

Chapter 7.6

ELICITATION OF ISOFLAVAN PHYTOALEXINS

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Lotus japonicus makes a good model legume species for the study of induced isoflavonoid phytoalexin biosynthesis. As part of its defence response against pathogens, Lotus leaves have been reported to produce a number of isoflavans, a specific class of isoflavonoids. Model systems for Lotus elicitation have been set up using the thiol reagent reduced glutathione as elicitor and the accumulation of phytoalexins has been monitored by analysis of vestitol, which is excreted into the media. In this article, we present two protocols for the elicitation of Lotus leaves and three methods for the analysis of the induced isoflavan vestitol.

INTRODUCTION

In plants, the flavonoid pathway has been well-characterised and involves numerous branches leading to the accumulation of a wide range of end products with a spectrum of functions: colourful anthocyanins, antimicrobial isoflavonoids, UV protecting flavonols, deterrent condensed tannins (Koes *et al.*, 1994, Aoki *et al.*, 2000; Iwashina, 2000). They have also been the object of significant interest as many flavonoid compounds exhibit commercially relevant properties (Di Carlo *et al.*, 1999; Humphreys and Chapple, 2000).

In legumes, flavonoids also play an important role in the interactions between plants and micro-organisms (Shirley, 1996; Harborne and Williams, 2000). Flavones and chalcones, for example, are important inducers in the mechanisms of nodulation by symbiotic bacteria, a process almost specifically confined to legumes (Stafford, 1997). Isoflavonoids, which represent a different branch of the flavonoid pathway, are characterised by a migration of the phenyl ring and occur principally in legumes. They are involved in the defence response of plants against pathogens (phytoalexins) (Dixon *et al.*, 1995; Kuc, 1995). Recently, numerous articles have

been published describing the growing health-promoting potentials of these molecules (Dixon and Steele, 1999; Dixon *et al.*, 2002).

Phytoalexins from the Leguminosae have already been described and characterised from a range of legume plants (Ingham, 1982). Model experiment systems have also been developed where the defence response is generated by elicitors of biotic or abiotic origin such as cell wall preparations of pathogens, heavy metals or thiol reagents (Wingate *et al.*, 1988; Edwards *et al.*, 1991; Robbins *et al.*, 1995).

In *Lotus* species, the main phytoalexin biosynthesised is vestitol, which belong to the class of isoflavans (Figure 1). Vestitol has been described in *Lotus* seedlings after elicitation with a thiol reagent, reduced glutathione (Shimada *et al.*, 2000) while vestitol, and its methylated/demethylated versions sativan and demethylvestitol, were observed in *L. corniculatus* leaves and hairy root cultures (Bonde *et al.*, 1973; Robbins *et al.*, 1991). They have also been reported to be induced in *L. corniculatus*, *L. uliginosus*, *L. hipidus*, *L. edulis* and *L. angustissimus* leaves following treatment with fungal pathogens (Ingham, 1977; Ingham and Dewick, 1979; Ingham and Dewick, 1980).

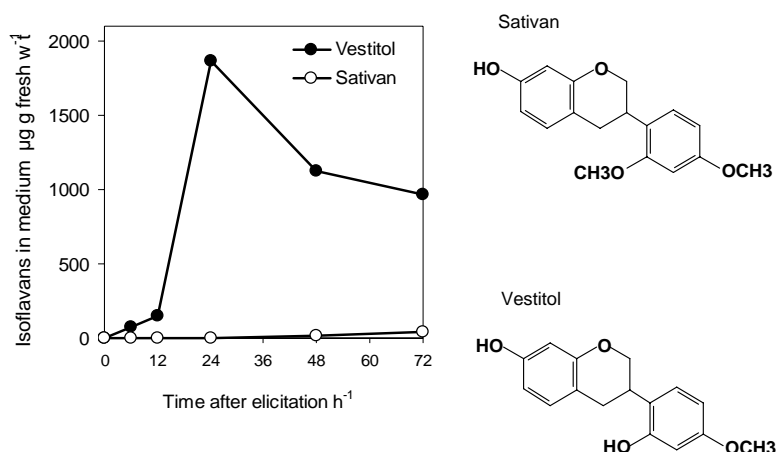


Figure 1. Kinetics of glutathione induced vestitol and sativan accumulation in the incubation medium following elicitation of *Lotus japonicus* leaves.

Glutathione elicitation of *Lotus* is a good model system to study isoflavonoid phytoalexin biosynthesis and its regulation because of the existence of a collection of mutants (Perry *et al.*, 2003), an EST database (www.kazusa.or.jp/en/plant/lotus/EST/, 21/09/03) and the amenability of *Lotus* to transformation (Handberg and Stougaard, 1992; Lombardi *et al.*, 2003). It is also a good plant model for genetic manipulation of legume flavonoid genes (Weisshaar and Jenkins, 1998; Forkmann and Martens, 2001).

This article describes two protocols for elicitation of *Lotus* leaves and three methods of analysis of vestitol and sativan: High Pressure Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC) and chemometric analysis of FT-IR spectra. The first protocol describes the elicitation of *Lotus* leaves with reduced glutathione

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and the quantification of vestitol and sativan by HPLC with an example of the kinetics of phytoalexin accumulation. The second protocol describes a high throughput method allowing the qualitative analysis of phytoalexin biosynthesis in a large number of plants used as part of a screening program for phytoalexin mutants.

PROCEDURES

Material

Plants are mature, non-flowering greenhouse grown *Lotus cv* GIFU, although similar results have also been obtained with seedling leaves. However, seedling roots and stems have so far failed to elicit isoflavans following glutathione elicitation. Reduced glutathione (Sigma G-4251) is prepared as a 100mM stock solution, filter sterilised and then used immediately. SDS is used as a 10% stock at a final concentration of 0.05% and is necessary to act as a wetting agent.

Kinetics of elicitation of leaves

Elicitation

Two grams of trifoliolate leaves are excised from each plant and collected in a glass dish containing sterile water. Three 0.5g leaf aliquots are randomly sampled and placed into three 250 ml sterile Erlenmeyer flasks containing 50 ml of 10 mM glutathione and 0.05% SDS. The flasks are incubated at 25°C in room light and shaken at ca 150 rpm for up to 72 h.

Extraction

Following incubation, leaf tissue is removed and briefly rinsed and the medium samples passed through a glass microfibre filter. The filtered medium is loaded onto activated Sep-Pak C₁₈ cartridges (Waters Ass) and eluted drop-wise. The column is then washed with 2 ml distilled water and the flavonoids eluted with 4 ml 100% methanol into evaporation-proof vials, which are stored at 4°C until analysis. For HPLC or TLC, 400 µl of each extract is taken to dryness in a centrifugal vacuum evaporator (Jouan RC 10.22).

High through put screen for vestitol induction in leaves

Elicitation

For high throughput screening, 100 to 200 milligrams of trifoliolate leaves are placed in 1.8 ml of distilled water in wells of a 5x5 Repli-dishes (Sterilin 103). 200 µl of glutathione stock solution and 10 µl of stock SDS solution are added to each well. The plates are sealed with parafilm and incubated with shaking at ca 80 rpm in the dark at 25°C for 24 h.

Extraction

The leaves are then removed and allowed to drain and 1 ml of a 20 mg/ml suspension of Waters C₁₈ preparative resin is added (Waters WAT020594), this resin had been pre-activated with 100% methanol and diluted to 5% methanol. After 30 min incubation, the medium is separated from the resin using a 96 well vacuum filtration unit. Alternatively, the medium is separated from the resin using a small diameter suction pipette leaving the resin in the plate wells. Isoflavans are eluted by adding 200 µl of 100% methanol to each well and gently mixing. These extracts are then transferred to a 96 well plate and allowed to dry. A second elution is performed with 200 µl 100% methanol and pooled with the first extract.

Analysis methods

High Pressure Liquid Chromatography (HPLC)

Aliquots of the extracts are dried and resuspended in 25 µl 100% methanol. They are then separated on a Waters reverse phase µ-Nova-Pak C₁₈ (8x10) column in a Radial Compression Module using a Waters Millennium 32[®] system.

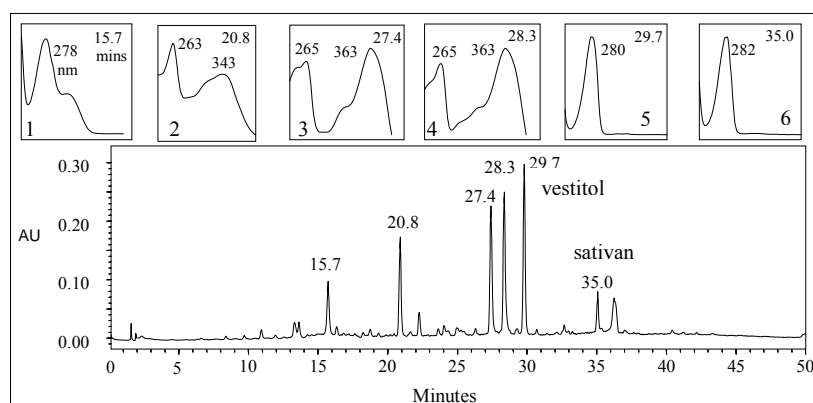


Figure 2. HPLC of medium from glutathione-elicited *Lotus japonicus* leaves.

Elution from the column is carried out with a linear gradient from 0 to 100% methanol in 50 min. The second solvent is 5% acetic acid and the flow rate 2 ml/min. The elution is monitored by a programmable 996 PDA detector (Waters) at 280 nm (isoflavans have a typical λ_{\max} at 280 nm, Figure 2) Data is collected from 240 to 400 nm and analysed with Millennium software (Waters, Milford, MA). Vestitol retention time is *ca* 29 min while sativan is eluted after *ca* 35 min (Figure 2).

Chemometric analysis with FT-IR

Extracts are resuspended in 20 µl 100% methanol and loaded onto 96 well aluminium plates that are dried at 60°C for 30 mins. Plates are scanned in reflection

mode from 500 to 4000 cm^{-1} on an Equinox 55 FT-IR spectrometer (Bruker Optics Ltd) using a HTS XT microplate attachment and data analysed using OPUS software. Vestitol is quantified against a standard curve obtained with different quantities of vestitol standard spiked into negative control samples following integration of the C-H band (Figure 3).

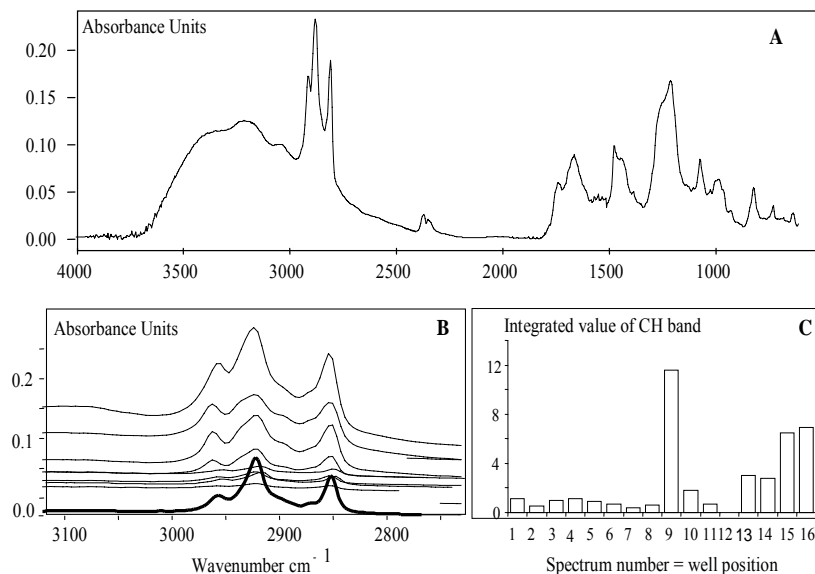


Figure 3. High throughput screening of medium from elicited Lotus leaves for vestitol using FT-IR spectroscopy. (A) FT-IR spectrum of vestitol. (B) C-H Stretching range, reference spectra vestitol (bold). (C) Integrated value of C-H band, wells 1-11 20 μl dried medium samples, wells 13-16 = 2 or 5 μg vestitol as a standard.

Thin Layer Chromatography (TLC)

TLC is carried out on 0.25mm Silica gel G plates (Macherey-Nagel) developed in CHCl_3 :MeOH (15:1). Isoflavans are visualised by spraying with Gibbs reagent (0.4% dichloroquinone 4, chloroimide in methanol (SIGMA D-6511) followed by 20% Na_2CO_3) or with 1% diazotised p-nitroaniline. Vestitol spots immediately turn blue at rf 0.2-0.3 with Gibbs reagent. If only vestitol is of interest, a higher ratio of methanol (CHCl_3 : MeOH (50:6)) is utilised to increase its rf value. Other isoflavans develop slowly with a blue or purple colour. Sativan is not visible with Gibbs reagent but appears yellow with diazotised p-nitroaniline at an rf value of 0.6-0.7.

DISCUSSION

Leaves of Lotus were elicited in order to identify and quantify the isoflavan phytoalexins biosynthesised and to investigate the kinetic characteristics of the response in this tissue (Figure 1). Analysis of the elicitation medium was sufficient

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as the isoflavans excreted into the medium accounted for more than 90% of the total isoflavan content (data not shown). The main isoflavan identified was vestitol, with maximum accumulation after 24 h. Other isoflavans were also identified: sativan was detected after 48 h incubation and its presence coincides with a decrease in vestitol concentration. It is probable that sativan, (methylated vestitol - Figure 1), was synthesised from vestitol. Smaller amounts of other isoflavans were also detected after 72 h, which have yet to be identified. Other classes of flavonoids were also observed on the HPLC chromatogram. Lotus leaves contain constitutively high concentration of kaempferol and quercetin glycosides, which are also lost from the leaves on treatment with glutathione and SDS (see spectra 2 to 4 in Figure 2).

This model system could be utilised for the identification (and/or cloning) of the flavonoid genes involved in defence response as reported by Shimada (Shimada, et al., 2000). It would also allow the investigation of the control mechanisms that direct flux toward the synthesis of specific classes of flavonoids. In particular, several genes from this pathway are present in plants as gene families: phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase. It has been proposed that individual members of these gene families are specifically involved in the biosynthesis of some flavonoids. By identifying which gene family members are specific to the defence response; it might be possible to genetically engineer new plants where the concentration of flavonoids of interest would be modulated. The second protocol describes a method to rapidly investigate the phytoalexin induction of a high number of plants and would be ideal for the screening of mutants or transgenics with modified phytoalexin responses (Figure 3).

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REFERENCES

- Aoki T, Akashi T, and Ayabe S. (2000) **Flavonoids of leguminous plants: Structure, biological activity, and biosynthesis.** *Journal of Plant Research* 113, 475-488.
- Bonde MR, Millar RL, and Ingham JL (1973) **Induction and identification of sativan and vestitol as two phytoalexins from *Lotus corniculatus*.** *Phytochemistry* 12, 2957-2959.
- Di Carlo G, Mascolo N, Izzo AA, and Capasso F. (1999) **Flavonoids: Old and new aspects of a class of natural therapeutic drugs.** *Life Sciences* 65, 337-353.
- Dixon RA, Harrison MJ, and Paiva NL (1995) **The isoflavonoid phytoalexin pathway - from enzymes to genes to transcription factors.** *Physiologia Plantarum* 93, 385-392.
- Dixon RA and Steele CL (1999) **Flavonoids and isoflavonoids - a gold mine for metabolic engineering.** *Trends in Plant Science* 4, 394-400.
- Dixon RA, Achnine L, Kota P, Liu CJ, Reddy MSS, and Wang LJ. (2002) **The phenylpropanoid pathway and plant defence - a genomics perspective.** *Molecular Plant Pathology* 3, 371-390.
- Edwards R, Blount JW, and Dixon RA. (1991) **Glutathione and elicitation of the phytoalexin response in legume cell-cultures.** *Planta* 184, 403-409.

A.J. Márquez (Editorial Director). 2005. *Lotus japonicus* Handbook. pp. 355-362.
<http://www.springer.com/life+sci/plant+sciences/book/978-1-4020-3734-4>

- Forkmann G, and Martens S. (2001) **Metabolic engineering and applications of flavonoids**. *Current Opinion in Biotechnology* 12,155-160.
- Handberg K, and Stougaard J. (1992) ***Lotus japonicus*, an autogamous, diploid legume species for classical and molecular-genetics**. *The Plant Journal* 2, 487-496.
- Harborne JB, and Williams CA. (2000) **Advances in flavonoid research since 1992**. *Phytochemistry* 55, 481-504.
- Humphreys JM, and Chapple C. (2000) **Molecular 'pharming' with plant P450s**. *Trends in Plant Science* 5, 271-272.
- Ingham JL (1977) **Isoflavan phytoalexins from *Anthyllis*, *Lotus* and *Tetragonolobus***. *Phytochemistry* 16, 1279-1282.
- Ingham JL, and Dewick PM. (1979) **A new isoflavan phytoalexin from leaflets of *Lotus hipidus***. *Phytochemistry* 18, 1711-1714.
- Ingham JL, and Dewick PM. (1980) **Isolation of a new isoflavan phytoalexin from 2 *Lotus* species**. *Phytochemistry* 19, 2799-2800.
- Ingham JL (1982) **Phytoalexins of the Leguminosae** In: *Phytoalexins*, (Bailey JA, and Mansfield, JW Ed.). Glasgow: Blackie. pp. 21-80.
- Iwashina T. (2000) **The structure and distribution of the flavonoids in plants**. *Journal of Plant Research* 113, 287-299.
- Koes RE, Quattrocchio F, and Mol JNM. (1994) **The flavonoid biosynthetic-pathway in plants - Function and evolution**. *Bioessays* 16, 123-132.
- Kuc J. (1995) **Phytoalexins, stress metabolism, and disease resistance in plants**. *Annual Review of Phytopathology* 33, 275-297.
- Lombardi P, Ercolano E, El Alaoui H, and Chiurazzi M. (2003) **A new transformation-regeneration procedure in the model legume *Lotus japonicus*: root explants as a source of large numbers of cells susceptible to *Agrobacterium*-mediated transformation**. *Plant Cell Reports* 21, 771-777.
- Perry JA, Wang TL, Welham TJ, Gardner S, Pike JM, Yoshida S, and Parniske M. (2003) **A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume *Lotus japonicus***. *Plant Physiology* 131, 866-871.
- Robbins MP, Hartnoll J, and Morris P. (1991) **Phenylpropanoid defense responses in transgenic *Lotus corniculatus* .1. Glutathione elicitation of isoflavan phytoalexins in transformed root cultures**. *Plant Cell Reports* 10, 59-62.
- Robbins MP, Thomas B, and Morris P. (1995) **Phenylpropanoid defense responses in transgenic *Lotus corniculatus* .2. Modelling plant defence responses in transgenic root cultures using thiol and carbohydrate elicitors**. *Journal of Experimental Botany* 46, 513-524.
- Shimada N, Akashi T, Aoki T, and Ayabe S. (2000) **Induction of isoflavonoid pathway in the model legume *Lotus japonicus*: molecular characterization of enzymes involved in phytoalexin biosynthesis**. *Plant Science* 160, 37-47.
- Shirley BW. (1996) **Flavonoid biosynthesis: 'New' functions for an 'old' pathway**. *Trends Plant Science* 1, 377-382.
- Stafford HA. (1997) **Roles of flavonoids in symbiotic and defence functions in legume roots**. *Botanical Reviews* 63, 27-39.
- Weisshaar B and Jenkins GI. (1998) **Phenylpropanoid biosynthesis and its regulation**. *Current Opinion in Plant Biology* 1, 251-257.
- Wingate VPM, Lawton MA, and Lamb CJ. (1988) **Glutathione causes a massive and selective induction of plant defence genes**. *Plant Physiology* 87, 206-210.

A.J. Márquez (Editorial Director). 2005. *Lotus japonicus* Handbook. pp. 355-362.
<http://www.springer.com/life+sci/plant+sciences/book/978-1-4020-3734-4>