Isoflavone Glycoside Formation in Transformed and Non-Transformed Suspension and Hairy Root Cultures of *Lupinus polyphyllus* and *Lupinus hartwegii*

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Transformed cell suspension and hairy root cultures were established by infecting seedlings of *Lupinus polyphyllus* and *L. hartwegii* with various wild type strains of *Agrobacterium tumefaciens* and *A. rhizogenes*. Transformation of the cultures was confirmed either by their phytohormone autotrophy, detection of opines or southern analysis. Glucosides of genistein and 2′-hydroxygenistein, were found to be the main secondary metabolites in normal and transformed suspension cultures as well as in hairy root cultures. Although some of the isoflavone glycosides of the cultures were apparently new constituents of *Lupinus,* they were afterwards also found in young seedlings.

Introduction

Many secondary pathways are not well expressed in plant cell suspension cultures. Techniques such as screening, selection, media variation or elicitation improved the product formation greatly in some cases but failed in many other systems (see for review [1]). Therefore it is anticipated that such recalcitrant pathways may only be expressed in undifferentiated cells after genetic manipulation. Quinolizidine alkaloid biosynthesis, for example, must be regarded as a recalcitrant pathway since all efforts failed to accumulate distinct levels of these alkaloids in suspension cultures of *Lupinus* and other Fabaceae [2]. The proposed biosynthetic sequence from lysine to the ring structure of the quinolizidine alkaloids *via* a two-step pathway [3] makes this an interesting target for genetic manipulation.

Transformation of dicot plants *via* infection with *Agrobacterium* strains has been reported for numerous species. However, the experimental expenditure for obtaining transformed cells of the various species seems to be quite different. Transformation of *Lupinus* species by *A. tumefaciens* has to our knowledge not yet been reported. Transformation of *L. albus* with *A. rhizogenes* was mentioned in one report [4], while Wink and Witte [5] stated that they could not establish hairy cultures of *L. albus* and *L. polyphyllus*. Thus the aim of the present investigation was to establish the conditions for the transformation of sterile grown seedlings of *Lupinus* species with wild type strains of *Agrobacterium* and to analyze whether the transformation process itself would have any effects on the expression of the quinolizidine alkaloid pathway. As no appreciable amounts of alkaloids and cadaverine were detected in any white or green transformed suspension or in hairy root cultures, it is reasonable to assume that this pathway is not affected by transformations with wild type strains of *Agrobacterium*.

Studies on the secondary metabolite production by cell cultures of *Lupinus* have concentrated on quinolizidine alkaloids. However, *Lupinus* plants are also known to be a rich source of isoflavonoids [6, 7], some of which are highly fungitoxic [8, 9]. Indeed suspension and hairy root cultures of *Lupinus* were found to accumulate rather high levels of isoflavone glucosides of which some had not previously been described.

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Materials and Methods

Bacterial strains

The strain *Agrobacterium tumefaciens* DSM 30150 was obtained from DSM – Deutsche Sammlung Mikroorganismen, Braunschweig. The strains *A. tumefaciens* B6S3 (octopine) and C58 (nopaline) [10] were provided by the Max-Planck-Institut für Züchtungsforschung, Köln. The strain *A. rhizogenes* 15834 [11] was obtained originally from Dr. Schieder, Berlin.

Plant material

Surfaced sterilized seeds of *L. polyphyllus* and *L. hartwegii* were germinated on Murashige-Skoog (MS) agar medium [12].

a) Establishment of transformed cultures

Three- to six-week-old seedlings were used for infection with the various *Agrobacterium* strains. Either the stem of a seedling or cut pieces of all parts of seedlings were inoculated by the use of a syringe needle. Inoculated pieces were transferred after two days to phytohormone-free medium (MS) containing 250 µg cefotaxime (Claforan™)/ml to prohibit bacterial growth. In the case of heavy bacterial growth each piece was washed by shaking several times before transfer to fresh agar plates. Treatment with the antibiotic had to be repeated in some cases for up to 6 months (6–12 passages). Roots or calli were detected first after 2 to 3 weeks of which only those showing good and continuous growth on the phytohormone-free media were used further. When tumors (roots or callus) appeared at the inoculated sites of seedlings they were removed after 2 to 3 weeks and transferred to phytohormone-free agar plates containing the antibiotic.

b) Establishment of normal callus culture

Pieces of seedlings were placed on MS agar with 2 µM 2,4-D, and as control, also on phytohormone-free medium.

c) Maintenance of transformed and non-transformed cultures

Transformed calli and roots were maintained on phytohormone-free solid or liquid MS medium. Standard inoculum was 2 g fresh mass/70 ml medium. Subcultivation was performed every 10 days in the case of suspensions, while root cultures were transferred after 10 to 20 days. Normal suspension cultures were maintained under the same conditions in the presence of 2 µM 2,4-D.

Analytical methods

a) Isolation of plant DNA

DNA of the root and suspension cultures were isolated as essentially described by Dellaporta *et al.* [13]. 2.5 g plant material, ground under liquid nitrogen, were incubated with 15 ml TEN* extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, pH 8.0), 12 µl mercaptoethanol and 1 ml 20% SDS solution at 65 °C for 10 min. After addition of 5 ml of 5 M CH_3COOK solution the suspension was incubated on ice for 20 min and centrifuged at 3600 × *g* at 4 °C. The supernatant was filtrated through Kleenex paper and DNA was precipitated with 10 ml isopropanol at –20 °C. The centrifuged pellet was taken up in 700 µl TE (50 mM Tris-HCl, 10 mM EDTA, pH 8.0), extracted with an equal volume of C_6H_5OH:$CHCl_3$:isoamylalcohol, 25:24:1 and precipitated with 1/10 vol. 3 M CH_3COONa and 7/10 vol. isopropanol. The pellet was dissolved in 600 µl 10 mM Tris-HCl, 0.5 mM EDTA, 10 mM NaCl, pH 8.0, and incubated with 6 µl RNAse (10,000 U/ml Tl RNAse and 5 mg/ml RNAse A) for 1 h and with 6 µl proteinase K (5 mg/ml) for another hour. The DNA solution was extracted 3 times with C_6H_5OH/$CHCl_3$/isoamylalcohol, then with $CHCl_3$, precipitated with ethanol and finally taken up in 300 µl of the last buffer.

b) Southern analysis of plant DNA

Total plant DNA (20 µg) was incubated with *Eco*RI overnight and subjected to electrophoresis on 0.8% agarose gel in TBE (Tris-borate:EDTA buffer, pH 8.0) [14]. DNA fragments were transferred under alkaline conditions to Hybond N membranes (Amersham) and cross-linked with UV light. Prehybridizations and hybridizations were performed in 50% formamide, 2 × SSPE, 0.2% SDS, 5 × Denhardt's and 100 µg *E. coli* *t*-RNA/ml at 42 °C according to Sambrook *et al.*

* Abbreviations of buffers are according to Sambrook *et al.* [14].

[14]. For hybridizations of DNA of bacteria and plant cells transformed with *A. tumefaciens* strains DSM 30150, C58 and B6S3, respectively, the *Hind*III fragment 23 of pTiT37 [15] and the *Hpa*I fragment 14 of pGV0201 [16] were used. DNA of root cultures was hybridized with *Eco*RI fragments 3a and 15 of pRiHRI, isolated from cosmid pLJ1 [17]. Probes were labelled by random primed DNA labelling (Boehringer, Mannheim) in the presence of (α)-[^{32}P]dCTP (Amersham). The probes were purified on a Push Column (Stratagene) and used at concentrations of 10^6 cpm/ml hybridization solution. Filters of the overnight hybridizations were washed several times in 0.1 × SSC, 0.2% SDS at 42, 52 and 56 °C and exposed to Kodak XAR films at −70 °C. Alternatively, the probes were labelled with the Flash-Prime-IT™ Random Primer Labelling Kit (Stratagene) as described by the manufacturer and DNA transfer, hybridization and detection were performed using the Flash™ membranes and detection system. The size of the DNA fragments were determined by comparison to lambda-*Hind*III length standards.

c) Opine analysis in transformed tissues

For nopaline and octopine analysis cells were extracted with MeOH : $CHCl_3$: H_2O, 12:5:3 as described [18] and either chromatographed on silica (50% *n*-propanol, 1% NH_4HCO_3) or cellulose (*n*-amylalcohol : pyridine : H_2O, 21:42:37) plates or electrophoresed on TLC plates (HCOOH : CH_3COOH : water, 1:3:16). Detection was performed with the phenanthrenequinone reagent [19] or in the case of cellulose with the Sakaguchi reagent [18]. For detection of agropine and mannopine root cells were analyzed as described by Saito *et al.* [20].

d) Purification and identification of secondary metabolites

I) The isoflavonoid glucosides were purified on a *semi*-preparative HPLC column including a silica gel TLC step: 30 g dry mass were extracted twice with 600 ml MeOH. The MeOH extract was reduced to 30 ml. During the concentration procedure the extract was kept from time to time at −30 °C for 1−2 h and the precipitate was filtered off before further evaporation of MeOH. One ml portions of this extract were then fractionated on a *semi*-preparative Multosorb RP_{18} (10 µm, 250 × 25 mm) column. Solvent A: 0.5% aq. CH_3COOH; solvent B: MeOH + 0.5% CH_3COOH; gradient: 15% B to 70% B in 40 min, 35 min at 70% B, then 25 min 100%; flow rate 4 ml/min; detection 280 nm. 40 ml fractions were collected for further analysis. The fractions containing the desired compounds were further purified on silica TLC plates (BuOH : CH_3COOH : H_2O, 4:1:5/upper phase). The major UV-absorbing zones were removed, eluted with MeOH and, if necessary, finally purified on the RP_{18} column using H_2O/MeOH gradients suitable for the compound in question.

For the identification of yellow coloured spots on TLC plates, 20 g root cells were extracted with 80% aq. MeOH. The extract concentrated to 20 ml (all precipitated material was discarded) was placed on a polyamide CC6 column (450 × 45 mm) and eluted stepwise with H_2O, 40% MeOH, 100% MeOH and 100% MeOH containing 0.1% NH_4OH. Detection was at 360 nm. The yellow fractions were checked on cellulose TLC plates (BuOH : CH_3COOH : H_2O, 4:1:5, upper phase). Three fractions were further purified on a Sephadex LH-20 column (1100 × 25 mm) by stepwise elution with 50%, 80%, and 100% MeOH. The yellow fractions were then chromatographed on cellulose plates as above, scraped off and eluted with 80% MeOH.

The structure of the purified compounds were identified by ^{1}H NMR spectroscopy and FAB-MS spectrometry (further details see Table I).

e) Analytical HPLC for quantitative analysis

25−50 mg dry mass were extracted with 2.5 ml 80% aq. MeOH with stirring. 5−20 µl of the extracts were chromatographed on a Lichrosorb RP_{18} column (7 µm, 250 × 4 mm) with gradient 15% aq. MeOH − 70% aq. MeOH (0.5% CH_3COOH) in 20 min, flow rate 2 ml/min, detection 280 nm. In the case of root extracts the gradient was held at 70% for 4 min. Isoflavonoid content was calculated from a standard curve of authentic genistein.

Results and Discussion

Initiation of transformed cultures

Over a period of 5 years several cell lines of *Lupinus* transformed with various wild type strains of *A. tumefaciens* and *A. rhizogenes* have been

established in our laboratories. As such strains did not contain any selective marker, callus/tumor formation on phytohormone-free medium was the first indicator of transformation. This was a reliable indicator, as uninfected pieces of *Lupinus* seedlings did not show continuous growth on phytohormone-free medium in the many control experiments performed. Some initial swellings, callus growth or root elongations of uninfected explants ceased within 1 to 2 weeks. Compared to our experience with *Nicotiana* [21] and *Peganum* (in preparation), transformants of *Lupinus* were obtained at much lower rates. The percentage of the number of transformants/inoculated pieces was initially between 0–2%. We did not succeed in transforming root explants. Stem and hypocotyl material gave rather unpredictable responses. Very often the infected pieces became dark brown and this had negative effects on the formation of transformed callus. In contrast, for initiation of normal phytohormone-dependent cultures stem pieces were the most convenient plant material. In our hands, leaves of 4- to 8-week-old seedlings were the most responsive material for transformation. The best results were obtained when the infection was set at the site of petiole/leaf ground. Sometimes up to 25% of the inoculated pieces showed tumor formation after 2–3 weeks. However, not all of these tumors resulted later in callus or suspension cultures which showed rapid growth on phytohormone-free medium. As the establishment of transformed cell cultures growing as rapidly on phytohormone-free medium as normal phytohormone-dependent lines had priority, the development or morphogenetic potential of poorly growing, seemingly encapsulated tumors in the presence of phytohormone was not analyzed.

Overall, the three wild type strains of *A. tumefaciens* C58, B6S3 and DSM 30150 were comparably efficient in transforming *Lupinus* explants. However, when both the number of tumors and also the growth rates of the developing tumors on phytohormone-free medium was the criterium for the success of the transformation, then strain 30150 yielded the best lines. Most transformed callus and suspension lines became green when maintained under a light regime, while others remained yellowish-white.

Despite the fact that we did not see distinct differences in the transformation of *Lupinus* by the above wild type strains, it seems to be clear that the transformation efficiency of *Agrobacterium* strains is not only dependent upon the explant but also upon the virulence of the bacteria [22]. Thus, we failed to obtain any transformed *Lupinus* line using disarmed *Agrobacterium* strains containing co-integrative or binary vectors carrying marker genes and/or a bacterial lysine decarboxylase gene, although these strains gave very high numbers of transformants with tobacco [21].

In the case of *A. rhizogenes* 15834, 5–10% of the inoculated explants regularly developed rapidly growing root cultures. Some growth curves of the most vigorously growing root culture Lupo 15834C are given in Fig. 1. Even at extreme low inocula of 500 mg fresh mass/l up to 20-fold increases of biomass were obtained within 12–14 days. The appearance of the various root cultures was quite different. Thus we obtained a green callus root line of which only the callus material was transferred over a period of 2 years. Even after this time, roots reappear after a few days. While most root cultures of *L. polyphyllus* were yellow-white and consisted of long, branched (hairy) roots, *L. hartwegii* root cultures were more brownish and the roots were short and twisted.

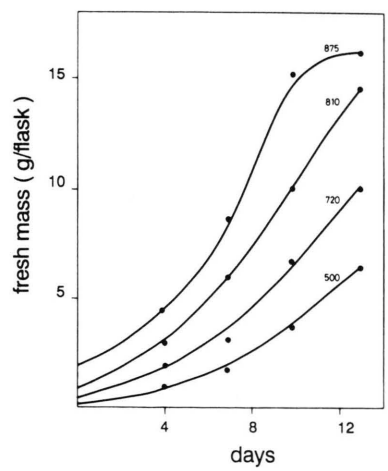


Fig. 1. Growth of the hairy root culture Lupo 15834C with different inocula from 250–2000 mg fresh mass/70 ml MS medium. In the presence of 2% sucrose dry mass up to 900 mg were found.

Biochemical and molecular characterization of the transformation

To prove that transformation had occurred in the various *Lupinus* lines the cultures were first analyzed for opine formation (Fig. 2). No opines were detected even in freshly initiated lines derived from strains DSM 30150 and B6S3 by electrophoresis or TLC while C58 cell cultures showed the presence of an unidentified opine with a R_f value between nopaline and octopine during the first 3 months of cultivation. Later this compound was only found when 5 mM arginine was fed to C58 cell cultures. Arginine feeding resulted in the accumulation of a phenanthrenequinone- [19] and a Sakaguchi-positive [20] compound below octopine in *Lupinus* 30150 lines, while this compound was not regularly found in transformed B6S3 suspensions. Non-transformed lines never accumulated such "opine-like" compounds after feeding of arginine. As *Lupinus* C58 lines did not accumulate nopaline but an opine with a lower R_f, the accumulation of an opine below octopine in *Lupinus* 30150 and sometimes in *Lupinus* B6S3 was regarded as indication that the biochemically and molecularly uncharacterized strain DSM 30150 might be more related to octopine strains. However, as the transformed suspension cultures accumulated neither nopaline nor octopine, opine analyses gave only partial evidence for transformation. In the case of the hairy roots neither agropine nor mannopine were detected in *Lupinus* 15834 cultures.

For further characterization of the transformed *Lupinus* lines and of the strain DSM 30150, southern analyses were performed. DNA of *A. tumefaciens* strains C58, B6S3 and DSM 30150, as well as of *A. rhizogenes* 15834, were hybridized with two probes specific for nopaline (Fig. 3) and octopine Ti-plasmids, respectively. Fig. 3 shows that the *Hind*III 23-fragment of pTiT37 [15] hybridized strongly to two homologous *Hind*III-*Bam*HI fragments of 2.2 and 1 kb and weakly to a larger fragment of strain C58. Only weaker hybridization signals were obtained with the strains DSM 30150, B6S3 and 15834 (Fig. 3). The restriction pattern confirmed that DSM 30150 is more related to B6S3 than to C58 but is not identical with B6S3. The fact that the *Hpa*I fragment 14 of

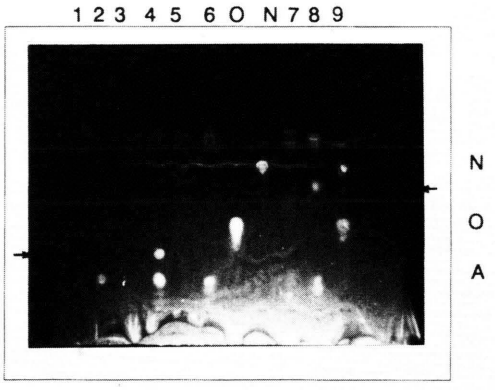


Fig. 2. TLC chromatogram (1% NH_4HCO_3 in 50% propanol) of extracts of normal and transformed *Lupinus* suspension cultures with and without feeding of 5 mM arginine (A) for 3 d, and detection of opines by the phenanthrenequinone reagent [19]. 1 = Luha, 2 = Luha + A, 3 = Luha 30150, 4 = Luha 30150 + A, 5 = Luha B6S3, 6 = Luha B6S3 + A, 7 = Luha C58, 8 = Luha C58 + A, 9 = Luha B6S3 + O + N. A = arginine, N = nopaline, O = octopine. The arrows mark the location of the unknown "opines" formed in transformed cultures after feeding of arginine.

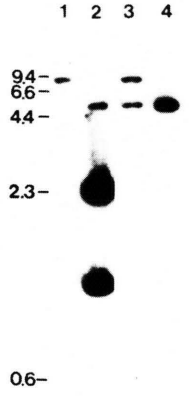


Fig. 3. Southern blottings of *Bam*HI/*Hind*III fragments of the DNA isolated from *A. tumefaciens* 1 = B6S3, 2 = C58, 3 = DSM 30150 and from *A. rhizogenes* 4 = 15834 with the *Hind*III 23 probe of pTiT37 [15]. The sizes (kb) of some *lambda-Hind*III fragments are indicated by bars.

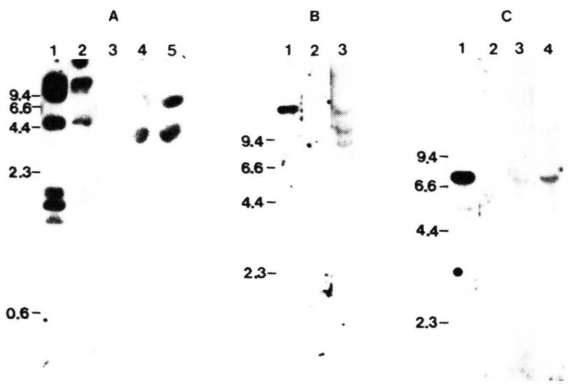


Fig. 4. Southern hybridization of *Eco*RI-restricted bacterial (0.2–0.5 μg/slot) or plant (20 μg) DNA. A: 1 = pLJ1 and 2 = *A. rhizogenes* 15834 hybridized with pLJ1 [17]; 3 = Luha non-transformed, 4 = Lupo 15834, and Luha 15834 hybridized with *Eco*RI fragments 3a and 15 of pRiHRI from pLJ1; B: 1 = *A tumefaciens* C58, 2 = Luha 30150, and 3 = Luha C58 hybridized with the *Hind*III 23 fragment of pTiT37 [15]; C: 1 = *A. tumefaciens* DSM30150, 2 = untransformed Luha, 3 = Luha 30150, and 4 = Lupo 30150 hybridized with *Hpa*I 14 of pGV0201 [16]; *Eco*RI 3a/15 of pRiHRI from pLJ1 [17].

pTiAch5 (octopine type) [16] showed strong hybridization with B6S3 (not shown) and DSM30150 (Fig. 4C) was further proof that both strains are closely related.

Consequently, *Eco*RI digests of the DNA of Luha C58 hybridized with the *Hind*III 23-fragment of pTiT37 while Luha 30150 did not give a signal (Fig. 4B). The fact that plant DNA fragments of 18 kb, 11 kb and 8.5 kb gave hybridizations signals showed the integration of 3 copies of the T-DNA into the genome of this transformant. As the *Hpa*I fragment 14 of pTiAch5 is an internal T-DNA region of octopine strains, this probe detected *Eco*RI fragments of the same size in DSM30150, Lupo 30150 and Luha 30150 (Fig. 4C) and B6S3 (not shown) while the DNA of non-transformed Luha and Lupo (not shown) gave no signals. Finally, the transformation of Luha 15834 and Lupo 15834 was shown by southern blottings using *Eco*RI fragments 3a and 15 of pLJ1 as probes [17] (Fig. 4A). Fragment 15 has been described as an internal fragment of the T_L-DNA from pRiHRI [17] and is responsible for the identical lower signals of Lupo 15834 and Luha 15834 (Fig. 4). The upper signals represent *Eco*RI fragments of the plant DNA which are larger or smaller than fragment 3a. Therefore they seem to be fragments which include the T-DNA border in the plant DNA.

Structural identification of the isoflavonoid glucosides

Since no alkaloidal compounds were detected in the suspension or root cultures, methanol extracts of several cell lines obtained by transformation with *A. tumefaciens* C58, B6S3, DSM30150 or *A. rhizogenes* 15834 were inspected by HPLC for their metabolite pattern at wavelengths between 230–310 nm. The chromatogram of the root culture with the highest specific content (Fig. 5) gives an impression of the metabolite pattern of a *Lupinus* culture. An exceptional line was one of the Lupo 30150 lines, not only because of its high content but also because of the quite different pattern with compound **1** accounting initially for more than 40% of all compounds absorbing at 280 nm.

Inspection of the various cell lines indicated that the highest producing suspension culture Lupo 30150 (for **1** and **2**) and the root culture Luha 15834 (Fig. 5) could be used as sources for the structural elucidation of the main metabolites. The peaks **1–10** isolated by HPLC and TLC and were

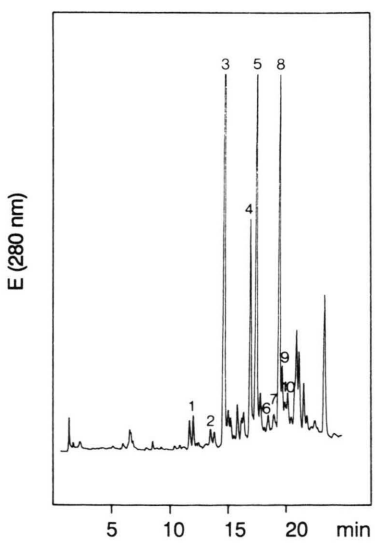
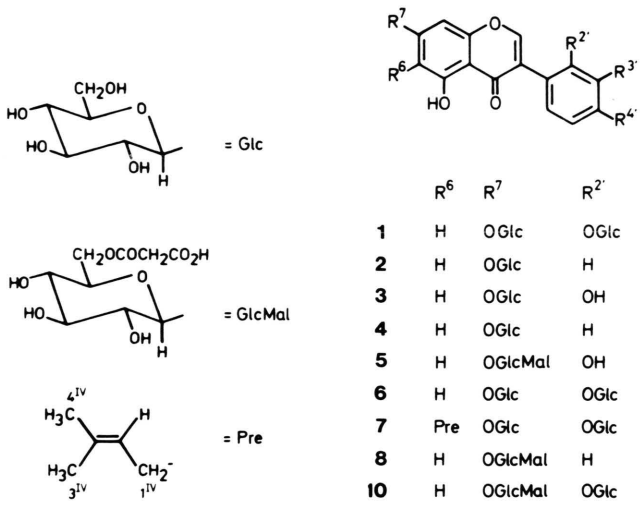


Fig. 5. HPLC diagram of a methanol extract of Luha 15834 root culture on a RP_{18}-Lichrosorb column (see Materials and Methods). The structures of the compounds **1–10,** established from the data in Table I, are given in Fig. 6.



	R^6	R^7	$R^{2'}$	$R^{3'}$	$R^{4'}$
1	H	OGlc	OGlc	H	OH
2	H	OGlc	H	H	OGlc
3	H	OGlc	OH	H	OH
4	H	OGlc	H	H	OH
5	H	OGlcMal	OH	H	OH
6	H	OGlc	OGlc	Pre	OH
7	Pre	OGlc	OGlc	H	OH
8	H	OGlcMal	H	H	OH
10	H	OGlcMal	OGlc	Pre	OH

Fig. 6. Genistein and 2′-hydroxygenistein glucosides identified from suspension and hairy root cultures of *L. polyphyllus* and *L. hartwegii*. **1** = 2′-hydroxygenistein-7,2′-di-O-glucoside, **2** = genistein-7,4′-di-O-glucoside, **3** = 2′-hydroxygenistein-7-O-glucoside, **4** = genistein-7-O-glucoside, **5** = 2′-hydroxygenistein-7-O-(6‴-malonylglucoside), **6** = 2′-hydroxy,3′-dimethylallylgenistein-7,2′-di-O-glucoside, **7** = 6-dimethylallyl,2′-hydroxygenistein-7,2′-di-O-glucoside, **8** = genistein-7-O-(6‴-malonylglucoside), **10** = 2′-hydroxy,3′-dimethylallylgenistein-7-(6‴-malonyl),2′-di-O-glucoside.

readily identified as derivatives of the aglyca genistein and 2′-hydroxygenistein which are well known as isoflavonoid constituents typical of *Lupinus* plants. The structures of the isoflavonoid glucosides, **1–8** and **10** (Fig. 6) were identified from a combination of ^{1}H NMR and MS data. To allow comparison all these data are reported in Table I. For compounds **5, 8** and **10** the presence of an acyl group at C-6 of one glucose unit was evident from the characteristic low field chemical

Table I. ^{1}H NMR and negative ion fast atom bombardment MS data for **1–10.**

^{1}H NMR data[a,b]:

Chemical shifts

Cpd	H-2	H-6	H-8	H-2′	H-3′	H-5′	H-6′	H-1″	H-1‴	H-6″A H-6‴A	H-6″B H-6‴B	H-2″–H-5″ H-2‴–H-5‴	H-1^{iv}	H-2^{iv}	H-3^{iv}	H-4^{iv}
1	8.158	6.575	6.773	–	6.740	6.715	7.217	5.101	4.95	3.958 3.953	3.760 3.758	3.54–3.24 3.54–3.24				
2	8.230	6.572	6.764	7.552	7.216	7.216	7.552	5.095	5.003	3.952 3.952	3.754 3.754	3.61–3.19 3.61–3.19				
3	8.128	6.570	6.764	–	6.444	6.416	7.097	5.098	–	3.956	3.758	3.6 –3.4				
4	8.193	6.563	6.753	7.431	6.895	6.895	7.431	5.097	–	3.956	3.762	3.6 –3.4				
5	8.126	6.561	6.754	–	6.445	6.418	7.100	5.076	–	4.590	4.316	3.6 –3.4, 3.807 (H-5″)				
6	8.193	6.607	6.806	–	–	6.840	7.052	5.110	4.995	3.957 3.932	3.753 3.749	3.62–3.41	3.62–3.41	5.310	1.838	1.694
7	8.147	–	6.847	–	6.744	6.719	7.218	5.134	4.956	3.964 3.953	3.763 3.758	3.7 –3.4	3.7 –3.4	5.308	1.843	1.693
8	8.158	6.539	6.722	7.427	6.890	6.890	7.427	5.055	–	4.592	4.314	3.6 –3.4, 3.797 (H-5″)				
10	8.198	6.591	6.806	–	–	6.839	7.052	5.093	4.997	4.568 3.957	4.316 3.758	3.6 –3.4, 3.812 (H-5″)	3.6 –3.4	5.312	1.837	1.693

Coupling constants

Cpd	6–8	2′–3′ + 2′–5′	3′–5′	5′–6′	1″–2″	1‴–2‴	5″–6″A 5‴–6‴A	5″–6″B 5‴–6‴B	6″A–6″B 6‴A–6‴B	1–2	2–3, 2–4
1	2.2		2.3	8.3	7.6	n.o.	2.0 1.9	5.2 5.5	12 12.2		
2	2.2	8.8			7.6	n.o.	small small	5.4 5.4	12.1 12.1		
3	2.1		2.4	8.3	7.3	–	2.2	5.7	12.2	–	–
4	2.0	8.5			6.8	–	small	5.6	11.9	–	–
5	2.1		2.3	8.2	7.2	–	1.9	7.0	11.9	–	–
6	2.0			8.5	7.2	7.6	2.2, 2.1	5.9, 5.5	12.7, 12.2	7.2	1, 0.6
7	–		2.3	8.3	7.7	7.4	2.2, 2.2	5.4, 5.9	12.2, 12.1	7.1	small
8	Peaks too broad to allow definition of couplings.										
10	~2			8.4	7.1	7.6	small, 2.2	6.8, 6.2	11.8, 12.2	7.1	small

shifts of the attached protons. That these are malonyl groups was ascertained from the MS data as the methylene protons of the NMR spectrum occur in the vicinity of the residual solvent signal and appear to readily exchange in CD_3OD. The position of the glucose units was determined from NOE data for **1–3, 5** and **6** (Table I), and by comparison of chemical shifts for the other compounds. The position of the isopentenyl system in **7** and **10** followed from the nature of the aromatic spin systems and from comparisons of chemical shifts. It is assumed that in **10** the malonyl system is on the glucose unit attached to C-7 of the aglycone from a comparison of the data of **5** and **8.** The structure of **9** could not be unambiguously identified as the ^1H NMR indicated a mixture of compounds. However, the MS spectrum was similar to **10** and ^1H NMR data indicated the presence of an aglycone, an isopentenyl system and two glucose units, one of which carried an acyl group which presumably was a malonyl system. The nature of the aromatic spin system could not be deduced from the signals in the region 6.5 and 7.5 ppm.

All cultures also contained several yellow compounds of which kaempferol, kaempferol-7-O-glucoside, kaempferol-3,4′-di-O-glucoside and kaempferol-3,7-di-O-glucoside were identified. The diglucosides were assigned to the minor peaks between isoflavonoids **3** and **4** (Fig. 5), the monoglucoside content is represented by the first small peak between **5** and **6.** The peaks after 20 min (Fig. 5) have not yet been identified, as their UV absorption indicated compounds other than isoflavonoids.

Comparison of isoflavonoids patterns and levels in suspensions, root cultures and plantlets

While product levels and patterns of suspensions and root cultures of *L. hartwegii* or *L. polyphyllus* varied quantitatively, the compounds identified were detectable in all extracts. One or two of the major peaks **3–5** and **8** were usually also major peaks of the other lines. Table II gives some total isoflavonoid levels found in various cultures. More detailed characteristics of some of the lines are given in the following paper [23]. Several conclusions may be drawn even if some lines were analyzed only for a short period with respect to their isoflavonoid content. While most suspension cultures accumulated only 0.2–0.5% isoflavonoids on a dry mass basis, some highly productive lines were found accumulating up to 2% even after being in suspension for more than 5 years. Though the highest productive suspension culture was a transformed line, no indication was found that transformed suspension cultures, while growing without phytohormones, might show a higher tendency for increased isoflavonoid accumulation. For example, the second best producing culture

Table I. (Continued.)

Nuclear Overhauser Enhancements					
Cpd	Irradiation position	Positive NOE[c]	Cpd	Irradiation position	Positive NOE[c]
1	1″	3′, only	5	1″	6, 8
	1‴	6, 8		2	6′
2	1″	3′, 5′	6	4[iv]	2[iv]
	1‴	6, 8		1‴	none
3	1″	6, 8			
	2	6′			

Negative ion FAB MS data[d]:
1: 609 $[M-H]^-$; **2:** 593 $[M-H]^-$; **3:** 448 $[M]^-$, 447 $[M-H]^-$, 285 $[aglycone-H]^-$; **5:** 534 $[M]^-$, 533 $[M-H]^-$, 498 $[M-CO_2H]^-$, 285 $[aglycone-H]$
6: 678 $[M]^-$, 677 $[M-H]^-$, 515 $[M-Glc-H]^-$, 353 $[M-2Glc-H]^-$; **7:** 678 $[M]^-$, 677 $[M-H]^-$, 515 $[M-Glc-H]^-$, 353 $[M-2Glc-H]^-$; **8:** 518 $[M$
269 $[aglycone-H]^-$; **9** and **10:** 764 $[M]^-$, 763 $[M-H]^-$, 719 $[M-CO_2H]^-$, 557 $[M-CO_2H-Glc]^-$, 515 $[M-249]^-$, 353 $[M-249-Glc]^-$.

[a] All NMR data were recorded on Bruker NMR spectrometers locked to the major deuterium resonance of the solvent, CD_3OD. In the table Cpd = Compound number and H-1″–H-6″ refer to the protons of the glucose moiety on C-7 of the aglycone and H-1‴–H-6‴ to those of the glucose moiety attached to the aglycone B-ring.

[b] For **9** see text.

[c] Nuclear Overhauser Enhancements to sugar ring protons are not reported.

[d] Negative ion FAB MS were recorded on a Finnigan MAT 8430 spectrometer with glycerol as matrix. A satisfactory spectrum was not obtained for **4. 8** showed additional peaks at 519 and 520 presumably due to the presence of deuterium as the mass spectra were recorded subsequent to the NMR spectra.

Table II. Yields of isoflavone glucosides of some normal and transformed suspensions or callus (B6S3/C58/30150) and roots (15834) of *Lupinus* cultures (expressed as genistein equivalents).

Cell line	Number of lines analyzed	Genistein equivalents [mg/g dry mass]
Lupo	3	2– 3
Lupo 30150	3	5–19
Lupo 15834	3	7–10
Luha	4	2–15
Luha 30150	2	1–10
Luha C 58	2	2– 5
Luha B6S3	2	2– 7
Luha 15834	2	15–22

was a good growing but rather aggregated normal Luha line. Indeed, aggregated *Lupinus* cultures seemed to synthesize more isoflavonoids than the fine suspensions. As aggregated lines usually change with time into fine suspensions, many of the initially highly productive lines may then show distinctly reduced accumulation of isoflavonoids. Fine cell suspension cultures such as a Lupo 30150 [23] accumulating 1–2% isoflavonoid aglyca (corresponding to 2–4% glucosides) on a dry mass basis after several years of cultivation may thus be regarded as variant lines.

Despite the fact that some highly productive suspension cultures were found, it was evident that the average *Lupinus* root culture contained higher isoflavone glucoside levels than the average suspension culture. While the productivity of suspension cultures decreased when permanently maintained in liquid medium [23], the productivity of the root cultures was stable over many years under such conditions. Also the pattern of the various root cultures did not change over the years.

The number of isoflavone glucosides described for *Lupinus* plants is very small [24]. However, it was soon evident that the isoflavonoid glucosides identified from Lupo 30150 and Luha 15834 were not restricted to cultured cells, as they were also detected in extracts of plantlets of *L. polyphyllus* and *L. hartwegii*. With respect to the isoflavone glucosides only quantitative differences were found between cultures and the various organs of the plantlets. In the case *L. hartwegii* the main components of the plantlets were also the main components of the cultures (Table III). The largest difference were between leaf extracts and the cultures, as the leaf extracts contained two major unknown components. The same held true for *L. polyphyllus* cultures and plantlets with the exception of the above mentioned line Lupo 30150. As the isoflavonoid glucosides are present in all parts of the *Lupinus* plants, the patterns found in root or suspension cultures could not be related to specific organs of the plantlets.

In conclusion, transformed suspension and hairy root cultures of *Lupinus* species can readily be obtained using wild type strains of *Agrobacteria*, some of which may form high levels of a variety of isoflavone glucosides and could thus be a useful source for further biochemical studies, *e.g.* for the prenylation step [25] or for comparative investigations on the different glucosyl transferases.

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Table III. Comparison of isoflavonoid pattern of 4 week-old sterile-grown *Lupinus hartwegii* plantlets von MS agar, normal and transformed cell suspensions (7 d) and hairy root cultures (14 d). The isoflavone glucosides are numbered as in Fig. 5.

Tissue	Ranking of isoflavone glucosides according to levels I.	II.	III.	IV.	Remarks
Plantlet roots	**8**	**4**	**5**	**3**	
hypocotyls	**8**	**4**	**3**	**5**	
cotyledons	**3**	**8**	**7**	**4**	
stems	**3**	**8**	**5**	**4/7**	
leaves	**8**	**7**	**4**	**3**	major unknown peaks
Suspensions normal	**3**	**5**	**8**	**4**	
transformed	**8**	**4**	**3**	**5**	
Hairy roots line A	**3**	**5**	**4**	**8**	
Hairy roots line B	**5**	**3**	**8**	**4**	

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