Activation of Isoflavone Biosynthesis in Excised Cotyledons of *Lupinus* Seedlings by Jasmonoids and Excess Light

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Exogenous jasmonic acid (JA) and methyl jasmonate (MJ) induced accumulation of isoflavone constituents in cotyledons prepared from imbibed seeds of white lupin (*Lupinus albus* L.). Exogenous 0.2 mM MJ enhanced the levels of 7-*O*-(6″-*O*-malonyl)glucosylgenistein and 7-*O*-glucosylgenistein in the cotyledons of etiolated seedlings that had been incubated in the dark for 48 h. Regarding isoflavone induced by excision and slicing in the cotyledons as background level, the effect of light was 2- to 3-fold higher than that of 0.2 mM MJ. Cotyledons exposed to MJ along with a 24-h light period displayed a higher level of isoflavone accumulation than that of light alone. Total molar amounts of isoflavone accumulated in the cotyledons treated with MJ under continuous light were approximately the sum of those induced by MJ alone and light alone, respectively. The additive-like effect of MJ and light on isoflavone accumulation in lupin tissues suggested the presence of two different signaling systems independently responsible for those two stimuli. Excised cotyledons from etiolated yellow lupin (*L. luteus* L. cv. Topaz) seedlings also supported this hypothesis. The cotyledons could accumulate both an isoflavone and a flavone, and MJ selectively increased some of the isoflavone constituents, whereas light enhanced the levels of both. The selective accumulation mechanism of isoflavonoids in cotyledons, in which jasmonoids are involved, clearly differed from that activated by light.

Introduction

Isoflavonoids (with a skeleton of 1,2-diphenylpropane, distinguished from other flavonoids having a 1,3-diphenylpropane skeleton) are localized in leguminous and some other plants. Lupins (*Lupinus*: Leguminosae) produce prenylated isoflavones (**5**, **6**, **7** and so on, see Figs. 1 and 2) as defensive (Harborne *et al.*, 1976; Ingham *et al.*, 1983) and other functional (Gagnon *et al.*, 1995a) secondary metabolites. Both white (*L. albus* L.) and yellow (*L. luteus* L.) lupins produce a prenylated isoflavone luteone (**5**) constitutively (Fukui *et al.*, 1973) and inducibly (Ingham and Dewick, 1980; Shibuya *et al.*, 1992) as the most predominant antifungal compounds. Healthy seedlings of white lupin, on the contrary, show a remarkably simple isoflavonoid compositions, in which large amounts of 7-*O*-glucosylgenistein (**2**) and its 6″-*O*-malonyl derivatives (**1**) are accumulated in green cotyledons (Katagiri *et al.*, 2000). We have recently found that seedlings of yellow lupin contain substantial amounts of 8-*C*-glucosylgenistein (**3**, an isoflavone) and 7-*O*-*ß*-(2-*O*-*α*-rhamnosyl)-glucosylapigenin (**4**, a flavone) in the green cotyledons (Zapesochnaya and Laman, 1977; Franski *et al.*, 1999).

When white lupin is exposed to a biotic and/or an abiotic stress, compound **5** and other antifungal isoflavones are biosynthesized (Gagnon and Ibrahim, 1997; Wojtaszek and Stobiecki, 1997) along with activation of phenylalanine ammonia-lyase (PAL) which is a key enzyme of flavonoid pathway. It is also known that light irradiation increases flavonoid contents along with PAL activation (Ward and Buzzel, 1983 and cited therein). This light-induced flavonoid accumulation is thought to

Abbreviations: JA, jasmonic acid; MeCN, acetonitrile; MJ, methyl jasmonate; PAL, phenylalanine ammonia-lyase; SD, standard deviation.

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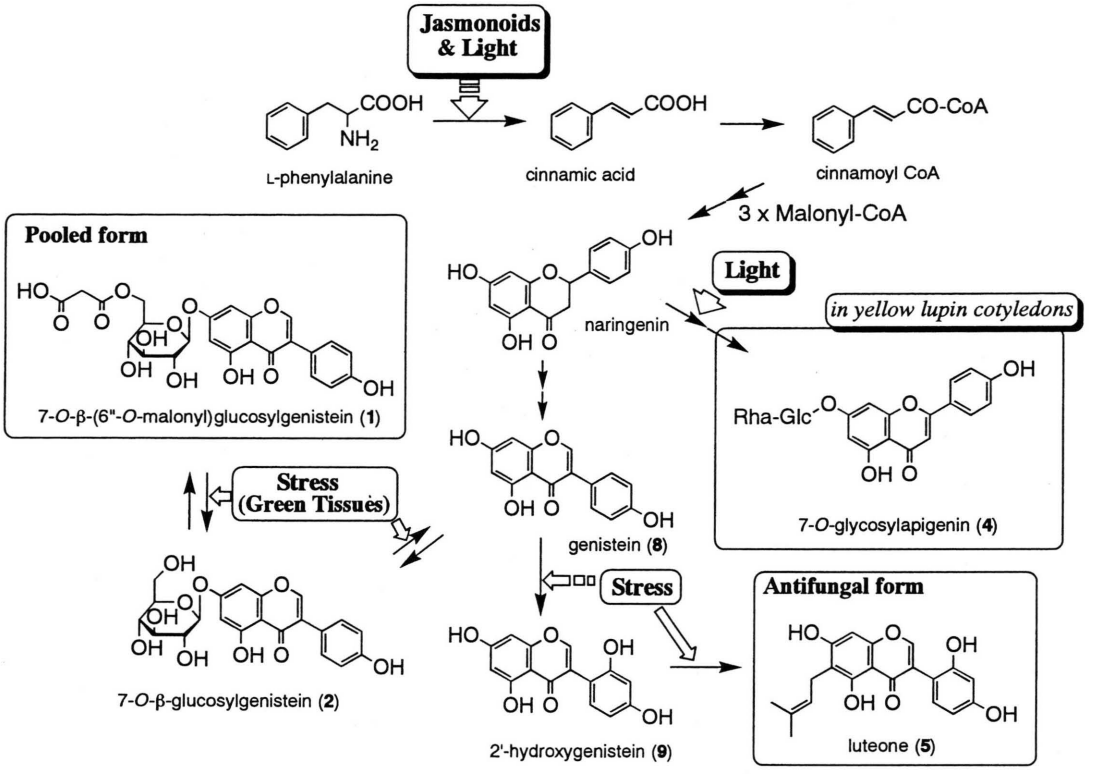


Fig. 1. Metabolic pathway of isoflavonoids in white and yellow lupin cotyledons. The flavonoids induced in cotyledons of white and yellow lupins with MJ-treatment/light and other stresses, including excision, mechanical slicing, treatment with $CuCl_2$ and fungal infection, are shown. These simple flavonone/isoflavones and their glucosides accumulated by MJ or light had no antifungal activity, but further stimulation with heavy metal and/or fungal infection led to conversion of the pooled isoflavones into major antifungal isoflavone (**5**) hydroxylated and prenylated at C-2′ and C-6 positions, respectively.

be involved in photoprotection systems of higher plants. It has also been reported that jasmonic acid (JA), an endogenous signal transducer, enhances the transcription level of PAL gene and induces flavonoids in some plants (Gundlach *et al.*, 1992; Graham and Graham, 1996; Rakwal *et al.*, 1996).

In order to know the phytochemical response of lupin seedlings in association with signal transduction, we investigated the effects of exogenous (±)-jasmonic acid (JA) and methyl jasmonate (MJ) and light radiation on isoflavonoid metabolism, particularly the quantitative responses. In this paper, we describe jasmonoid-mediated isoflavone accumulation in excised lupin cotyledons which are epigeal and autotrophic, and discuss a significance of the activation system in biosynthesis mediated by jasmonoids and light.

Materials and Methods

General

A Waters HPLC system consisting of a 600E multisolvent delivery system, a 996 photodiode-array detector, and a U6K injector were used for qualitative and quantitative analyses of the lupin isoflavonoids. The plants were grown in growth chambers, Biotron NC 220 (NK system, Osaka) at 23 °C.

Chemicals

Synthetic (±)-JA used in the preliminarily experiments was donated from Dr. S. Tamogami and Prof. O. Kodama, Faculty of Agriculture, Ibaraki University. MJ was purchased from Wako Pure Chemical Industries Co. These compounds were

previously dissolved in acetone to be 200 mM, and the acetone solution was 1×10^3 times diluted with water. A chloroaniline Schiff base-type PAL inhibitor, S-84702 was provided by Dr. T. Kato and Dr. K. Kamoshita, Sumitomo Chemical Industries Co. Flavone (2-phenylchromone) used for an internal standard in quantitative HPLC was from Tokyo Chemical Industry Co. Ltd. As the standard solution, 10 mM 2-phenylchromone in MeOH was used. 8-*C*-Glucosylgenistein(**3**) and 7-*O*-*β*-(2-*O*-*α*-rhamnosyl)-glucosylapigenin (**4**) from yellow lupin were identified by comparing the ^{1}H- and ^{13}C-NMR spectra with those reported previously (van Heerden *et al.*, 1980; Rao and Rao, 1982). Other isoflavonoids mentioned in the present paper were previously isolated and identified in our laboratory (Hashidoko *et al.*, 1986; Tahara *et al.*, 1984; 1989).

Plant material

Seeds of white lupin (*L. albus* L. cv. Kievskij Mutant) and yellow lupin (*L. luteus* L. cv. Topaz) were harvested at the experimental farm in Hokkaido University. When the cotyledons of the imbibed seeds were used, they were prepared from soaking mature seeds in air-bubbled water at 37 °C for 6 hours. When the cotyledons from seedlings were used, the seedlings were grown as follows. The mature seeds soaked in running tap water overnight were allowed to germinate in a bed of clean and moistened vermiculite in growth chambers under 0- and 16-h photoperiods under condition at 10,000 *lux* at 23 °C. Cotyledons from the imbibed seeds or the seedlings grown for set periods, were excised with a razor blade. To exclude the effect of light, the excision and following treatments of the cotyledons were performed in a darkroom under intensive green light (from a Toki green light bulb, 7 W).

Extraction of the total isoflavonoids from cotyledons and cleanup of crude extracts

The tissues sliced and treated with each test-solution were immediately homogenized in cooled MeOH or were put into a 20 ml capped vial filled with 10 ml of MeOH to stock at −20 °C. After an optional volume of standard solution was added, the tissues with the solvent were completely transferred from the vial into a 50 ml Falcon tube and homogenized by a Polytoron (model CH-6010, Kinematica) followed by centrifugation at 3,000 × *g* to give crude methanol extracts of lupin isoflavonoids as the supernatant. Each extract portion (100 μl) was diluted with 4 ml of water to be loaded on a Bond Elut C18 (reversed-phase silica gel) cartridge column (Varian) previously washed with acetone, MeCN and subsequently with water. The cartridge adsorbed the samples was first washed with 5 ml of 5% MeCN-buffer (50 mM H_3PO_4-KOH, pH 3.0) and successively 5 ml of water. Flavonoid constituents were then eluted from the cartridge with 3 ml of 86% MeCN-water. The eluates were concentrated *in vacuo* up to dryness, and the resulting residues were re-dissolved in 200 μl of 50% MeCN to give a sample solution for HPLC analyses.

Hydrolyses of isoflavone glycosides and cleanup

To quantify malonylated glucosylisoflavone, 100 μl of a crude methanolic extract was dissolved in an excess volume of 0.4 M NH_4OH and then kept at 37 °C for 90 min. The resulting solution was diluted and then subjected to the cleanup process described above. An estimated value of an isoflavone glucoside increasing after the mild alkaline hydrolysis was regarded those from the hydrolysed malonylated derivative **1**. For quantification of total isoflavone in the cotyledons, only simple isoflavone aglycons obtained by complete acid hydrolysis were analyzed. Each extract portion was taken to a 10 ml capped glass tube, and then removed the solvent *in vacuo*. The residues re-dissolved in 200 μl of EtOH were mixed with equal volume of 2 M aq. HCl, and then kept in boiling water for 1 h (Markham, 1989). The hydrolyzed mixture diluted with 4 ml of water was then charged on a conditioned Bond Elut C18 cartridge column and processed for cleanup as described above to obtain isoflavone aglycons.

High-performance liquid chromatography and quantitative analyses of isoflavonoids

Analytical conditions for the total isoflavones by a reversed-phase HPLC system were principally based on our previous method (Katagiri *et al.*, 2000). For analysis of simple isoflavones obtained by acid hydrolysis, a LiChrospher 100 RP-18 (5 μm, 125 mm × 4 mm i.d., Merck) column was used with an isocratic solvent system, 1 ml/min of

MeOH-MeCN-THF-water = 95:160:53:492. It took 15 min for quantitative analysis of one sample.

Treatment of imbibed seeds or excised cotyledons with JA and other chemicals

Imbibed five seeds of white lupin were first removed their seed coats and radicles, and then each cotyledon was sliced into five pieces. The sliced tissues were put together in a glass petri dish (60 mm i.d.), to which was added 4 ml of 1 mM JA solution. Shaking slowly at 23 °C in the dark, the tissues were pre-incubated for 24 h (Kauss *et al.*, 1992), at which point the JA solution was replaced with the same volume of a fresh 1 mM JA or 1.5 mM $CuCl_2$ solution to continue incubation for another 48 h. As the control, water with acetone was used instead of JA solution.

When the seedlings were used, green or etiolated cotyledons were excised and collected from five seedlings grown for 4 days under a 16-h photoperiodic condition or in the dark. The excised cotyledons, all cut into two pieces at the center of midrib, were bedded on a filter paper in a petri dish (60 mm i.d.). A test solution (5 ml) was then poured therein to allow the cut face wet with the solution. As a control, the excised and sliced cotyledons were treated with water containing 0.1% acetone.

Quantification of isoflavonoids in cotyledons incubated for 48-h under light with or without JA treatment

For more precise quantification, five seeds of white lupin were set as one group. Then, cotyledons of the imbibed seeds or the etiolated seedlings from one-day-old to five-day-old were respectively excised. Treatments of the excised cotyledons as described above (A; water/dark, B; MJ/dark, C; water/light, and D; MJ/light) were triplicated. Excised cotyledons of each stage accordingly involved 12 groups. The averaged weights and standard deviation (SD) of the tissues from five seeds/seedlings were as follows: zero day (from imbibed seeds); 3.53 g (SD ± 0.39), one-day-old; 3.69 g (± 0.20), 2-day-old; 3.69 g (± 0.34), 3-day-old; 3.82 g (± 0.23), 4-day-old; 3.81 g (± 0.38) and 5-day-old; 3.62 g (± 0.28). Isoflavonoids in the treated tissues incubated for 48 h were extracted, hydrolyzed and analyzed as described above.

Time course isoflavone accumulation in etiolated cotyledons treated with JA and/or light

Ten pieces of cotyledons were excised from etiolated 4-day-old seedlings, and their fresh weights were recorded. The cotyledons were cut into two pieces along midrib and then put in 5 ml water or 0.2 mM MJ solution, followed by incubation for 0, 12, 24, 36 and 48 hours in the dark or under 24-h photoperiodic conditions at 10,000 *lux*. Total amounts of the isoflavonoids in the tissues were monitored along the time course. All of the treatments were triplicated. The averaged weight and SD of the tissues from five seedlings were as follows: 0 h (without incubation); 3.79 g (± 0.31), 12 h; 3.53 g (± 0.27), 24 h; 3.86 g (± 0.31), 36 h; 3.72 g (± 0.33) and 48 h; 3.94 g (± 0.34).

Quantitative analysis of light-induced and MJ-induced flavonoids in yellow lupin cotyledons

Cotyledons excised from etiolated 4-day-old seedlings of yellow lupin were sliced and treated as did toward white lupin cotyledons. The resulting cotyledon tissues were homogenized in MeOH, and the methanolic extracts were subjected to the cleanup process, directly or after mild alkaline hydrolysis as described above. The calibration factors at 263 nm were 2.40 and 0.89 for 8-*C*-glucosylgenistein (**3**) and 7-*O*-β-(2-*O*-α-rhamnosyl)glucosylapigenin (**4**), respectively. The factors for other compounds are used with previous values (Katagiri *et al.*, 2000).

Results

The cotyledons excised from imbibed seeds and allowed incubation for 48 h accumulated isoflavones, and the isoflavonoid profiles were remarkably changed in quality and quantity with exogenous chemicals (Fig. 2). In the cotyledons sliced and then treated with 1.5 mM $CuCl_2$, total amounts of isoflavones were found nearly unchanged but isoflavone aglycons relatively increased. This indicated that constitutive genistein glycosides (**1** and **2**) were apparently hydroxylated at C- 2′ and then prenylated at C-6- or C-3′-positions followed after the hydrolysis, to accumulate modified compounds

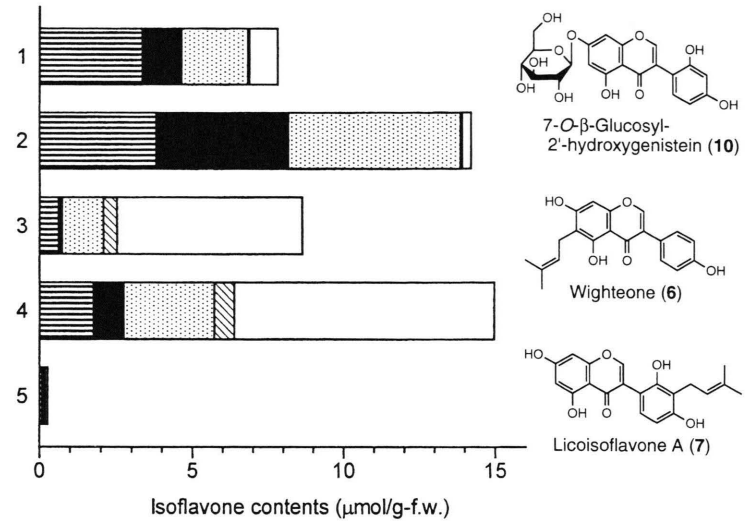


Fig. 2. Effects of exogenous JA, PAL inhibitor and $CuCl_2$ for isoflavonoid constituents in sliced cotyledons from imbibed seeds of white lupin. The excised and sliced cotyledons from five seeds soaked in bubbling water were put on a first solution for 24 h and then transferred onto a new second solution for another 48 h. The first and second solutions are 1) water and water; 2) 1 mM JA and 1 mM JA; 3) 1.5 mM $CuCl_2$ and 1.5 mM $CuCl_2$; 4) 1 mM JA and 1.5 mM $CuCl_2$; and 5) 1 mM JA containing 0.5 mM PAL-inhibitor and 1.5 mM $CuCl_2$, respectively. All of the cotyledons were incubated under 16-h photoperiodic conditions at 23 °C. After the incubation, all of the treated cotyledons were homogenized in 30 ml of 80% MeOH and then filtered to obtain methanolic filtrates. Showing of each bar is as follows, hatched; 7-*O*-(6″-*O*-malonyl)-glucosylgenistein (**1**), black solid; 7-*O*-glucosylgenistein (**2**), dotted; 7-*O*-glucosyl-2′-hydroxygenistein (**10**), diagonal down hatched; 2′-hydroxygenistein (**9**), and white solid; prenylated isoflavones (**5**, **6** and **7**). Structures of compounds **6**, **7** and **10**, all of which are not in Fig. 1, are shown here.

9, **5** and **7**, respectively. Whereas, 1 mM JA enhanced the isoflavone level, allowing the tissues to accumulate a *ca.* 2-fold-higher amount of isoflavone conjugates, in which 7-*O*-glucosylgenistein (**2**) and 7-*O*-glucosyl-2′-hydroxygenistein (**10**) were involved. JA-treatment, however, did not accumulate corresponding aglycons (**8** and **9**) and prenylated isoflavones (**5**–**7**). This stimulating effect of exogenous JA on the isoflavone biosynthesis was also observed under a co-presence of 1.5 mM $CuCl_2$, in which 2-fold-accumulations of isoflavonoids occurred in parallel with similar modification of the isoflavones caused by the $CuCl_2$-treatment alone. On the contrary, applying 0.5 mM of the phenylalanine ammonia-lyase (PAL)-inhibitor S-84702 together with 1 mM JA solution resulted in complete inhibition of the isoflavone accumulation with JA. Thus, exogenous JA certainly had an activity to induce isoflavone accumulation, in associated with an enhanced transcription level of PAL gene in the tissues (Gundlach *et al.*, 1992).

When excised cotyledons from etiolated white lupin seedlings of 4-day-old were used, 0.2 mM solutions of JA and MJ separately displayed an equivalent activity to a 2 mM concentration to enhance the total isoflavone level. Hence, 0.2 mM MJ was used for the following experiments due to an expectation that MJ is more stable than JA. Greening cotyledons of 3-day- or more-old white lupin seedlings grown under 16-h photoperiodic condition had already accumulated remarkable amounts of isoflavonoids, mainly genistein glucosides **1** and **2** (Gagnon *et al.*, 1995b). The level of concentration of total isoflavone in 15-day-old seedlings grown at 16-h photoperiod reached to *ca.* 10 μmol/g (Katagiri *et al.*, 2000). Moreover, when cotyledon parts excised from the seed that had been allowed imbibition for 6 h in the dark but not yet germinated were incubated in water under a 24-h photoperiod, the tissues also began to accumulate isoflavonoids within 48 h, similar to intact cotyledons from green seedlings. Similar response was also observed on etiolated cotyledons

excised from 4-day-old white lupin seedlings. When the latter cotyledons were treated with 0.2 mM S-84702 inhibitor under the 24-h photoperiodic condition, isoflavone accumulation caused by light was completely inhibited (unpublished data). Thus, light at a 24-h photoperiod allowed etiolated cotyledons to induce a high level of isoflavone accumulation.

Both light and treatment with exogenous jasmonoids induced accumulation of isoflavone glycosides **1** and **2** along with PAL activation. However, their physiological significance in the self-defense is likely to be different. Jasmonoids mediate signal transduction to defend local infection (Gundlach, *et al.*, 1992; Kauss, *et al.*, 1992), whilst light-induced flavonoids accumulation is thought to be involved in photoprotection of higher-plants (Reddy *et al.*, 1994 and cited therein). Accordingly, we carried out quantitative analysis of total isoflavones in the cotyledons in order to characterize each phytochemical response (Fig. 3). Etiolated cotyledons set for the experiment were either dipped in water (groups A and C) or 0.2 mM MJ (groups B and D). Groups A and B were incubated in the dark, and C and D were kept under a 24-h light period. The cotyledons were prepared from imbibed seeds and seedlings from 1-day-old to 5-day-old, respectively, because cotyledons in different stages might express different sensitivity toward exogenous stimuli, especially toward excess light.

Compared with the corresponding zero time controls (without incubation, I in Fig. 3), all samples incubated with water in the dark (A in Fig. 3) displayed increases of total isoflavones due to their physical damages by the excision and the slicing. Considering the endogenous isoflavone level in A as the background, light (C – A) enhanced the isoflavone level approximately 2- to 3-fold higher than 0.2 mM MJ (B – A) throughout all of the stages investigated. Cotyledons exposed to 0.2 mM MJ and a 24-h light period for the same time (D in Fig. 3) displayed a higher isoflavone accumulation than with light-irradiation alone (C). Accordingly, increased amounts of the isoflavone in the cotyledons exposed to MJ under 24-h photoperiodic conditions (D – A) approximated the sum of those induced by MJ alone (B – A) and by light alone (C – A). This additive-like effect of light and MJ is expressed by the equation, D = B + C – A.

MJ-treatment led to a continuous increase of the isoflavone level up to 48 h (Fig. 4). After a 36 h-incubation, the isoflavone level caused by 0.2 mM MJ in the dark was approximately 2-fold of that of cotyledons incubated in water in the dark (9.2 ± 0.3 and 4.6 ± 0.5 μmol/g, respectively). Whilst, the tissues exposed to water under a 24-h photoperiod after 36 h reached a 3-fold higher level (16.8 ± 1.7 μmol/g) vs. the cotyledons with water in the dark. Irradiation of 24-h light period led to maximal isoflavone level after 36 h, and then decreased. On the other hand, isoflavone level in the tissues exposed to 0.2 mM MJ under a 24-h photoperiod for 36 h was nearly identical with those increased by the MJ treatment in the dark plus those accumulated by irradiation of 24-h

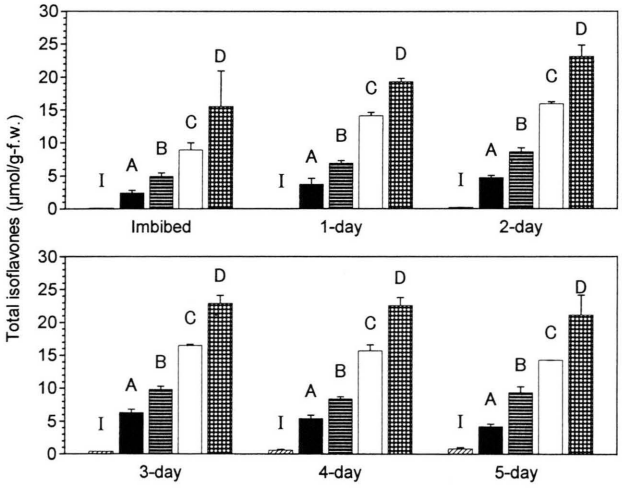


Fig. 3. Effects of exogenous JA and/or light-irradiation for total amounts of isoflavones on excised cotyledons of white lupin. The cotyledons were prepared from imbibed seeds (as zero-days-old materials) and etiolated seedlings of one to five-day-old. After slicing into two pieces the cotyledons were treated as follows: A) in water in the dark; B) in 0.2 mM MJ in the dark; C) in water under a 24-h photoperiod; and D) in 0.2 mM MJ under a 24-h photoperiod. All of the incubation was done at 23 °C for 48 h. Zero time controls without incubation (I) were also set up from imbibed seeds and the seedlings. The cotyledons after incubation were homogenized in 10 ml of MeOH and then centrifuged at 6,800 × *g* to obtain methanolic supernatant. Each experiment consisting of cotyledons from five seeds was triplicated.

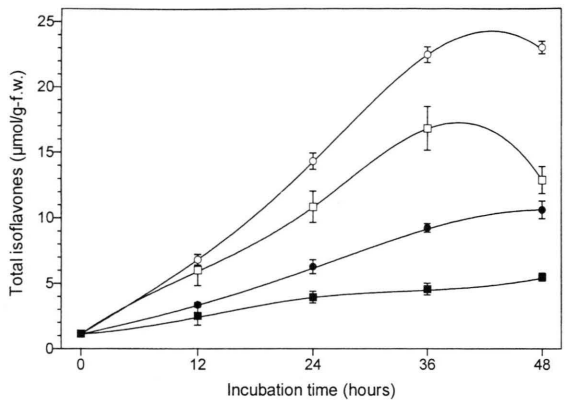


Fig. 4. Time courses of isoflavone accumulations in excised and sliced white lupin cotyledons treated with 0.2 mM MJ and/or light. Cotyledons excised from etiolated seedlings of 4-days-old and then sliced into two pieces. These were exposed to 0.2 mM MJ under a 24-h photoperiod (-○-); 0.2 mM MJ in the dark (-●-); water under a 24-h photoperiod (-□-); and water in the dark (-■-). All of the incubation was done at 23 °C. Incubation time was set up as 0, 12, 24, 36 and 48 h for each treatment, and resulting cotyledon tissues were homogenized in 10 ml of MeOH and then centrifuged at 6,800 × *g* to obtain a methanolic supernatant. Each experiment including cotyledons from five seeds was triplicated.

photoperiod in water. A simple calculation to demonstrate this additive effect (MJ + light − back ground) gave 21.4 μmol/g, a concentration almost equivalent to the practical value for the MJ-and-light obtained by our quantitative HPLC analysis (22.4 ± 0.5 μmol/g). Similar additive effects were also observed at 12 and 24 h. Isoflavone levels of the MJ-and-light treatment turned to decrease at 48 h, which likely reflects a change of the phytochemical response in the tissues under the 24-h photoperiod.

Excised cotyledons of etiolated yellow lupin seedlings treated with 0.5 mM exogenous jasmonoid showed a qualitatively distinguishable response as variation of accumulated flavonoids from those treated under 24 h light conditions. Namely, the light irradiation resulted in increasing the level of genistein 8-*C*-glucoside (**3**, an isoflavone), genistein 7-*O*-glucoside (**2**) and apigenin 7-*O*-rhamnosylglucoside (**4**, a flavone). In contrast, exogenous jasmonoid treatment led to an enhanced level of only isoflavones (**2** and **3**, in Table 1). Total molar amounts of the flavonoids, which include both flavone and isoflavone, induced by light and by jasmonoid were approximately the same.

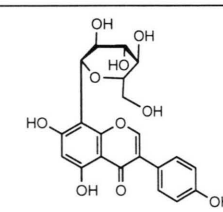
Discussion

Since mature seeds of white lupin contain no flavonoids (Katagiri *et al.*, 2000), PAL gene expression in the cotyledon tissues must be the most significant regulatory factor in *de novo* synthesis of isoflavonoids (Camm and Towers, 1973). Nevertheless etiolated cotyledons put under the 24-h

Table I. Compositional change of flavonoid and isoflavonoids in cotyledons excised from etiolated yellow lupin seedlings (μmol/g-f.w.)[a]

Constituents	0 Cont	Water[b]	MJ[b,c]	$CuCl_2$[b,c]	Light
2′-Hydroxygenistein 7-*O*-glucoside (**10**)	tr[d]	0.1±0.02	0.2±0.02	0.2±0.01	0.5±0.19
Genistein 8-*C*-glucoside (**3**)	0.2±0.01	0.3±0.02	0.6±0.04	0.3±0.04	0.4±0.04
Genistein 7-*O*-glucoside (**2**)	tr	tr	0.3±0.07	tr	0.5±0.08
2′-Hydroxygenistein (**9**)	0.1±0.10[e]	0.1±0.12[e]	0.2±0.18	0.7±0.28	0.1±0.01
Genistein (**8**)	tr	tr	0.2±0.03	0.1±0.03	0.1±0.02
Prenylated isoflavones (**5, 6** and **7**)	tr	1.0±0.15	0.8±0.12	3.3±0.12	1.2±0.11
Total isoflavones	0.4±0.17	1.7±0.29	2.2±0.25	4.7±0.25	2.7±0.39
Apigenin 7-*O*-rhamnosylglucoside (**4**)[f]	0.6±0.05	0.7±0.12	0.8±0.10	0.7±0.08	1.7±0.09

a) All values were given by average with their standard deviation (*n* 3–6).
b) The tissues treated with water, MJ and $CuCl_2$ were incubated in the dark at 23 °C, respectively.
c) MJ and $CuCl_2$ solution used are 0.5 mM and 1 mM, respectively.
d) tr = less than 0.1 μmol/g-f.w.
e) Because compound **2** was detected only in a part of the samples, each deviation became larger value than the average.
f) Identified flavonoid is apigenin 7-*O*-rhamnosylglucoside alone. Other peaks of flavones were not observed.



8-*C*-β-glucosylgenistein (**3**)

photoperiodic condition for 36 h accumulated a maximal level of light-induced isoflavone, parallel treatment with MJ resulted in further accumulation of isoflavones in the cotyledons (Fig. 3). This fact indicated an additive effect of jasmonoid alone and light alone to induce isoflavone accumulation, respectively.

Increase of a transcription level of PAL gene by light and following accumulation of flavonoids or phenylpropanoids has also been reported in seedlings of *Glycine max* (Graham and Graham, 1996) and some other plants (Reddy *et al.*, 1994; Frohnmeyer *et al.*, 1997) in association with their self-defensive response against photodamage as observed in cotyledons of *Lupinus* seedlings. In fact, Cramer *et al.* (1989) have discovered the presence of a small gene family encoding PAL in genomic library from cultured cells of a kidney bean. Moreover, Estabrook and Sengupta-Gopalan (1991) have practically demonstrated that soybean roots expressed a symbiosis-specific PAL-gene during early nodulation stages but this PAL gene was never activated by other biotic or abiotic stresses that generally enhanced the level of PAL gene expression in other parts of the tissues. We hence speculate the presence of independent systems in white lupin to stimulate *de novo* synthesis of PAL, which is compatible with a hypothesis of selective expression of signal-specific PAL genes. It is necessary to characterize molecular species of PAL genes that are activated by jasmonoid and light, respectively.

In excised cotyledons of etiolated yellow lupin seedlings, on the contrary, total molar amounts of the flavonoids induced by light and by jasmonoid were almost the same levels in quantity, indicating that both stimuli enhanced approximately the same level of PAL. However, phytochemical responses of yellow lupin to the stimuli were qualitatively significance, compared with white lupin that is more evident in the quantity (Table I). Characteristic accumulation of flavone in yellow lupin by light is likely a typical photoprotection against excess photo-irradiation, which have also been reported in rice and other plants (Reddy *et al.*, 1994 and cited therein). Whereas, increase of isoflavone glucosides (**2** and **3**) by jasmonoid is involved in chemical defense against phytopathogens (Gundlach *et al.*, 1992). Chappell and Hahlbrock (1984) have examined in cell suspension culture of parsley (*Petroselinum hortense*) expression of PAL gene that was equally induced by UV irradiation and by treatment with fungal elicitor to yield totally different products by these stimuli. Phytochemical response of yellow lupin toward light and MJ is quite similar with that of parsley cell culture. In isoflavone biosynthesis of yellow lupin, key regulatory enzyme is probably isoflavone synthase (Fig. 2) that is selectively activated by jasmonoids.

In flavonoid pathway of *Lupinus* plants, physiological significance of the different key enzymes between white and yellow lupin is not clearly known yet, and further studies of their mechanisms to regulate key enzymes of isoflavone biosynthesis are awaited. Our approach to monitor the metabolic dynamisms of isoflavonoid directly on HPLC chromatograms is promised in the study of signal transduction using some legume plants.

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