Over-expression of cinnamate 4-hydroxylase leads to increased accumulation of acetosyringone in elicited tobacco cell-suspension cultures

Abstract Cell-suspension cultures were produced from transgenic tobacco (Nicotiana tabacum L.) plants harboring a constitutively expressed alfalfa cinnamate 4-hydroxylase (C4H) transgene. Increased levels of C4H enzyme activity in the transgenic cultures were observed only following exposure of the cells to yeast elicitor, although alfalfa C4H transcripts were expressed at a high level from the cauliflower mosaic virus 35S promoter in the absence of elicitation. Increased expression of C4H in elicited cell-suspension cultures had no appreciable effect on the HPLC profiles of soluble phenolic compounds. However, levels of one compound, subsequently identified as 3,5-dimethoxy-4-hydroxy acetophenone (acetosyringone), were strongly elevated in the wall-bound phenolic fraction. The results are discussed in relation to the correlation between C4H activity and the synthesis of 3,5-dimethylated hydroxycinnamic acid derivatives in tobacco.

Keywords Acetosyringone biosynthesis · Metabolic engineering · Nicotiana (cell-suspension culture) · Phenylpropanoid pathway

Introduction

The phenylpropanoid pathway leads to a wide variety of bioactive plant natural products, and has recently become the target for a number of studies aimed at increasing the production of such metabolites by genetic engineering (Dixon et al. 1996; Dixon and Steele 1999). These studies have either employed over-expression of enzymes at the entry point into the pathway or its various branch pathways (Howles et al. 1996; Blount et al. 2000), or have exploited manipulation of transcriptional regulators to effect more global control through coordinated regulation of series of pathway enzymes (Tamagnone et al. 1998; Borewitz et al. 2001).

The first reaction committed to phenylpropanoid metabolism is the deamination of L-phenylalanine to trans-cinnamic acid, catalyzed by L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5). Over-expression of PAL from a bean PAL transgene in tobacco results in a proportional increase in the levels of the major soluble phenylpropanoid compound chlorogenic acid (caffeoyl quinic acid) in leaf tissue, and to a smaller increase in the flavonoid rutin (Howles et al. 1996). PAL over-expression also results in increased production of salicylic acid, formed via chain-shortening of cinnamic acid, particularly following infection (Felton et al. 1999). Salicylic acid is a signal molecule for local and systemic disease resistance responses, and increasing its production by engineering PAL expression is accompanied by increased resistance to viral pathogens (Felton et al. 1999), although this is associated with increased susceptibility to insect herbivory (Felton et al. 1999).

The second reaction of the phenylpropanoid pathway is catalyzed by the membrane-associated cinnamic acid 4-hydroxylase (C4H, EC 1.14.13.11), a cytochrome P450 enzyme. Down-regulation of C4H expression in transgenic tobacco harboring an antisense alfalfa C4H construct results in reduced levels of chlorogenic acid and the phenylpropanoid polymer lignin (Sewalt et al. 1997; Blount et al. 2000), effects which are also observed following PAL down-regulation (Bate et al. 1994).
reduction in C4H activity appears to cause a corresponding decrease in PAL activity, possibly via a negative feedback loop involving sensing of cinnamic acid levels (Blount et al. 2000). However, whereas strong reduction in PAL activity leads to reduced lignin levels with an increased lignin syringyl/guaiacyl ratio (S/G ratio, recording the relative proportions of dimethylated S units and monomethylated G units in the lignin), down-regulation of C4H results in a similar reduction in lignin levels but a vastly reduced S/G ratio as a result of a near complete blockage in the synthesis of S units (Sewalt et al. 1997). It is not clear why down-regulation of consecutive enzymes at the entry point into the phenylpropanoid pathway should have such different impacts on downstream end products.

The metabolic consequences of transgenic over-expression of C4H have been less well studied than the effects of PAL over-expression. An accumulation of chlorogenic acid in leaves has been described (Blount et al. 2000). In the present manuscript, we describe the behavior of cell-suspension cultures derived from tobacco plants with increased C4H activity resulting from expression of an alfalfa C4H transgene. Such cell lines produce significantly larger quantities of a wall-bound phenolic compound following exposure to yeast elicitor than do wild-type cultures. This compound was characterized as acetosyringone, a dimethylated acetoephonone with the same ring substitution pattern as the S lignin unit.

Materials and methods

Transgenic cell-suspension cultures

All recombinant DNA techniques in *Escherichia coli* (strains HB101 and DH5α) were carried out according to standard procedures (Sambrook et al. 1989).

The BamHI/SalI fragment of the W2A yeast expression vector harboring the alfalfa C4H transgene in the sense orientation (Fahrenhodt and Dixon 1993) was cloned in place of the β-glucoronidase (GUS) gene in the binary vector pB121 under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator. This binary vector construct was used to transform *Agrobacterium tumefaciens* strain LBA4404 using a freeze-thaw method (Chen et al. 1994). Transgenic tobacco (*Nicotiana tabacum L.* cv. Xanthi) plants harboring the alfalfa C4H cDNA in the sense orientation were generated by *Agrobacterium*-mediated leaf-disc transformation (Masoud et al. 1993). After co-cultivation, leaf disks were plated on BM medium (1–1: 4.3 g l–1 glucose, 4.3 g Murashige and Skoog salts, 30 g sucrose, 100 mg inositol, 10 mg thiamine, 1 mg nicotinic acid, 1 mg pyridoxine, 250 mg casamino acids, 4 g phytof. 14CPhenylalanine-feeding experiment

Dark-grown cell cultures (10-ml batches) were treated with yeast elicitor or water (control) at 3 days post-subculture, and immediately labeled with 75 kBq of L-[U-14C]phenylalanine (17 GBq mmol−1; Amersham Pharmacia, Bucks., UK). Cells were harvested 24 h after treatment, and methanolic extracts prepared as described previously for HPLC analysis (Howles et al. 1996). Ten-microliter samples were applied to silica gel TLC plates, which were developed in toluene:ethyl acetate:methanol:petroleum ether (6:4:0.5:3, by vol.), and the positions of labeled spots determined by autoradiography.

HPLC analysis of phenolic compounds

One-gram batches of frozen tissue were extracted and analyzed for soluble phenolics by HPLC as previously described (Howles et al. 1996), monitoring with a diode array detector at 254 nm, 270 nm, and 310 nm. The wall-bound phenolics were extracted from the cell residues previously extracted for soluble phenolics as previously described (Blount et al. 2000), separated by HPLC as above, and monitored at 254 nm, 270 nm, and 310 nm. The unidentified compound with a retention time (RT) of 31 min in the wall-bound phenolic fraction of elicited C4H over-expressing cells was purified by HPLC using a two-step process in which the analytical gradient of Howles et al. (1996) was the first step. The fraction was collected, concentrated and then re-injected onto the HPLC under isocratic conditions of 22% acetonitrile, 78% 1% phosphoric acid.