Phytochemistry 71 (2010) 751–759

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

The biosynthesis of acetovanillone in tobacco cell-suspension cultures

Jonathan Negrel *, Francine Javelle

INRA, UMR Plante-Microbe-Environnement, BP 86510, 17 Rue Sully, 21065 Dijon Cedex, France

ARTICLE INFO

Article history:
Received 26 November 2009
Received in revised form 19 January 2010
Available online 24 February 2010

Keywords:
Nicotiana tabacum
Solanaceae
Tobacco
Biosynthesis
Acetovanillone
Acetosyringone
Apocynin
Hydroxycinnamoyl-CoA derivatives
Jasmonic acid

ABSTRACT

A soluble enzyme, extracted from tobacco cell-suspension cultures 24 h after treatment with 100 μM methyl jasmonate, has been shown to synthesize acetovanillone (apocynin) from feruloyl-CoA in the presence of NAD. The enzyme displayed Michaelis–Menten kinetics with apparent K_m values of 5.6 μM for feruloyl-CoA and 260 μM for NAD and exhibited very high specificity for its substrates. The increase in acetovanillone synthase activity was followed by an increase in the concentration of both acetovanillone and acetosyringone in the culture medium. No intermediate could be detected when analysing the reaction medium by HPLC during the formation of acetovanillone in cell-free extracts. The apparent molecular mass estimated by gel permeation on an FPLC column was ca. 79 kDa. To our knowledge, this is the first report of an enzymic system catalysing the synthesis of an acetophenone. This work demonstrates that the biosynthesis of acetophenones in tobacco proceeds from hydroxycinnamic acids through a CoA-dependent β-oxidation pathway. Interestingly in methyl jasmonate-treated cells, which synthesize very large amounts of hydroxycinnamoylputrescines, inhibition of the synthesis of these conjugates increased the concentration of acetovanillone and acetosyringone in the culture medium, suggesting that the two metabolic pathways can compete for their common precursors, i.e. hydroxycinnamoyl-CoA thioesters.

1. Introduction

Acetophenones are known to be wound-induced plant compounds involved in the activation of the virulence genes of *Agrobacterium tumefaciens* (Stachel et al., 1985; Spencer and Towers, 1988; Duban et al., 1993). Although they have repeatedly been identified in cell cultures of the Solanaceae after elicitation (Threlfall and Whitehead, 1988; Miguel and Barroso, 1994; Mühlenbeck and Barz, 1996; De Rosa et al., 1996; Blount et al., 2002; Baker et al., 2002, 2005a) the exact pathway of their biosynthesis is still unknown. The ring substitution pattern of acetosyringone and acetovanillone suggests that they could derive from the corresponding hydroxycinnamic acids but the key chain-shortening reactions from the acids to the acetophenones have never been characterized (Spencer and Towers, 1991; Blount et al., 2002). The same situation holds with respect to the formation of acetophenones from cinnamic acids in microorganisms (Hilton and Cain, 1990; Shanker et al., 2007). By contrast the enzymes and genes involved in the biosynthesis of structurally more complex ketones, such as benzophenones, have already been identified (Liu et al., 2003).

In the course of a study of methyljasmonate-induced changes in the regulation of various secondary metabolites in tobacco cell-suspension cultures, we observed a massive accumulation of hydroxycinnamoylputrescines (HCPs) (*p*-coumaroyl-, caffeoyl- and feruloylputrescine) in the cells and a concomitant increase of acetovanillone and acetosyringone concentration in the culture medium. Surprisingly, inhibition of the synthesis of the putrescine conjugates resulted in an increase of the concentration of acetophenones in the medium, suggesting that the two families of phenolic compounds could compete for the same phenolic precursors. Since HCPs are formed from hydroxycinnamoyl-CoA derivatives (Meurer-Grimes et al., 1989; Negrel, 1989), we tested these thioesters as potential precursors of acetophenones in cell-free extracts of methyljasmonate (MeJa) treated cells. In this study we show that these extracts catalyse the synthesis of acetovanillone from feruloyl-CoA in the presence of NAD. This paper reports a preliminary characterization of the corresponding enzymatic activity, referred to as acetovanillone synthase (AVS), and which may catalyse a key step in the biosynthesis of acetophenones in the Solanaceae. To our knowledge, it is the first characterization of an enzymic system catalysing the synthesis of an acetophenone *in vitro*.

2. Results and discussion

2.1. Exogenously supplied MeJa induces a dramatic accumulation of HCPs in tobacco cell-suspension cultures and an increase of acetophenones concentration in the culture medium

In the course of a study of the effect of exogenous MeJa on secondary metabolism in tobacco cell suspensions, we observed a

* Corresponding author. Tel.: +33 (0)380693165; fax: +33 (0)380693753.
E-mail addresses: negrel@dijon.inra.fr, jmnegrel@free.fr (J. Negrel).

0031-9422/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2010.01.011

massive accumulation of HCPs in the treated cells (Fig. 1A). The three amides were readily detected by HPLC analysis of methanolic extracts of these cells and their concentration increased regularly during 3 days. Caffeoylputrescine accounted for ca. 75% of the mixture (Fig. 1A). Altogether, and using an optimal concentration of MeJa (100 μM), the total amount of HCPs reached 18.6 μmol per g fr. wt (ca. 10% of the dry wt) after 3 days, a figure comparable to the yield obtained in *p*-fluorophenylalanine resistant tobacco cells derived from the TX1 cell line used in this study, and which have been found to accumulate HCPs (Berlin et al., 1982). MeJa caused however severe growth inhibition of the treated cells (ca. 33% inhibition on a fresh wt basis after 3 days), confirming previous work on tobacco BY-2 cells (Swiatek et al., 2002). The accumulation of HCPs was not surprising since MeJa has been found to trigger the accumulation of putrescine conjugates in many plants or cell-suspension cultures (Chen et al., 2006 and references therein) and recently a transcription factor regulating the synthesis of these amides in tobacco in response to MeJa has been identified (Galis et al., 2006). In our experiments the amides accumulated within the cells but caffeoylputrescine was also detectable at low concentration in the culture medium of both control and MeJa-treated cells. Interestingly MeJa treatment decreased the concentration of caffeoylputrescine in the culture medium (Fig. 1B). In the course of the analysis of the extracellular medium of treated cells, we detected an increase in the concentration of two relatively hydrophobic products, with retention times exceeding those of HCPs. After extraction with ethylacetate, concentration and purification by HPLC, these two products were identified by GC–MS as acetovanillone and acetosyringone, their concentration in the culture medium reaching 40 and 5 μM, respectively after 72 h (Fig. 1B). Both ketones were undetectable in the cell extracts, at least in free form. These results confirm previous work describing the occurrence of both HCPs and acetophenones in the extracellular medium of tobacco cell suspensions (Baker et al., 2002, 2005a) and they demonstrate for the first time that the synthesis of acetophenones can be induced by MeJa.

2.2. DL-α-difluoromethylornithine (DFMO), a specific irreversible inhibitor of ornithine decarboxylase, inhibits the synthesis of HCPs in tobacco cells and reinforces the accumulation of acetophenones in the culture medium

MeJa is known to induce the expression of the ornithine decarboxylase gene in tobacco cell suspensions (Imanishi et al., 2000). While trying to inhibit the MeJa-induced HCPs synthesis using DFMO, a powerful irreversible inhibitor of plant ornithine decarboxylase (Kumar et al., 1997), we observed, as expected, a dramatic decrease of HCPs concentration (Fig. 1A) but also, and more surprisingly, a marked increase in acetovanillone and acetosyringone concentration in the culture medium (Fig. 1B). This observation led us to surmise that these acetophenones could derive from the same phenolic precursors than HCPs, i.e. from hydroxycinnamoyl-CoA thioesters. It seemed therefore possible that acetophenones could be formed from hydroxycinnamic acids through a CoA dependant β-oxidation pathway reminiscent of fatty acid β-oxidation, leading to phenyl-β-keto-propionic acids. Such β-keto

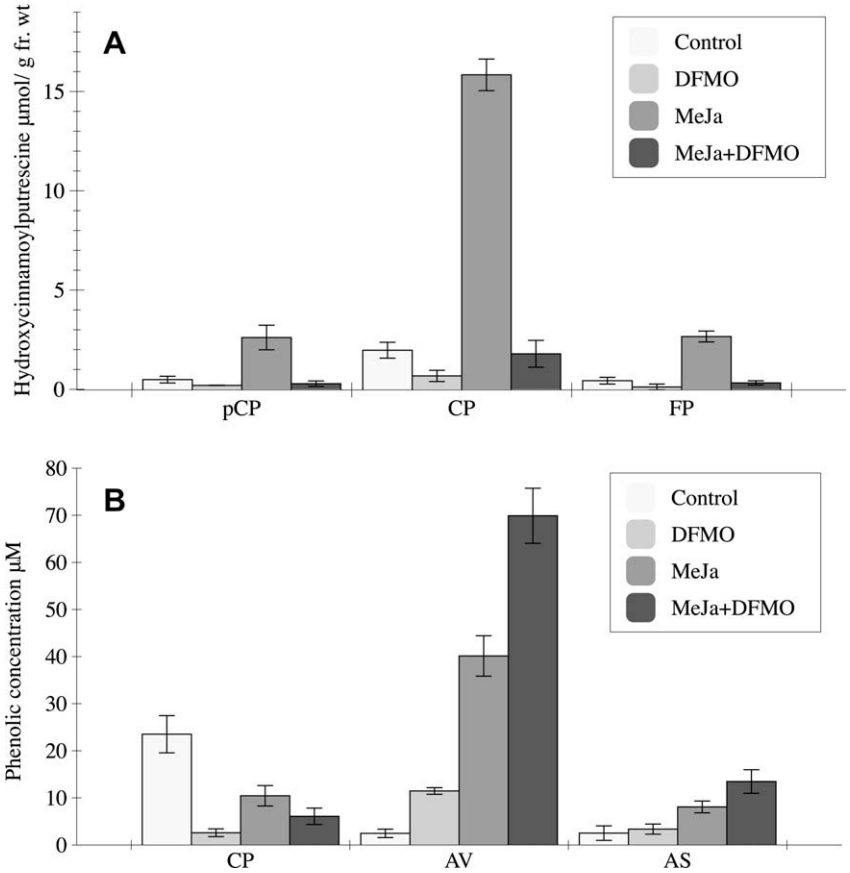

Fig. 1. Combined effects of MeJa and DFMO on the accumulation of putrescine conjugates and acetophenones in tobacco cell-suspension cultures. (A) Induction by MeJa and inhibition by DFMO of the accumulation of HCPs in the cells. pCP, *p*-coumaroylputrescine; CP, caffeoylputrescine; FP, feruloylputrescine B; effect of MeJa and DFMO on the concentration of caffeoylputrescine (CP), acetovanillone (AV) and acetosyringone (AS) in the culture medium of tobacco cells. Cell suspensions (75 ml) were treated with 75 μl 0.1 M MeJa and/or 75 μl 0.1 M DFMO 3 days after subculture and harvested 72 h later. Control cells were treated with 75 μl MeOH. *p*-Coumaroylputrescine and feruloylputrescine occurred only in trace amount in the medium and were not quantified. The data shown represent the results of three independent experiments.

acids, which are known to undergo facile decarboxylation (March et al., 1982) would then lead directly to the corresponding ketones. Although this pathway has to our knowledge never been demonstrated neither in plants nor in microorganisms, 3-keto-3-phenylpropionic acid has been shown to transiently accumulate in a mutant strain of an unclassified *Pseudomonas* sp. growing on cinnamic acid and converting it to acetophenone, suggesting that this conversion could proceed through a β-keto acid intermediate (Hilton and Cain, 1990). In order to test this hypothesis we therefore tried to incubate enzymic extracts obtained from MeJa-treated tobacco cells in the presence of different hydroxycinnamoyl-CoA derivatives with the coenzymes used for β-oxidation reactions, i.e. NAD or NADP, and to try to detect the formation of the corresponding acetophenones by HPLC.

2.3. Cell-free extracts of MeJa-treated tobacco cells catalyse the formation of acetovanillone from feruloyl-CoA and NAD

When crude enzymic extracts of tobacco cell-suspension cultures treated with 100 μM MeJA for 24 h were incubated with feruloyl-CoA in the presence of NAD in Tris HCl buffer at pH 8, formation of acetovanillone could be evidenced by HPLC analysis of aliquots of the incubation medium. A typical chromatogram obtained after 30 min incubation is shown in Fig. 2. In the absence of NAD, feruloyl-CoA was rapidly hydrolysed by thioesterases and phosphatases so that ferulic acid, dephosphoferuloyl-CoA, and feruloyl-phosphopantetheine were very rapidly formed (Negrel and Smith, 1984). In the presence of NAD, these side reactions were still very rapid but an additional peak could be detected on the chromatogram (Fig. 2). The corresponding product was purified by HPLC. It showed the same R_t, UV spectrum and R_f after TLC as authentic acetovanillone (see Section 4). Its identity was further confirmed by GC–MS analysis. No intermediate between feruloyl-CoA and acetovanillone could be detected in the incubation medium by analysing aliquots at different time intervals. No acetovanillone synthesis was detected when the enzyme, the substrate, or the coenzyme, was omitted, or when a heat-inactivated enzymatic extract was used. Moreover, when feruloyl-CoA was replaced by ferulic acid, no product formation occurred. The enzymic system catalysing acetovanillone synthesis was soluble (after centrifugation at 100,000 *g* for 1 h, all the activity was still in the supernatant). Maximal activity was found at pH 8 in Tris–HCl buffer. The same activity was detected at pH 8 in K–Pi buffer and half maximal activity was obtained at pH 7.1 and 8.8 in Tris–HCl buffer.

The enzymic system exhibited very high specificity towards feruloyl-CoA and NAD. All the other cinnamoyl-CoA derivatives tested (cinnamoyl-CoA, *p*-coumaroyl-CoA, caffeoyl-CoA and sinapoyl-CoA) were inactive as substrates. It is noteworthy that absolutely no activity was detected with sinapoyl-CoA, neither in crude nor in partially purified extracts although acetosyringone was rapidly formed *in vivo*. Moreover we did not detect any change in the substrate specificity in cells taken at different time intervals after treatment with MeJa (24, 48 or 72 h). No activity was detected when using NADP as coenzyme. Moreover NAD/NADP mixtures were always less effective than NAD alone (36% inhibition in the presence of 1 mM NADP in the standard photometric assay). Further work is however required to assert that the enzymic system exhibits absolute specificity towards feruloyl-CoA and NAD, since the activity of other potential substrates such as 5-hydroxy-feruloyl-CoA has not yet been tested.

The proposed pathway of acetovanillone biosynthesis from feruloyl-CoA is shown in Fig. 3, the overall reaction being summarised by the following equation:

$$\text{Feruloyl-CoA} + \text{NAD} + 2\text{H}_2\text{O} \rightarrow \text{acetovanillone} + \text{NADH} + \text{CoA} + \text{CO}_2$$

The formation of NADH in the incubation medium could be detected by HPLC at 340 nm, but only transiently. Rapid degradation of NADH and coenzyme A occurred in crude enzyme extracts. We did not attempt to detect the formation of CO_2 in the course of this preliminary work since this would have required the synthesis of [carboxy-^{14}C]-feruloyl-CoA. The fact that the reaction requires NAD suggests that it involves hydration of the CoA thioester and oxidation of the hydroxyl group to a ketone (Fig. 3). Acetovanillone hence probably arises by the decarboxylation of 4-hydroxy-3-

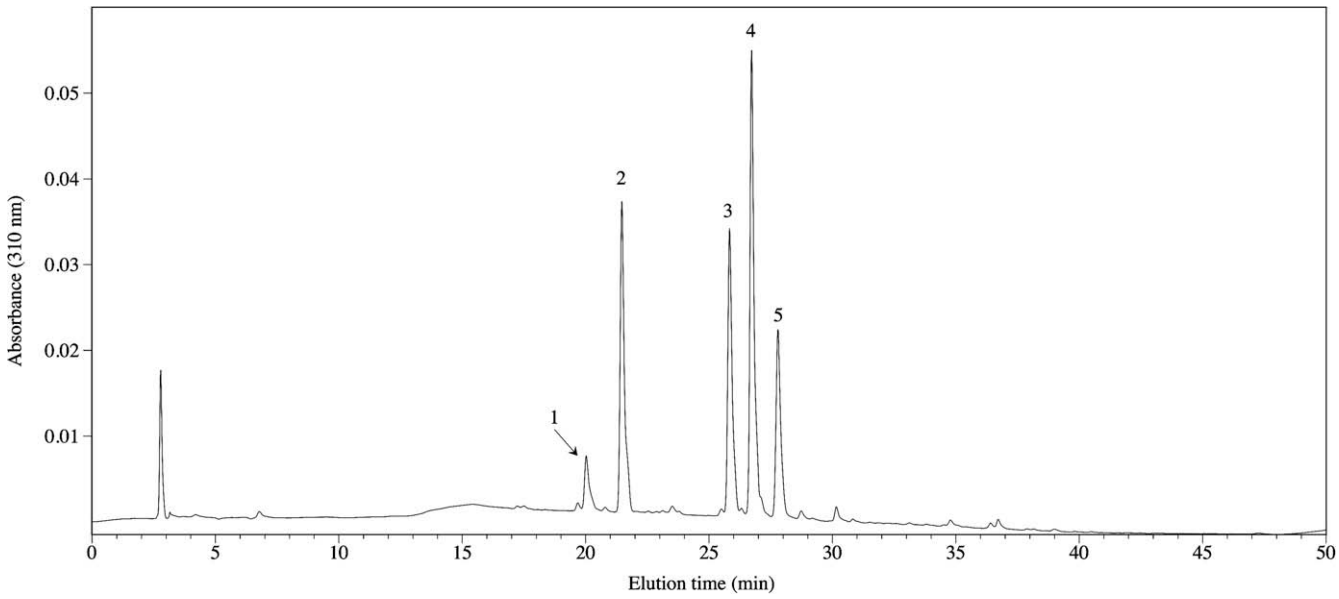

Fig. 2. Formation of acetovanillone in enzymatic extracts of MeJa-treated tobacco cells: HPLC analysis of the incubation medium after 30 min reaction time with feruloyl-CoA and NAD as substrates. (1) Acetovanillone, (2) ferulic acid, (3) feruloyl-CoA, (4) feruloyl-dephospho-CoA and (5) feruloyl-phosphopantetheine. Formation of NADH could be transiently detected at 340 nm (R_t 4.27 min). Compounds 2, 4 and 5 are also formed from feruloyl-CoA in the absence of NAD (data not shown). After 2 h reaction time acetovanillone and ferulic acid were the only products detectable at 280, 310 or 340 nm. No formation of vanillin or vanillic acid could be detected. After 1 h in the absence of NAD, feruloyl-CoA was completely hydrolysed to ferulic acid.

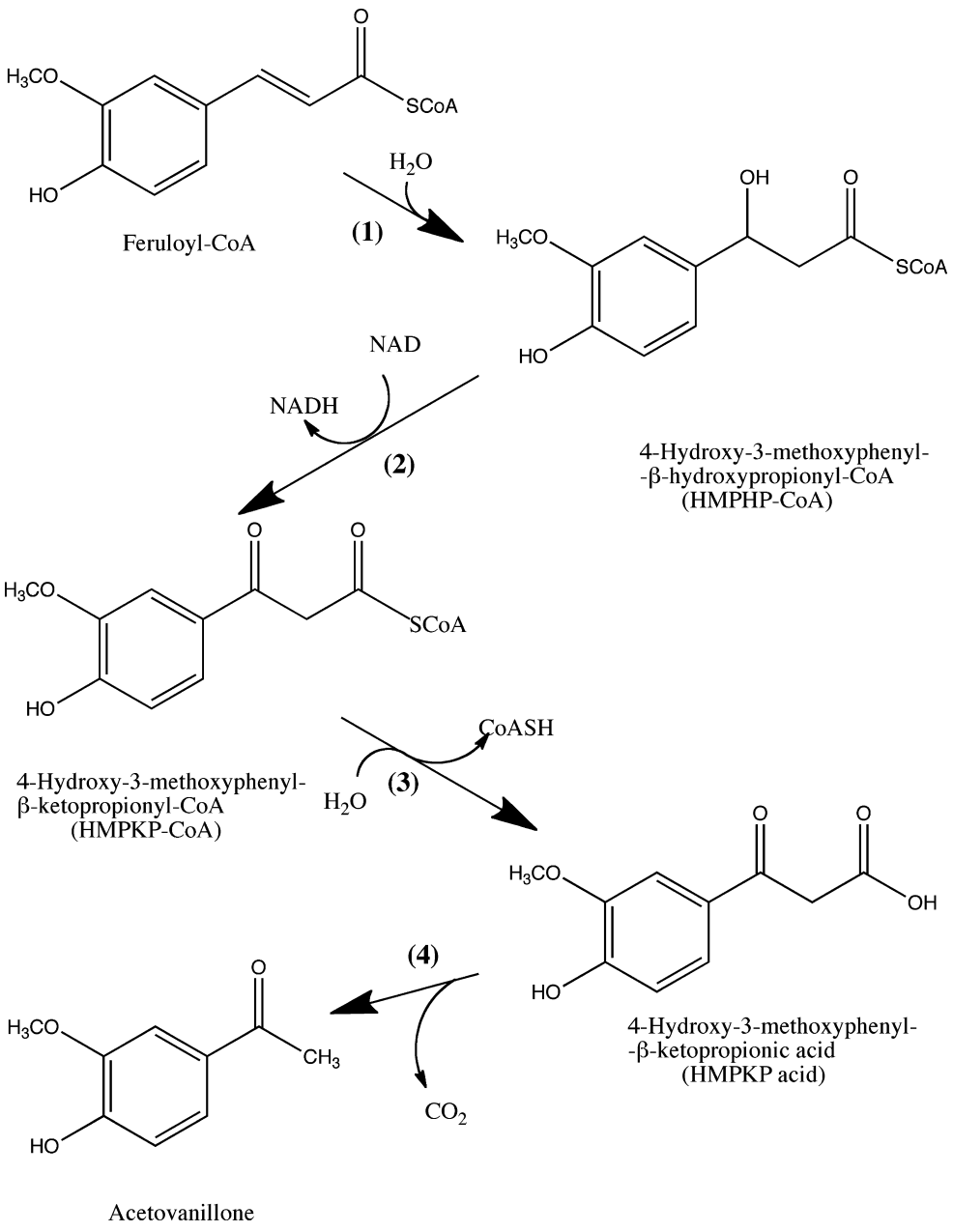

Fig. 3. Proposed pathway of acetovanillone formation from feruloyl-CoA in *Nicotiana tabacum*. (1) feruloyl-CoA hydratase activity, (2) HMPHP-CoA dehydrogenase activity, (3) HMPKP-CoA thioesterase activity, (4) HMPKP acid decarboxylase activity.

methoxyphenyl-β-ketopropionic acid. No accumulation of unidentified products, which may have corresponded to the putative intermediates described in Fig. 3, was observed during kinetic analysis of acetovanillone formation from feruloy-CoA and NAD. The detection of such intermediates may of course be very difficult in crude enzymic extracts containing high concentration of feruloyl-CoA hydrolysis products. It is nevertheless likely that these labile intermediates are not released in the incubation medium (Mitra et al., 1999).

The formation of acetovanillone could be monitored spectrophotometrically at 350 nm. The change in absorbance at this wavelength is the result of the decrease in the absorbance of feruloyl-CoA when it is transformed to acetovanillone and of the increase in absorbance of NAD when it is reduced to NADH. Best results were however obtained at 380 nm. At this wavelength at pH 8 the absorbance of NADH and acetovanillone is negligible so that the decrease in absorbance is only due to the NAD-dependent disappearance of feruloyl-CoA. The enzymatic activity was therefore calculated from the initial velocity of the reaction monitored at 380 nm (see Section 4). Good correspondence was observed between the activity measurements based on the photometric assay and the quantification of acetovanillone formation in the same extracts by HPLC. This was important since incubation of feruloyl-CoA in an unpurified extract could also have led to the formation of vanillic acid in the presence of NAD (Abd El-Mawla and Beerhues, 2002; Wildermuth, 2006). We therefore used the photometric assay, which theoretically measures the feruloyl-CoA hydratase/4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA (HMPHP-CoA) dehydrogenase activity (Fig. 3), to determine the

properties of AVS and to monitor its activity. Using this assay AVS displayed Michaelis–Menten kinetics with apparent K_m values of 5.6 μM for feruloyl-CoA and 261 μM for NAD.

At present we do not know the number and the structure of the enzyme(s) which could catalyse the reactions depicted in Fig. 3. It seems however highly unlikely that they could be catalysed by independent enzymes. Plant multifunctional fatty acid oxidation proteins belonging to the enoyl-CoA hydratase superfamily possess L-3-hydroxyacyl dehydrogenase activity (Rylott et al., 2006 and references therein). Moreover the last two steps, i.e. hydrolysis of the thioester and decarboxylation of the β-keto acid, may practically correspond to simultaneous reactions, due to the ease of decarboxylation of the free β-keto acid (March et al., 1982). Moreover we have used anion exchange and gel permeation chromatography columns to attempt to partially purify the AVS activity. During these preliminary experiments good correspondence was again observed between the activity detected with the photometric assay and the detection of acetovanillone formation in the different fractions by HPLC. So far we therefore failed to separate even partially the feruloyl-CoA hydratase/HMPHP-CoA dehydrogenase activity from the overall AVS activity. The apparent molecular mass of native AVS was thus estimated by chromatography on a FPLC-Superose-12 HR column and found to be ca. 79 kDa (see Section 4). This relatively low molecular mass seems to exclude the possibility that AVS is a large multifunctional enzyme complex.

2.4. The AVS activity detected in tobacco cell suspensions after treatment with MeJa parallels the accumulation of acetovanillone in the culture medium

Fig. 4A shows the time course of changes in AVS activity, measured with the photometric assay, in tobacco cell-suspension

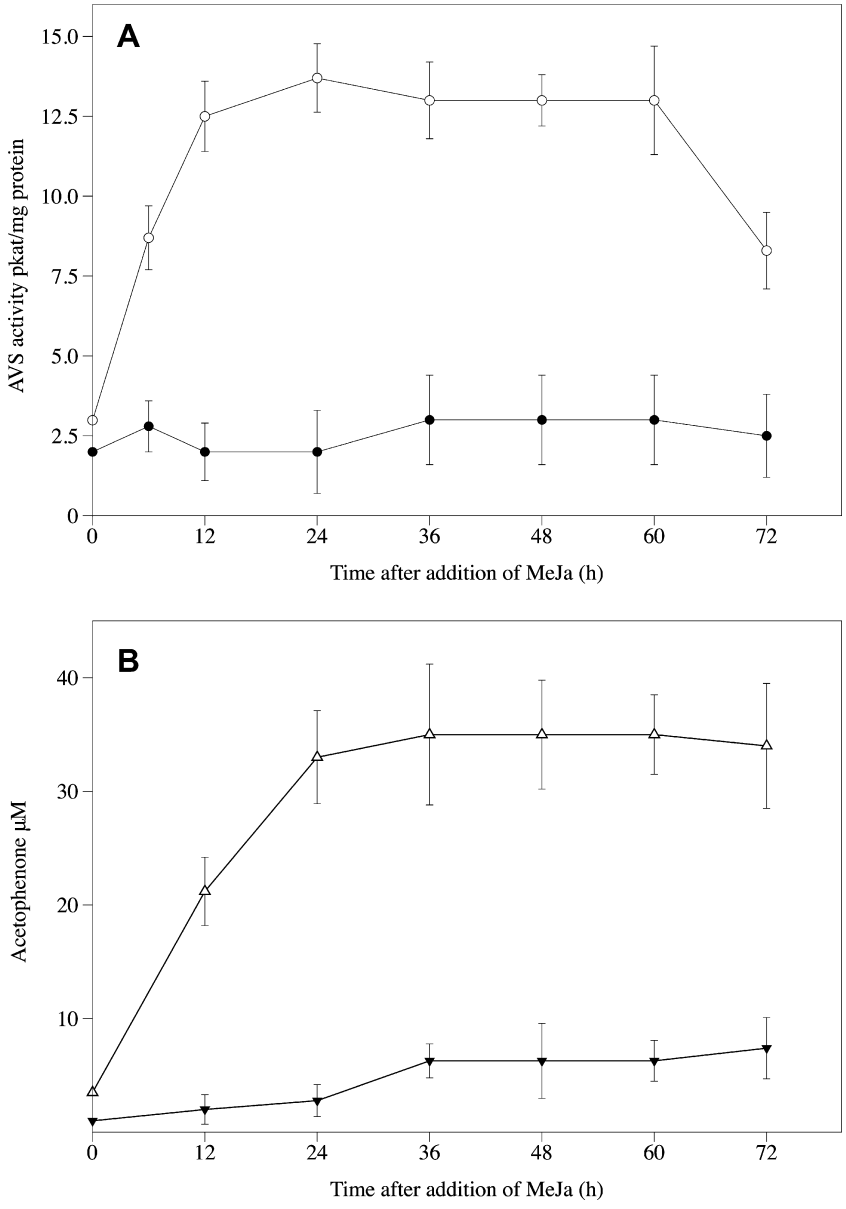

Fig. 4. Time course of acetophenone biosynthesis in MeJa-treated tobacco cell-suspension cultures. (A) Time course of changes in AVS activity measured with the photometric assay. Cell suspensions (75 ml) were treated 3 days after subculture with 75 μl 0.1 M MeJa (○) or 75 μl MeOH (●, control cells). (B) Accumulation of acetovanillone (△) and acetosyringone (▼) in the culture medium of MeJa-treated cells. The data shown represent the results of one experiment with 2 replicates. Values obtained at 6, 24, 48 and 72 h were checked several times during independent experiments and similar results were obtained. The concentration of acetovanillone and acetosyringone in the culture medium of control cells remained very low, below 5 and 2 μm, respectively.

cultures following treatment with 100 μM MeJa. The maximum enzyme activity was reached after 12 h and remained stable during 48 h, before declining slowly. The specific activity remained relatively low, never exceeding 15 pkat/mg protein in ammonium sulphate concentrated extracts. A faint activity could also be detected in control cells confirming that tobacco cell suspensions slowly synthesize acetophenones in the absence of MeJa treatment (Baker et al., 2005a). In the culture medium of MeJa-treated cells the concentration of acetovanillone increased very quickly during the rise of AVS activity (Fig. 4B). The pattern of accumulation of acetosyringone was quite different, its concentration rising slowly but continuously (Fig. 1B). The intensity of AVS induction and of acetophenone accumulation was directly connected to the age of the suspension cultures at the time of MeJa addition. Best results were obtained by treating the cell suspensions 1–3 days after subculture. Treatment of tobacco cells 1 week after subculture led to much lower specific activity (data not shown). Interestingly it is known that in plant/*Agrobacterium* co-cultivations only actively growing plant cell cultures are able to stimulate efficient *vir* gene expression (Stachel et al., 1985) and that in the entire plant acetophenones are mainly synthesized by metabolically active wounded cells (Stachel et al., 1985; Spencer and Towers, 1991).

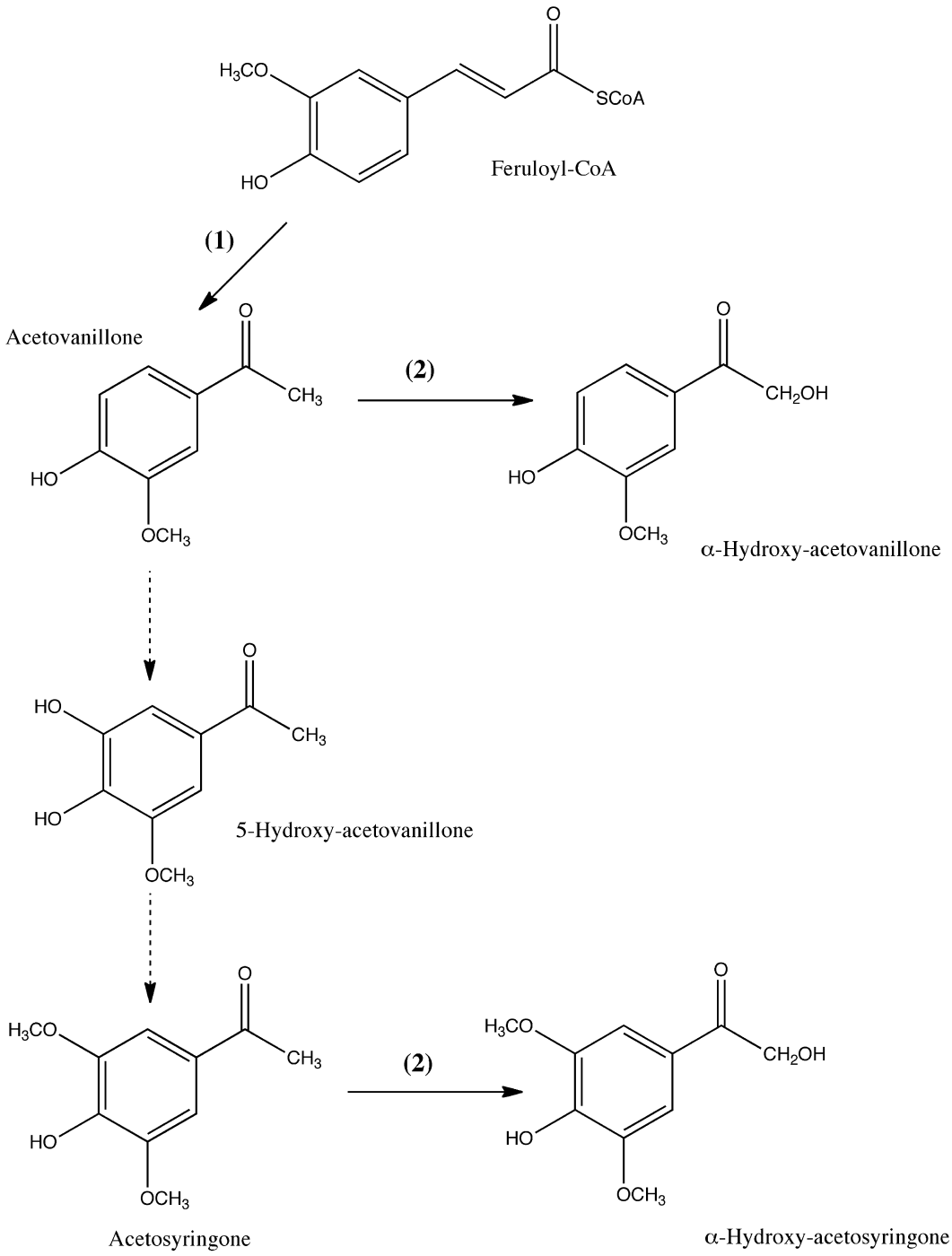

Fig. 5. Possible biosynthetic route to α-hydroxy-acetosyringone from feruloyl-CoA. Broken arrows indicate unknown conversions. (1) AVS activity (2) cytochrome-P450-dependant monooxygenase.

We also attempted to measure AVS activity in tobacco plants grown in pots in a greenhouse using the photometric assay. AVS activity was detectable only in root extracts. The specific activity (ca. 10 pkat/mg protein) was close to that measured in MeJa-treated cell suspensions. By contrast no activity could be detected in leaves or stems. HPLC analyses confirmed the occurrence of acetovanillone and acetosyringone in roots (13 nmol and 59 nmol/g fr. wt, respectively) and their absence from leaves and stems.

2.5. Acetosyringone may be synthesized via 5-hydroxyacetovanillone

One of the surprising results obtained during this study is that no formation of acetosyringone could be evidenced *in vitro* using sinapoyl-CoA as substrate, even in enzymatic extracts prepared from tobacco roots or cell cultures rapidly accumulating acetosyringone in the culture medium. It is of course possible that the β-oxidation of sinapoyl-CoA was too slow to be detected in the extracts and under the conditions we have used. An alternative explanation however is that acetosyringone is actually synthesized from acetovanillone. A product occurring at low concentration in the extracellular medium of cultured tobacco cells has previously been tentatively identified as 5-hydroxyacetovanillone (Baker et al., 2005a). 5-Hydroxyacetovanillone could be synthesized directly from acetovanillone and serve as an intermediate in the synthesis of acetosyringone, in which case AVS would play an important metabolic role since it would channel ferulic acid from the general phenylpropanoid pathway into the acetophenone biosynthetic route (Fig. 5). Alternatively, 5-hydroxyacetovanillone could be synthesized from the phenylpropanoid pathway by a parallel metabolic route. Tobacco *p*-coumarate:CoA ligase accepts *p*-coumarate, caffeate and ferulate as substrates but not sinapate, sinapoyl-CoA being synthesized from 5-hydroxyferuloyl-CoA (Pinçon et al., 2001). 5-Hydroxyacetovanillone could therefore also be formed directly from 5-hydroxyferuloyl-CoA. Work is in progress in our laboratory to test these different hypotheses.

3. Conclusions

Despite the relatively simple structure of acetophenones and despite their widespread occurrence among the Solanaceae (Spencer and Towers, 1991), the biochemical pathway leading to their formation has remained largely unknown nearly 25 years after the discovery of their role in the *Agrobacterium*–plant interactions (Stachel et al., 1985). The only enzyme involved in the biosynthesis of acetophenones characterized so far was a cytochrome-P450-dependant monooxygenase from *Solanum khasianum* catalysing the α-hydroxylation of acetovanillone (Fig. 5) (Mühlenbeck and Barz, 1997). This work therefore fills a gap in our understanding of the biosynthetic pathway of acetophenones from phenylpropanoids and opens the way to the purification of the enzymes involved in the chain-shortening reactions. The finding that feruloyl-CoA is a precursor of acetovanillone is also a new illustration of the versatility of hydroxycinnamoyl-CoA thioesters as intermediates in the biosynthesis of plant phenolics.

The finding that MeJa induces the synthesis of acetophenones in tobacco cell cultures is a new example of the importance of jasmonate signalling in the biosynthesis of secondary metabolites (Gundlach et al., 1992; Memelink et al., 2001; Sasaki-Sekimoto et al., 2005). This induction is consistent with the roles of jasmonates in the plant response to wounding (Leon et al., 2001; Koo and Howe, 2009). It is well established that in crown gall disease *Agrobacterium tumefaciens* enters plant tissues through wound sites in the host plant and that wounded cells are highly susceptible to transformation by the tumor-inducing plasmid. It seems therefore logical that jasmonates could mediate the wound-induced synthesis of signal molecules activating T-DNA transfer. Although acetophenones have been associated so far mainly with the plant/*Agrobacterium* interactions, recent work suggests that they could have a broader role in plant/pathogen interactions because of their antioxidant properties in the apoplasm (Baker et al., 2005b). Interestingly acetovanillone, which is also known as apocynin, is a potent inhibitor of NADPH oxidase activation in neutrophils (Stolk et al., 1994; Beukelman et al., 1995). Apocynin has also recently been shown to induce the synthesis of nitric oxide in corn (Tossi et al., 2009). Taken together these data suggest that acetophenones could exhibit biological activities independent from their known activity as signal molecules in crown gall disease. In this context the preliminary characterization of AVS activity described in this work should prove useful to monitor acetovanillone biosynthesis in plants responding to different stresses or interacting with microorganisms, especially among the Solanaceae. Further work is however necessary to purify and better characterize the enzyme(s) involved in this synthesis to validate the proposed pathway and to elucidate the biosynthetic route of acetosyringone.

4. Experimental

4.1. Plant material

The tobacco TX1 cell line (*Nicotiana tabacum* L. cv. Xanthi) used in this study was available in our laboratory (Negrel et al., 1992). The stock culture was originally provided by Dr. J. Berlin (Braunschweig, Germany). Cell-suspension cultures were grown in Murashige and Skoog medium containing 2 mg/l 2,4-D. 12.5 ml (approximately 2.5 g fr. wt) of the suspension were transferred to 62.5 ml fresh medium at 1-week intervals. Cells were treated with MeJa (75 µl of a 0.1 M solution in MeOH) 3 days after subculture and collected after 1–3 days by vacuum filtration. During initial experiments, different concentrations of MeJa were tested, from 25 to 200 µM. Highest concentration of HCPs and acetophenones were obtained at 100 µM so this concentration was used for all subsequent work. Just before collecting the cells an aliquot of the culture medium was centrifuged in an Eppendorf tube and frozen at −20 °C. The filtrated cells were frozen in liquid nitrogen and stored at −80 °C. Plants used to measure AVS activity (*Nicotiana tabacum* L. cv. Xanthi n.c.) were grown in a greenhouse for 7 weeks before use.

4.2. Chemicals and substrates

MeJa, DFMO, acetovanillone and acetosyringone were purchased from Sigma–Aldrich. Chemically synthesized HCPs used as standards for the HPLC analyses were available in our laboratory. Hydroxycinnamoyl-CoA thioesters were prepared by transesterification of hydroxycinnamoyl-*N*-hydroxysuccinimide esters with coenzyme A, as described previously (Stöckigt and Zenk, 1975; Negrel and Smith, 1984). Dephosphoferuloyl-CoA and feruloylphosphopanthotheine were prepared from feruloyl-CoA using a crude barley seedling extract and purified by preparative TLC using a published protocol (Negrel and Smith, 1984).

4.3. HPLC

Phenolics were separated by RP-HPLC using a Waters (Milford, MA) chromatography system equipped with a dual wavelength absorbance detector usually set at 310 and 340 nm. The same method was used to analyse phenolics accumulating in the culture medium, in tobacco cells, or detect the formation of acetovanillone from feruloyl-CoA in enzymic extracts. Good separation of the

different hydrolysis products of feruloyl-CoA was achieved using this protocol, adapted from a method used to analyse caffeoylputrescine in the intercellular fluid of tobacco leaves (Langebartels et al., 1991). Products were separated on a Nova Pack C_{18} column (3.9 × 300 mm, 4 μm) using a flow rate of 0.8 ml min^{-1}. The following conditions were used: 90% solvent A (4 g ammonium formiate and 20 ml formic acid per l of MilliQ water, final pH 2.85) and 10% solvent B (100% MeoH) for 5 min followed by a linear gradient elution within 30 min from 10% to 90% solvent B. Retention times were caffeoylputrescine 12 min, *p*-coumaroylputrescine 16.06 min, feruloylputrescine 18.23 min, acetovanillone 20.02 min, acetosyringone 21.27, ferulic acid 21.46 min, feruloyl-CoA 25.83 min, feruloyl-dephospho-CoA 26.72 min, feruloyl-phosphopantetheine 27.79 min.

4.4. Identification of phenolics

HCPs were extracted by leaving 1 g fr. wt of filtrated cells in 2.5 ml MeOH in the dark at 4 °C for 48 h. After centrifugation the supernatant was stored at −20 °C before being analysed by HPLC. HCPs have been well characterized in previous work in the TX1 cell line (Berlin et al., 1982) and were identified by comparison of their R_t and their UV spectra with those of chemically synthesized standards which were available in our laboratory. Acetophenones were extracted twice with 200 ml ethyl acetate from 1 l of culture medium collected 3 days after treatment with 100 μM MeJa. The combined organic phases were then evaporated under reduced pressure and the residue redissolved in 0.5 ml MeOH. Aliquots (20 μl) were then analysed on the same chromatographic column described above. Separation was performed at 300 nm within 30 min at a flow rate of 0.8 ml min^{-1} with a linear gradient elution from 10% to 90% solvent B (MeOH) in solvent A (1% AcOH in H_2O). Retention time for acetovanillone 21.54 min, for acetosyringone 22.48 min. Both peaks were collected and after repeated injections the purified products were evaporated under reduced pressure to remove MeOH. Residual H_2O was then eliminated by lyophilisation of the frozen sample to avoid excessive loss of the relatively volatile acetophenones. The two products showed the same R_t in HPLC, UV spectra (acetovanillone: λ^{MeOH} nm: 277,300 sh.; KOH 340, acetosyringone λ^{MeOH} nm: 298; KOH 360) and R_f after TLC on Kieselgel 60 F-254 (Merck) in EtOAc-*iso*PrOH (9:1) as authentic acetovanillone (R_f = 0.55) and acetosyringone (R_f = 0.52). Both products reacted with dinitrophenylhydrazone. Their identification was further confirmed by GC–MS. Mass spectra of underivatized samples were compared with the spectra of authentic acetophenones. GC–MS analyses were performed using a Focus DSQ Thermo Electron (30 m DB5 capillary column, EI mode).

Acetovanillone. GC–MS 70 eV, *m/z* (rel. int.): 166 $[M]^+$ (47), 151 $[M-Me]^+$ (100), 136 (2), 123 (23), 108 (10), 93 (4), 77 (5), 65 (5), 51 (7), 43 (10).

Acetosyringone. GC–MS 70 eV, *m/z* (rel. int.): 196 $[M]^+$ (46), 181 $[M-Me]^+$ (100), 153 (17), 138 (4), 123 (3), 110 (5), 93 (4), 78 (9), 65 (9), 51 (2), 43 (18).

Ferulic acid, dephospho-feruloyl-CoA and feruloyl-phosphopanthotheine were identified by comparison of their R_t and of their UV spectra with that of authentic standards.

4.5. Enzyme preparation

All work was done at 4 °C. Frozen TX1 cells were homogenized in a mortar with sand and 50 mg activated charcoal/g fr. wt in 0.2 M Tris–HCl buffer pH 7.5 (2 ml per g fr. wt) containing 10 mM ME, 1 mM EDTA and 20 g/l ascorbic acid. The extract was then centrifuged at 20,000 *g* for 15 min. Solid $(NH_4)_2SO_4$ was then added to 65% saturation and stirred for 1 h. After centrifugation, the precipitate was dissolved in a minimum volume of extraction buffer and desalted by dialysis against 0.01 M Tris–HCl buffer at pH 8 (0.1 mM EDTA, 10 mM ME).

4.6. Spectrophotometric assay

AVS activity was measured spectrophotometrically at 30 °C using feruloyl-CoA and NAD as substrates. The incubation mixture contained 50–200 μl protein extract, 50 μM feruloyl-CoA, 1 mM NAD and 0.1 M Tris–HCl buffer pH 8 in a final volume of 1 ml. The reference cell contained the same mixture, without NAD. The decrease in absorbance at 380 nm was measured over 10 min and the activity was quantified, after determination of the initial velocity, using the experimentally measured extinction coefficient of feruloyl-CoA at 380 nm in 0.1 M Tris HCl buffer at pH 8 $\varepsilon_{380} = 0.95 \times 10^4\ M^{-1}\ cm^{-1}$. For the study of the properties of AVS and for the determination of the apparent molecular mass by gel filtration the activity was measured in parallel by HPLC. In this case the reaction was stopped in the cell by adding 50 μl acetic acid. After precipitation of the proteins at 4 °C in an Eppendorf tube and centrifugation, a 10 μl aliquot was analysed by HPLC. The amount of acetovanillone detected after HPLC was then compared to the enzymatic activity calculated from the photometric assay. For the study of the specificity of AVS, the photometric assay was used at 380 nm for caffeoyl-CoA and sinapoyl-CoA, at 350 nm for *p*-coumaroyl-CoA and 313 nm for cinnamoyl-CoA. No activity was detected with these thioesters. This was confirmed by comparing the chromatograms obtained after injection of aliquots of the incubation media with control chromatograms corresponding to reference cells (without NAD).

4.7. Characterization of the reaction product

The enzymatically formed product showed the same R_t in HPLC, UV spectrum (λ^{MeOH} nm: 277, 300 sh.; KOH 340) as authentic acetovanillone. The identification was confirmed by MS: 2 ml of a crude enzymatic extract obtained after ammonium sulphate concentration and dialysis were incubated for 1 h in the presence of feruloyl-CoA and NAD in the conditions described above for the photometric assay but on larger scale (total incubation medium volume of 10 ml). After precipitation of the proteins and centrifugation, the supernatant was extracted twice with 10 ml ethyl acetate. After evaporation under reduced pressure at 35 °C, residual water was removed by lyophilisation and the enzymatically formed product was dissolved in 100 μl MeOH before being analysed by GC–MS: 70 eV, *m/z* (rel. int.): 166 $[M]^+$ (51), 151 $[M-Me]^+$ (100), 136 (4), 123 (25), 108 (8), 93 (2), 77 (7), 65 (8), 51 (9), 43 (16).

4.8. Protein

Protein concentration was determined according to Bradford using bovine serum albunin as a standard (Bradford, 1976).

4.9. FPLC

In order to estimate the molecular mass of native AVS, the enzyme was first prepurified on a DEAE Trisacryl M column (Pall BioSepra, Cergy-Saint-Christophe, France) (2.4 × 15 cm) preequilibrated in 0.01 M Tris–HCl buffer at pH 8.5 (10 mM ME). The extract obtained from 20 g of frozen cells was loaded after $(NH_4)_2SO_4$ precipitation and dialysis onto the column. The column was then washed with 250 ml loading buffer before applying a 0 to 0.5 M linear NaCl gradient at a flow rate of 1 ml/min. Elution of proteins was monitored at 280 nm. Ten milliliters fractions were collected and assayed for AVS activity using the photometric assay. The most active fractions (tubes n°12 and 13) were pooled and concentrated

to ca. 2 ml using an Amicon YM10 ultrafiltration membrane. The molecular mass of native AVS was then estimated by chromatography on a FPLC-Superose-12 HR 10/30 column (GE Healthcare), using a calibration curve obtained with catalase, bovine serum albumin (monomer and dimer), ovalbumin, chymotrypsinogen A and ribonuclease as standards. The column was equilibrated with 0.01 M Tris–HCl buffer at pH 8.5 containing 0.15 M NaCl and 10 mM ME. 200 µl of the partially purified AVS solution were injected into the column. The elution volume of AVS was determined by monitoring AVS activity with the photometric assay and checking the formation of acetovanillone by HPLC.

Acknowledgements

We are grateful to Dr. Denis Bouchu (Centre Commun de Spectrométrie de Masse, Université Claude Bernard, Lyon) for the mass spectral data.

References

Abd El-Mawla, A.M.A., Beerhues, L., 2002. Benzoic acid biosynthesis in cell cultures of *Hypericum androsaemum*. Planta 214, 727–733.

Baker, C.J., O'Neil, N.R., Deahl, K., Lydon, J., 2002. Continuous production of extracellular antioxidants in suspension cells attenuates the oxidative burst detected in plant–microbe interactions. Plant Physiol. Biochem. 40, 641–644.

Baker, C.J., Whitaker, B.D., Roberts, D.P., Mock, N.M., Rice, C.P., Deahl, K.L., Aver'yanov, A.A., 2005a. Induction of redox sensitive extracellular phenolics during plant–bacterial interactions. Physiol. Mol. Plant Pathol. 66, 90–98.

Baker, C.J., Mock, N.M., Whitaker, B.D., Roberts, D.P., Rice, C.P., Deahl, K.L., Aver'yanov, A.A., 2005b. Involvement of acetosyringone in plant–pathogen recognition. Biochem. Biophys. Res. Commun. 328, 130–136.

Berlin, J., Kukoschke, K.H., Höfle, G., Witte, L., 1982. Biochemical characterization of two tobacco cell lines with different levels of cinnamoylputrescines. J. Nat. Prod. 45, 83–87.

Beukelman, C.J., Van den Berg, A.J.J., Kroes, B.H., Labadie, R.P., Mattsson, E.E., Van Dijk, H., 1995. Plant-derived metabolites with synergistic antioxidant activity. Immunol. Today 16, 2.

Blount, J.W., Masoud, S., Sumner, L.W., Huhman, D., Dixon, R.A., 2002. Overexpression of cinnamate 4-hydroxylase leads to increased accumulation of acetosyringone in elicited tobacco cell-suspension cultures. Planta 214, 902–910.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Chen, H., Jones, D., Howe, G.A., 2006. Constitutive activation of the jasmonate signaling pathway enhances the production of secondary metabolites in tomato. FEBS Lett. 580, 2540–2546.

De Rosa, S., De Giulio, A., Tommonaro, G., 1996. Aliphatic and aromatic glycosides from the cell cultures of *Lycopersicum esculentum*. Phytochemistry 42, 1031–1034.

Duban, M.E., Lee, K., Lynn, D.G., 1993. Strategies in pathogenesis: mechanistic specificity in the detection of generic signals. Mol. Microbiol. 7, 637–645.

Galis, I., Simek, P., Narisawa, T., Sasaki, M., Horiguchi, T., Fukuda, H., Matsuoka, K., 2006. A novel R2R3 MYB transcription factor NtMYBJS1 is a methyl jasmonate-dependant regulator of phenylpropanoid-conjugate biosynthesis in tobacco. Plant J. 46, 573–592.

Gundlach, H., Müller, M.J., Kutchan, T.M., Zenk, M.H., 1992. Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc. Natl. Acad. Sci. 89, 2389–2393.

Hilton, M.D., Cain, W.J., 1990. Bioconversion of cinnamic acid to acetophenone by a pseudomonad: microbial production of a natural flavor compound. Appl. Environ. Microbiol. 56, 623–627.

Imanishi, S., Nakakita, M., Yamashita, K., Furuta, A., Utsuno, K., Muramoto, N., Kojima, H., Nakamura, K., 2000. Aspirin and salicylic acid do not inhibit methyl jasmonate-inducible expression of a gene for ornithine decarboxylase in tobacco BY-2 cells. Biosci. Biotechnol. Biochem. 64, 125–133.

Koo, A.J.K., Howe, G.A., 2009. The wound hormone jasmonate. Phytochemistry 70, 1571–1580.

Kumar, A., Taylor, M., Altabella, T., Tiburcio, A., 1997. Recent advances in polyamine research. Trends Plant Sci. 2, 124–130.

Langebartels, C., Kerner, K., Leonardi, S., Schraudner, M., Trost, M., Heller, W., Sandermann, H., 1991. Biochemical plant responses to ozone. Differential induction of polyamine and ethylene biosynthesis in tobacco. Plant Physiol. 95, 882–889.

Leon, J., Rojo, E., Sanchez-Serrano, J.J., 2001. Wound signalling in plants. J. Exp. Bot. 52, 1–9.

Liu, B., Falkenstein-Paul, H., Schmidt, W., Beerhues, L., 2003. Benzophenone synthase and chalcone synthase from *Hypericum androsaemum* cell cultures: cDNA cloning, functional expression, and site-directed mutagenesis of two polyketide synthases. Plant J. 34, 847–855.

March, M.V., Caldwell, J., Hutt, A.J., Smith, R.L., Horner, M.W., Houghton, E., Moss, M.S., 1982. 3-Hydroxy and 3-keto-3-phenylpropionic acids: novel metabolites of benzoic acid in horse urine. Biochem. Pharmacol. 31, 3225–3230.

Memelink, J., Verpoorte, R., Kijne, J., 2001. ORCAnization of jasmonate responsive gene expression in alkaloid metabolism. Trends Plant Sci. 6, 212–219.

Meurer-Grimes, B., Berlin, J., Strack, D., 1989. Hydroxycinnamoyl-CoA:putrescine hydroxycinnamoyltransferase in tobacco cell cultures with high and low levels of caffeoylputrescine. Plant Physiol. 89, 488–492.

Miguel, M.G., Barroso, J.G., 1994. Accumulation of stress metabolites in cell suspension cultures of *Hyoscyamus albus*. Phytochemistry 35, 371–375.

Mitra, M., Kitamura, Y., Gasson, M.J., Narbad, A., Parr, A.J., Payne, J., Rhodes, M.J.C., Sewter, C., Walton, N.J., 1999. 4-Hydroxycinnamoyl-CoA hydratase/lyase (HCHL) – an enzyme of phenylpropanoid chain cleavage from *Pseudomonas*. Arch. Biochem. Biophys. 365, 10–16.

Mühlenbeck, U., Barz, W., 1996. Formation of hydroxycinnamoylamides and α-hydroxyacetovanillone in cell cultures of *Solanum khasianum*. Phytochemistry 42, 1573–1579.

Mühlenbeck, U., Barz, W., 1997. Cytochrome-P450-dependent formation of α-hydroxyacetovanillone from acetovanillone in *Solanum khasianum*. Phytochemistry 44, 865–867.

Negrel, J., 1989. The biosynthesis of cinnamoylputrescines in callus tissue cultures of *Nicotiana tabacum*. Phytochemistry 28, 477–481.

Negrel, J., Smith, T.A., 1984. The phosphohydrolysis of hydroxycinnamoyl-coenzyme A thioesters in plant extracts. Phytochemistry 23, 31–34.

Negrel, J., Paynot, M., Javelle, F., 1992. Purification and properties of putrescine hydroxycinamoyltransferase from tobacco (*Nicotiana tabacum*) cell suspensions. Plant Physiol. 98, 1264–1269.

Pinçon, G., Chabannes, M., Lapierre, C., Pollet, B., Ruel, K., Joseleau, J.P., Boudet, A.M., Legrand, M., 2001. Simultaneous down-regulation of caffeic/5-hydroxyferulic acid-*O*-methyltransferase I and cinnamoyl-coenzyme A reductase in the progeny from a cross between tobacco lines homozygous for each transgene. Consequences for plant development and lignin synthesis. Plant Physiol. 126, 145–155.

Rylott, E.L., Eastmond, P.J., Gilday, A.D., Slocombe, S.P., Larson, T.R., Baker, A., Graham, I.A., 2006. The *Arabidopsis thaliana* multifunctional protein gene (MFP2) of peroxisomal β-oxidation is essential for seedling establishment. Plant J. 45, 930–941.

Sasaki-Sekimoto, Y., Taki, N., Obayashi, T., Aono, M., Matsumoto, F., Sakurai, N., Suzuki, H., Hirai, M.Y., Noji, M., Masuda, T., Takamiya, K., Shibata, D., Ohta, H., 2005. Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. Plant J. 44, 653–668.

Shanker, K.S., Kishore, K.H., Kanjilal, S., Misra, S., Murty, U.S.N., Prasad, R.B.N., 2007. Biotransformation of ferulic acid to acetovanillone using *Rhizopus oryzae*. Biocatal. Biotransform. 25, 109–112.

Spencer, P.A., Towers, G.H.N., 1988. Specificity of signal compounds detected by *Agrobacterium tumefaciens*. Phytochemistry 27, 2781–2785.

Spencer, P.A., Towers, G.H.N., 1991. Restricted occurrence of acetophenone signal compounds. Phytochemistry 30, 2933–2937.

Stachel, S.E., Messens, E., Van Montagu, M., Zambryski, P., 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature 318, 624–629.

Stöckigt, J., Zenk, M.H., 1975. Chemical syntheses and properties of hydroxycinnamoyl-coenzyme A derivatives. Z. Naturforsch. 30c, 352–358.

Stolk, J., Hilterman, T.J., Dijkman, J.H., 1994. Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. Am. J. Respir. Cell Mol. Biol. 11, 95–102.

Swiatek, A., Lenjou, M., Van Bockstaele, D., Inze, D., Van Onckelen, H., 2002. Differential effects of jasmonic acid and abscisic acid on cell cycle progression in tobacco BY-2 cells. Plant Physiol. 128, 201–211.

Threlfall, D.R., Whitehead, I.M., 1988. Co-ordinated inhibition of squalene synthetase and induction of enzymes of sesquiterpenoid phytoalexin biosynthesis in cultures of *Nicotiana tabacum*. Phytochemistry 27, 2567–2580.

Tossi, V., Cassia, R., Lamattina, L., 2009. Apocynin-induced nitric oxide production confers antioxidant protection in maize leaves. J. Plant Physiol. 166, 1336–1341.

Wildermuth, M.C., 2006. Variation on a theme: synthesis and modification of plant benzoic acids. Curr. Opin. Plant Biol. 9, 288–296.