Effect of Phenolic Glycosides on *Agrobacterium tumefaciens virH* Gene Induction and Plant Transformation

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O-Aryl-D-glucoside (**4**−**7**) and D-xyloside (**8**−**10**) derivatives were synthesized and tested on *Agrobacterium virH* gene induction and plant transformation. α- or *β*-Glycosides enhanced *vir* activity at concentrations above 250 *μ*M. The highest *vir* activity was observed with *β*-glucoside derivative **4** at 10 mM. A marked difference between phenol glucoside derivative **4** and the corresponding free phenol on the growth of transformants was observed. The regenerated transgenic tissues, after transformation on medium containing acetosyringyl *β*-glucoside **4**, grew at twice the rate of those on medium containing only free acetosyringone (**AS**). Compound **4** was less toxic for tobacco explants compared to the corresponding free phenol. However, the xyloside derivatives tested (**8**−**10**) were less effective for gene induction compared with corresponding free phenols.

Several compounds are known to influence gene expression. A plant cell becomes susceptible to *Agrobacterium* when it is wounded or is precultured on a phytohormone-containing medium.[1−6] Wounded plant cells produce an abundance of low molecular weight phenolic compounds[7] that are implicated in lignin biosynthesis or its degradation and act as specific inducers of *vir* gene expression.[2] Once the *vir* genes are expressed, the encoded protein products facilitate T-DNA transfer to the cytoplasm and nucleus,[8,9] which is followed by integration of T-DNA into the plant genome. The role of some phenolic compounds and of *VirA-VirG* activation to obtain high levels of *vir* gene induction and gene transfer have been reported.[7,10−12] Recently we have studied the effects of nine phenolic compounds on *Agrobacterium* virulence gene induction, on *Agrobacterium*-mediated gene transfer, and on both transient and stable transformation rates on *Petunia* and tobacco. We confirmed that virulence induction and transformation rates are increased by the use of phenolic *vir* inducers bearing an unsaturated lateral chain.[13] It has also been demonstrated that some monosaccharides such as D-glucose or D-galactose can interact with constitutive gene products, and activation of surface proteins leads to a synergestic *vir* gene induction in the presence of phenolic compounds.[14] Delmotte and co-workers synthesized phenolic glycosides and tested their effect on virulence of *Agrobacterium*.[15,16] They showed that *O*-aryl *β*-glycosides derived from L-fucoside, D-galactoside, D-glucoside, and D-maltoside were much less effective than the corresponding free phenols. Nevertheless, the *β*-maltoside derivative of syringic acid showed induction capacity equivalent to that of the free acid at 500 *μ*M and had induction capacity higher than that of syringaldehyde *β*-glucoside or *β*-galactoside derivatives.[15] These facts strongly suggest that the sugar residue may be a key determinant of the *virA-virG* virulence gene induction. It has been further shown that the toxicity of free phenolics on Agrobacteria was considerably reduced by glycosylation.[17]

In this paper, we report the effect of new *O*-aryl-D-gluco- and D-xylosides on *Agrobacterium tumefaciens virH* gene induction and plant transformation.

Results and Discussion

Previously, we have observed a higher activity of 4-(4-hydroxy-3,5-dimethoxyphenyl)but-3-en-2-one (**2**) and 1-(4-hydroxy-3,5-dimethoxyphenyl)-3-(4-hydroxyphenyl)prop-2-enone (**3**) than acetosyringone (**1**), which was attributed to a better stabilization due to the mesomeric forms involved in the molecular mechanism of *VirA* activation by phenols.[13] Here, we have synthesized their glycosidic derivatives following three methods of *O*-aryl *β*-glycosylation (Figure 1). The *β*-D-glucosides derivatives **4**,[18] **5**, and **6** were synthesized according to Michael reaction,[19] the α-D-glucoside **7** was prepared from a D-glucopyranosyl phenylsulfoxide,[20] and the *β*-D-xylosides **8**−**10** were prepared via a stereoselective one-pot synthesis involving 1,2-cyclic sulfite derivatives from unprotected xylose.[21]

Action of Glycosylated Compounds on *virH* Gene Induction. At a concentration of 100 *μ*M, acetosyringone (**1**) and benzalacetone (**2**) have a high *vir* gene induction (Figures 2 and 3). At the same concentration *O*-aryl *β*-xyloside derivatives (**8**, **9**, and **10**), acetosyringyl α-glucoside (**7**), and *β*-glycosides (**4**, **5**) (Figure 1) showed a strong inhibition of *vir* gene induction (Figures 2 and 3). The three acetosyringyl glycosides (**4**, **7**, and **8**), the *β*-xyloside derivatives (**9** and **10**), and the *β*-glucoside (**5**) gave *vir* induction equal to the control. This was probably due to the masked phenol function in the glycosides.

We have previously shown[17] that a *β*-glucosyl ester of syringic acid has the same *vir* induction effect at a concentration of 100 *μ*M as syringic acid. The loss of an active phenol function appeared to be the most important parameter in the inhibition of *vir* induction observed by the three forms of glycosidic phenolic compounds. We have also shown that *β*-glycosides of phenolic compounds have

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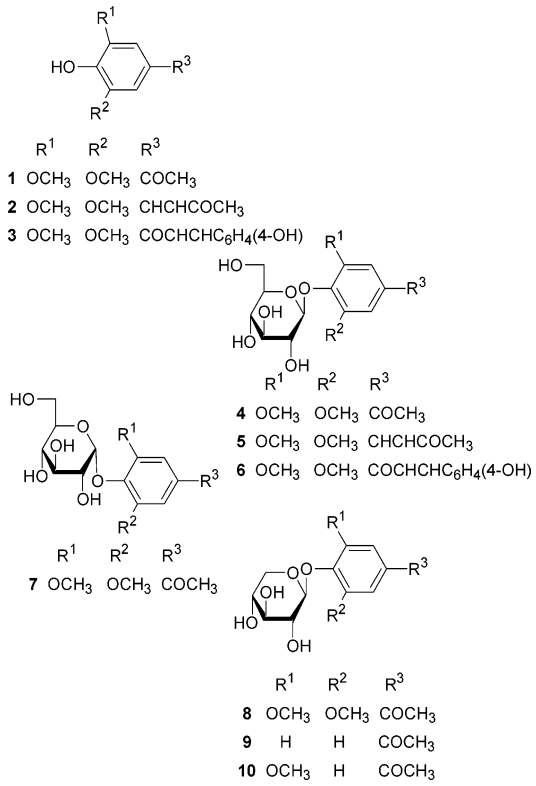

Figure 1. Structure of tested phenolic compounds: **4**−**6**, *β*-glucosides; **7**, α-glucoside; **8**−**10**, *β*-xyloside derivatives.

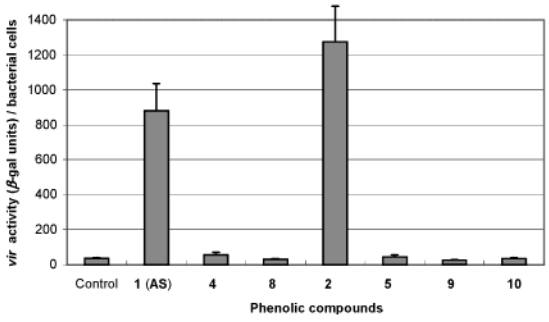

Figure 2. Effect of aryl D-glycoside (*β*-glucosides **4** and **5**) and *β*-xyloside (**8**, **9**, and **10**) derivatives on *virH* gene induction compared with free phenols **AS** (**1**) and **2**. All compounds were tested at 100 *μ*M. Values are averages (n = 9) ± SE. Probability showing differences between media was 1.00. The *β*-galactosidase activity units were calculated using the formula $A_{420} \times 1000/A_{600}$ and are described as units per bacteria cells.

no effect on *vir* gene induction at an effective concentration of the free forms (100 *μ*M) and a relatively strong action at a high concentration (∼10 mM).[17]

Action of Different Concentrations of Glycosylated Phenolic Compounds on *virH* Induction. As shown in Figure 3, when the concentration of acetosyringone (**1**) was increased, the *vir* gene induction decreased after 250 *μ*M and a toxicity and null effect were observed at 5 mM. Comparatively, the *vir* activity of the three tested glycosides, α- and *β*-glucosides and *β*-xyloside, on *vir* genes was very different at concentrations between 0.25 and 20 mM. At 0.5 mM, the effect of glycosides derivatives **7** and **8** on *vir* induction reached about 25% of maximal action of **1**. At 1 mM, the *vir* activity of **8** reached 50% of the max **1**. The *β*-glucoside **4** had a marked effect on *vir* induction at 10 mM. However, the maximal activity of these compounds varied with their concentrations: about 2.5−5.0 mM for *β*-xyloside (**8**) and α-glucoside (**7**) and about 10.0−15.0 mM for *β*-glucoside (**4**).

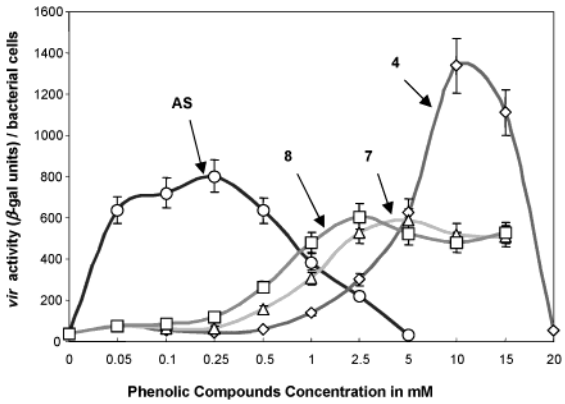

Figure 3. Effect of various concentrations of phenolic glycosides **4**, **7**, and **8** on gene *virH* induction compared to **AS**. Values represent the average of 5 repetitions (mean ± SE). The *β*-galactosidase activity units were calculated using the formula $A_{420} \times 1000/A_{600}$ and are described as units per bacteria cells.[22]

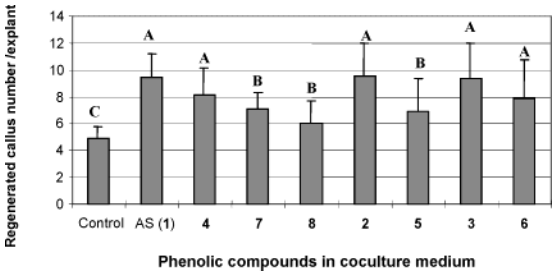

Figure 4. Effect of phenolic compounds (**1**−**3**) and aryl D-glycosides (**4**−**8**) on tobacco transformation, based on number of calli and buds regenerated per leaf explant on selection medium 4 weeks after co-cultivation. Compounds were tested at 100 *μ*M in the co-culture medium. 30 to 40 leaf explants were used for each measurement (n = 3). Values are average ± SE. Probability showing differences between media was >0.9. Newman-Keuls test classification of phenolic compounds' statistical effect is indicated by letters A, B, and C.

Delay et al.[16] investigated the activities of endogenous glycosylhydrolases of *Agrobacterium tumefaciens* A348 pSM243 cd and A348 pSM358 harboring virB::lacZ and virE::lacZ fusion plasmids and showed that the naturally occurring α- and *β*-glucosidases have high activities and *β*-xylosidase a low activity. If similar glycosidase activities in our A348 pSM219 strain are present, it may explain the high *vir* activities observed at 2.5−15 mM glycosides (Figure 3): these compounds could have been hydrolyzed and thus liberate free acetosyringone **1** and sugar moieties. It is known that a synergistic effect exists between some monosaccharides such as glucose and simple phenolic compounds.[14] These free molecules can cooperate and generate the high *vir* induction rates as observed in the present study.

Action of Glycosylated Phenolic Compounds on Plant Transformation. Glycosylation led to a complete loss of *vir* induction property of the compounds at the concentration of 100 *μ*M in Figure 1. A similar result on plant transformation with glycosides could be expected. We tested some of these glycosides on *Agrobacterium*-mediated genetic transformation of tobacco. Indeed, we observed (Figure 4) that these glycosides significantly increased transformation rates as compared with the control. Comparatively, these transformation rates were nearly the same as those obtained with the corresponding free compounds. Many secondary metabolites of plants are transported and stored in tissues as glycosides, and plants contain the necessary enzymes to hydrolyze these glycosides and then release the active metabolite forms.[22] We measured (data not shown) the natural activity of glyco-

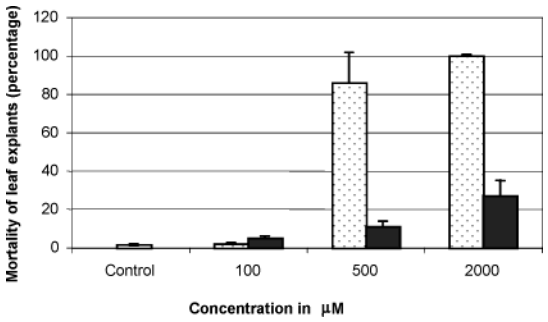

Figure 5. Effect of acetosyringyl β-D-glucoside (**4**) (■) and acetosyringone (**1**) (□) on the mortality of leaf explants. Values represent average (2 repetitions of 40−45 explants) mortality (%) of leaf explants, measured 7 days after bacterial co-culture.

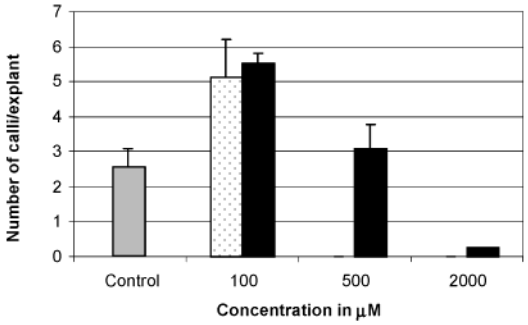

Figure 6. Effect of acetosyringyl β-D-glucoside (**4**) (■) and acetosyringone (**1**) (□) on plant transformation using increased phenolic compound concentrations. Transformation frequency is expressed as the number of calli/explant. Values are average (3 repetitions of 40−45 explants) of callus on selection medium measured 4 weeks after bacterial co-culture. Control explants were cultivated on a medium without phenolic compound.

sylhydrolases in tobacco and observed a high activity of β-glucosidase and β-xylosidase and low activities of α-glucosidase. Therefore, during co-culture, β-glycosides could be hydrolyzed and released as free active phenols. However, we observed a notable increase in transformation rate in the presence of acetosyringyl α-glucoside (**7**), with an intermediate result between control and acetosyringyl β-glucoside (**4**).

Delay et al. studied glycosylhydrolase activity in *Agrobacterium* strains and made a correlation between these activities and the strain virulence.[16] No glycosylhydrolase activity was detected in culture supernatants of the various strains they investigated. They suggested that there was a specific transport through the outer membrane and the periplasmic space.[15,16] Our study may suggest that the host plants also have the capacity to release free active *vir* inducers from their glycosylated forms. This enzymatic activity in plants could have an impact on the susceptibility of tobacco to *Agrobacterium*-mediated transformation.

Protective Effect of Glycosylation against Toxicity of Phenolic Compounds. One of the general effects of glycosylation is to protect biological systems against toxicity of metabolites such as free phenolic compounds.[15,16] We observed a possible protection effect on *Agrobacterium* and also in tobacco plants.

The study of different concentrations of **1** and the corresponding acetosyringyl β-glucoside (**4**) on the number of surviving leaf explants (after 7 days of culture) and on transformation rates (after 4 weeks of culture on selection medium) showed that efficiency of leaf protection by glucosylation of **1** was high (Figures 5 and 6). At 500 μM, **1** showed a high toxicity, whereas compound **4** was less toxic. Compound **4** induced high toxicity only at higher

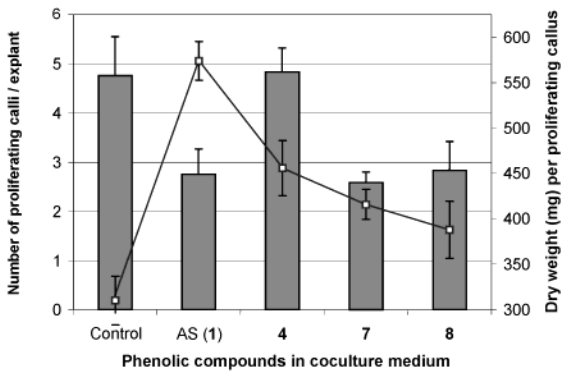

Figure 7. Influence of acetosyringyl glycosides (**4**, **7**, and **8**) and free **AS** (**1**) on transformation frequency of tobacco leaf explants. Transformation frequency is expressed as number (□—□) and the size of calli/explant. Sizes are represented by measures of callus growth (shaded) (dry weight in mg) after 21 days in culture. Compounds were tested at 100 μM. Weight values are averages of 310−574 putative transformed calli.

concentrations. Whereas 2 mM **1** killed all leaf explants (within 3 to 4 days), the explants survived for several weeks at the same concentration of β-glucosylated (**4**). While at 500 μM, compound **4** had no significant effect on transformation rate (Figure 6), the same concentration of free **1** had a detrimental effect. Nevertheless, compound **4** at 500 μM had a qualitative positive effect on transformed calli compared to the control, as they were greener and more vigorous.

Effect of Glycosylation on Callus Formation and Plant Regeneration. Explants of tobacco leaves co-cultivated on media with acetosyringyl β-glucoside (**4**) formed a transgenic callus more quickly, and buds/plantlets were developed more rapidly. In addition, the transformed calli were bigger than those on medium with free **1**. The difference between the two media was further confirmed from dry weight analysis of transformed calli. For example, transformed calli obtained on medium with acetosyringyl β-glucoside (**4**) weighed the same as those on the control medium (without any compound) (Figure 7), but had twice the dry weight of those obtained on other media. Thus, leaf explant survival and transgenic callus development (and consequently transformation efficiency of tobacco leaf explants) were strongly influenced by the phenolic compounds.

Furthermore, to compare the effect of **1** and its glycoside derivatives on *Agrobacterium tumefaciens*-mediated transformation, these compounds were tested for *vir* gene induction. Although the tested phenolic compounds had poor effect on *vir* gene induction, these compounds had significantly promoted transformation rates in tobacco leaf explants (Figure 6). Two hypotheses could best explain the results obtained by glycosylated phenolic compound on transformation rates. First, plant glycosylhydrolases release in the culture medium free phenolic compounds from the glycosides, may induce significant bacteria virulence, and avoid a toxic effect on plant cells of a high initial concentration of free phenolics. Second, the plant glycosylhydrolases also release free monosaccharides such as β-glucose, which can be directly assimilated by plant cells.

In conclusion, our investigations showed that some of these new phenolic compounds were very effective in *Agrobacterium*-mediated transformation. We observed that acetosyringyl β-D-glucoside (**4**) promotes a better growth of transformed calli and shoots as compared to acetosyringone and has a highly reduced toxicity at high concentrations. Therefore, its quantitative and qualitative effect on

the transformation efficiency of other plant species, particularly recalcitrant ones, should be investigated.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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