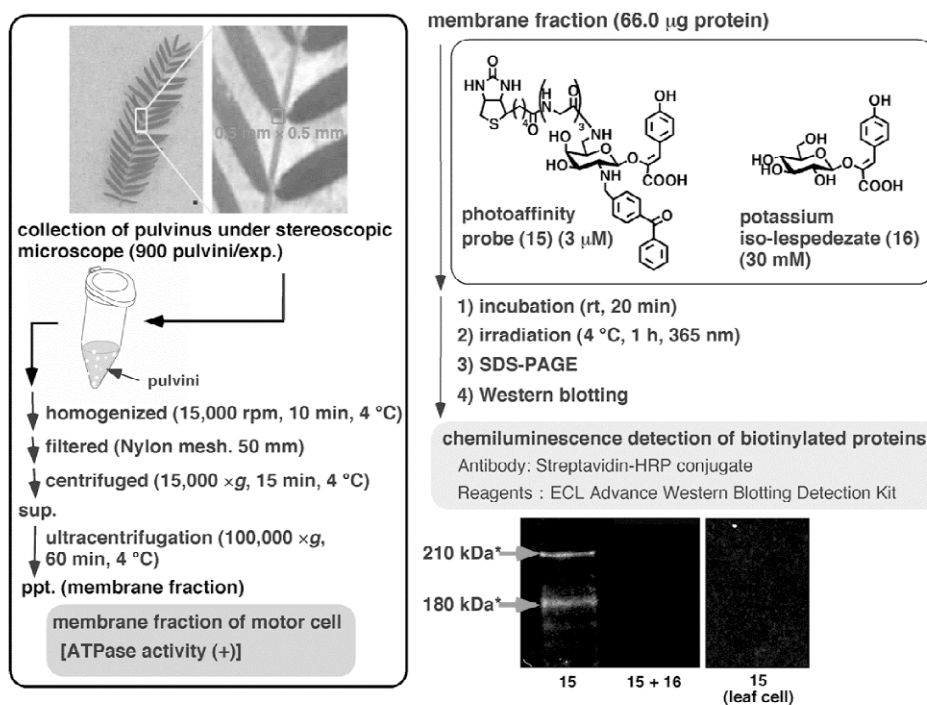
Fig. 5 Schematic presentation of the photoaffinity-labeling study using the membrane fraction of motor cells from *A. saman*. After Western blotting of biotinylated protein using Streptavidin-HRP conjugate, specific binding of the probe **15** was visualized by chemiluminescence with ECL advance Western blotting detection kit. Left lane; using membrane fraction of pulvini and 3 μM solution of photoaffinity probe **15**, center lane; in the presence of excess potassium iso-lespedezate **16** (30 mM) as a competitor, right lane; using membrane fraction of leaves instead of pulvini.

CARNIVOROUS PLANTS AND "MEMORY" IN HIGHER PLANTS

The Venus flytrap (*Dionaea muscipula* Ellis) is an insectivorous plant that catches insects using a trap consisting of large modified 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 [40].

Interestingly, a kind of "memory" appears to be involved in leaf closure of *Dionaea* (Fig. 6). Rapid closure of the trap requires two stimuli within 30 s of each other on the sensory hairs, which are located on the internal surface of the trap leaves. Leaf closure never occurs when only a single stimulus is applied. Clearly, *Dionaea* has a mechanism for "remembering" the first stimulus. We have hypothesized that a bioactive substance is involved in this "memory" process.

If secretion of the relevant metabolite is stepwise and occurs in response to each stimulus, the "memory" response could be triggered by the stepwise accumulation of the secreted bioactive substance. Thus, a study was initiated to isolate the endogenous metabolite responsible for triggering the closure of traps in *Dionaea*. 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 gradually be secreted after multiple stimulations until its concentration in vivo exceeds that of 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.
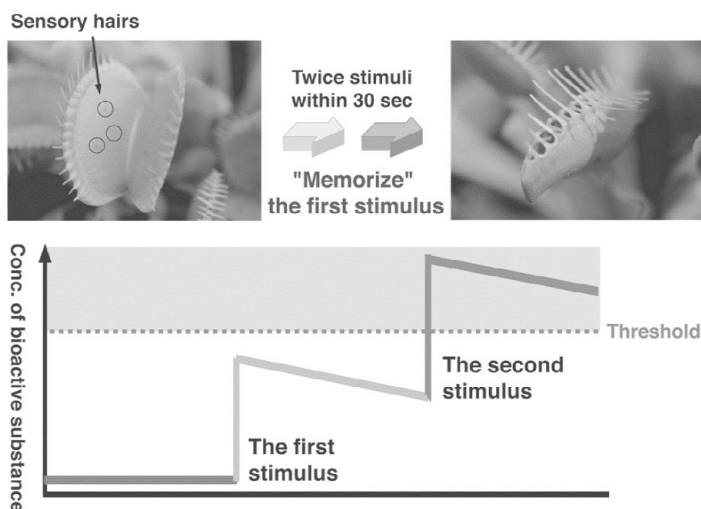
Fig. 6 Overview of possible mechanism for memory of the Venus flytrap in two-stage stimulus.

A bioassay for leaf-closing activity offers an approach for identifying and isolating such a substance. Bioassays using the leaves or plant body of *Dionaea* generally have low reproducibility because of individual differences between plants. However, bioassays performed on genetically uniform clones of *Dionaea* have resulted in much more reproducible and reliable outcomes [41]. Using this method, we were able to identify and isolate a bioactive fraction, one that had the capacity to induce closure of *Dionaea* leaves without the requirement for external stimuli.

Using this bioassay, we demonstrated the existence of a threshold in the quantity of accumulated bioactive factor required to induce trap closure. Aqueous solutions of 10 and 20 g/L of *Dionaea* extract were prepared and added to trap leaves by transpiration. Trap leaves were observed to close when they adsorbed over 80 µL of the 10 g/L solution without stimuli, and when they adsorbed over 40 µL of 20 g/L solution. These results clearly showed the existence of a threshold effect involving the quantity of the bioactive substance in the extract for inducing trap closure, and strongly supported our hypothetical mechanism of "memory".

Bioassay-guided separation of the extract led to the identification of an endogeneous bioactive polysaccharide consisting of α-arabinofuranoside, α-galactopyranoside, and α-xylopyranoside moieties (Fig. 7). This polysaccharide has the capacity to induce the closure of traps, without external stimuli, at very low concentrations, approximately 2 ng/leaf. Although this compound has only been isolated at very low concentrations, some information on the polysaccharide nature of the compound has been elucidated using 500 MHz cryoprobe NMR analysis.

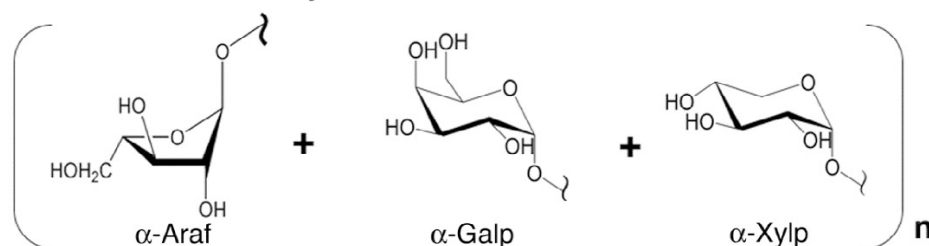## Monosaccharide components in bioactive substance

Fig. 7 Three components of polysaccharide stimulating the memory of the Venus flytrap.

The mechanism for the "accumulation of the chemical substance resulting in ion channel activation resulting in action potential generation" observed in *Dionaea* closely resembles the stimulation transmission mechanisms found in higher-order animals [3,42]. Interestingly, the leaf trap movement observed in *Dionaea* was also induced using high concentrations (ca. 0.1–1 g/L) of the common neurotransmitters found in higher-order animals, such as norepinephrine, DOPA, and glutamate. There is also some evidence for the "memory" substance in *Dionaea* also possessing neurotransmitter activity.

The studies reviewed here revealed that "memory" in the trap movement of *Dionaea* can be explained by the stepwise accumulation of a unique bioactive metabolite. Elucidation of the full structure of the relevant bioactive agent has meant that there are real prospects now for exploring the chemical basis of this "memory" phenomenon. This would enable chemists to design molecular tools and probes that could be applied to deduce the detailed mechanisms of these intriguing physiological processes.

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