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O-methyltransferase(s)-suppressed plants produce lower amounts of phenolic *vir* inducers and are less susceptible to *Agrobacterium tumefaciens* infection

Stéphane Maury · A. Delaunay · F. Mesnard · D. Crônier · B. Chabbert · P. Geoffroy · M. Legrand

Abstract The first step *of Agrobacterium tumefaciens*/plant interaction corresponds to the activation of a transduction pathway of the bacterium by plant exudate. Phenolic compounds rapidly secreted by wounded plant cells induce the expression of bacterial virulence (*vir*) genes; however, little is known about their biosynthesis in plant. Here we show that inoculation of an *Agrobacterium tumefaciens* virulent strain on orthodiphenol-*O*-methyltransferases-suppressed tobacco plants leads to significantly smaller tumors compared to control plants. These transgenic plants are inhibited for caffeic acid *O*-methyltransferase class I or II (OMT; EC 2.1.1.6) and/or caffeoyl-coenzyme A *O*-methyltransferase (CCoAOMT; EC 2.1.1.104) that are involved in monolignol biosynthesis. The significant decrease of tumor size could be suppressed by the pre-activation of bacterial virulence, before inoculation, using acetosyringone a known *vir* inducer. Total soluble phenolic amounts and cell wall composition analyzed by FT-IR analysis did not show significant differences between transgenic and control plants. The potential of phenolic extracts from control and OMT-suppressed plants to induce virulence was evaluated using an *Agrobacterium tumefaciens* reporter strain carrying a *vir::LacZ* gene fusion plasmid. Lower *vir*-inducing activities were recorded for plants that show inhibition to caffeic acid *O*-methyltransferase activity. HPLC analysis confirmed that the levels of several phenolic compounds were differently affected by wounding and/or by bacterial inoculation. Statistical correlations were established between tumor sizes, *vir*-inducing activities, *O*-methyltransferases proteins accumulations and the levels of various soluble phenolic compounds such as acetosyringone. These results demonstrate the role of the *O*-methyltransferases of the phenylpropanoid pathway in the early production of soluble *Agrobacterium tumefaciens vir* inducers.

Keywords Acetosyringone · *Agrobacterium* · Caffeic acid *O*-methyltransferase · Caffeoyl-coenzyme A *O*-methyltransferase · HPLC · *Nicotiana*

Abbreviations

CCoAOMT	Caffeoyl-coenzyme A *O*-methyltransferase
COMTI	Caffeic acid *O*-methyltransferase of class I
COMTII	Caffeic acid *O*-methyltransferase of class II
FT-IR	Fourier transformed-infrared
HPLC	High-performance liquid chromatography
OMT	Orthodiphenol-*O*-methyltransferases
Vir	Virulence

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Introduction

The phenylpropanoid pathway that provides many phenolic compounds to the plant cell, such as salicylic acid, flavonoids, coumarins and lignin-building units, is strongly activated upon infection by pathogens or treatment with elicitors (Dixon and Paiva 1995; Dixon et al. 2002; Ferrer et al. 2008). Plant phenolics play important roles in the outcome of plant/microbe interactions. On the one hand, in infected plants, the deposition of phenylpropanoid compounds participates in cell wall reinforcement that restricts pathogen invasion (Vance et al. 1980; Nicholson and Hammerschmidt 1992; Dixon and Paiva 1995) by cross-linking with cell wall constituents (Iiyama et al. 1994). On the other hand, phenolic compounds act as primary signals for several bacteria such as *Rhizobium* and *Agrobacterium* (Gelvin 2006; McCullen and Binns 2006) to activate an early transduction pathway controlling the expression of bacterial genes involved in symbiosis or pathogenesis.

Wounded plant cells secrete exudate with high concentration of various phenolics (Walker et al. 2003a, b) that induce *Agrobacterium tumefaciens* virulence (*vir*) genes expression. *Agrobacterium tumefaciens* is a Gram-negative soil bacterium that causes crown gall disease. It affects many woody and herbaceous plants belonging to 140 genera of more than 60 families and particularly fruit trees of economic interest. This bacterium has the capacity to transfer genetic material (T-DNA) into the plant cell genome after *vir* genes induction by plant phenolics (Gelvin 2006; McCullen and Binns 2006; Dafny-Yelin et al. 2008). The specific composition of phenolic compounds in plant exudates may influence the host specificity of *Agrobacterium tumefaciens.*

The best characterized and most effective *vir* inducers are monocyclic phenolics such as acetosyringone (Stachel et al. 1985a), coniferyl and sinapyl alcohol (Melchers et al. 1989); diphenolic compounds such as dehydrodiconiferyl alcohol aglycone and glucosides (Lynn et al. 1987; Orr and Lynn 1992; Teutonico et al. 1991); hydroxycinnamic acid amides (Berthelot et al. 1998); sugars or acidic pH (Shimoda et al. 1990; Zhu et al. 2000) and GABA (Chevrot et al. 2006). The *vir* regulon is coordinately induced in response to host phenolic compounds in combination with monosaccharides and extracellular acidity in the range of pH 5–5.5. *Vir* inducers are not detected or detected at very low levels in uninjured plants, but their amounts increase rapidly in wounded plant cells (Stachel et al. 1985a; Teutonico et al. 1991; Tamagnone et al. 1998; Chevrot et al. 2006). These chemical stimuli are detected by the transmembrane two-component sensor kinase VirA (Lee et al. 1995; Gelvin 2006). This sensor contains four functional domains (periplasmic, linker, kinase and receiver). Its periplasmic domain is required for detection of a sugar binding protein named ChvE, while the linker domain allows the detection of phenolic compounds. Then, VirA can phosphorylate the response regulator VirG that positively regulates all *vir* promoters. It was recently shown that salicylic acid could attenuate the function of the VirA kinase domain (Yuan et al. 2007).

Most of the phenolic *vir* inducers are mono- or di-methoxylated compounds, the biosynthesis and relative importance for the establishment of tumors of which are poorly understood. Two S-adenosyl-*L*-methionine (SAM)-dependent *O*-methyltransferases (OMTs) methylate the phenyl ring of monolignol precursors: caffeic acid OMTs (COMTs) and caffeoyl-CoA OMTs (CCoAOMTs). COMT methylates caffeoyl- and 5-hydroxyferuloyl-containing acids, aldehydes and alcohols in vitro but displays a kinetic preference for the alcohols and aldehydes over the free acids, while CCoAOMT accepts only the CoA-ester as a substrate (Maury et al. 1999; Hoffmann et al. 2001; Do et al. 2007; Ferrer et al. 2008). Furthermore, transgenic tobacco plants suppressed for the different orthodiphenol-*O*-methyltransferases (OMTs) involved in the phenylpropanoid pathway were produced and the impact on lignin biosynthesis and plant growth was studied (Atanassova et al. 1995; Pinçon et al. 2001a). These plants show inhibition to caffeic acid orthodiphenol-*O*-methyltransferase (COMT) of class I or II and/or caffeoyl-coenzyme A orthodiphenol-*O*-methyltransferase (CCoAOMT) activities by antisense RNA expression. The activation of the OMTs during plant/pathogen interaction such as tobacco/TMV has been reported (Maury et al. 1999; Toquin et al. 2003), but no data were available about their possible role in the production of *vir* inducers during infection of tobacco by *Agrobacterium tumefaciens*. Here, we show that OMT-suppressed transgenic plants inoculated with a virulent *Agrobacterium tumefaciens* strain developed smaller tumors than those of control plants. This phenotype could be suppressed by the pre-activation of bacterial virulence with acetosyringone before inoculation. Lower *vir* gene induction activities were measured using an *Agrobacterium tumefaciens* reporter strain carrying a *vir::LacZ* gene fusion in plants expressing *COMTI*, *COMTII* and *COMTI::CCoAOMT*. Finally, HPLC analysis demonstrated that a few phenolic compounds such as acetosyringone are major *vir* inducers that are produced in lower amounts in OMT-suppressed tobacco plants compared to controls upon infection. These results demonstrate the dual role of OMTs of the phenylpropanoid pathway in monolignol synthesis and in the early production of soluble *Agrobacterium tumefaciens vir* inducers.

Materials and methods

Bacterial strain and culture conditions

Agrobacterium tumefaciens strain A348 harboring the octopine type Ti plasmid (pTiA6) was used for plant inoculation (Anand et al. 2007). We used an A348 strain harboring the pSM219 plasmid that carries *lacZ* under control of the *virH* promoter in *trans* to the wild-type pTiA6 plasmid for virulence induction measurements (Stachel et al. 1985b). Bacteria were cultured in Luria–Bertani medium supplemented with kanamycin (50 μg ml^{-1}) and rifampicin (10 μg ml^{-1}) at 28°C (Anand et al. 2007). Overnight cultures were centrifuged and resuspended in culture medium to obtain 0.6 absorbance unit ml^{-1} at 660 nm before use.

Plant material and *in planta* tumor assay

Production of antisense OMT-suppressed tobaccos (*Nicotiana tabacum* L. cv Samsun NN; kindly provided by IBMP du CNRS, Strasbourg, France) with the tobacco COMTI sequence (named COMTI plants; accession number AF484252; Atanassova et al. 1995), the tobacco COMTII sequence (named COMTII plants; accession number X74453; Martz 1997), the tobacco CCoAOMT sequence alone (accession number U62735) or transcriptionally fused to COMTI (named CCoAOMT and dAS plants, respectively; Pinçon et al. 2001a) and control plants transformed with an empty plasmid (Atanassova et al. 1995; Martz 1997; Pinçon et al. 2001a) has been published previously. Plants were grown in the greenhouse under controlled conditions at 22°C with a 16-h/8-h photoperiod.

Stems of 6-week-old plants were wounded at the level of the same internode using a needle and inoculated with 10 μl of a culture at 10^9 CFU ml^{-1} of virulent *Agrobacterium tumefaciens* strain A348 induced or not with commercial acetosyringone (Sigma-Aldrich, Saint-Quentin Fallavier, France) according to Anand et al. (2007). Briefly, overnight cultures were centrifuged, washed and resuspended in agro-induction medium supplemented or not with acetosyringone (150 μg ml^{-1}) at 24°C for 15 h. The cultures were washed with sterile distilled water and resuspended in 0.9% NaCl at 10^9 CFU ml^{-1} for *in planta* tumor assay. Two types of control plants were used: wounded plants inoculated with 10 μl of bacterial media alone and unwounded plants. At 6 h after infection, treated and unwounded stem tissues of half of the plants were harvested for further analyses. Thus, previously published data on agro-inoculated tobacco BY-2 cell suspensions suggest a model in which tobacco undergoes a response in the first 6 h (Veena et al. 2003). The other half of the plants were grown in the greenhouse for 1 more month to obtain tumors. Tumors were pictured with a numeric camera, separated from stem tissues using a scalpel, weighed and frozen in liquid nitrogen. The numeric pictures were analyzed using imaging software (ImageTool for Windows version 3.00) to determine the area of each tumor. Two independent experiments with at least 20 individuals of the same clone by genotype and treatment were done.

Callus induction and proliferation

Callus induction and proliferation were carried out to check if the response to growth regulators (similar to those produced in crown galls) of transgenic tissues was affected or not compared to control plants. Callus induction was realized according to a standard protocol (Atanassova et al. 1995). Briefly, leaves and shoots from transgenic and control plants from 2-week-old in vitro seedling were cultivated separately on MS medium supplemented with 2 mg l^{-1} of 6-benzylaminopurine and 0.05 mg l^{-1} of naphthalene acetic acid (Sigma-Aldrich). Subcultures were performed every 2 weeks during 6 weeks. Calli obtained for each type of plant were initiated with the same number of starting explants (size homogenized between plants). At the end of the 6 weeks, the total fresh mass of callus was determined by weighing each Petridish. Eight replicates and two independent experiments were done for each type of plant and explant.

SDS-PAGE and immunoblotting

The basic procedures for electrophoresis under denaturing conditions, immunoblotting and OMT antibodies dilutions have been described previously (Maury et al. 1999). The blots were scanned and the protein amounts were quantified using imaging software (ImageTool for Windows version 3.00).

Extraction and quantification of phenolic compounds and *vir* gene induction assay

Tobacco stems (60 mg) were ground in liquid nitrogen and extracted twice with 70% methanol (1/20) (v/v). After centrifugation at 3,000*g* for 15 min, supernatants were collected, evaporated and resuspended in 1 ml of 70% methanol. Spectrophotometric quantification of total phenolic compounds was done using Folin-Ciocalteu's reagent (Carlo Erba Reagenti, Rodano, Italy) with an adapted method from Causevic et al. (2005). Catechin (0–10 μg μl^{-1}) was used as a standard. Bioassays for vir-inducing activity were performed according to Stachel et al. (1985b). Briefly, overnight cultures of strain A348 (pSM219) were centrifuged and resuspended in culture medium. As much as 100 μl of material to be tested for *vir*-inducing activity was inoculated with 900 μl of bacteria culture at 0.1 absorbance unit ml^{-1} at 600 nm and incubated for 15 h at 28°C. The reporter

β-galactosidase activity was measured, and the results were expressed in specific units calculated as previously described (Miller 1972; Stachel et al. 1985b). All experiments were performed in triplicate and controls with known amounts of acetosyringone or without inducer were included.

HPLC analysis

Methanolic extracts from stem were analyzed using high-performance liquid chromatography (HPLC). Analyses were performed using C18 columns (Kromasyl, 4.6 cm × 25 cm, 5 µm Phenomenex, Le Pecq, France and repeated on X-terra, 4.6 cm × 15 cm, 5 µm, INTERCHIM, Montluçon, France). The peaks were identified according to their retention time and spectral properties compared with commercial standards (Sigma-Aldrich). Eluted peaks were collected as independent fractions and tested for their *vir*-inducing potential.

FT-IR spectroscopy

FT-IR spectra were recorded between 4,000 and 400 cm^{-1} at 4 cm^{-1} resolution on a Nicolet spectrophotometer using KBr discs containing 1% of the dry samples (lyophilized tissues) and corrected for the KBr background. The window between 800 and 2,000 cm^{-1}, which shows information on polysaccharides and lignin was selected to compare cell wall modifications between samples. All spectra were baseline corrected, then normalized at 1,650 cm^{-1} prior to spectra comparison. Average spectra were obtained from the cell wall analysis of the three replicates.

Statistical analysis

Statistical analysis was done using SPSS statistical software package (SPPS version 11.0.1 PC, Chicago, IL, USA). Genotypic and treatment effects were evaluated by two-way ANOVA (GLM procedure) using the following model: $Y_{ijk} = \mu + G_i + T_j + (G_i \times T_j) + \varepsilon_{ijk}$; where Y_{ijk} are individual values, μ is the general mean, G_i is the effect of genotype i, T_j is the effect of treatment j, $G_i \times T_j$ is the genotype by treatment interaction and ε_{ijk} is the error. All statistical tests were considered as significant at $*P \leq 0.05$, $**P \leq 0.01$ or $***P \leq 0.001$.

Results

Induction of OMTs upon infection by *Agrobacterium* is decreased in antisense tobacco lines

Immunodetection of OMTs in stems of agro-inoculated controls (transformed with the empty plasmid) or OMT-suppressed plants using antibodies raised against COMTI, COMTII or CCoAOMT revealed four bands (Fig. 1a). The 39-kDa and 42-kDa isoforms corresponded to COMT proteins of class I and class II, respectively, while the 27-kDa and 32-kDa isoforms revealed two CCoAOMT isoforms (Maury et al. 1999). Quantification of these four isoforms in wounded–mock inoculated, wounded–agro-inoculated or unwounded transgenic stems 6 h after the treatment is shown in Fig. 1b. A genotypic effect was

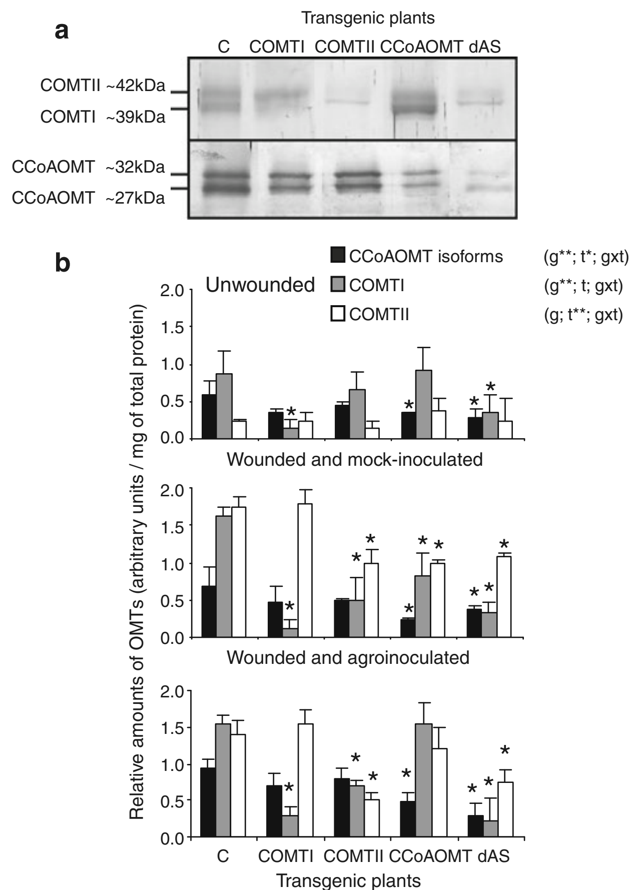


Fig. 1 Accumulation of OMT proteins in tobacco stems of various genotypes upon wounding and agro-infection. Treated stem tissues were harvested 6-h post-inoculation. Samples were immunoblotted with antibodies recognizing COMTI (*gray bars*), COMTII (*white bars*) or CCoAOMT (*black bars*). Protein amounts were quantified by scanning immunoblots. **a** Western blots showing the different OMT isoforms and their corresponding mass in kDa in agro-inoculated stems of transgenic plants. *C* control plants transformed with an empty plasmid. Antisense transgenic tobaccos were suppressed for COMTI (*COMTI*), COMTII (*COMTII*), CCoAOMT (*CCoAOMT*) or for both COMTI and CCoAOMT (*dAS*). **b** Amounts of the three types of OMT. The calculated protein amounts represent the mean values ± SE of two independent experiments using three replicates. Significant differences between control and transgenic plants are indicated at $*P \leq 0.05$ or $**P \leq 0.01$ To evaluate genotypic and treatment effects, two-way ANOVA analysis was performed: for each graph. *g* indicates the genotype effect, *t* the treatment effect and $g \times t$ the genotype by treatment interaction. Significant effects are indicated at $*P \leq 0.05$ or $**P \leq 0.01$

observed for CCoAOMT and class I COMT isoforms, while a treatment effect was evidenced for CCoAOMT and class II COMT isoforms. As expected for OMTs involved in lignin biosynthesis, a high basal level of expression was measured for CCoAOMT and class I COMT in unwounded control stems. The wounding and inoculation processes rapidly increased the amounts of all OMT proteins in stems. In OMT-suppressed plants the accumulation of the corresponding OMT proteins was decreased in healthy and in wounded and infected tissues compared to control plants. In a few cases, the inhibition of one OMT type in antisense plants affected the expression of another OMT type: for example, class II COMT was down-regulated in dAS plants, explaining the absence of genotypic effect.

Repression of the phenylpropanoid pathway decreases the virulence of *Agrobacterium tumefaciens*

Typical tumors that developed on stems of agro-inoculated control and OMT-suppressed plants are shown in Fig. 2a. The mass and area reached by the tumors 1 month after inoculation were used to evaluate bacterial virulence (Fig. 2b, c). These two variables were shown to be strongly correlated ($r = 0.91$ at $P < 0.05$). Transgenic plants inhibited for COMTI, COMTII or COMTI and CCoAOMT, but not CCoAOMT alone, exhibited tumors with significantly smaller size and mass compared to those developing on control plants.

To find the causes of tumor size decrease on transgenic plants, two hypotheses were tested: (i) the ability of *OMT*-suppressed cells to divide. In vitro callus proliferation was measured and it was confirmed that transgenic tissues respond to growth regulators (similar to those produced in crown galls) in a similar way as control cells (Fig. 3a); and (ii) the ability of *OMT*-suppressed plants to activate *Agrobacterium* virulence. *In planta* tumor assays were performed with activated (or not) bacteria using acetosyringone before inoculation (Fig. 3b). In each plant, the *vir* induction was followed by an increase of tumor area (about 10–20%). The significant decrease of tumor size using non-activated bacteria was confirmed on transgenic plants inhibited for COMTI, COMTII or COMTI and CCoAOMT, but was suppressed using *vir*-induced bacteria compared to control plants.

To confirm that the effect on tumor development was mediated by a decrease in the biosynthesis of phenolic signals implicated in the activation of *vir* genes, we compared *vir*-inducing activities of phenolic stem extracts from the different tobacco genotypes (Fig. 4). An *Agrobacterium tumefaciens* strain carrying a *pinF::lacZ* fusion gene was used as a reporter strain. In this strain, β-galactosidase expression is controlled by the *vir*

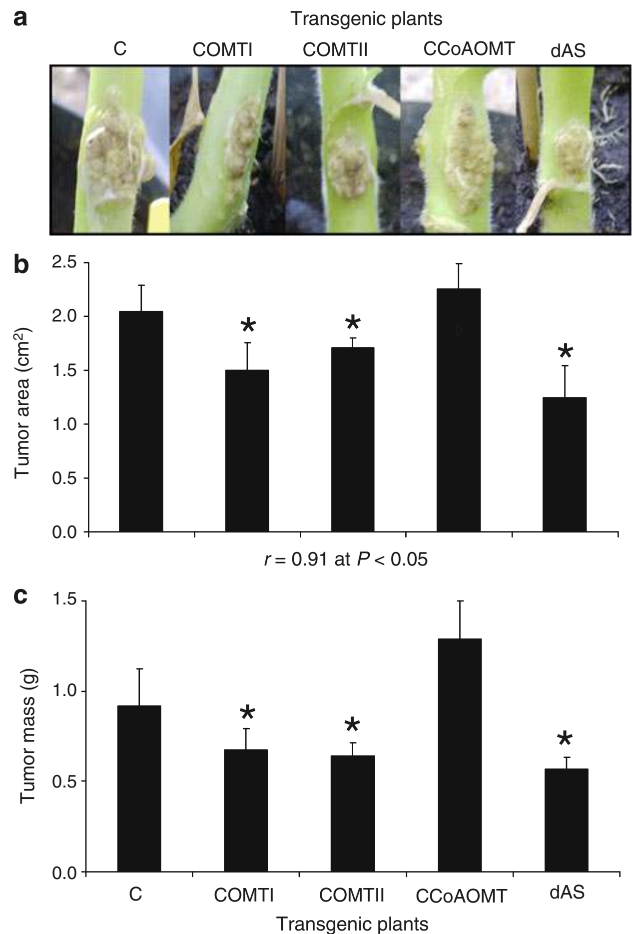


Fig. 2 Impact of OMT repression on the development of *Agrobacterium tumefaciens* tumors. **a** Phenotype of typical tumors; genotypes and abbreviations are as in Fig. 1. **b** Tumor area. **c** Tumor mass values. Mean values ± SE ($n = 40$) are shown. Abbreviations used for genotypes are as in Fig. 1. Two independent experiments were performed. Significant differences between control and transgenic plants are indicated at $^*P \leq 0.05$. Pearson coefficient (r) is given for the linear correlation between tumor area and weight values

locus and the relative amount of induced activity reflects the relative amount of *vir*-inducing activity to which the bacterial cell has been exposed (Stachel et al. 1985b). A stimulation of the *vir* activity was noticed in agro-inoculated control plants compared to unwounded ones (Fig. 4). Only a genotypic effect was observed for all transgenic plants. Transgenic plants inhibited for COMTI, COMTII or COMTI and CCoAOMT, but not CCoAOMT alone, showed a decreased of their *vir*-activities in healthy and/or agro-inoculated stems compared to control plants. The lack of treatment effect reflected the absence of stimulation of the *vir* activity in OMT-suppressed plants. Linear correlations were established between tumor areas, *vir*-inducing activities and COMTI accumulation (Table 1).

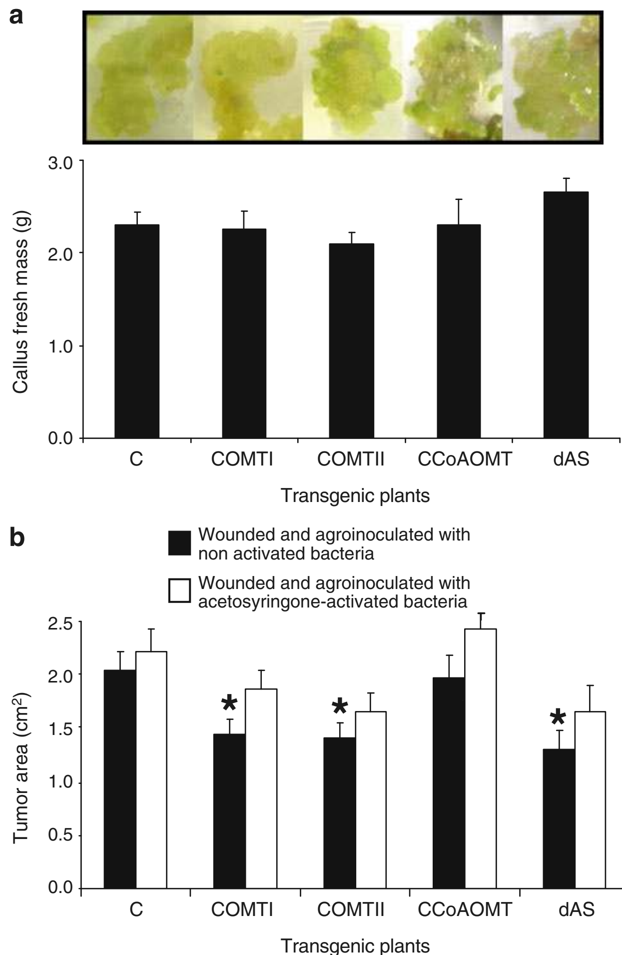


Fig. 3 Analysis of potential causes of the reduction of tumor size on *OMT*-suppressed plants (see Fig. 1 for abbreviations). Significant differences between control and transgenic plants are indicated at $*P \leq 0.05$ for at least two independent experiments. **a** Callus induction and proliferation in vitro of transgenic and control explants. Callus fresh mass (in g) was determined 6 weeks after induction. Pictures of calli and mean values of fresh mass ± SE ($n = 16$) are shown. **b** Tumor area after agro-inoculation with *Agrobacterium* cultures that have been *vir* activated (by acetosyringone) or not. Mean values ± SE ($n = 40$) are shown. Significant differences with mean tumor area obtained with non-activated bacteria on control plants are indicated at $*P \leq 0.05$

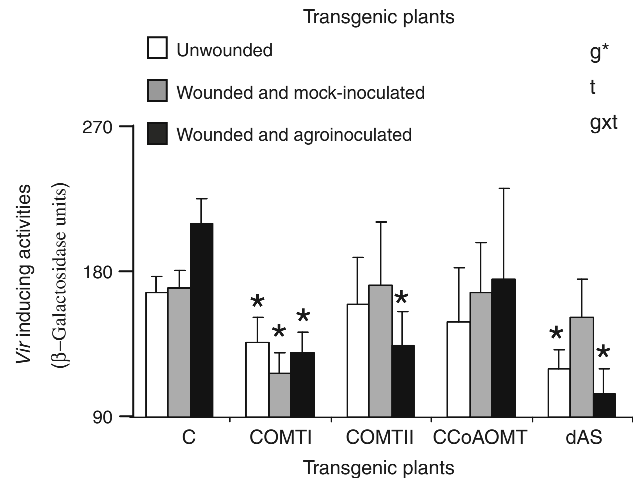


Fig. 4 *Vir*-inducing activity of soluble phenolic extracts from transgenic tobacco stems upon wounding and agro-infection. V*ir*-inducing activity was measured as *β*-galactosidase activity using an *Agrobacterium tumefaciens* reporter strain carrying a *pinF::LacZ* fusion plasmid. Phenolic extracts were prepared from 6-h post-treatment tobacco stems of unwounded (*white bars*), wounded and mock inoculated (*gray bars*) or wounded and inoculated with *Agrobacterium tumefaciens* (*black bars*). Positive controls with known amounts of acetosyringone (100 μM) or without inducer were established at 286 and 45 *β*-galactosidase units, respectively. Abbreviations used for genotypes are as in Fig. 1. Mean values ± SE ($n = 4$) are presented. Two independent experiments were performed. Significant differences between control and transgenic plants are indicated at $*P \leq 0.05$. In order to evaluate genotypic and treatment effects, two-way ANOVA analysis was performed: for each graph, *g* indicates the genotype effect, *t* the treatment effect and $g \times t$ the genotype by treatment interaction. Significant effects are indicated at $*P \leq 0.05$ or $**P \leq 0.01$

Identification of phenolic inducers of bacterial *vir* genes in control and transgenic plants

As *vir*-inducing activity inhibition paralleled the decrease in OMT stimulation in transgenics, this prompted us to analyze the phenolic composition of cell walls using FTIR spectroscopy and to estimate the total soluble phenolic fraction from stems (Suppl. materials 1 and 2, respectively). Several bands indicative of the main cell wall compounds such as polysaccharides (1,050 cm^{-1}), proteins (1,560 cm^{-1} and 1,650 cm^{-1}), esters (1,700 cm^{-1}) and aromatic skeleton (1,420 cm^{-1}) were observed by FTIR (Suppl. material 1). Overall, the fingerprint profile of FTIR spectra from the walls of 6-h post-treatment stems showed little variations compared to that of unwounded samples. Nevertheless, an increase of polysaccharides was observed in wounded control and in agro-inoculated dAS plants. In agro-inoculated CCoAOMT and dAS plants, an additional band was visible at 1,270 cm^{-1} and could possibly correspond to syringyl units. A genotypic effect was observed for total soluble phenolic contents but no treatment effect was detected (Suppl. material 2). dAS plants exhibited higher level of soluble content than control plants.

Soluble phenolic extracts from the different transgenic stems were separated by HPLC and 26 peaks were quantified in the extracts (Fig. 5). In control plants, the amounts of about half of them were affected either by wounding (peaks 4, 24 and 26), agro-inoculation (peaks 5, 7 and 13) or both treatments (peaks 6, 10, 14, 15, 18, 19, 21 and 23). Variations among transgenic plants were also observed for several peaks such as peak 1 that was increased in COMTI-inhibited plants. Peak 15 was identified as acetosyringone, one of the major known *vir* inducer (Stachel et al. 1985a, b). This compound was not detected in COMTI and dAS

Table 1 Pearson's coefficients (r) from linear correlations calculated between tumor areas, *vir* inducing activities, OMT proteins accumulations and acetosyringone amounts in 6-h post-treatment (unwounded, wounded and mock inoculated or wounded and *Agrobacterium tumefaciens* infected) transgenic plants (control, COMTI, COMTII, CCoAOMT and dAS plants; $n = 80$ except for acetosyringone where $n = 25$ corresponding to the number of plants with detectable amounts of this compound among the 80 samples)

	Tumor areas	Vir inducing activities	COMTI	COMTII	CCoAOMT	Acetosyringone
Tumor areas	1	0.86*	0.88*			
Vir inducing activities		1	0.78***			0.70*
COMTI			1			0.50*
COMTII				1		
CCoAOMT					1	0.88**
Acetosyringone						1

Only significant linear correlations are indicated at *$P \leq 0.05$, **$P \leq 0.01$ or ***$P \leq 0.001$

antisense plants (Fig. 5b, e) but was present in CCoAOMT-repressed tissues, thus demonstrating the function of COMTI in acetosyringone biosynthesis. In plants producing significant amount of acetosyringone, its quantities scaled positively with *vir*-inducing activities, COMTI and CCoAOMT levels (Table 1). Plants that do not produce detectable amounts of acetosyringone, such as COMTI and dAS plants (Fig. 5f), still exhibit *vir*-inducing activity due to unidentified compounds. Extracts from unwounded and wounded-agro-inoculated control or dAS transgenic genotypes were fractionated by HPLC to identify differences in active phenolic compounds. Each fraction was tested for its *vir*-inducing activity and the difference between values obtained for infected and unwounded plants was calculated (Fig. 6). Increased *vir* activities were found in control plants for fractions number 1, 2, 6 and 10, with highest changes in activity upon infection being recorded for fractions number 2 and 6. The compound responsible for inducing activity of fraction 2 could not be identified, but the active compound in fraction 6 was shown to be acetosyringone on the basis of elution time and absorbance spectrum compared to that of standard. Interestingly, some fractions issued from dAS profile displayed significant activity, while the corresponding control fractions were inactive (fractions number 7, 8 and 9; Fig. 6). These data suggest that OMT inhibition provoked the appearance of metabolic intermediates with inducing activity. The nature of these products remains to be elucidated.

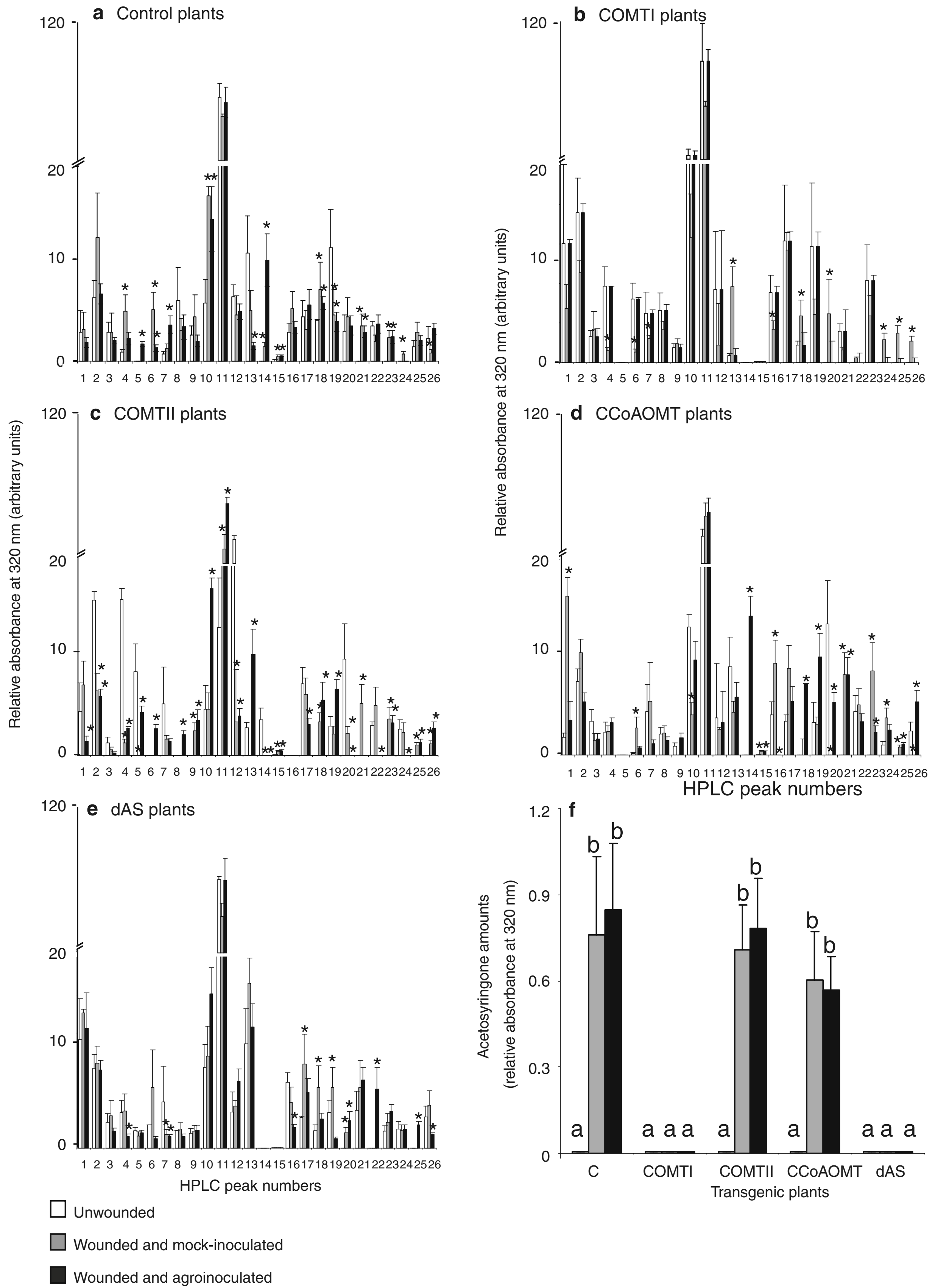
Discussion

OMTs of the phenylpropanoid pathway are involved in wounding and agro-infection responses of tobacco plants

COMTI and/or CCoAOMT-inhibited transgenic tobacco plants have been previously produced and characterized (Atanassova et al. 1995; Martz 1997; Pinçon et al. 2001a). Their analysis showed different impacts on lignin synthesis that were changes in lignin composition with a decrease of syringyl units and/or a decrease of total lignin content. Similar effects of OMT inhibition were confirmed in other plant species (Doorsselaere et al. 1995; Zhong et al. 1998; Do et al. 2007). In plants transformed with a double antisense construct (CCoAOMT/COMTI), stronger cumulative effects were obtained (Pinçon et al. 2001a; Do et al. 2007). Down-regulation of OMT isoforms was confirmed in the present work in the stem of each type of unwounded transgenic plants. Furthermore, inhibition of one OMT isoform was accompanied, in some cases, by the co-repression of another OMT isoform by an unknown mechanism. A similar phenomenon has been reported for several enzymes of the phenylpropanoid pathway including OMTs (Pinçon et al. 2001a, b). Here, we show that all OMT isoforms (COMTI, COMTII and CCoAOMT) are strongly and early induced in agro-infected stems of control plants. This result is in agreement with the model proposed by Veena et al. (2003) suggesting that tobacco responds in the first 6 h of infection, while *Arabidopsis* responds after 48 h (Ditt et al. 2006). In transgenic tobacco plants, the targeted OMT(s) was down-regulated even after infection. Up-regulation of the expression of genes encoding enzymes of the phenolic metabolism such as CCoAOMT by *Agrobacterium* infection has been shown in tobacco cells and *Arabidopsis* (Ditt et al. 2006; Veena et al. 2003). Similar early induction was previously reported for tobacco OMTS in wounded or TMV-infected leaves (Jaeck et al. 1992; Pellegrini et al. 1993; Martz et al. 1998; Maury et al. 1999; Toquin et al. 2003). These results suggest a general involvement of OMTs in the responses of plants to wounding or pathogen attack.

Down-regulation of OMTs results in lower susceptibility of tobacco plants to agro-infection by decreasing virulence of *Agrobacterium tumefaciens*

In transformed cells, the expression of T-DNA genes that are involved in the synthesis of plant hormones results in


a Control plants
b COMTI plants
c COMTII plants
d CCoAOMT plants
e dAS plants
f
Relative absorbance at 320 nm (arbitrary units)
HPLC peak numbers
Acetosyringone amounts (relative absorbance at 320 nm)
C
COMTI
COMTII
CCoAOMT
dAS
Transgenic plants
Unwounded
Wounded and mock-inoculated
Wounded and agroinoculated

◀ **Fig. 5** HPLC analysis of soluble phenolic compounds from wounded or agro-infected transgenic tobacco stems. Phenolic extracts were prepared from transgenic tobacco stems of unwounded (*white bars*), wounded and mock inoculated (*gray bars*) or wounded and inoculated with *Agrobacterium tumefaciens* (*black bars*). **a** Control plants transformed with an empty plasmid. **b** COMTI-inhibited transgenic tobaccos. **c** COMTII-inhibited transgenic tobaccos. **d** CCoAOMT-inhibited transgenic tobaccos. **e** dAS, transgenic tobaccos suppressed for both COMTI and CCoAOMT. Treated stem tissues were harvested 6-h post-inoculation. Total soluble phenolic compounds were extracted, and fractionated by HPLC in 26 peaks that were quantified by their absorbance at 320 nm. Mean values ± SE ($n = 4$) are shown. Two independent experiments were performed. Significant differences for a specific peak in a given transgenic plant between unwounded and wounded mock inoculated or agro-inoculated are indicated at *$P \leq 0.05$. **f** Acetosyringone amounts (HPLC peak no. *15*) in the various extracts. *Scale* has been expanded for better readability. Values marked with *distinct letters* are significantly different among them (at $P \leq 0.05$) as determined by one-way ANOVA

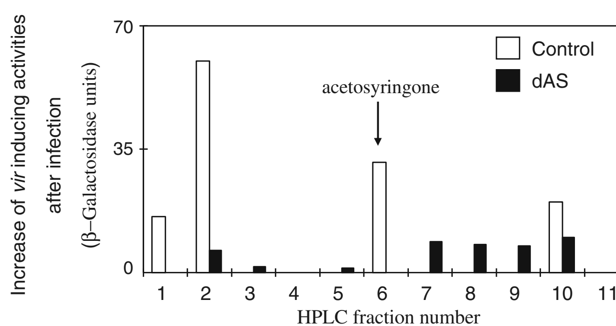


Fig. 6 Fractionation of *vir*-inducing activity produced by tobacco stems infected with *Agrobacterium tumefaciens.* Extracts from unwounded or *Agrobacterium tumefaciens*-infected stems of control plants (C, *white bars*) and CCoAOMT/COMTI-suppressed (dAS, *black bars*) plants were fractionated by HPLC and tested for their ability to induce *vir* activity. The differences between values obtained for infected and unwounded plants of each genotype were calculated and are presented for each HPLC fraction. Acetosyringone was identified in fraction number *6* on the basis of its retention time and UV absorption spectrum

the growth of tumors on the host plant (Gelvin 2006; McCullen and Binns 2006). We found that tumors developing on COMT-suppressed plants were significantly smaller than those on control plants. This phenotype was not related to an alteration of transgenic cells to grow and divide. Furthermore, the phenotype was suppressed if *Agrobacterium* was artificially activated with acetosyringone before inoculation. As few methoxylated phenolic compounds were known to be strong *vir* inducers (Stachel et al. 1985a; Melchers et al. 1989; Lynn et al. 1987; McCullen and Binns 2006), we tested the ability of phenolic extracts from agro-infected transgenic and control plants to induce *vir* genes. A strong inhibition of the *vir* induction was measured in COMT-suppressed plants and particularly in double transgenic plants, as has been previously shown for lignin biosynthesis (Pinçon et al. 2001a). This result indicated that COMTI inhibition resulted in a strong decrease of *vir*-inducer biosynthesis. In CCoAOMT-inhibited plants, no reduction of the *vir*-inducing activities was found, in good agreement with the lack of effect on the development of tumors on these transgenics. The strong impact in double transgenic plants could be due to the co-repression of COMTs and CCoAOMTs leading to the lowest residual accumulation of all OMTs as previously observed (Pinçon et al. 2001a). Interestingly, in most of the transgenic plants, *vir*-inducing activities of wounded extracts were significantly different from the *vir*-inducing activities of agro-inoculated extracts, but not from those of unwounded controls. The linear correlation we established between COMTI accumulation, tumor size and *vir*-inducing activities suggests that COMTI suppression affects the production of *vir* inducers synthesized in response to *Agrobacterium* infection and consequently decreases the susceptibility of the plant to the bacteria.

OMTs are involved in the early production of acetosyringone

OMTs from the phenylpropanoid pathway are known to synthesize, upon infection by pathogens, methoxylated phenolics either soluble or cross-linked to the cell walls (Nicholson and Hammerschmidt 1992; Iiyama et al. 1994; Dixon et al. 2002; McCullen and Binns 2006). However, little is known about the partitioning between the soluble and cell wall-bound fractions in the early stages of infection or wounding. In this frame, FT-IR spectra analysis did not show significant cell wall cross-linking 6 h after wounding or agro-inoculation. It is likely that such phenomenon would take several hours or days as it has been shown in some cases (De Ascensao and Dubery 2003; Hawkins and Boudet 2003; Hano et al. 2006). Nevertheless, an increased incorporation of syringyl lignin units was observed in relation to CCoAOMT inhibition (CCoAOMT and dAS plants) after agro-inoculation in accordance with the results in *Arabidopsis* (Do et al. 2007).

Thus, we concentrated our efforts on the analysis of the soluble phenolic fraction. Quantitative variations were observed between transgenic plants but without significant impact on the treatment (Suppl. material 2). This result, at odds with the variations of *vir*-inducing activities upon wounding and infection, prompted us to perform HPLC separation of soluble phenolic extracts from treated plants. We found that the production of several phenolic compounds was stimulated in response to wounding and/or agro-infection, in good agreement with their *vir*-inducing activities.

The biosynthesis of acetosyringone was shown to be dependent on COMTI and to be particularly inhibited in

plants repressed for both COMTI and CCoAOMT. This is certainly correlated to the presence of two methoxylated groups in acetosyringone molecule that may be introduced sequentially by the two OMTs, as demonstrated for the synthesis of syringyl units of lignin (Atanassova et al. 1995; Pinçon et al. 2001a). Nevertheless, inhibition of COMTI is sufficient to obtain a significant decrease in acetosyringone content and this is reminiscent of the decrease in syringyl units observed in COMTI-suppressed plants (Atanassova et al. 1995; Doorsselaere et al. 1995). Similarly, altered sinapoyl malate biosynthesis was found in an *Arabidopsis* COMTI–CCoAOMT double mutant (Do et al. 2007). All these results demonstrate a clear biosynthetic relationship between acetosyringone and sinapate derivatives. We have previously characterized COMT and CCoAOMT substrate specificities toward monolignol precursors (Maury et al. 1999; Hoffmann et al. 2001) and it will be now interesting to measure their kinetic values toward 3,4,5-trihydroxyacetophenone and 5-methoxy, 3,4-dihydroxyacetophenone to validate the putative involvement of the enzymes in acetosyringone biosynthesis. Another possibility would involve a decarboxylation step of sinapate derivatives (sinapic acid, sinapoyl-CoA, sinapaldehyde or sinapyl alcohol). It is noteworthy here that inhibition of cinnamoyl-CoA reductase (which accepts sinapoyl-CoA as substrate) in CCR-suppressed transgenic tobacco leads to the incorporation of acetosyringone in plant cell walls (Chabannes et al. 2001). Acetosyringone is usually not detectable in tobacco cell walls, but can be found, for example, in wheat straw after alkaline wet oxidation (Klinke et al. 2002). It was suggested that CCR inhibition has led to a change in the metabolic flux through the phenylpropanoid pathway.

The biological significance of phenolic release during wounding is poorly understood. It was proposed that acetosyringone could be related to lignin (cell wall?) repair in damaged cells or potentially toxic to other soil pathogens (Stachel et al. 1985a). This last assertion is supported by the recent report of inhibitory effects of acetosyringone on *Pseudomonas syringae* virulence (Baker et al. 2005) and aflatoxin biosynthetic genes of *Aspergillus flavus* (Hua et al. 1999; Hua 2001). This suggests that *Agrobacterium tumefaciens* has evolved an acquired resistance to acetosyringone. It was also recently shown that other phenolics like salicylic acid could attenuate the function of the VirA kinase domain and participate in defenses against *Agrobacterium* infection (Yuan et al. 2007). On the other hand, several monocotyledonous plants have been shown to contain *vir* gene inducing factors as ethylferulate (Messens et al. 1990), although they are recalcitrant to transformation by *Agrobacterium tumefaciens*. It has been proposed that this could be due to the local secretion around the root axis of MDIBOA (2-hydroxy-4,7-dimethoxybenzoxazin-3-one), a potent and specific inhibitor of the VirA-mediated induction of *vir* gene expression (Zhang et al. 2000).

Agrobacterium tumefaciens affects few monocotyledonous and a large array of dicotyledonous plants, particularly fruit trees of economic interest. In our experiments, down-regulation of *vir* inducers in tobacco has led to an increased resistance to infection suggesting that this strategy could be applied to plants of economic interest. However, recent analyses of COMTI and CCoAOMT-inhibited double transgenic tobacco have shown that they developed larger necrotic lesions on tobacco mosaic virus infection (Maury et al. 2000; Legrand et al. unpublished results). This increase of lesion size reflects a decrease of plant resistance to virus infection and suggests that some soluble and/or cell wall-bound phenolic compounds are synthesized by OMTs and are important elements of plant defense against tobacco mosaic virus. These phenolic compounds will have to be identified and quantified in transgenic tobacco plants. Altogether, these results demonstrate the complexity and the variety of the roles of phenolic compounds in plant adaptation to its environment.

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