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Detection of an *O*-methyltransferase synthesising acetosyringone in methyl jasmonate-treated tobacco cell-suspensions cultures

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ABSTRACT

Acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) is a well-known and very effective inducer of the virulence genes of *Agrobacterium tumefaciens* but the precise pathway of its biosynthesis in plants is still unknown. We have used two tobacco cell lines, cultured in suspension and exhibiting different patterns of accumulation of acetosyringone in their culture medium upon treatment with methyl jasmonate, to study different steps of acetosyringone biosynthesis. In the two cell lines studied, treatment with 100 μM methyl jasmonate triggered a rapid and transient increase in acetovanillone synthase activity followed by a progressive increase in *S*-adenosyl-*L*-methionine: 5-hydroxyacetovanillone 5-*O*-methyltransferase activity which paralleled the rise in acetosyringone concentration in the culture medium. This *O*-methyltransferase displayed Michaelis–Menten kinetics with an apparent K_m value of 18 μM for 5-hydroxyacetovanillone and its activity was magnesium-independent. Its molecular mass was estimated by gel permeation on an FPLC column and was found to be of ca. 81 kDa. 5-Hydroxyacetovanillone was the best substrate among the different *o*-diphenolic compounds tested as methyl acceptors in the *O*-methyltransferase assay. No formation of 5-hydroxyacetovanillone could be detected *in vitro* from 5-hydroxyferuloyl-CoA and NAD in the extracts used to measure acetovanillone synthase activity, indicating that 5-hydroxyacetovanillone is probably formed by direct hydroxylation of acetovanillone rather than by β-oxidation of 5-hydroxyferulic acid. Taken together our results strongly support the hypothesis that acetosyringone biosynthesis in tobacco proceeds from feruloyl-CoA via acetovanillone and 5-hydroxyacetovanillone.

Introduction

Acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) is a very effective inducer of the virulence genes of *Agrobacterium tumefaciens* which is often used to increase the efficiency of *A. tumefaciens* mediated plant transformation procedures (Gelvin, 2003; Hansen and Wright, 1999; Hiei et al., 1997). Despite this important role in plant biotechnology and although it has been known for more than 25 years that in the Solanaceae acetosyringone synthesis can be induced by wounding (Spencer and Towers, 1991; Stachel et al., 1985) or by treatment of cell cultures with elicitors (Negrel and Javelle, 2010, and references therein), the precise pathway of its biosynthesis is still unknown. We previously characterised an enzyme, acetovanillone synthase, synthesising acetovanillone (3′-methoxy-4′-hydroxyacetophenone) from feruloyl-CoA and NAD from tobacco cell-suspension cultures treated with methyljasmonate (MeJa), thus demonstrating that the biosynthesis of acetovanillone in tobacco proceeds from ferulic acid through a CoA-dependent β-oxidation pathway (Negrel and Javelle, 2010). The increase in acetovanillone synthase activity was followed by an increase in the concentration of both acetovanillone and acetosyringone in the culture medium but no formation of acetosyringone could be evidenced *in vitro* in enzymatic extracts incubated in the presence of sinapoyl-CoA and NAD. This led us to surmise that acetosyringone might be synthesised from acetovanillone via 5-hydroxyacetovanillone (Negrel and Javelle, 2010). A straightforward approach to confirm this hypothesis would have been to feed [^{14}C]-acetovanillone *in vivo* to MeJa-treated tobacco cell suspensions and to monitor the labelling of 5-hydroxyacetovanillone and acetosyringone. Unfortunately [^{14}C]-acetovanillone was not commercially available. In an alternative approach we have synthesised 5-hydroxyacetovanillone chemically using a published protocol and we have used it as a substrate to try to directly detect the formation of acetosyringone in enzymatic extracts prepared from MeJa-treated tobacco cells. We report here that acetosyringone is very rapidly synthesised from 5-hydroxyacetovanillone and *S*-adenosyl-*L*-methionine (SAM) in these extracts and we describe some properties of the corresponding *O*-methyltransferase (OMT): *S*-adenosyl-*L*-methionine: 5-hydroxyacetovanillone 5-*O*-methyltransferase (5-HAV-OMT). To our knowledge, this is the first report of an OMT catalysing the synthesis of

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acetosyringone. The comparison of the time course of changes in AVS and 5-HAV-OMT activities in two tobacco cell lines synthesising different amounts of acetosyringone suggests that both enzymes are probably involved in acetosyringone biosynthesis *in planta*.

Results and discussion

Time course of accumulation of acetosyringone in the culture medium of MeJa-treated tobacco cell suspensions

In the course of the study of acetovanillone biosynthesis in tobacco cell-suspension cultures, we tested different tobacco cell lines that were available in our laboratory to compare the rate of accumulation of acetosyringone in their culture medium upon treatment with MeJa. During this work we observed that one of these cell lines (TX4) released high amounts of acetosyringone in the medium even in the absence of MeJa treatment. This cell line was originally obtained by selection of p-fluorophenylalanine resistant cells (Berlin and Widholm, 1977) from the TX1 cell line used for the characterisation of acetovanillone synthase (Negrel and Javelle, 2010) and originally accumulated very high levels of cinnamoylputrescines, this accumulation being associated with a reduced growth rate (Berlin, 1981; Berlin and Widholm, 1977). After several years of subculture in our laboratory on solid Murashige and Skoog medium in the absence of selective pressure, this TX4 cell line stopped accumulating cinnamoylputrescines and grew again in liquid medium at the same speed than the TX1 cell line (unpublished result). When we tested this line (renamed OTX4, O standing for "old" TX4), we found that unexpectedly, the control, untreated cell culture spontaneously released high amounts of acetosyringone in the medium. Treatment of this cell line with MeJa nevertheless further increased the concentration of acetosyringone in the culture medium. Although the genetic origin of the constitutive activation of acetosyringone synthesis in this cell line was undefined, it seemed interesting to use it as an additional model to study the enzymology of acetosyringone biosynthesis, besides the TX1 cell line in which the synthesis of acetophenone derivatives must be induced by addition of MeJa to the culture medium. The concentration of extracellular acetophenones in the two TX1 and OTX4 cell lines was therefore first monitored by direct HPLC analysis of the culture medium (Fig. 1). Basal levels of acetosyringone showed little variation in the medium of the control TX1 cell line whereas it increased steadily in the OTX4 line (Fig. 1). With both cell lines however this concentration markedly increased during 96 h following treatment with MeJa to reach nearly 15 µM in the medium of the OTX4 cell line compared to about 8 µM in the medium of the TX1 cells (Fig. 1). MeJa also triggered a transient increase in acetovanillone concentration in the medium of the TX1 cells (Fig. 2), confirming previous results (Negrel and Javelle, 2010). Interestingly a transient increase in 5-hydroxyacetovanillone concentration, of much lower intensity, could also be detected (Fig. 2). A similar increase of 5-hydroxyacetovanillone concentration in the extracellular fluid of tobacco cell suspensions, preceding the rise in acetosyringone concentration, has previously been detected after placing the suspension cells in a fresh assay buffer (Baker et al., 2005). Trace amounts of acetovanillone and 5-hydroxyacetovanillone were also detected in the medium of the OTX4 cells but they showed little variation after addition of MeJa (data not shown). It is possible that this difference between the two cell lines is due to the fact that the enzymes involved in the synthesis of acetophenone derivatives are constitutively active in the OTX4 cells, so that there is no transient accumulation of intermediates in this cell line upon MeJa treatment (see Section "Time course of acetosyringone biosynthesis in MeJa-treated tobacco cell-suspension cultures").

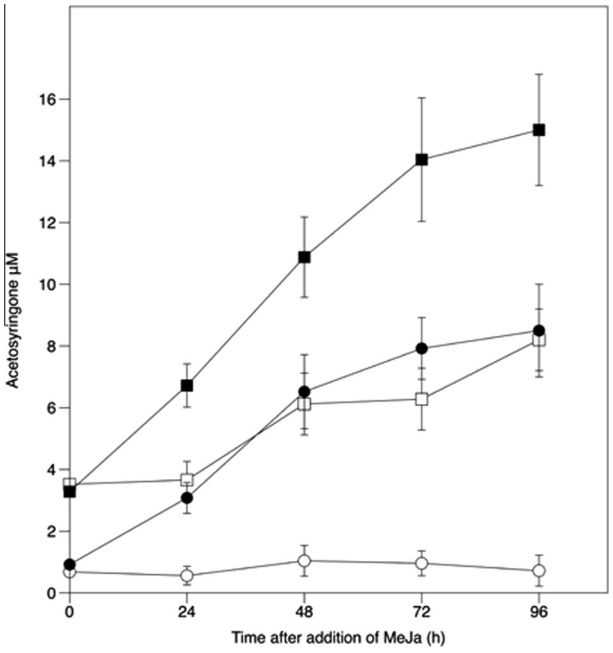

Fig. 1. Time course of accumulation of acetosyringone in the culture medium of MeJa-treated tobacco cell-suspension cultures. Cell suspensions (75 ml, TX1 or OTX4 cell lines) were treated 3 days after subculture with 75 µl MeOH (TX1 ○, OTX4 □) or 75 µl 0.1 M MeJa (TX1 ●, OTX4 ■). Aliquots (1 ml) of each suspension were taken at different time intervals and analysed by HPLC after centrifugation. The data shown represent the results of one experiment with 3 replicates. Cell density raised from ca. 40 mg ml^{-1} to ca. 120 mg ml^{-1} at 96 h with both cell lines. The experiment was repeated twice with similar results.

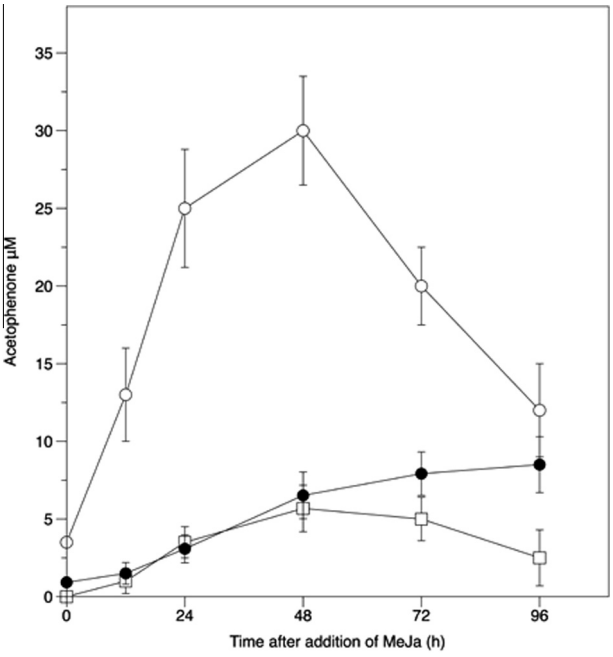

Fig. 2. Variation in the concentration of acetophenone derivatives in the culture medium of tobacco cell-suspension cultures upon MeJa treatment. Cell suspensions (75 ml, TX1 cell line) were treated with 75 µl 0.1 M MeJa 3 days after subculture. The data shown represent the results of one experiment with 3 replicates. (○: acetovanillone, □: 5-hydroxyacetovanillone, ●: acetosyringone). The experiment was repeated twice with similar results.

Cell-free extracts of MeJa-treated tobacco cells catalyse the formation of acetosyringone from 5-hydroxyacetovanillone and SAM

When crude enzymatic extracts prepared from the TX1 cell cultures previously treated with 100 μM MeJa for 72 h were incubated with 5-hydroxyacetovanillone and SAM in Tris–HCl buffer at pH 8.5, rapid formation of acetosyringone could be evidenced by HPLC analysis of the incubation medium. A typical chromatogram obtained after 30 min incubation is shown in Fig. 3B. When using a boiled enzymatic extract (Fig. 3A), or in the absence of SAM (chromatogram not shown), no formation of acetosyringone could be detected and no other transformation of 5-hydroxyacetovanillone could be evidenced. Enzymatically formed acetosyringone was first identified by co-migration with the commercial product. After purification by HPLC it showed the same UV spectrum and R_f after TLC as authentic acetosyringone. Its identity was confirmed by HR mass spectrometry (see Experimental Section). Maximal activity was detected at pH 8.5, half activity being obtained at pH 7 and pH 9.5. The apparent molecular mass of native 5-HAV-OMT was estimated by chromatography on an FPLC-Superose 12 HR column and found to be ca. 81 kDa (Supplementary data, Fig. 1). It displayed Michaelis–Menten kinetics with apparent K_m value of 18 μM for 5-hydroxyacetovanillone in the presence of saturating concentrations of SAM (Table 1) and an apparent K_m for SAM of 8 μM. 5-HAV-OMT did not require the addition of divalent cations (Mg^{2+} or Ca^{2+}) for maximal activity. Identical results (optimum pH, effect of divalent cations and molecular mass) were obtained when the properties of the OMT were studied using crude enzymatic extracts prepared from MeJa-treated OTX4 cell suspensions.

Table 1
Kinetic data of the *o*-diphenolic compounds tested as substrates in the OMT assay.

Substrate	V_{max} (pkat/mg pr.)	K_m (μM)	V_{max}/K_m
5-Hydroxyacetovanillone	15 (3)	18	0.83
5-Hydroxyvanillin	22 (12)	87	0.25
5-Hydroxyferulic acid	102 (92)	280	0.36
Caffeic acid	30 (27)	262	0.11
Catechol	0 (0)	–	–
Chlorogenic acid	0 (0)	–	–

V_{max} and K_m values were measured using crude enzymatic extracts prepared from TX1 tobacco cells collected 72 h after MeJa treatment, except for values inside brackets corresponding to the V_{max} values measured with extracts prepared from MeOH-treated cells. Data were obtained using variable concentrations of each substrate (5 μM to 2 mM) and a constant SAM concentration (200 μM). V_{max} and K_m values were calculated from Lineweaver–Burk plots.

The activity of different *o*-diphenolic substrates was tested in the same OMT assay, using crude enzymatic extracts prepared from MeJa- or MeOH-treated TX1 cell cultures (Table 1). The methylation of the different substrates was monitored by HPLC, by directly measuring the formation of the corresponding products. Although this method is time-consuming by comparison with the usual radioactive assay for OMTs with [^{14}C]-SAM, it allowed a precise identification and quantification of the different products formed *in vitro*, using variable substrate concentrations. 5-Hydroxyferulic acid, caffeic acid, and 5-hydroxyvanillin were very rapidly

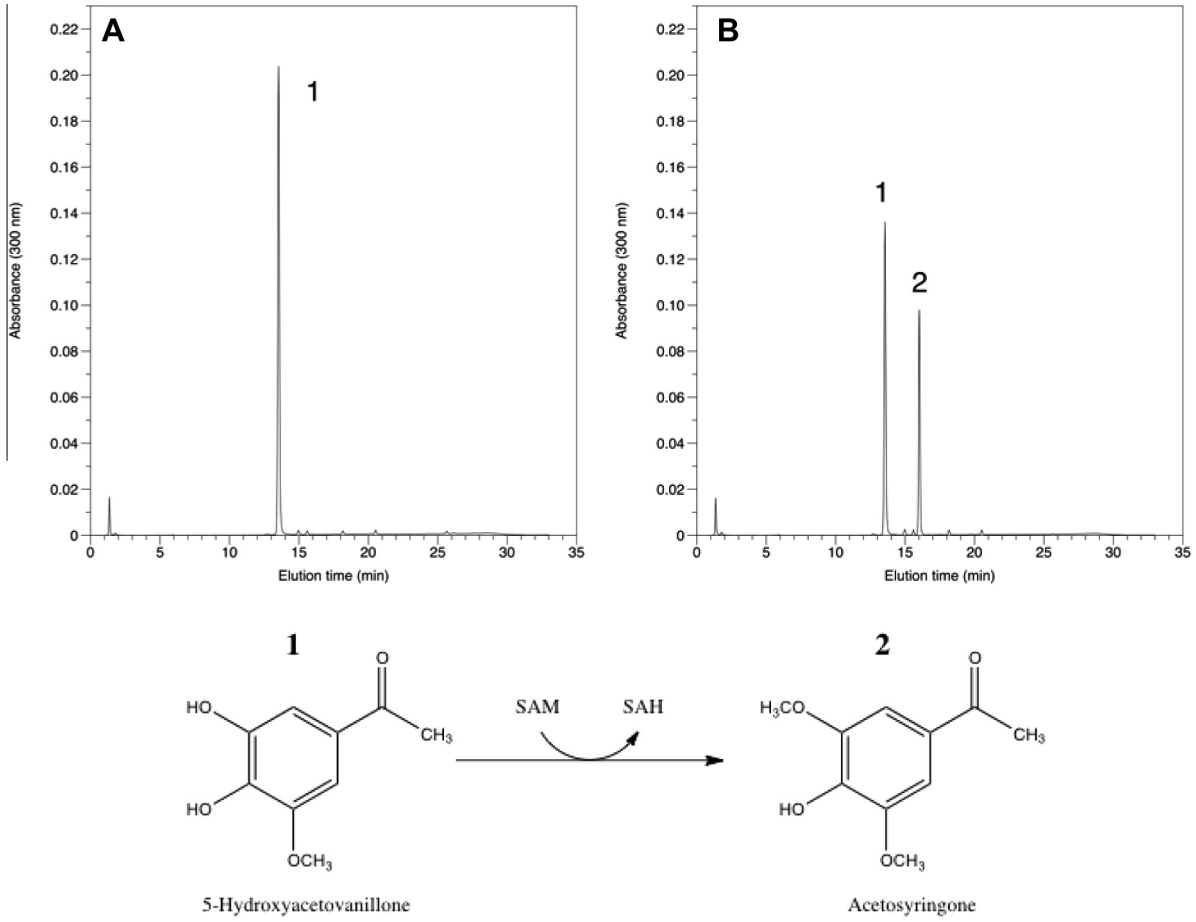

Fig. 3. *In vitro* formation of acetosyringone from 5-hydroxyacetovanillone. HPLC analyses of the incubation medium after 30 min reaction time in the presence of 5-hydroxyacetovanillone and SAM. (A) boiled extract, (B) non-boiled extract 1: 5-hydroxyacetovanillone; 2: acetosyringone; SAM: *S*-adenosyl-*L*-methionine; SAH: *S*-adenosyl-*L*-homocysteine.

methylated, but appeared as poorer substrates than 5-hydroxyacetovanillone when the V_{max}/K_m ratios were compared (Table 1). No methylation of catechol or chlorogenic acid could be detected. The activity of caffeoyl-CoA and 5-hydroxyferuloyl-CoA was not tested, since these thioesters were very rapidly degraded in the crude enzymatic extracts, as previously described in tobacco leaf extracts (Negrel and Smith, 1984). Competition experiments confirmed the presence in the enzymatic extracts of an OMT exhibiting high affinity for 5-hydroxyacetovanillone. Addition of 200 μM catechol or 200 μM 5-hydroxyvanillin to the standard incubation medium resulted in only 1 and 21% inhibition, respectively, of acetosyringone formation from 5-hydroxyacetovanillone. Interestingly MeJa treatment markedly increased the specific activity measured using 5-hydroxyacetovanillone as substrate, whereas by comparison the specific activity measured with 5-hydroxyferulic or caffeic acid showed little variation (Table 1). Thus MeJa does not induce a general increase in OMT activity, but strongly increases the activity measured with 5-hydroxyacetovanillone, and to a lower extent with 5-hydroxyvanillin. 5-Hydroxyvanillin is a known substrate of alfalfa caffeic acid 3-*O*-methyltransferase (Kota et al., 2004). Since it is a close analogue of 5-hydroxyacetovanillone it may also be methylated by the tobacco OMT synthesising acetosyringone. These results therefore suggest that several OMTs are present in the crude extract prepared from cell suspensions and that MeJa-treatment increases the activity of the isoform, or isoforms, involved in acetosyringone synthesis.

Time course of acetosyringone biosynthesis in MeJa-treated tobacco cell-suspension cultures

Fig. 4 shows the time course of changes in AVS and 5-HAV-OMT activities in tobacco cell-suspension cultures following treatment with 100 μM MeJa. AVS activity was measured in the crude enzymatic extracts using feruloyl-CoA and NAD as substrates. Although feruloyl-CoA was rapidly degraded in these extracts, the initial velocity of the reaction could be readily measured spectrophotometrically, making monitoring of the activity possible (Negrel and Javelle, 2010). The two enzymes displayed distinct patterns of induction, AVS activity increasing rapidly but transiently after addition of MeJa (Fig. 4A), whereas 5-HAV-OMT activity increased slowly but continuously (Fig. 4B). In the TX1 cell line, maximum AVS activity was reached after 12–24 h and declined progressively to reach the basal level 96 h after addition of MeJa (Fig. 4A), confirming previous results (Negrel and Javelle, 2010). In the OTX4 cell line a similar kinetic was observed but the basal level before addition of MeJa was 2–3-fold higher than in the TX1 cell line, demonstrating that acetophenones synthesis is taking place constitutively in this cell line (Fig. 4A). Moreover the activity in OTX4 cells increased during 48 h even in control, MeOH-treated cells, before slowly declining between 48 and 96 h. While measuring AVS activity, we systematically also tested 5-hydroxyferuloyl-CoA and sinapoyl-CoA as potential substrates of acetovanillone synthase, using the same spectrophotometric assay. Sinapoyl-CoA was inactive as substrate in both TX1 and OTX4 extracts, confirming results obtained with the TX1 cell line (Negrel and Javelle, 2010). No activity could be detected either in the presence of 5-hydroxyferuloyl-CoA. With both CoA thioesters the reaction mixtures were also analysed by HPLC to make sure that no formation of acetosyringone or 5-hydroxyacetovanillone had occurred. This seems to rule out the possibility that 5-hydroxyacetovanillone could be synthesised by β-oxidation of 5-hydroxyferulic acid and it seems therefore much more likely that it could be formed by direct hydroxylation of acetovanillone. The activity of 5-HAV-OMT was measured using the same enzymatic extracts (Fig. 4B): it was very low in control TX1 cells, but increased continuously for 3 days, after a lag period of about 12 h, following MeJa treatment. A similar pattern of induction was observed in the OTX4 cell line after addition of MeJa but the basal level in control, MeOH-treated cells, was again much higher (c.a. 5-fold) than in the TX1 cell line (Fig. 4B).

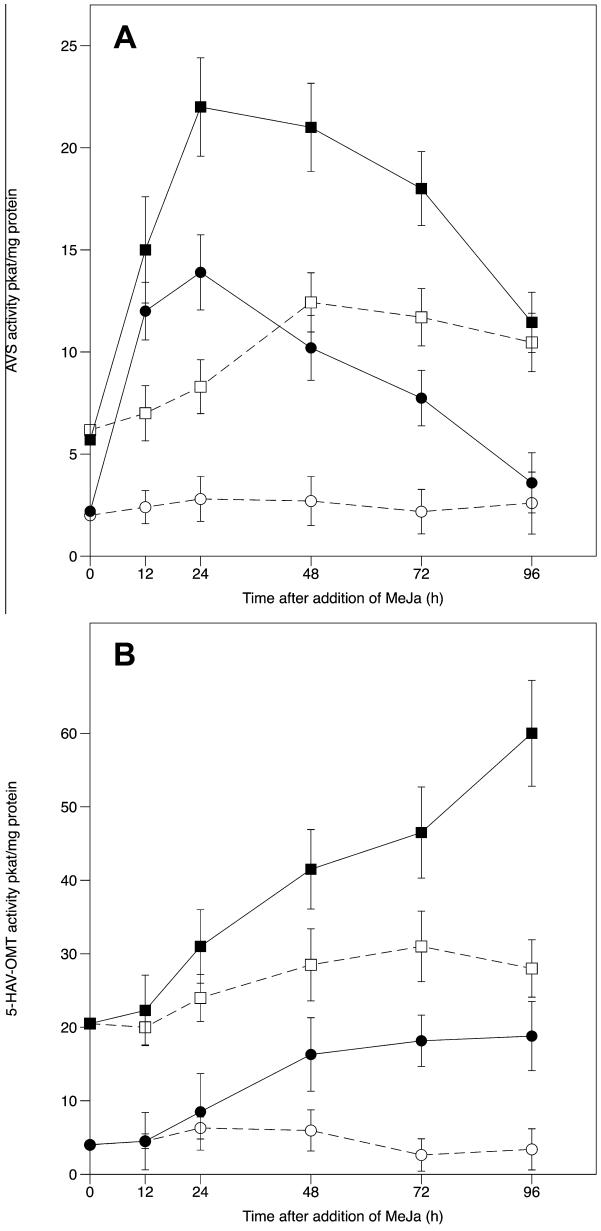

Fig. 4. Time course of induction of AVS (A) and 5-HAV-OMT (B) activities in MeJa-treated tobacco cell-suspension cultures (TX1 and OTX4 cell lines). Cell suspensions (75 ml) were treated 3 days after subculture with 75 μl MeOH (TX1 ○, OTX4 □) or 75 μl 0.1 M MeJa (TX1 ●, OTX4 ■). The data shown represent the mean of 3 replicates ± SD.

Immunodetection of OMT isoforms in MeJa-treated tobacco cell-suspension cultures

Different OMT isoforms, with variable efficiency towards plant *o*-diphenolic substrates, and belonging to different OMT classes, have been characterised in tobacco (Lam et al., 2007). cDNA sequence analysis has shown that tobacco contains two classes of caffeic acid OMTs (COMTs, EC 2.1.1.6, COMT I and II) and 3 classes of caffeoyl-CoA OMTs (CCoAOMTs EC 2.1.1.6) (Hermann et al., 1986; Maury et al., 1999; Pellegrini et al., 1993; Pinçon et al., 2001). The 3 CCoAOMT classes encode isoforms of 27 and 32 kDa

whereas COMTs are known to be dimeric enzymes with subunit molecular masses around 40 kDa (39.5 for COMT I, 42 and 43 kDa for COMT II) (Maury et al., 1999). In order to try to correlate the induction of 5-HAV-OMT activity with the increase of the intensity of an immunoreaction band on Western blots, and to try to determine which OMT isoform could be involved in the synthesis of feruloyl-CoA used by AVS during acetovanillone biosynthesis, we attempted to detect these different OMTs using antibodies recognising the different isoforms of CCoAOMTs and COMTs.

CCoAOMT isoforms were readily detected in tobacco cell suspension extracts by Western blot analysis (Fig. 5A), confirming that feruloyl-CoA biosynthesis is taking place constitutively in these cells. Little variation in the expression of the different isoforms was detected in TX1 cells during the first 24 h following MeJa treatment (Fig. 5A), i.e. when AVS activity is induced (Fig. 4A). This result was confirmed by directly measuring caffeoyl-CoA methylation in enzymatic extracts, using short incubation times to limit caffeoyl-CoA degradation (Fig. 5B). By contrast an increase in activity was detected after 48 h (Fig. 5B), this increase corresponding with an intensification of the band at 27 kDa on Western blots (Fig. 5A). The increase in CCoAOMT activity in MeJa-treated TX1 cells (Fig. 4B) therefore appears to occur later than the activation of AVS (Fig. 4A), a finding which raises the question of the origin of feruloyl-CoA and of the regulation of its synthesis during acetovanillone biosynthesis. This result must however be interpreted with care since it may be difficult to detect a specific activation of caffeoyl-CoA methylation linked to acetophenones synthesis when MeJa is known to trigger the synthesis of several feruloyl-CoA derived phenolic compounds, such as feruloylputrescine (Negrel and Javelle, 2010).

Detection of COMT isoforms on Western blots in the tobacco cell suspension extracts was more difficult, especially when the antibody raised against COMT I was used. Very long incubation times in the presence of the alkaline phosphatase substrate (BCIP/NBT) were required to detect immunoreactive bands at the expected size for tobacco COMTs with this antibody, whereas a band at 66 kDa was systematically and rapidly detected in all the extracts analysed (data not shown). A faint band corresponding to the size of COMT I (39 kDa) could however be detected on Western blots in MeJa-treated TX1 and OTX4 cells extracts and was also detectable in control OTX4 cell extracts. This immunoreactive band, which probably corresponds to a COMT I isoform was readily detected with the anti-COMT II antibody (Fig. 6). We did not attempt in the course of this preliminary work to determine whether this band could correspond to the OMT synthesising acetosyringone. Interestingly however, we repeatedly observed that addition of anti-COMT II antibody to crude enzymatic extracts inhibited 5-HAV-OMT activity by more than 80%, when the anti-COMT I antibody was far less effective (10–15%) and the anti-CCoAOMT antibody completely inactive in the same conditions (see Experimental Section).

Comparatively little work has been devoted to the characterisation of OMTs occurring in tobacco cell-suspension cultures. Early work on tobacco OMTs has shown that enzymatic extracts prepared from cell-suspension cultures could catalyse the *O*-methylation of various phenolic substrates, including caffeic acid, 5-hydroxyferulic acid, coumarins and flavonoids (Kuboi and Yamada, 1976; Tsang and Ibrahim, 1979), the activity of caffeic acid OMT being correlated with cell aggregation and lignification (Yamada and Kuboi, 1976). Our results bring new information and demonstrate that MeJa-treated tobacco cells contain a SAM-dependent OMT able to synthesise acetosyringone very efficiently from 5-hydroxyacetovanillone, and which exhibits a high affinity for this substrate. Most significantly the time course of changes in 5-HAV-OMT activity corresponds with the time course of accumulation of acetosyringone in the culture medium of the MeJa-treated cell suspensions, suggesting that it could play a direct role in acetosyringone synthesis *in vivo*. We did not attempt in the course of this work to purify and clone this OMT so it is not possible to know precisely to which OMT class it could belong. The apparent molecular mass of 5-HAV-OMT and the fact that its activity is not dependent on magnesium both indicate however that it could belong to one of the COMT classes. This is also supported by the inhibition of 5-HAV-OMT activity by anti-COMT antibodies. In tobacco COMT I is highly expressed in lignifying tissues and is considered primarily associated with lignin biosynthesis whereas COMT II is regarded as a pathogenesis-related enzyme involved phenylpropanoid metabolism associated with defense responses (Pinçon et al., 2001). Interestingly the promoter of the COMT II gene has been shown to be inducible by various chemicals, including jasmonic acid (Toquin et al., 2003). Other examples of MeJa-inducible OMTs have been described in the plant kingdom (Frick and Kutchan, 1999; Lee et al., 1997). In tobacco COMTs are

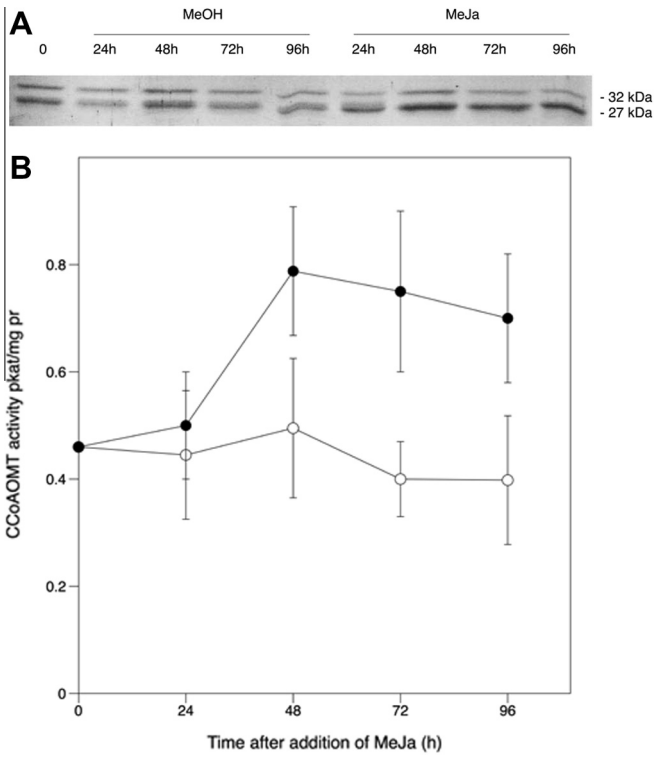

Fig. 5. Kinetics of CCoAOMT expression in MeJa-treated tobacco cell-suspension cultures (TX1 cell line). Extracts were prepared from cell suspensions and used both to detect CCoAOMT isoforms on immunoblots and to assay CCoAOMT activity. (A) Western blot showing the different isoforms of CCoAOMT extracted from MeOH- or MeJa-treated cells. The corresponding masses are indicated in kDa. (B) Time course of changes in CCoAOMT activity in cell suspensions treated 3 days after subculture with 75 µl MeOH (○) or 75 µl 0.1 M MeJa (●). The data shown represent the mean of 3 independent replicates ± SD.

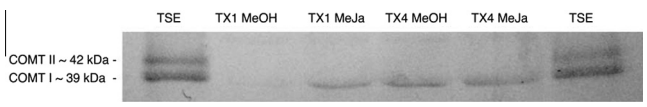

Fig. 6. Immunodetection of COMTs in TX1 and OTX4 extracts. Protein extracts were prepared from tobacco cell suspensions (TX1 and OTX4 cell lines) harvested 72 h after treatment with MeJa or MeOH. After separation of proteins by SDS–PAGE, COMTs isoforms were detected on immunoblots using antibodies recognising the different isoforms of COMTs (COMTI and COMTII). A tobacco stem extract (TSE) containing both COMT I and COMT II (Maury et al., 2010) was used as a control.

not encoded by single genes but they form multigene families. The COMT II gene family for example is composed of four to six members (Pellegrini et al., 1993; Toquin et al., 2003). The specificity of the different known tobacco OMTs has been compared after purification from TMV inoculated leaves and after purification of the corresponding recombinant proteins (Collendavelloo et al., 1981; Hoffmann et al., 2001; Maury et al., 1999), but to our knowledge 5-hydroxyacetovanillone has never been tested as a substrate of these transferases. The two known tobacco COMTs which have been purified both accept catechol as substrate (Hermann et al., 1986; Maury et al., 1999) whereas rather surprisingly no methylation of catechol could be detected in the extracts of MeJa-treated tobacco cells, even at high concentration (2 mM). This suggests that the 5-HAV-OMT that we have detected may correspond to a new and yet undescribed COMT isoform, although it can be misleading to compare the specificity of an enzyme in a plant cell extract with that of purified recombinant proteins (Maury et al., 1999). Moreover the problem may be complicated by the fact that COMTs are dimeric enzymes (Zubieta et al., 2001) and that interaction between subunits is known to influence the specificity of OMTs (Frick and Kutchan, 1999; Frick et al., 2001).

The characterisation of an OMT synthesising acetosyringone from 5-hydroxyacetovanillone strongly supports the hypothesis that acetosyringone biosynthesis in tobacco proceeds from feruloy-CoA via acetovanillone (Negrel and Javelle, 2010). This hypothesis implies that the two methoxy groups in acetosyringone are introduced sequentially by two OMTs, the first one methylating caffeic acid or caffeoyl-CoA to form feruloyl-CoA, and the second one synthesising acetosyringone from 5-hydroxyacetovanillone. Interestingly OMT(s)-suppressed plants have been shown to produce lower amounts of acetosyringone and to be less susceptible to *A. tumefaciens* infection (Maury et al., 2010). A significant decrease in acetosyringone content was detected in antisense plants transformed with the COMT I sequence alone or fused with the CCoAOMT sequence. Inhibition of COMT I, which is able to methylate *in vitro* both CoA esters such as 5-hydroxycaffeoyl-CoA and caffeoyl-CoA, and small molecules such as catechol and protocatechuic aldehyde (Maury et al., 1999), was sufficient to obtain a strong decrease in acetosyringone concentration. Acetosyringone content was however unaffected in antisense CCoAOMT plants, a result which raises again the question of the regulation of feruloyl-CoA formation during acetosyringone biosynthesis.

Another interesting question raised by our work concerns the origin of the constitutive activation of acetosyringone biosynthesis in the OTX4 cell line. One possible explanation is that it was constitutively activated in the TX4 cell line from the beginning following selection on p-fluorophenylalanine and that this activation has remained stable, whereas the activation of cinnamoylputrescines synthesis has eventually been lost after the long period of culture in the absence of selective pressure. The genetic status of p-fluorophenylalanine-resistant cell lines is unclear but in light of recent research on retrotransposons activation in tobacco under stress conditions and during tissue culture (Grandbastien, 1998; Takeda et al., 1999) it seems likely that the mutation(s) controlling this resistance could result from the activation of retrotransposons. Since both cinnamoylputrescines and acetophenone synthesis are induced by MeJa in tobacco cell suspensions (Negrel and Javelle, 2010), one can wonder whether the resistance to p-fluorophenylalanine could directly or indirectly be linked to the expression of jasmonic acid-induced genes.

Conclusions

In conclusion we have shown that an OMT catalysing the synthesis of acetosyringone and which exhibits a high affinity for 5-hydroxyacetovanillone is induced in MeJa-treated tobacco cell suspensions. To our knowledge, this is the first report of an OMT catalysing the synthesis of acetosyringone. This result supports the hypothesis that acetosyringone biosynthesis in tobacco proceeds from feruloyl-CoA via acetovanillone and 5-hydroxyacetovanillone. Further work is necessary to purify and clone this OMT to study its specificity and the expression of the corresponding gene. A molecular and genetic approach is needed to identify unambiguously the different OMT isoforms involved in acetosyringone biosynthesis. It will be interesting to compare the sequence of the OMT methylating 5-hydroxyacetovanillone with that of other OMTs involved in the synthesis of small molecules such as catechol-OMT, which has recently been identified in tomato (Mageroy et al., 2012). Further work is also now necessary to characterise the enzyme synthesising 5-hydroxyacetovanillone from acetovanillone. Practically the characterisation of an OMT synthesising acetosyringone could be useful to synthesise [^{14}C]-acetosyringone from [^{14}C]-SAM in order to undertake metabolic studies *in vivo*. This could be useful to determine whether acetosyringone, which has repeatedly been found associated with cell walls (Blount et al., 2002; Chesson et al., 1997; Piquemal et al., 1998), is metabolised in tissues in which it is synthesised. The tobacco cell-suspension cultures that we have used in this work may be a good model to undertake these studies.

Experimental

Plant material

Suspension cultures of tobacco (*Nicotiana tabacum* L. cv. Xanthi) were grown in Murashige and Skoog medium containing 2 mg l^{-1} 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 1–4 days by vacuum filtration. Two tobacco cell lines were used, namely the TX1 cell line previously used for the characterisation of acetovanillone synthase (Negrel and Javelle, 2010) and the TX4 cell line which was also available in our laboratory. The two cell lines were originally provided in December 2000 by Dr. J. Berlin (Braunschweig, Germany).

Chemicals and substrates

MeJa, acetovanillone, acetosyringone, 5-hydroxyferulic acid, 5-hydroxyvanillin (3,4-dihydroxy-5-methoxybenzaldehyde) and SAM were purchased from Sigma–Aldrich. Ferulic acid, sinapic acid, syringaldehyde, chlorogenic acid, catechol and guaiacol were available in our laboratory. 5-Iodoacetovanillone was synthesised from acetovanillone (Lee et al., 1992). Feruloyl-CoA and 5-hydroxyferuloyl-CoA were prepared enzymatically from ferulic acid and 5-hydroxyferulic acid respectively, using recombinant tobacco 4-coumarate:coenzyme A ligase (Beuerle and Pichersky, 2002). Sinapoyl-CoA was prepared by transesterification of sinapoyl-*N*-hydroxysuccinimide ester as previously described (Negrel and Smith, 1984). CoA thioesters were purified using C_{18} solid phase extraction cartridges (Beuerle and Pichersky, 2002).

5-Hydroxyacetovanillone

5-Hydroxyacetovanillone was prepared from 5-iodoacetovanillone essentially as described by Banerjee et al. (1962): 5-iodoacetovanillone (0.584 g, 0.2 mmol), hydrated copper sulphate (0.32 g), and 4 N sodium hydroxide (15.2 ml) were refluxed overnight under nitrogen. After acidification to pH 3–4 with concentrated HCl and dilution with water to 100 ml the mixture was extracted twice with 100 ml EtOAc. The organic phases were

mixed, evaporated and redissolved in a minimum volume of MeOH. This mixture was applied onto a silica gel chromatography column (Kieselgel 60, Merck, 2.5 × 15 cm) and eluted using $CHCl_3$–MeOH (4:1) as mobile phase. The different fractions were analysed by TLC on Kieselgel 60 F-254 plates (Merck) in EtOAc-*iso*PrOH (9:1). 5-Hydroxyacetovanillone reacted with both acidic dinitrophenylhydrazine and aqueous ferric chloride. The fractions containing 5-hydroxyacetovanillone were pooled, evaporated under reduced pressure, redisssolved in 1 ml MeOH and stored at −20 °C. On standing at this temperature, part of the product crystallised (mp 163–166 °C, 31% from 5-iodoacetovanillone). Its molecular formula was established as $C_9H_{10}O_4$ on the basis of HR-ESIMS *m/z* 205.0468 $[M+Na]^+$ (calcd for $C_9H_{10}NaO_4$ 205.0471) and its structure was confirmed by 1H NMR (400 MHz, MeOD): δ 7.19 (s, 1H), 7.17 (s, 1H), 3.91 (s, 3H), 2.53 (s, 3H). For routine OMT activity measurements, and to avoid the crystallisation step, aliquots of the MeOH solution were purified by RP-HPLC before using the product in enzymatic assays. The concentration of the substrate was then calculated from the absorbance at 298 nm (e_{298} = 1.18 × 10^4 M^{-1} cm^{-1}).

Mass spectrometry and NMR

HR-ESIMS analyses were performed using a microToF QII Bruker Daltronics mass spectrometer, operating in positive mode and calibrated using a sodium formiate solution.

The NMR spectrum was recorded on a Bruker Avance III HD 400 spectrometer, using a 5 mm QNP probe.

HPLC and TLC

The same HPLC method was used to analyse phenolics in the culture medium and to detect the formation of acetosyringone from 5-hydroxyvanillone in enzyme assays. Phenolics were separated by RP-HPLC using a Waters (Milford, MA) chromatography system equipped with a dual wavelength absorbance detector set at 280 and 300 nm. Products were separated on a Nova Pack C_{18} column (3.9 × 150 mm, 4 μm) using a flow rate of 0.8 ml min^{-1}. The following conditions were used: 90% solvent A (milliQ water containing 1 ml l^{-1} acetic acid) 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 5-hydroxyacetovanillone 13.7 min, acetovanillone 15.63 min, acetosyringone 16.10 min.

TLC was on Kieselgel 60 F-254 plates (Merck) using the following solvents: (1) EtOAc-*iso*PrOH (9:1). R_f acetovanillone 0.55, 5-hydroxyacetovanillone 0.52, acetosyringone 0.52; (2) $CHCl_3$–MeOH (9:1). R_f acetovanillone 0.52, 5-hydroxyacetovanillone 0.34, acetosyringone 0.59.

Identification and quantification of acetophenones in the culture medium

The concentration of extracellular acetophenones in the TX1 and OTX4 cell lines was monitored by direct HPLC analysis of the culture medium (Baker et al., 2005). One-millilitre samples of tobacco cell suspensions were centrifuged at 12,000*g* for 5 min and stored at −20 °C prior to HPLC analysis. 20 μl aliquots were injected onto the column. Acetovanillone and acetosyringone were identified as previously described (Negrel and Javelle, 2010). To identify 5-hydroxyacetovanillone, 500 ml of culture medium was collected 48 h after treatment with 100 mM MeJa and extracted twice with 200 ml EtOAc. EtOAc was evaporated under reduced pressure and the residue redissolved in 0.5 ml MeOH. Aliquots (20 ml) were then analysed by HPLC as described above and the 5-hydroxyacetovanillone peaks were collected. The product was then identified by co-chromatography (HPLC, TLC) and comparison of its UV spectrum with the synthetic standard. Its identity was confirmed by checking that it was converted into acetosyringone when used as substrate in the 5-HAV-OMT assay.

Enzyme extraction

All work was done at 4 °C. Frozen tobacco cells were homogenised 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^{-1} ascorbic acid. The extract was then centrifuged at 20,000*g* for 15 min. Solid $(NH_4)_2SO_4$ was then added at 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). After centrifugation the desalted extract was used in the different enzyme assays and to measure the apparent molecular mass of 5-HAV-OMT.

Enzyme assays

AVS activity was measured spectrophotometrically at 30 °C using feruloyl-CoA and NAD as substrates as previously described (Negrel and Javelle, 2010). The same assay was used to try to detect the formation of 5-hydroxyacetovanillone or acetosyringone from 5-hydroxyferuloyl-CoA or sinapoyl-CoA, respectively: in this case the incubation mixture contained 200 μl protein extract, 50 μM 5-hydroxyferuloyl-CoA (or sinapoyl-CoA), 1 mM NAD and 0.1 M Tris HCl buffer pH 8 in a final volume of 1 ml. No decrease of the absorbance at 380 nm could be detected after 30 min. HPLC analysis of aliquots of the reaction medium confirmed that no formation of 5-hydroxyacetovanillone or acetosyringone had occurred after 30 min.

5-HAV-OMT activity was measured by incubating a mixture containing 150 μl protein extract, 335 μl 0.1 M Tris–HCl pH 8.5, 5 μl 20 mM SAM and 10 μl 5-hydroxyacetovanillone 10 mM at 30 °C in an Eppendorf tube. After 30 min, the reaction was stopped with 50 μl acetic acid. After precipitation of the proteins at 4 °C and centrifugation, a 10 μl aliquot was analysed by HPLC and the amount of acetosyringone formed during the reaction was quantified. When more than 30% of the substrate was consumed, a shorter reaction time was used. This assay was used to determine the optimum pH, using KPi, Tris–HCl and $NaHCO_3$-Na_2CO_3 buffers. The effect of cations on the activity was tested by adding $MgCl_2$ or $CaCl_2$ (1 mM final concentration) to the incubation medium. Inhibition of 5-HAV-OMT activity by anti-COMT antibodies was tested by mixing protein extracts with 10% (v/v) serum at room temperature for 10 min before assaying residual activity as described above.

CCoAOMT activity was determined essentially as described by Ye et al. (1994). 100 μl protein extract was mixed with 25 μl 2.5 mM cafeoyl-CoA, 5 μl 25 mM SAM and 370 μl 50 mM Tris–HCl buffer pH 7.5 containing 0.2 mM $MgCl_2$, 2 mM DTT, 10% glycerol and 0.2 mM PMSF. The reaction mixture was incubated at 30 °C for 5 min only, to avoid excessive degradation of caffeoyl-CoA, and stopped by the addition of 55 μl 5 N NaOH. After hydrolysis of the CoA esters during 15 min at 40 °C and acidification with 62 μl 6 N HCl, proteins were removed by centrifugation and ferulic acid was extracted from the surnageant with 1 ml EtOAc. 500 μl of the EtOAc phase was then evaporated *in vacuo* and redissolved in 100 μl MeOH. A 10 μl aliquot was then analysed by HPLC to quantify the amount of ferulic acid formed during the reaction.

Characterisation of the reaction product in the OMT assay

The enzymatically formed product in the 5-HAV-OMT assay showed the same R_t in HPLC, UV spectrum (λ^{MeOH} nm: 298; KOH 360) and R_f in TLC as authentic acetosyringone. 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 5-hydroxyacetovanillone and SAM in the conditions described above for the standard enzymatic assay but on larger scale (total incubation medium volume of 10 ml). After precipitation of the proteins at 4 °C and centrifugation, the supernatant was extracted twice with 10 ml EtOAc. After evaporation under reduced pressure, acetosyringone was dissolved in a minimum volume of MeOH and analysed by MS after HPLC purification. The molecular formula of the enzymatically formed product was established as $C_{10}H_{12}O_4$ on the basis of HR-ESIMS *m/z* 219.0627 $[M+Na]^+$ (calcd for $C_{10}H_{12}NaO_4$ 219.0628).

Activity of the o-diphenolic substrates in the OMT assay

In order to compare the activity of the different *o*-diphenolic substrates in the OMT assay, the V_{max} and K_m values corresponding to each substrate were determined using the incubation conditions used for 5-hydroxyacetovanillone. The corresponding products were separated and quantified by HPLC as described above. The different products were identified by co-chromatography with authentic standards and comparison of their UV spectra. Retention times were: catechol 5.66 min, guaiacol 14.33 min, 5-hydroxyvanillin 12.69 min, syringaldehyde 15.83 min, caffeic acid 13.37 min, ferulic acid 16.48 min, 5-hydroxyferulic acid 14.33 min, sinapic acid 16.53 min, chlorogenic acid 17.74 min. All substrates and products were detected at 300 nm except catechol which was detected at 280 nm.

Protein, SDS–PAGE and immunoblotting

The protein content was determined by the method of Bradford (1976). PAGE of proteins was carried out under denaturating conditions at 10% concentration using minigels, 50 μg protein being loaded in each well. Proteins were electrotransferred onto nitrocellulose membranes and the different OMTs were immunodetected using antibodies recognising the different isoforms of CCoAOMTs and COMTs. The alkaline phosphatase labelled secondary antibody was detected using the BCIP/NBT substrate.

FPLC

The molecular mass of native OMT was 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. 100 μl aliquots of the dialysed protein extract (ca. 2.5 mg pr) were injected onto the column.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.12.013.

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