The Enzymatic Transformation of Leaf-Movement Factor in *Lespedeza cuneata* G. Don Controlled by a Biological Clock

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We have shown that enzymatic transformation of potassium lespedezate (leaf-opening substance) to potassium 4-hydroxyphenylpyruvate in a nyctinastic plant, *Lespedeza cuneata* G. Don, controls the leaf-movement of the plant. By this transformation in the evening, the balance of concentration between leaf-opening substance and leaf-closing substance is inversed through a day. A biological clock should control the leaf-movement by activation or expression of the enzyme concerned in this step.

Nyctinastic plants, such as *Mimosa pudica* L. and *Cassia mimosoides* L., are well known for the movement of their leaves according to the circadian rhythm. Schildknecht *et al.* have isolated bioactive substance causing leaf-closing movement from *Mimosa pudica* L., *Acacia karoo etc.;* [1,2] and named it turgorin. They also insisted that all nyctinastic movements are controlled by the change in the concentration of their turgorin. Recently, however, we have identified different leaf-closing factors from different nyctinastic plants. These results strongly suggested that different leaf-closing and -opening substances exist in each nyctinastic plant.[3-9] Moreover, we have discovered that the nyctinastic movement is not controlled by the concentration of the leaf-closing factor, but by the balance of concentration between leaf-closing and leaf-opening substances.[7] We considered that these bioactive substances are not accumulated intact, but it was to be metabolized in the course of time. The enzymatic transformation in this metabolism should be controlled by a biological clock. Thus, chemical studies on this transformation should give us the important clue for the chemical control of leaf-movement by a biological clock.

The fresh whole plants of *Lespedeza cuneata* G. Don collected in the daytime and at night (3.0 kg and 3.4 kg each) were immersed in methanol (20 L each) for two weeks and concentrated *in vacuo*. The concentrated aqueous extracts were partitioned with ethyl acetate. Two diluted aqueous layers showed inverse bioactivity depending on the time of collection. The extract collected in the daytime showed leaf-opening activity and another one collected at night showed leaf-closing activity. The balance of concentration between **1** and **2** (or **3**, *cis*-isomer of **2**) should be reversed in these two extracts.[7]

Table 1. Concentration of **2**, **3** and **4** in *Lespedeza cuneata* G. Don.

Time of collection	concentration (mol/L)		
	2	**3**	**4**
daytime	2.7×10^{-5}	4.1×10^{-5}	2.5×10^{-5}
night	2.2×10^{-5}	1.8×10^{-5}	1.2×10^{-4}

Water content of the plant material is calculated to be 80% of total weight.

The HPLC analysis using Develosil ODS HG-5 column confirmed this hypothesis. Good separation on the HPLC analysis was achieved when acidic mobile phase (10% MeOHaq. containing 0.1% TFA) was used. The extract collected in the daytime contained twofold as much **2** and **3** as the extract collected at night (Table 1). This twofold increase in the content of **2** and **3** is sufficient to inverse the bioactivity of the extract from our previous research on the competition experiment between **1** and **2** (or **3**).[7] Thus, **2** and **3** should be metabolized in the evening, and biosynthesized in the morning. Moreover, we discovered that the content of **2** and **3** in the plant is inversely proportional to that of potassium 4-hydroxyphenylpyruvate (**4**). **4** was effective for leaf-opening movement at 5×10^{-5} M. Because **2** (or **3**) is effective at 8×10^{-7} M, **2** (or **3**) loses its bioactivity to the extent of one-hundredth by the action of glucosidase. The extract collected at night contained fivefold as much **4** as the extract collected in the daytime.[10] This result strongly suggests that **2** is biosynthesized from **4** in the morning, and is metabolized enzymatically to **4** in the evening (Figure 1). The identification of each peak was established by the retention time confirmed by the co-injection of the authentic samples and

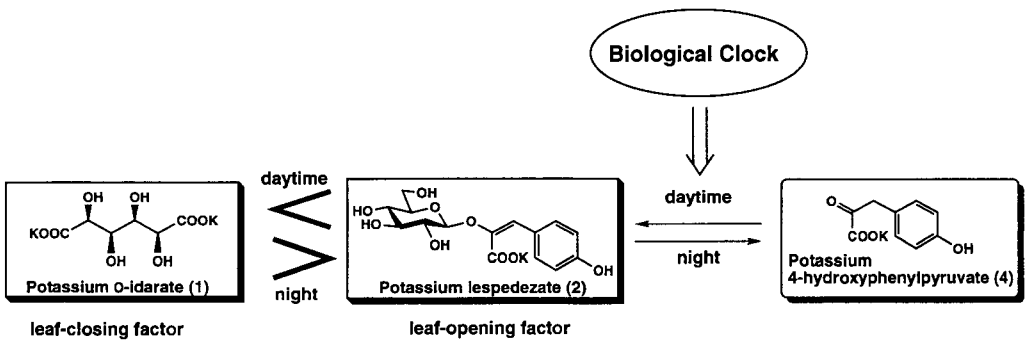

Figure 1. The chemical control of leaf-movement in *Lespedeza cuneata* G. Don.

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UV-vis spectra observed by HPLC equipped with a photodiode array detector.

This hypothesis was strongly supported by the measurement of the β-glucosidase activities of the crude enzyme prepared from plants collected in the daytime and in the evening. The crude enzyme was prepared according to the method by Watanabe *et al.*[11] We collected the leaf of *Lespedeza cuneata* G. Don. and prepared acetone powder. We used **2** and **3** as the substrate of the enzyme,[12] and quantitatively analyzed **4** produced by the treatment with the acetone powder by HPLC. The HPLC analysis was carried out under the same condition as previously described. The β-glucosidase activity was observed only in the acetone powder prepared from the plant collected in the evening (Table 2). However, the activity of it from the plant collected in the daytime was almost as low as that of the blank sample.

We have shown that enzymatic transformation of leaf-movement factor **2** and **3** in a nyctinastic plant, *Lespedeza cuneata* G. Don, controls the leaf-movement of the plant. By this transformation, the balance of concentration between **1** and **3** is inversed through a day. A biological clock should control the leaf-movement by activation or expression of the enzyme concerned in this step.

Now, there are two plausible mechanisms on the control of the balance between leaf-closing and leaf-opening substances.; 1) The biosynthesis and metabolism of both leaf-closing and leaf-opening substances are to be under the control of biological clock., 2) That of only leaf-opening substance is to be under the control of biological clock. We will further attempt to study on the metabolism of the leaf-closing substance of *Lespedeza cuneata* G. Don.

Table 2. The result of enzymatic tranformation of **2** to **4** with acetone powder prepared from the leaf of *Lespedeza cuneata* G. Don.

Time of collection	concentration (mol/L)	
	2	**4**
daytime	1.0×10^{-3}	ND
evening	1.0×10^{-3}	2.2×10^{-5}
blank	1.0×10^{-3}	ND

To 1.0 mL of 1.0 mM **2** solution in 0.1 M citrate buffer (pH 5.0), 1.0 g of acetone powder was added. Then, the reaction mixture was incubated at 37 °C for two hours. After quenching the reaction, the reaction mixture was analyzed by HPLC.

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10 The increase of **4** cannot be explained only by the hydrolysis of **2** and **3**. But, **4** is known as one of the important intermediate of many secondary metabolites. We suppose that the increase of **4** is also affected by the control of other biosyhthetic pathways. In addition, There is another possibility that potassium *O*-D-glucopyranosyl-lespedezate and -isolespedezate, which will show much lower activity as described in ref. 6, coexist together with **2** and **3**.
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12 The leaf-opening substance **2** used in the enzymatic transformation is transformed into a mixture of **2** and **3** at room temperature on the reaction (see ref. 6).