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# Phenylpropanoid defence responses in transgenic *Lotus corniculatus* 1. Glutathione elicitation of isoflavan phytoalexins in transformed root cultures

**M. P. Robbins, J. Hartnoll, and P. Morris**

Cell Manipulation Group, Institute of Grassland and Environmental Research, WPBS Plas Gogerddan, Aberystwyth, Dyfed, SY23 3EB, UK

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**Summary.** When *Agrobacterium rhizogenes* transformed root cultures of *Lotus corniculatus* were treated with glutathione, isoflavan phytoalexins accumulated in both tissue and culture medium. This accumulation of phytoalexins was preceded by a transient increase in the activity of phenylalanine ammonia lyase (PAL). Elicitation of PAL occurred throughout the growth curve of *Lotus* 'hairy roots' and in different sectors of transformed root material.

## Introduction

Legume species characteristically accumulate phenylpropanoid phytoalexins under conditions of biological stress such as pathogen attack, wounding etc. A number of groups have used legume callus and cell suspension culture systems as models to study mechanisms controlling phytoalexin biosynthesis. A variety of species have been studied including soybean (Ebel et al. 1984), chickpea (Barz et al. 1989), French bean (Robbins et al. 1985), alfalfa (Dalkin et al. 1989) and white clover (Gustine 1981).

We have chosen to study *Agrobacterium rhizogenes* transformed root cultures of legumes and were interested to see whether we could elicit phytoalexin biosynthesis in these organised tissue culture systems. We have initiated this work with *Lotus corniculatus* which is a species particularly amenable to tissue culture and plant transformation protocols (Webb et al. 1990). Initial work has used glutathione (GSH) as an abiotic elicitor. This compound has been reported to be a particularly potent inducer of plant defence genes in cell suspension cultures of bean (Wingate et al. 1988).

We report the characterisation of PAL induction with GSH in transformed *Lotus* root cultures and the identification of a number of inducible isoflavan phytoalexins.

## Materials and Methods

*Source and characterisation of* Lotus *root cultures.* All experiments were carried out using *Lotus* line E33A. This line was derived from a single transformed root tip of *L. corniculatus* cv. Leo transformed with *Agrobacterium rhizogenes* C58C1 harbouring pRi 15834. Genetic characterisation and growth kinetics of this line have been recently reported (Morris and Robbins 1991).

*Culture of* Lotus *root cultures.* 0.3 g of *Lotus* root tips were routinely subcultured into 50 ml of B5 basal medium supplemented with 3% sucrose in 250 ml flasks. Flasks were incubated on a rotary shaker at 133 rpm in the dark at 25°C. Experiments were routinely carried out with 12-day root cultures. This time point corresponded to mid-growth phase with a typical cell mass of ca. 2.5 g fresh weight/flask.

*Elicitation of* Lotus *root cultures.* Glutathione (Sigma Chemical Company) was prepared as a 100 mM stock, filter sterilised and then used immediately. Appropriate volumes of stock solution were added directly to flasks containing root cultures under sterile conditions. After elicitation, tissues were filtered, frozen in liquid nitrogen and stored at -70°C. Medium of elicited flasks was filtered through 2 layers of Miracloth and then immediately applied to a Waters C18 Sep-Pak cartridge.

*Elicitation of* Lotus *leaf material.* 0.2 g samples of excised leaflets were harvested from greenhouse-grown *Lotus* plants. Leaflets were placed in 9 cm Petri dishes containing 20 ml of sterile water and an appropriate volume of 100 mM GSH. In addition 0.001% SDS was included in the incubation medium as this treatment successfully optimises PAL elicitation of whole plant material with GSH (Robbins and Hartnoll, unpublished observations). Samples were incubated at 25°C in the light, harvested by filtration, frozen in liquid nitrogen and then stored at -20°C.

*Assay of phenylalanine ammonia lyase.* Root tissue was extracted with 4 volumes of 50 mM Tris pH 8.5, 14.4 mM 2-Mercaptoethanol in the presence of 5% Dowex 1 X 2, 5% PVP. Recent work in the laboratory has indicated that the inclusion of Dowex and PVP as tannin protectants permits the convenient and rapid assay of PAL from *Lotus* root cultures (Robbins, unpublished data). Assays were carried out at 40°C for one hour as described by Edwards and Kessman (1991). Assays were linear with up to 200 µg of protein extract. Protein determinations were carried out according to the method of Bradford (1976). Assays were carried out in duplicate and mean values calculated in µkat/kg, with assay error estimated as ± 10%.

*Analysis of phytoalexins.* Medium extracts from root cultures were applied to activated Waters C18 Sep-Pak cartridges. After washing the

*Offprint requests to:* M. P. Robbins

cartridges with 10 ml of distilled water, medium extracts were eluted with 2 ml of 100% MeOH and stored at 4°C in evaporation-proof vials. Tissue samples (1 g FW) were extracted four times with 10 ml quantities of 95% MeOH in a pestle and mortar, filtered and then concentrated on a rotary evaporator. 10 ml of distilled water was added to the concentrate and then samples were loaded onto activated Sep-Pak cartridges, washed with 4 ml of distilled water and eluted dropwise with 2 ml of 100% MeOH and stored at 4°C as previously described.

Extracts were subjected to both TLC and HPLC. TLC was carried out on 0.25 mm Silica gel G plates (Macherey-Nagel) developed in $CHCl_3$: MeOH (50:2) and isoflavans visualised with Gibbs reagent (0.4% 2,6,dichloroquinone 4,chloroimide in MeOH, followed by 20% $Na_2CO_3$) and with diazotized p nitroanaline reagent. The measurement of sativan and vestitol was performed by HPLC on a μBondapak C18 (0.4 x 10 cm) column in a Radial Compression Module (Waters Ass.), isocratically at 2 ml/min with MeOH:HOAc (5%) (60:40) at 280 nm. Vestitol (5.4 mins) and sativan (11.5 mins) were quantified against authentic standards obtained from Plantech (UK) based on extinction coefficients for sativan of loge = 3.62 at 284 nm and for vestitol loge = 3.63 at 281 nm (Bonde et al. 1973). Vestitol and sativan were confirmed by co-TLC and co-HPLC and by comparison of UV spectra (± NaMeO) of fractions collected from semi-preparative HPLC.

## Results

### *Optimal GSH concentration for PAL elicitation*

Transformed *Lotus* root cultures were treated with different amounts of GSH ranging in final concentrations between 0 and 10 mM (Fig. 1). Induced PAL activities were compared with control *Lotus* leaves elicited with GSH over the same concentration range, 6 hours after elicitation. PAL activities in root cultures increased approximately three-fold with GSH treatment peaking at between 2 and 4 mM but with higher concentrations of GSH also resulting in enhanced extractable PAL activities. In contrast, PAL was induced over four-fold in GSH treated *Lotus* leaves with an optimum at approximately 1 mM with higher concentrations proving relatively ineffective with regard to PAL elicitation. In view of these observations, 2 mM GSH was selected as a convenient elicitor concentration for the elicitation of PAL in *Lotus* root cultures.

### *Time course for PAL elicitation*

Duplicate *Lotus* root cultures were treated either with sterile water or 2 mM GSH. Extractable PAL activities were determined at times up until 24 hours post-elicitation (Fig. 2). Control PAL activities showed little change throughout the period of the experiment whereas PAL was transiently induced in elicitor-treated roots. PAL activities peaked 8 hours after the addition of elicitor after a lag phase of approximately 3 hours. After 24 hours, PAL activities in elicitor-treated roots had returned to control values. Interestingly, although basal PAL activities were relatively high (ca. 50 μkat/kg) in *Lotus* root cultures, elicited values for PAL were maximally 150 μkat/kg above corresponding control levels.

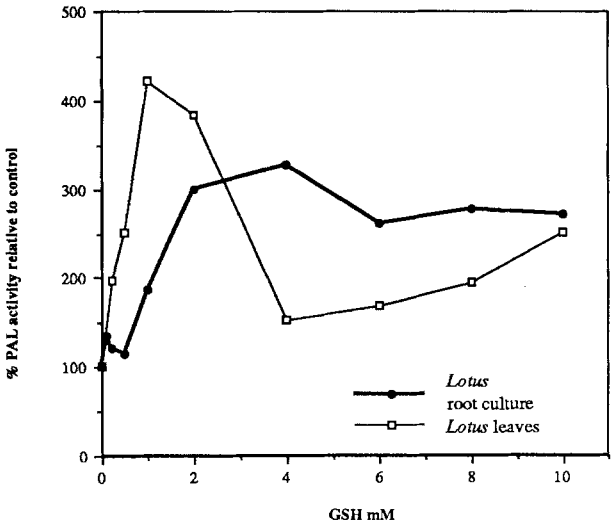


**Fig 1.** Dose response curve for PAL elicitation with GSH in *Lotus* root cultures. Control specific activities were 52 μkat/kg (*Lotus* root cultures), 31 μkat/kg (*Lotus* leaves). Values calculated from means of duplicate treatments.

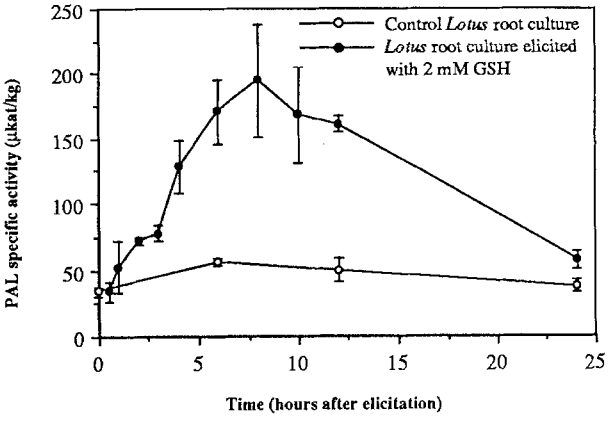


**Fig 2.** Time course for PAL elicitation of root cultures with GSH. Values represent mean ± sem from duplicate flasks.

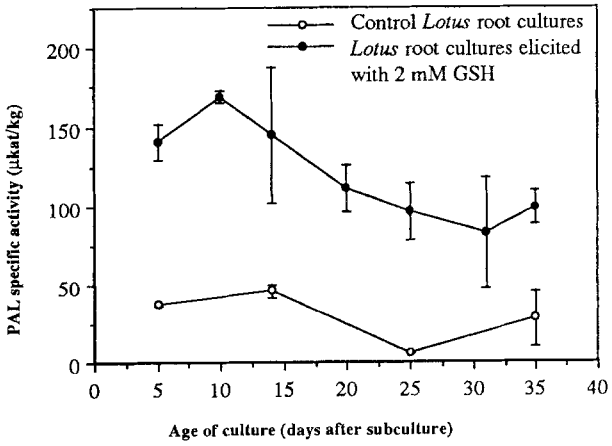


**Fig. 3.** Effect of age of culture on PAL elicitation with GSH. PAL determinations carried out 6 hours after elicitation. Values represent mean ± s.e.m. from duplicate flasks.

*Effect of culture age on elicitation*

Previous work (Morris and Robbins 1991) has shown that this culture line exhibits reproducible growth kinetics up to 40 days after subculture. In order to study the effect of age of culture on elicitation, duplicate flasks were elicited at 5 day intervals between day 5 and day 35 following subculture and PAL activities determined 6 hours after elicitation (Fig. 3). Control values were low and at no time point exceeded 50 µkat/kg. Mean elicited values were in every case higher than corresponding control values and generally showed at least three-fold PAL elicitation irrespective of age of culture. Therefore it appears that elicitation of PAL with GSH is independent of culture age in this system.

*Elicitation in different sectors of* Lotus *root*

In contrast to callus suspension cultures, transformed root cultures grow as an organised tissue mass. In addition, during culture in flasks, we have found that *Lotus* tissue exists in a distinct age profile with older tissue in the centre of the flask and younger tissue including root tips at the edge of the culture flask. This arrangement of plant material has permitted us to dissect out individual transformed *Lotus* roots (Fig. 4a).

Therefore we decided to study PAL elicitation in three sectors of differing ages of *Lotus* root. Flasks were elicited with either 0, 2 or 10 mM GSH and roots were dissected 6 hours after elicitation. Three root sectors were isolated, root tip (0-4 days old), intermediate root (approx. 6-10 days old) and old root tissue (12+ days old). Figure 4b shows that basal PAL activities declined with age of tissue. However, treatment with 2 mM GSH resulted in PAL elicitation in all three sectors of root with increases in the order of 100 µkat/kg. It is striking, however, that a higher (10 mM) concentration of GSH appeared to selectively elicit older tissue sectors.

*Induction of phenylpropanoid phytoalexins*

Two major phenylpropanoid phytoalexins were accumulated 24 hours after elicitation of root cultures with glutathione (Table 1). Vestitol (7,2'-dihydroxy-4'methoxyisoflavan) was the main compound detected, but low levels of sativan (7-hydroxy-2',4'dimethoxyisoflavan) were also detectable. Accumulation of vestitol and sativan was concentration dependent with higher levels accumulating with 10 mM than with 2 mM GSH, possibly reflecting the differential sensitivity of old and young roots to GSH (Fig. 4). Interestingly, between 60 and 70% of vestitol was found in culture medium and maximal yields 24 hours after elicitation amounted to 675 µg/flask.

## Discussion

To our knowledge, this is one of the first reports of phytoalexin defence responses in a transgenic plant system; GSH being

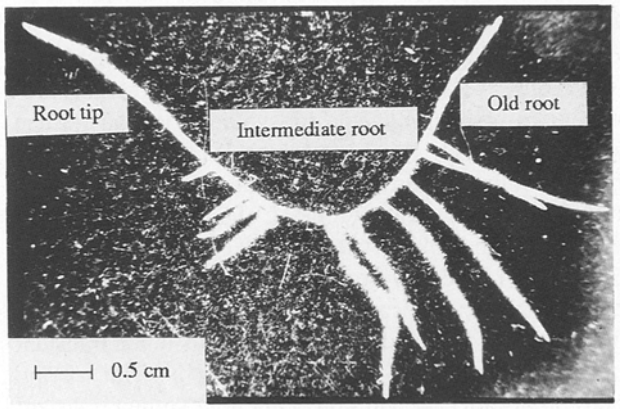


**Fig. 4a.** Single transformed root of *L. corniculatus* 12 days after subculture. Sectors selected for dissection are indicated. Side roots were removed prior to dissection of individual root sectors.

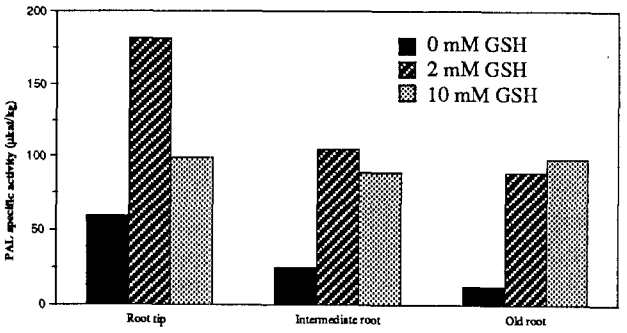


**Fig. 4b.** Elicitation of PAL in different sectors of *Lotus* roots. PAL determinations carried out 6 hours after elicitation. Values are means of triplicate tissue samples.

| | Vestitol | | | | Sativan | | | |
|---|---|---|---|---|---|---|---|---|
| | µg/g fresh wt | µg/ml medium | Total mg/l culture | % in medium | µg/g fresh wt | µg/ml medium | Total mg/l culture | % in medium |
| Control | nd | 0.67±0.085 | 0.67±0.085 | - | nd | nd | nd | - |
| 2 mM GSH | 58.2±5.9 | 5.77±0.4 | 8.29±0.4 | 69.8±5.8 | nd | tr | tr | - |
| 10 mM GSH | 159.6±33 | 7.76±0.39 | 12.4±1.2 | 63.7±5.3 | nd | 0.05±0.02 | 0.05±0.02 | - |

**Table 1.** Phytoalexin accumulation in elicited root cultures of *Lotus corniculatus*. 12 day old root cultures in 50 ml medium elicited with GSH for 24 hours. Values are mean ± SEM (n ≥ 3 or 5 replicate flasks). Tr (trace amount detected). nd (less than detection limit).

found to be an effective elicitor of PAL in transformed *Lotus* root cultures. Similar levels of elicitation have been noted with GSH in bean cell suspension cultures (Wingate et al. 1988). Interestingly, basal PAL activities in *Lotus*, ca. 50 μkat/kg, are higher than those reported for bean cell suspensions, 2 - 22 μkat/kg; this may reflect higher levels of constitutive phenylpropanoid biosynthesis in transformed *Lotus* roots, which accumulate high levels of condensed tannins in culture (Morris and Robbins 1991). In *Lotus*, the optimal GSH concentration for PAL elicitation was found to be between 2 and 4 mM, whereas with bean, lower levels of GSH (0.01 - 1.0 mM) were effective; the significance of the greater sensitivity of bean cultures to GSH is unclear.

In this study, the addition of GSH results in the transient induction of PAL with peak extractable activities being noted 8 hours after the addition of elicitor. Similar transient PAL induction kinetics have been noted in other elicitation systems such as alfalfa cell suspensions (Dalkin et al. 1989) and bean cell suspensions (Robbins et al. 1985) when treated with biotic elicitor preparations. Data from bean cell suspensions (Ellis et al. 1989) suggests that this transient rise and fall in PAL actitives follows transient accumulation of PAL transcripts; and the induction kinetics in *Lotus* root cultures suggests that there may be transcriptional control of PAL expression in this system also.

PAL elicitation in *Lotus* root cultures appears to be independent of culture age, but it is also clear that root cultures are made up of tissues of different ages, a situation that is not obvious in callus suspension systems. Root tissue of different ages showed differential sensitivity to GSH and other data from this laboratory indicate that the GSH optimum for *Lotus* root tips is 2 mM, while for older root tissue the optimal GSH concentration for PAL elicitation is 10 mM (Wilkes and Robbins, unpublished data). It would be interesting to see whether these differences relate to differences in patterns of phenylpropanoid accumulation in root tissues of different ages.

Elicitation of *Lotus* root cultures results in the accumulation of isoflavan phytoalexins both in elicited tissue and also external culture medium. Vestitol and sativan have previously been identified in *Lotus corniculatus* leaves after inoculation with a spore suspension of *Helminthosporium turcicum* (Bonde et al. 1973). Of note, is the observation that *Lotus* leaves accumulate high levels of both vestitol, 551 - 826 μg/g FW, and sativan, 1615 - 1974 μg/g FW, whereas transformed *Lotus* root cultures accumulate mainly vestitol and only trace quantities of sativan. It is clear therefore that *Lotus* root cultures have the biosynthetic capacity of both vestitol and sativan biosynthesis; the low levels of sativan noted in *Lotus* root cultures may reflect low inducible activities of vestitol 2'0-methyl transferase in the experiments reported here. The kinetics of induction of this late enzyme are currently being investigated. However, the data presented here suggest that transgenic *Lotus* tissues are able to respond to elicitation in a similar manner to non-transformed *Lotus* plants. It is possible that transformed root cultures may be interesting model systems for the study of the regulation of phenylpropanoid defence responses in legumes, and their genetic modification.

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