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ORIGINAL ARTICLE

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4-Hydroxycinnamoyl-CoA hydratase/lyase, an enzyme of phenylpropanoid cleavage from *Pseudomonas*, causes formation of C_6-C_1 acid and alcohol glucose conjugates when expressed in hairy roots of *Datura stramonium* L.

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Abstract 4-Hydroxycinnamoyl-CoA hydratase/lyase (HCHL), a crotonase homologue of phenylpropanoid catabolism from *Pseudomonas fluorescens* strain AN103, led to the formation of 4-hydroxybenzaldehyde metabolites when expressed in hairy root cultures of *Datura stramonium* L. established by transformation with *Agrobacterium rhizogenes*. The principal new compounds observed were the glucoside and glucose ester of 4-hydroxybenzoic acid, together with 4-hydroxybenzyl alcohol-*O*-*β*-D-glucoside. In lines actively expressing HCHL, these together amounted to around 0.5% of tissue fresh mass. No protocatechuic derivatives were found, although a trace of vanillic acid-*β*-D-glucoside was detected. There was no accumulation of 4-hydroxybenzaldehydes, whether free or in the form of their glucose conjugates. There was some evidence suggesting a diminished availability of feruloyl-CoA for the production of feruloyl putrescine and coniferyl alcohol. The findings are discussed in the context of a diversion of phenylpropanoid metabolism, and the ability of plants and plant cultures to conjugate phenolic compounds.

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Abbreviations CPL: chorismate pyruvate lyase · HCHL: 4-hydroxycinnamoyl-CoA hydratase/lyase · PAL: phenylalanine ammonia-lyase

Introduction

The expression of bacterial genes that encode enzyme activities not normally present in a plant may cause the modification or diversion of plant metabolic pathways. This offers new tools to study metabolic regulation and may create valuable opportunities to synthesise new products. Examples of the modification of plant metabolism by the expression of bacterial genes include the introduction of the capacity for polyhydroxybutyrate synthesis into *Arabidopsis thaliana* and *Gossypium hirsutum* (Poirier et al. 1992; John and Keller 1996); the formation of uncharacteristic cadaverine-derived alkaloids and hydroxycinnamate conjugates in plants and hairy-root cultures of *Nicotiana* species expressing a bacterial lysine decarboxylase (Herminghaus et al. 1996; Berlin et al. 1998); enhancement of the production of the anthraquinone, alizarin, in hairy roots of *Rubia peregrina* by the introduction of isochorismate synthase (Lodhi et al. 1996); and finally, the formation of 4-hydroxybenzoate glucoside conjugates in plants and cell cultures of *Nicotiana tabacum* as a result of expressing chorismate: pyruvate lyase (CPL) from *Escherichia coli* (Siebert et al. 1996; Li et al. 1997).

The phenylpropanoid pathway is a principal metabolic route in plants, leading inter alia to flavonoids and lignin (Hahlbrock and Scheel 1989), and hence is an attractive target for intervention. Its molecular genetics and transcriptional regulation, and those of its branch

pathways, continue to be investigated intensively (Dixon and Paiva 1995; Holton and Cornish 1995; Whetten and Sederoff 1995; Mol et al. 1996; Winkel-Shirley 1999). Increasingly, it is possible to direct the extent and tissue- and organ-specificity of phenylpropanoid pathway expression (Martin 1996; Grotewold et al. 1998; Tamagnone et al. 1998). Due to the diversity and importance of phenylpropanoid-derived substances, the pathway offers a number of potential opportunities for metabolic engineering by heterologous gene expression. A classical example is the introduction of the synthesis of the phytoalexin, resveratrol, into *Nicotiana tabacum* by the expression of stilbene synthase from *Vitis vinifera* (Hain et al. 1990).

There remain important phenylpropanoid-derived metabolites for which the biosynthetic routes, enzymology and molecular genetics are still incompletely resolved. One class comprises the benzaldehydes, benzoates and benzyl alcohols and their derivatives, and includes the signalling compound, salicylic acid (Lee et al. 1995), the phytoalexin and naphthaquinone precursor, 4-hydroxybenzoate (Schnitzler et al. 1992; Löscher and Heide 1994), a range of floral scent compounds (Dudareva et al. 1998) and the flavour compound, vanillin (Rao and Ravishankar 2000). These are assumed to be derived by a 2-carbon chain-shortening of phenylpropanoid precursors and, in the case of 4-hydroxybenzoate formation, evidence suggestive of two cleavage mechanisms differing in coenzyme A requirement and 4-hydroxybenzaldehyde involvement was presented some years ago (Yazaki et al. 1991; Schnitzler et al. 1992; Löscher and Heide 1994). Recently, on the other hand, it has been reported that benzaldehyde is not an intermediate in the biosynthesis of benzoic acid and salicylic acid in *Cucumis sativus* and in *Nicotiana attenuata* (Jarvis et al. 2000).

The first reported enzymic and molecular genetic characterisation of phenylpropanoid chain-cleavage to a benzaldehyde was in *Pseudomonas fluorescens* strain AN103, a bacterium isolated from soil on the basis of its ability to catabolise ferulic acid (4-hydroxy-3-methoxy-*trans*-cinnamic acid) as sole carbon source (Gasson et al. 1998; Narbad and Gasson 1998; Mitra et al. 1999). The enzyme 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) is a crotonase homologue that hydrates 4-hydroxycinnamoyl-CoA thioesters and then catalyses a retro-aldol cleavage to produce the corresponding 4-hydroxybenzaldehydes, together with acetyl-CoA (Fig. 1). In *P. fluorescens* strain AN103, an NAD^+-linked dehydrogenase then oxidises the 4-hydroxybenzaldehydes to the corresponding 4-hydroxybenzoic acids.

Quite apart from the question of whether HCHL is a model for corresponding phenylpropanoid cleavage reactions in plants, this enzyme offers a novel opportunity to divert plant phenylpropanoid metabolism since the 4-hydroxycinnamoyl-CoA thioesters that are its substrates are intermediates of the plant phenylpropanoid pathway. Here, we describe the effects of expressing HCHL in hairy-root cultures of *Datura stramonium* established by genetic transformation with *Agrobacterium rhizogenes*. We report the formation of new soluble phenolic compounds and the correlation between gene expression, enzyme activity and the levels of these new end-products. We also comment on the potential of HCHL expression in organ cultures to divert the phenylpropanoid pathway towards the formation of useful compounds.

The effects of HCHL expression in aerial tissues of plants of *Nicotiana tabacum* are reported elsewhere (Mayer et al. 2001).

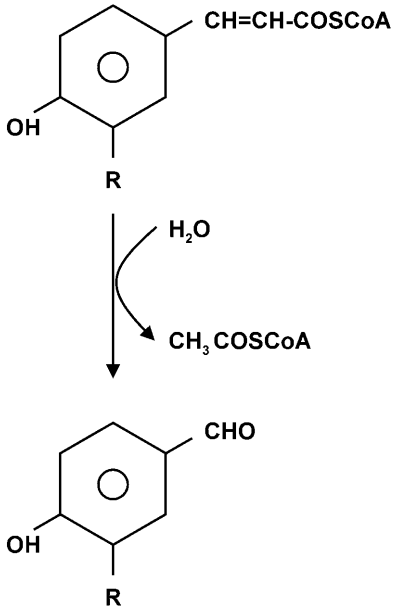

Fig. 1 The reaction catalysed by HCHL. R = H, 4-coumaroyl-CoA, 4-hydroxybenzaldehyde; R = OH, caffeoyl-CoA, protocatechuic aldehyde; $R = OCH_3$, feruloyl-CoA, vanillin

Materials and methods

Chemicals

Chemicals were generally obtained from Sigma-Aldrich Chemical Co. (Poole, Dorset, UK) and were of the highest quality commercially available. Feruloyl-CoA was prepared as indicated previously (Semler et al. 1987; Gasson et al. 1998). 4-Hydroxybenzoic acid-β-D-glucoside and glucose ester were kindly provided by Prof. L. Heide, Universität Tübingen, Germany. 4-Hydroxybenzyl alcohol-4-*O*-β-D-glucoside and vanillic acid-β-D-glucoside (as its methyl ester) were supplied by Takasago International Corporation, Tokyo, Japan. Caffeoyl and feruloyl putrescines were supplied by Dr. J Negrel, INRA, Dijon, France. Acetylated coniferyl and sinapyl alcohols were prepared as described by Beerhues and Berger (1994).

Plant transformation

Two-month-old, glasshouse-grown *Datura stramonium* L. plants were used for transformation. Detached leaves were surface-sterilised and their midribs were infected with *Agrobacterium rhizogenes*, essentially as described by Hamill and Lidgett (1997).

A. rhizogenes was transformed by triparental mating (Bevan 1984), either with the empty pBin19 vector, or with pBin19 containing pmHCHL under the control of the cauliflower mosaic virus (CaMV) 35S promoter, as described elsewhere (Mayer et al. 2001). The construct pmHCHL incorporated a 1-bp change after the start codon (ATG**A**GC to ATG**G**GC) to improve the translational initiation context for plant expression (Mayer et al. 2001). Individual roots emerging from the wound sites were then established in Gamborg's B5 medium (Melford Laboratories, Ipswich, UK) containing 250 μg ml^{-1} of ampicillin and 25 μg ml^{-1} of kanamycin, and subsequently subcultured routinely at 3-week intervals.

Nucleic acid isolation and Southern and RNA gel-blot analyses

Genomic DNA from root tissue (ca. 1 g) frozen in liquid nitrogen was isolated essentially according to Dellaporta et al. (1983) and restriction-digested using *Eco*RI (Promega, Southampton, UK). The restriction fragments were separated by agarose gel electrophoresis and capillary-blotting of the gel was performed as described by Sambrook et al. (1989), using Hybond-N hybridisation membrane (Amersham-Pharmacia, Aylesbury, Bucks., UK). Total RNA was extracted from frozen roots essentially as described by Verwoerd et al. (1989), except that SDS was replaced by *N*-laurylsarcosine. Total RNA (15 μg) was electrophoresed through 1.5% agarose/formaldehyde gels and transferred to Hybond-N membrane by capillary blotting (Sambrook et al. 1989). RNA size markers were from Gibco-BRL, Life Technologies, Paisley, Scotland. Equality of RNA gel loading was checked by ethidium bromide staining. The complete HCHL open reading frame was used as a probe to detect the transgene and transcript. It was radiolabelled by random priming with [^{32}P]dCTP (Amersham-Pharmacia), using High Prime (Roche Diagnostics, E. Sussex, UK), and used for hybridisation as described elsewhere (Mayer et al. 2001). The filters were washed at high stringency.

Preparation of cell-free extracts

Fresh root tissue (1 g) was frozen in liquid nitrogen and ground into powder in the presence of 100 mg of insoluble polyvinylpyrrolidone (Polyclar AT), using a chilled mortar and pestle. The powder was then mixed thoroughly with 3 ml of chilled extraction buffer [100 mM Tris-HCl (pH 8.5), containing 10 mM EDTA and 20 mM DTT]. After clarification of the extract by centrifugation at 0–4 °C, the extract was passed at 0–4 °C through a Sephadex G25 column (PD-10; Amersham-Pharmacia) pre-equilibrated with extraction buffer. The extract was then divided into small aliquots, frozen in liquid nitrogen and held at –70 °C prior to assay. No loss of enzyme activity was detected after storage at –70 °C.

Immunochemical techniques

HCHL, purified from ferulate-grown *Pseudomonas fluorescens* strain AN103 by ion-exchange chromatography followed by chromatofocusing (Mitra et al. 1999), was used to raise polyclonal anti-HCHL antibodies in rabbits, essentially as outlined by Robins (1994). Three successive injections of HCHL protein (50 μg) were made at 3-week intervals and rabbits were bled at 8 weeks.

Discontinuous SDS-PAGE of protein extracts was performed essentially as described by Laemmli (1970), with a resolving gel containing 12% (w/v) acrylamide, and using an Atto AE 8210 apparatus (Genetic Instrumentation, Great Dunmow, Essex, UK). Proteins resolved by SDS-PAGE were electroblotted onto Immobilon-P transfer membrane (Millipore UK, Watford, Herts.) with a semi-dry Milliblot transfer system used according to the manufacturer's directions (Millipore), blotted standard proteins (Gibco-BRL, high-molecular-weight range) being included and visualised by Coomassie staining. Western analysis using the polyclonal antibodies raised to HCHL was performed essentially as described by Karakas Sen et al. (1999).

HCHL activity assay

HCHL activity was detected by a stopped HPLC assay using feruloyl-CoA as substrate, as described by Mitra (1999). The standard reaction mixture contained extract (20 μl; approx. 3 μg of protein) and 0.15 mM feruloyl-CoA in 100 mM Tris-HCl buffer (pH 8.5) in a reaction volume of 100 μl. Reaction mixtures were routinely incubated at 30 °C for 5 min and stopped by adding 100 μl of 2 M acetic acid in methanol. Control reaction mixtures (100 μl) routinely lacked extract. Vanillin determination by HPLC (50 μl injection vol.) was carried out using a Lichrosorb 10 μm C18 column (25 cm long, 4.6 mm i.d.; Phase Sep, Deeside, Clwyd, UK), with absorbance detection at 310 nm by means of a SpectraFOCUS scanning diode-array detector (ThermoSeparation Products, Stone, Staffs., UK). Linear gradient elution was used at 1.2 ml min^{-1}; solvent A was 20 mM sodium acetate (pH 6.0), solvent B was methanol and the elution gradient was as follows: $t = 0$ min, 100% A; $t = 15$ min, 90% A; $t = 40$ min, 50% A; $t = 45$ min, 30% A; $t = 50$ min, 100% A. The response factor for authentic vanillin was 390 Detector Area Response Units $pmol^{-1}$ and the detection limit (per injection) was 10 pmol.

Assay of phenylalanine ammonia-lyase (PAL)

PAL was assayed at 36 °C in 100 mM Tris-HCl buffer (pH 8.5) containing 20 mM L-phenylalanine and 15 μl of extract (approx. 2 μg of protein) in a total volume of 100 μl. Reactions were stopped after 15 min by adding 100 μl of 2 M acetic acid in methanol. Control reaction mixtures lacked extract or contained boiled extract. Cinnamic acid was determined by HPLC using a Columbus 5 μm reverse phase C-18 column (25 cm long, 4.6 mm i.d.; Phenomenex, Herts, UK), with linear gradient elution and detection at 290 nm, using an authentic standard for calibration; the flow rate was 1 ml min^{-1}; solvent A was 1 mM aqueous trifluoroacetic acid and solvent B was acetonitrile; the gradient profile was as follows: $t = 0$ min, 75% A; $t = 10$ min, 0% A; $t = 15$ min, 75% A.

Assay of protein

Protein was assayed by the method of Bradford (1976), using commercially prepared reagent (Bio-Rad, Hemel Hempstead, Herts., UK) and bovine serum albumin (Sigma) as a standard.

Extraction and analysis of soluble phenolic compounds and alkaloids

Powdered root tissue (1 g) was added to methanol (70%, v/v; 5 ml) and vortex-mixed. After clarification by centrifugation, the supernatant solution was diluted ×2 with water and analysed by HPLC (20 μl injection vol.) using a Columbus 5 μm column (Phenomenex) with gradient elution. Gradient conditions were: solvent A, 1 mM trifluoroacetic acid; solvent B, acetonitrile; flow rate, 1 ml min^{-1}; at $t = 0$ min, A = 100%; at $t = 50$, 60 and 65 min, A = 60%, 30% and 100%, respectively. A SpectraFOCUS scanning diode-array detector (ThermoSeparation Products) was used to identify the major phenolic compounds. Quantification was based on absorbance measurements at 255 nm for 4-hydroxybenzoic acid-β-D-glucoside and glucose ester, at 210 nm for 4-hydroxybenzyl alcohol glucoside, at 290 nm for vanillic acid-β-D-glucoside, at 325 nm for caffeoyl putrescine and feruloyl putrescine and at 210 nm for the tropane alkaloid, hyoscyamine. Authentic standards were available (see *Chemicals*) for all these compounds and were used to calculate HPLC response factors. Tropane alkaloids were extracted and determined according to Payne et al. (1987).

Extraction of cell-wall material

Cell-wall material was isolated from frozen root tissues essentially according to the method of Parr et al. (1996). Root tissue (ca. 1 g of

fresh mass) was ground to powder in the presence of liquid nitrogen. The powder was blended with 1.5% (w/v) SDS containing 5 mM sodium metabisulphite and stirred for 10 min at room temperature. The homogenate was filtered through 50-μm nylon mesh and the residue was washed with cold 0.75% aqueous SDS containing 3 mM sodium metabisulphite. The washed residue was ball-milled (Pascall ball mill, 0.5 l capacity; Fisher Scientific, Loughborough, UK) at 2–3 °C in 0.5% (w/v) SDS, containing 3 mM sodium metabisulphite, for 2 h at 60 rpm, filtered, resuspended in cold 3 mM sodium metabisulphite and again filtered. The residue was resuspended and re-filtered a further three times until the cell-wall residue was free of starch granules as assessed by light microscopy and iodine/potassium iodide staining. The cell-wall residue was further extracted with boiling ethanol, washed three times with acetic anhydride and finally air-dried in the dark at room temperature. Approx. 15–30 mg of cell-wall material was obtained per gram of root tissue.

Extraction and HPLC analysis of lignin fragments

Cell-wall material was analysed for lignin fragments by the DFRC (Derivatisation Followed by Reductive Cleavage) method of Lu and Ralph (1997). Isolated cell-wall material (15 mg) was first solubilised and acetylated, by suspending in 3 ml of acetyl bromide/acetic acid (1:4, v/v) and stirring for 3 h at 50 °C. The solvents were removed by evaporation under vacuum at 40 °C. Reductive cleavage of β-aryl ethers was achieved by stirring the residue, dissolved in dioxane/acetic acid/water (5:4:1, by vol.), with zinc dust (50–100 mg) for 30 min at room temperature. After filtration, the filtrate was evaporated to dryness at 40 °C under vacuum. The residue, acetylated lignin fragments, was dissolved in 0.2–1.0 ml of methanol and analysed using a Spherisorb 5 chiral (25 cm long, 4.6 mm i.d.) column (Phase Sep HPLC), with 50 μl sample injection vol., 1 ml min^{-1} flow rate and linear gradient elution with 1 mM aqueous trifluoroacetic acid (solvent A) and acetonitrile (solvent B). Gradient conditions were: $t = 0$ min, 100% A; $t = 50$ min, 65% A; $t = 65$ min, 0% A. Detection was achieved with a SpectraFOCUS (ThermoSeparation Products) scanning UV detector, with quantitation by integration of peak areas at 255 nm and calibration using acetylated coniferyl and sinapyl alcohols prepared according to Beerhues and Berger (1994).

Mass spectrometry

Mass spectrometry/liquid chromatography analysis of soluble phenolic compounds was performed as described by Mayer et al. (2001).

Results

Transformation of *Datura stramonium*

A range of hairy-root lines was established by transformation of *D. stramonium* with *A. rhizogenes* LBA 9402 harbouring the pmHCHL or pBin19 vector, followed by selection for kanamycin resistance. Five transgenic lines (H-4, H-6, H-8, H-10 and H-11) were established by transformation with *A. rhizogenes* LBA 9402 containing pHCHL, whilst four control lines (C-2, C-5, C-6 and C-7) were similarly produced by transformation with *A. rhizogenes* containing the unmodified pBin19 vector. All the transgenic lines were shown by Southern analysis to contain the HCHL gene, whereas the gene appeared absent from control lines (Fig. 2).

All these lines showed a profusion of root hairs and a high degree of lateral branching. Growth rates were variable, but generally rapid, typically with a doubling time of ca. 4 days. There was no consistent difference between the growth rates of transgenic and control lines (data not shown). There was, however, a clear colour difference between the control and transgenic lines towards the end of the culture cycle (3 weeks), with all the transgenic lines developing a reddish coloration.

HCHL gene expression

Transcription of the HCHL gene was revealed by RNA gel-blot analysis (Fig. 3a). Clear signals were observed when root culture RNA was probed under high-stringency conditions with the HCHL open reading frame. The transcript size (ca. 1,000 nucleotides) was as predicted and high levels of HCHL transcript were noted in three lines (H-6, H-10 and H-11), though levels in lines H4 and H8 were much lower. There was no evidence of transcript expression in control lines. Transcript levels measured at intervals throughout the culture cycle, in line H-10, appeared constant (data not shown).

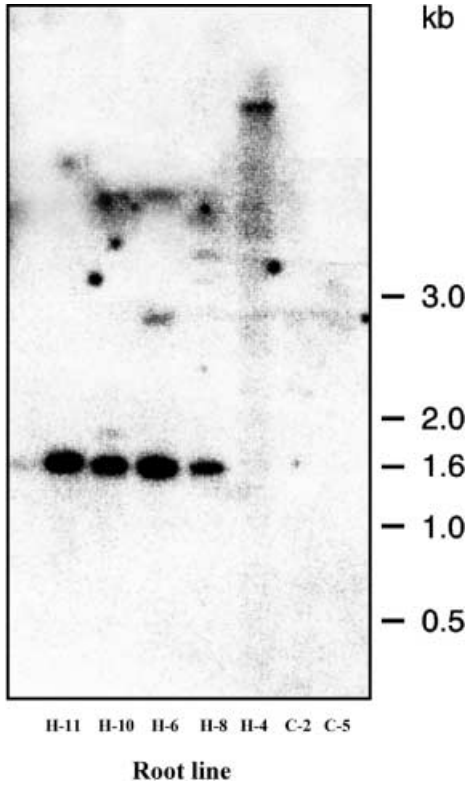

Fig. 2 Genomic Southern analysis of the HCHL gene in HCHL-transgenic (*H-4*, *H-6*, *H-8*, *H-10* and *H-11*) and control (*C-2* and *C-5*) lines of *Datura stramonium* hairy roots. Experimental details are as described in *Materials and methods*. Genomic DNA was restricted with *Eco*RI. Restriction sites were –731 bp and 841 bp from the start of the HCHL gene, at the 3′ end of the 35S promoter and of the terminator, respectively, of the construct pmHCHL (Mayer et al. 2001). Lanes contained ca. 10 μg of genomic DNA

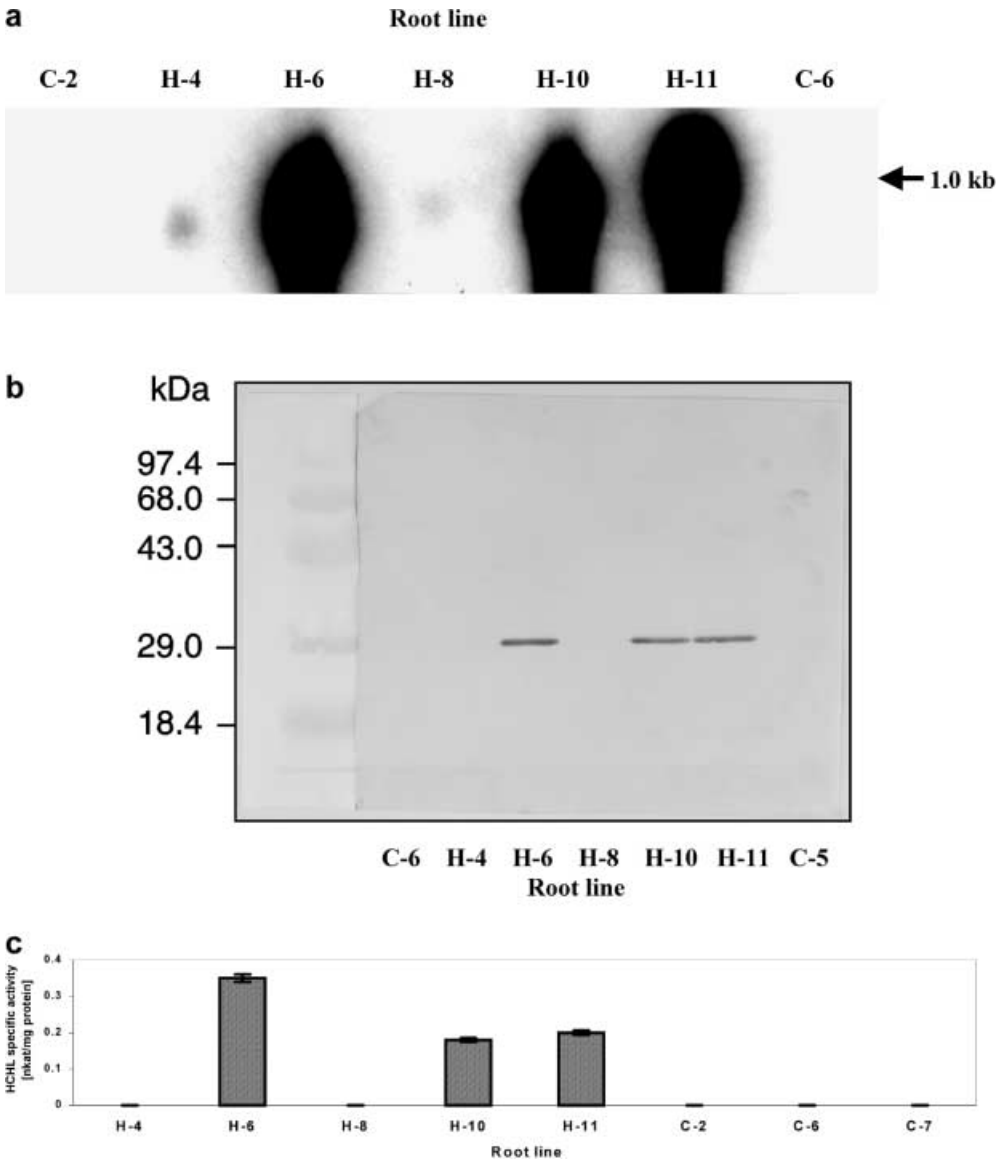

Fig. 3 **a** RNA gel-blot analysis of HCHL gene expression in total RNA extracted 21 days from subculture from HCHL-transgenic (*H-4*, *H-6*, *H-8*, *H-10* and *H-11*) and control, empty-vector-transformed (*C-2* and *C-6*) lines of *D. stramonium* hairy roots. **b** Immunoblot of protein extracted 21 days from subculture from HCHL-transgenic (*H-4*, *H-6*, *H-8*, *H-10* and *H-11*) and control (*C-5* and *C-6*) lines, using a polyclonal antibody raised against HCHL purified from ferulate-grown *Pseudomonas fluorescens* AN103. **c** Specific activity of HCHL (nkat mg^{-1} of extract protein) determined 21 days from subculture in HCHL-transgenic (*H-4*, *H-6*, *H-8*, *H-10* and *H-11*) and control (*C-2*, *C-6* and *C-7*) lines. HCHL activity was determined at pH 8.5 and 30 °C using the stopped HPLC assay described and values are the means ± SD of at least three independent determinations

Immunoblot analysis using polyclonal antibodies raised against HCHL enzyme purified from *P. fluorescens* strain AN103 revealed a single band of the appropriate molecular mass (31 kDa) in lines H-6, H-10 and H-11, but not in lines H-4 and H-8 or in control lines (Fig. 3b). This correlated broadly with the results of RNA gel-blot analysis, where only very weak evidence of transcription in lines H-4 and H-8 was observed. Similarly, there was no HCHL enzyme activity detectable in lines H-4 and H-8, or in control lines. The specific activity in line H-6, determined 21 days after subculture, was ca. 0.35 nkat mg^{-1} of extract protein, at 30 °C; values in lines H-10 and H-11 were somewhat lower – ca. 0.2 nkat mg^{-1} (Fig. 3c). In line H-10, and in agreement with the RNA gel-blot analysis, there was little variation in HCHL activity determined at different stages in the culture cycle (see Fig. 5d). For comparison, the specific activity of the endogenous PAL in these extracts was not substantially greater and ranged between 0.4 nkat mg^{-1} of protein (lines C-2 and H-8) and 2.2 nkat mg^{-1} of protein (line H-10), determined at 36 °C (data not shown).

Soluble phenolic compounds

Soluble phenolic compounds present in the roots were analysed by diode-array HPLC. The principal soluble phenolic components in extracts of control lines were caffeoyl putrescine and feruloyl putrescine (Fig. 4); 4-coumaroyl putrescine was not detected. The total content of these two substances was ca. 0.08–0.15 mg g^{-1} fresh mass of roots. In the HCHL-transgenic lines, there was some evidence of a decline in the levels of feruloyl putrescine, whilst levels of caffeoyl putrescine remained essentially unaffected. The level of feruloyl putrescine in line H-8, which expressed no measurable HCHL activity, was equivalent to that in the three control lines.

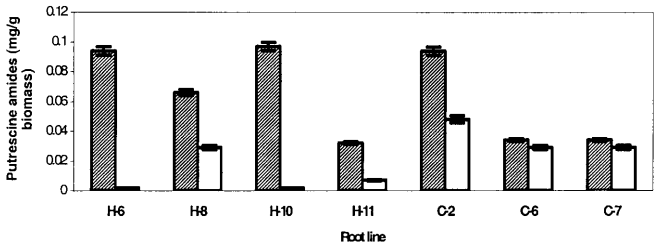

Fig. 4 Levels of caffeoyl putrescine (*hatched bars*) and feruloyl putrescine (*open bars*) 21 days from subculture in HCHL-transgenic (*H-6*, *H-8*, *H-10* and *H-11*) and control (*C-2*, *C-6* and *C-7*) lines of *D. stramonium* hairy roots. Values are means ± SD of at least three independent determinations

In extracts of lines expressing HCHL, several uncharacteristic compounds became the principal soluble phenolic components (Fig. 5a, b; Table 1). Analysis revealed the formation of glucosides of 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol (with glucose conjugated to the phenolic hydroxyl group) at mg g^{-1} of fresh mass levels, together with 4-hydroxybenzoic acid glucose ester and, in much smaller amounts (Table 1 and data not shown), the glucoside of vanillic acid (4-hydroxy-3-methoxybenzoic acid). 3,4-Dihydroxy-substituted (protocatechuic) derivatives were not detected. The identity of these compounds, including the position of glucose conjugation, was confirmed by diode-array UV-spectral analysis in comparison with authentic standards and by electrospray mass spectrometry, as described in detail elsewhere (Mayer et al. 2001). These compounds were detected at appreciable levels only in those lines (H-6, H-10 and H-11) that were actively expressing HCHL (Table 1); levels appeared much lower, or undetectable, in weakly expressing (H-4 and H-8) and control lines. All these compounds appeared to be present entirely in the root tissue and were not detectable in the culture medium. Free 4-hydroxybenzaldehydes, 4-hydroxybenzoic acids and 4-hydroxybenzyl alcohols were not detected in any of the root lines.

Levels of the tropane alkaloid, hyoscyamine, were found to be characteristic of *D. stramonium* root cultures (Payne et al. 1987) and unaffected by the expression of HCHL (data not shown).

Lignin analysis

Cell-wall material of cultures 21 days from subculture was extracted by ball-milling and analysed for lignin by the DFRC procedure of Lu and Ralph (1997). By far the major lignin fragment detected in extracts from control lines was acetylated coniferyl alcohol, together with traces of acetylated sinapyl alcohol. The levels of acetylated coniferyl alcohol in control lines (C-2 and C-6) and in an HCHL-transgenic line that did not express detectable HCHL activity (H-8) ranged between 0.2 and 0.7 mg g^{-1} of cell-wall material, appreciably higher than the levels (< 0.1 mg g^{-1}) in the HCHL-transgenic lines H-6, H-10 and H-11 (Fig. 6), suggesting that HCHL expression diminished the formation of lignin in these root cultures. Recoveries of cell-wall material by these procedures are not accurately known, however, so whilst comparative estimates are meaningful, precise quantitative values are not obtainable.

Variation of HCHL expression and product levels during culture cycle

In the comparatively high-expressing line H10, the content of 4-hydroxybenzoic acid-β-D-glucoside and related glucose conjugates (Fig. 5c and data not shown) did not vary greatly throughout the culture cycle, broadly consistent with near-constant HCHL activity (Fig. 5d). Over a 28-day period, the activity of HCHL was throughout 0.06–0.07 nkat g^{-1} of fresh mass (Fig. 5d) and the levels of 4-hydroxybenzoic acid-β-D-glucoside were between 2.5 and 3.8 mg g^{-1} of fresh mass (Fig. 5c); the levels of the other glucose conjugates were similarly near-constant (data not shown). Over this period, the fresh weight of the culture increased from ca. 0.3 g to 4.7 g (Fig. 5d), with the roots beginning to develop a reddish coloration around day 15 and entering stationary phase around day 30.

These determinations enable an approximate comparison to be drawn between the measured HCHL activity and the accumulation of 4-hydroxybenzaldehyde derivatives. In line H10, soluble glucose-conjugated 4-hydroxybenzaldehyde derivatives accounted in total for ca. 0.5% of the fresh mass (Table 1). Therefore, over a 7-day period from day 7 to day 14, when the tissue fresh mass increased from 0.5 g to 3.2 g (Fig. 5d), an accumulation of ca. 40 μmol of glucose-conjugated 4-hydroxybenzaldehyde derivatives can be calculated, equating to a production rate of ca. 0.06 nmol s^{-1}. Over the same period, the HCHL activity (determined at 30 °C and with saturating feruloyl-CoA as substrate) was ca. 0.06 nkat g^{-1} fresh mass (Fig. 5d), equivalent to a time-averaged total tissue activity of ca. 0.12 nkat, assuming a time-averaged tissue fresh mass of ca. 2 g. The in vitro activity of HCHL was therefore of the order of twice the rate of production of 4-hydroxybenzaldehyde derivatives. For further comparison, the endogenous PAL activity that was measured in 21-day cultures of line H10 was around 0.3 nkat g^{-1} of fresh mass (assayed at 36 °C), around 5-fold higher than the measured HCHL activity and therefore some 5–10 times the production rate of 4-hydroxybenzaldehyde derivatives.

Discussion

HCHL under the control of the CaMV 35S promoter was actively expressed in hairy-root cultures of *Datura stramonium*, as confirmed by RNA gel-blot and enzyme-activity analyses. The measured activity of HCHL was comparable to the measured activity of endogenous

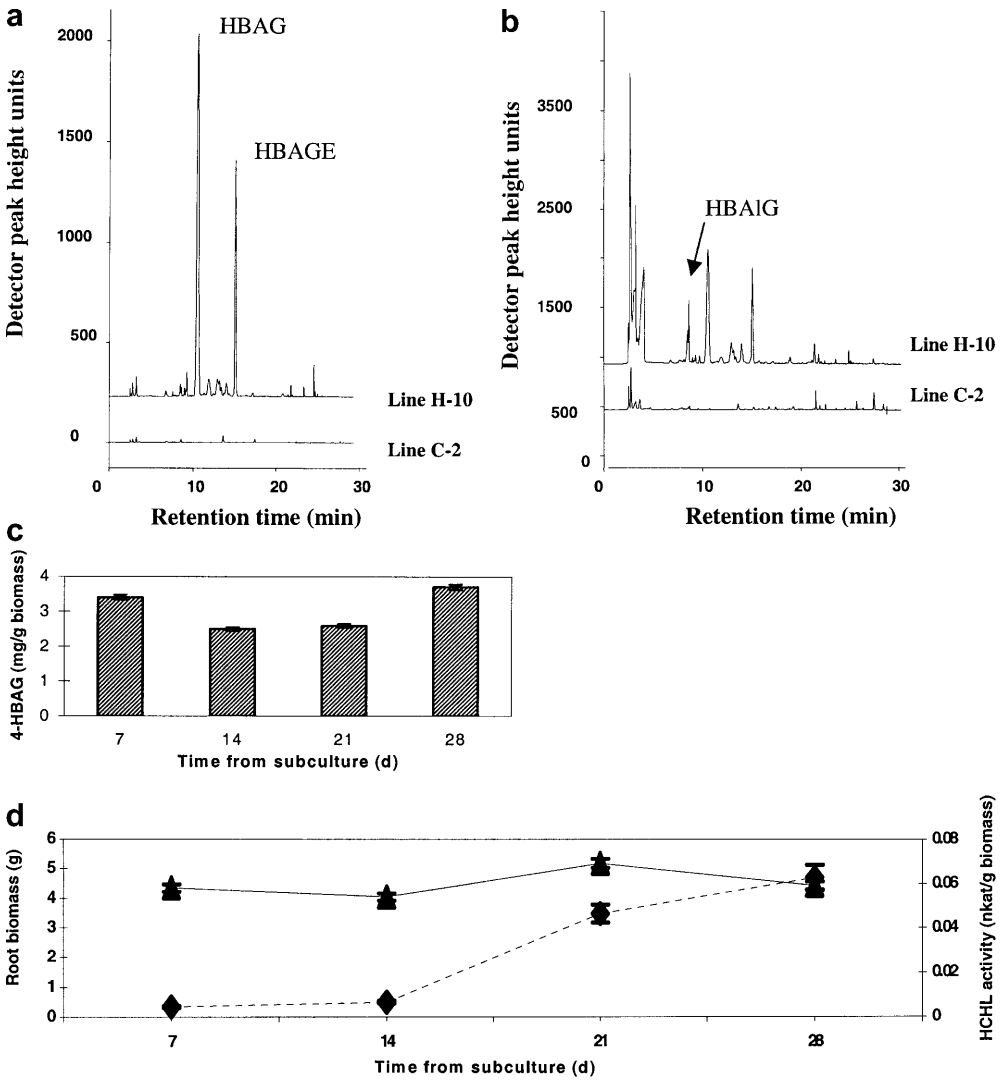

Fig. 5a–d Analysis of soluble phenolic glucose conjugates in HCHL-transgenic (*H-10*) and control (*C-2*) lines of *D. stramonium* hairy roots. **a** HPLC chromatogram of 21-day methanolic extract with detection at 255 nm. **b** HPLC chromatogram of 21-day methanolic extract with detection at 210 nm. **c** Content of 4-hydroxybenzoic acid-*β*-D-glucoside (*HBAG*) in line H-10 determined at 7, 14, 21 and 28 days from subculture. **d** HCHL activity and root biomass (*broken line*) in line H-10 at 7, 14, 21 and 28 days from subculture. Values in **c** and **d** are the means ± SD of at least three independent determinations. *HBAG* 4-Hydroxybenzoic acid-*β*-D-glucoside, *HBAGE* 4-hydroxybenzoic acid glucose ester, *HBAlG* 4-hydroxybenzyl alcohol-*β*-D-glucoside. Relative molar peak area response factors (HBAG and HBAGE determined at 255 nm; HBAlG determined at 210 nm) were 1.00 (HBAG), 1.70 (HBAGE) and 0.22 (HBAlG). Compounds were identified by liquid chromatography/mass spectrometry, including comparison with authentic standards, analysing full-scan UV spectra and the mass spectra of the ($[M + Na]^+$) adducts (mass 323, HBAG, HBAGE; mass 309, HBAlG), as described by Mayer et al. (2001)

PAL, suggesting the potential to cause an evident metabolic diversion of the phenylpropanoid pathway.

This diversion was, in the event, clearly observed. Calculation of an approximate rate of formation of 4-hydroxybenzaldehyde derivatives in line H-10 accounted for ca. one-half of the activity of HCHL measured with feruloyl-CoA as substrate (though at saturating substrate concentrations, the V_{max} of HCHL with 4-coumaroyl-CoA is twice the value with feruloyl-CoA – Mitra et al. 1999). By similar calculations, these soluble products of the HCHL reaction also have accounted for around 10–20% of the measured activity of PAL.

The findings invite obvious comparison with the effects of expression of the *ubiC* gene encoding chorismate:pyruvate lyase (CPL) in plants and cell cultures of *N. tabacum* (Siebert et al. 1996; Li et al. 1997) and in hairy-root cultures of *Lithospermum erythrorhizon* (Sommer et al. 1999). In *N. tabacum* plants expressing *ubiC* under the control of a constitutive promoter, with targeting of CPL to the plastid, the principal intracellular location of chorismate, the products of 4-hydroxybenzoic acid consisted almost entirely of the *β*-D-glucoside and the glucose ester, with the glucoside representing about 69% of the total (Siebert et al. 1996). These two compounds together amounted to ca. 0.52% of the dry mass of leaves. The measured CPL activity, at 37 °C, was in the same plants around 0.21 nkat mg^{-1} of protein. This is very comparable to the activity of HCHL measured here in line H-10 (0.18 nkat mg^{-1} of protein, at 21 days; 30 °C), yet in line H-10 the content of 4-hydroxybenzaldehyde derivatives was around 0.5% of the fresh mass of roots. This suggests that HCHL can be at least as effective in causing metabolic diversion as CPL. Results are not available for expression of CPL in hairy roots of *Datura* spp. or *Nicotiana* spp.; the most evident metabolic effect of expression in hairy roots of *L. erythrorhizon* was to cause an unexpected 5-fold increase in the formation of menisdaurin, a nitrile glucoside not obviously related to the reaction catalysed by CPL (Sommer et al. 1999).

HCHL has recently been expressed in tobacco plants (Mayer et al. 2001) and shown to lead in aerial parts to the formation of 4-hydroxybenzaldehyde derivatives, along with a range of, in general, deleterious phenotypic

Table 1 The contents of four glucose conjugates determined 21 days from subculture in several HCHL-transgenic (*H-4*, *H-6*, *H-8*, *H-10* and *H-11*) and control (*C-2*, *C-5*, *C-6* and *C-7*) lines of *D. stramonium* hairy roots. Determination was by HPLC. *HBAG* 4-Hydroxybenzoic acid-β-D-glucoside, *HBAGE* 4-hydroxybenzoic acid glucose ester, *HBAlG* 4-hydroxybenzyl alcohol-β-D-glucoside, *HMBAG* 4-hydroxy-3-methoxybenzoic acid-β-D-glucoside, *n.d.* not detectable

Root line	Glucose conjugates (μg g^{-1} fresh mass)			
	HBAG	HBAGE	HBAlG	HMBAG
H-4	40	5	110	n.d.
H-6	2,200	350	1,280	130
H-8	16	36	70	n.d.
H-10	2,800	180	1,730	140
H-11	4,400	930	2,760	200
C-2	4	2	40	n.d.
C-5	10	2	70	n.d.
C-6	2	4	n.d.	n.d.
C-7	3	6	n.d.	n.d.

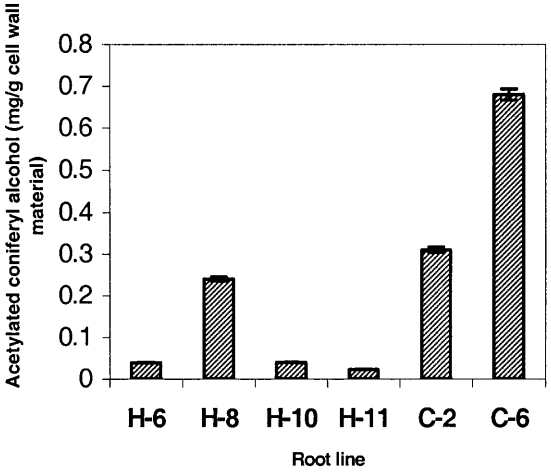

Fig. 6 Levels of acetylated coniferyl alcohol determined as a result of lignin analysis in HCHL-transgenic (lines *H-6*, *H-8*, *H-10* and *H-11*) and control (*C-2* and *C-6*) lines of *D. stramonium* hairy roots. Values are means ± SD of at least three independent determinations

effects. Interestingly, although HCHL activity levels in the transgenic tobacco leaves were similar to those reported here, the content of 4-hydroxybenzaldehyde derivatives was in general about 5-to 10-fold lower, similar to the values obtained by Siebert et al. (1996) in leaves of tobacco expressing CPL. It is unknown whether this might reflect a lower overall phenylpropanoid pathway flux in leaves than in root cultures. On the other hand, very high levels of 4-hydroxybenzaldehyde derivatives, even larger than those reported here, and including appreciable amounts of vanillic acid β-D-glucoside, were found in the seed capsules of one of the transgenic tobacco plants. Although the 4-hydroxybenzaldehyde derivatives formed in the tobacco plants were the same compounds as those observed here, they were produced in a somewhat different ratio, with an appreciably higher proportion of the alcohol conjugate, 4-hydroxybenzyl alcohol β-D-glucoside, being present in the *Datura* roots. The reason for this difference is unclear.

The phenolic reaction products of CPL and HCHL, 4-hydroxybenzoate and 4-hydroxybenzaldehyde, respectively, differ only in their state of oxidation; however, whereas chorismate is the sole physiological substrate for CPL, HCHL catalyses reactions of three substrates, 4-coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA, with broadly similar K_m and V_{max} values (Mitra et al. 1999). The prevalence of novel products with the 4-hydroxyphenyl substitution pattern – and the complete absence of those with the protocatechuic pattern – in lines expressing HCHL suggests that in these lines much lower concentrations of caffeoyl-CoA and feruloyl-CoA than of 4-coumaroyl-CoA were available to react with the HCHL enzyme. As indicated in Fig. 4, however, the major soluble phenolic compounds in control hairy-root lines of *D. stramonium* (in agreement with observations in *Hyoscyamus muticus* – Medina-Bolivar and Flores 1995) were the putrescine conjugates of caffeic and ferulic acids; and these conjugates, especially caffeoyl putrescine, were still made in the lines expressing HCHL (Fig. 4), indicating that their precursors caffeoyl-CoA and feruloyl-CoA must still have been produced and were available for conjugation with putrescine. In principle, HCHL might be expected to tend to deplete the availability of caffeoyl-CoA and feruloyl-CoA by cleaving 4-coumaroyl-CoA, since 4-coumaroyl-CoA is an immediate metabolic precursor of both, though a pathway to caffeoyl-CoA and feruloyl-CoA involving free 4-coumaric, caffeic and ferulic acids may also be functional (Whetten and Sederoff 1995). In any event, the findings presented here are suggestive of some type of metabolic channelling in the formation and utilisation of 4-hydroxycinnamoyl-CoA thioesters. In a number of other plant tissues, labelling experiments and studies with isolated microsomes have suggested channelling of the two initial reactions of the phenylpropanoid pathway, PAL and cinnamic acid 4-hydroxylase (reviewed by Winkel-Shirley 1999; see Rasmussen and Dixon 1999); and in the monolignol pathway, isoenzymes (notably of 4-coumaric acid: CoA ligase and caffeic acid *O*-methyltransferase) exhibiting differing substrate specificities (Ehlting et al. 1999; Grima-Pettenati and Goffner 1999; Maury et al. 1999) are likely to be important features of an organised network for the channelling and progressive ring-substitution of 4-hydroxycinnamic acids, their CoA thioesters and monolignols. It is important to recognise that there remains scant information on the regulation and compartmentation of the phenylpropanoid pathway and its branch pathways in roots.

There was only one visible phenotypic effect of HCHL expression: a red-brown coloration reminiscent of the changes in xylem lignin coloration variously observed in plants down-regulated for specific enzymes of the monolignol pathway, including cinnamyl alcohol dehydrogenase, 4-coumarate: CoA ligase and cinnamoyl-CoA reductase (reviewed by Piquemal et al. 1998).

This has also been observed in tobacco plants engineered to express HCHL (Mayer et al. 2001). It will be of interest to discover whether, as proposed in other cases, this coloration is caused by an accumulation of cinnamaldehydes or cinnamic acids within lignin. The results presented here (Fig. 6) throw no light on this, but indicate that expression of HCHL may diminish the incorporation of coniferyl alcohol residues into cell-wall lignified material. This is consistent with the suggestion of lower levels of feruloyl putrescine in lines expressing HCHL presented in Fig. 4, since both might plausibly result from a diversion of 4-coumaroyl-CoA towards 4-hydroxybenzaldehyde formation and away from feruloyl-CoA formation.

The formation of glucosides and glucose esters of hydroxybenzoic acids has been known for many years (Cooper-Driver et al. 1972; Klick and Herrmann 1988). Indeed, conjugation of phenolic compounds with glucose or other sugars is widespread if not ubiquitous in higher plants, where it may serve variously to increase water solubility, limit oxidation, permit transport, and protect the plant against toxicity or untimely physiological responses which would be caused by the free compounds (Lee et al. 1995; Chong et al. 1999; Hipskind and Paiva 1999). The free compounds are then assumed to be released by enzymic hydrolysis if or when required, for example as signal or defensive compounds in response to pathogen attack, or for incorporation into the cell wall. In addition to endogenous compounds, phenolic substances supplied exogenously to plant cell and organ cultures are also very actively glycosylated (Walton et al. 1999). Thus, this capacity for conjugation readily explains the absence of free C_6-C_1 metabolites observed here and is in agreement with the data of Siebert et al. (1996), who observed only very small amounts of free 4-hydroxybenzoate in leaves of plants of *N. tabacum* expressing CPL. Two constitutively expressed glucosyltransferases which could form, respectively, the β-D-glucoside and the glucose ester of 4-hydroxybenzoate, were detected in cell-free extracts of *N. tabacum* cell cultures (Li et al. 1997).

It is interesting to compare the absence of free 4-hydroxybenzaldehydes and their glucosides observed in the present work with the results of feeding vanillin exogenously to various cell cultures. Vanillin β-D-glucoside, along with vanillyl alcohol, was found to be an early product of vanillin fed to cell cultures of *N. plumbaginifolia* (Schroeder et al. 1997) whilst at later times, the two isomeric vanillyl alcohol β-D-glucosides were detected. Cultures of *Catharanthus roseus* produced as much as 1.54 g l^{-1} of vanillin β-D-glucoside (6% of the dry weight of the culture) within 24 h (Sommer et al. 1997). This suggests that the lack of accumulation of glucosides of 4-hydroxybenzaldehydes observed in the present work may be due to a form of metabolic channelling, the metabolism of endogenous 4-hydroxybenzaldehydes being quite distinct from that of the exogenous compounds supplied at high concentrations. In addition, as discussed above, the particular lack of accumulation of vanillin and its derivatives that we observed here very likely reflects a low availability of feruloyl CoA as a substrate for HCHL. In this connection, it is remarkable that immature pods of *Vanilla* spp. are able to accumulate very high concentrations (in excess of 2% of dry mass) of vanillin-β-D-glucoside (Rao and Ravishankar 2000; Dignum et al. 2001). This becomes hydrolysed to vanillin after harvest, during curing. The role of feruloyl-CoA in this biosynthesis, implied many years ago (Zenk 1965), has never in fact been established, although a two-carbon chain-shortening of a phenylpropanoid precursor, probably in the form of a CoA thioester and occurring via β-oxidation or retro-aldol cleavage (see Yazaki et al. 1991; Schnitzler et al. 1992; Löscher and Heide 1994; Mitra et al. 1999; Jarvis et al. 2000), seems certain to occur. The biosynthesis of vanillin-β-D-glucoside in *Vanilla* appears to be an unexpectedly complex process (Funk and Brodelius 1990a, b; Funk and Brodelius 1992; Dignum et al. 2001), belying the structural similarity between ferulic acid and vanillin and possibly involving several glucose conjugates (Kanisawa et al. 1994). Whatever the mechanism, however, the biosynthetic process occurring in *Vanilla* must limit or prevent oxidation or reduction of the vanillin aldehyde group once it is formed. Engineering into other plant species a capacity to accumulate vanillin-β-D-glucoside, as a source of vanillin flavour, would require both the precursor supply and the stability of the vanillin aldehyde group to be addressed, together with compartmentation to protect from endogenous β-glucosidases.

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