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INFLUENCE OF PHENOLIC COMPOUNDS ON *AGROBACTERIUM vir* GENE INDUCTION AND ONION GENE TRANSFER

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Key Word index—*Agrobacterium tumefaciens*; phenolic compounds; *vir* gene induction; *Allium cepa*; gene transfer induction.

Abstract—Eight chalcones and benzalacetones were tested for their virulence induction on *Agrobacterium tumefaciens*. With one exception, they had a strong action, and in particular 4-(3,5-dimethoxy-4-hydroxy-phenyl)-but-3-en-2-one (**10a**) was very effective with a virulence induction about 1.5–2 times that of acetosyringone (**AS**). When applied to *Agrobacterium*-mediated gene transfer of onion, both **10a** and **AS** at 250 μM, led to increased gene transfer of between 25 and 35% when compared with the control. Many derivatives of **AS** were tested and the indispensable chemical functions required to induce *vir* genes were determined; for example, the presence of at least one methoxy group and a carbonyl group as in acetyl, aldehyde and acid functions. The most effective *vir*-inducing compound used was the original **AS** with two methoxy groups and an acetyl function. By testing the corresponding β-glucosides and glucosyl esters (from acids), we established that a phenolic function was also essential for virulence induction. These glucosides led to a decreased toxicity to the bacteria in relation to the original product. However, the presence of a β-glucosylated phenol function led to the total loss of *vir* induction while the corresponding esters, particularly the glucosyl sysingate ester, conserved a good *vir* induction.

INTRODUCTION

During the last decade, genetic engineering of plants has become an important tool for crop improvement. Several molecular techniques are now used to introduce foreign genes into plants. However, *Agrobacterium tumefaciens*-mediated gene transfer has proved to be efficient, highly versatile and the most widely used vehicle for the genetic transformation of many dicots and a few monocots, which are natural hosts of this bacterium [1, 2].

During the course of infection, *A. tumefaciens* transfers a portion of its extra chromosomal tumour-inducing (Ti) plasmid, called the transferred DNA (T-DNA) into the cells of the host plant where it is integrated into the plant nuclear DNA. It is known that T-DNA transfer is highly regulated and triggered only in the presence of susceptible plant cells. Such plant cells are wounded and they produce an abundance of low M_r phenolic compounds, such as acetosyringone and hydroxy-acetosyringone, which are specific inducers of *vir* gene expression [3, 4]. The expression of the *vir* genes which are located on the Ti plasmid, outside the T-DNA region, initiates the mobilization and transfer of the T-DNA into the plant cell. Several commercially available plant metabolites have been shown to induce the *vir* genes of the *Agrobacterium* Ti plasmid [5–7]. Phenolics such as catechol, pyrogallic acid, sinapic acid and certain chalcone derivatives have been reported to induce *vir* genes [8, 9].

The lack or low level of *vir*-inducing substances in the exudates of monocots has been indicated as one of several possible hindrances to transformation by *Agrobacterium* [10]. However, there are reports that wheat cell suspensions and wheat and oat seedlings [11, 12] produce *vir* gene inducing compounds. Whether the type of inducer dictates host specificity of *Agrobacterium* remains largely unclear. A few monocots including *Asparagus officinalis* [13], *Oryza sativa* [14, 15], *Allium cepa*, [16] and *Zea mays* [17] have been transformed, indicating that other *vir* gene expression systems might be operating.

In an effort to explore the direct interaction between the phenolic compounds and transformation of a monocots, we have synthesized several new compounds and tested their effect on *vir* gene induction. More importantly we have correlated their effect on *Agrobacterium*-mediated gene transfer and expression of β-glucuronidase (GUS) gene in the cells of onion. We believed that it was necessary to establish a direct relationship between *vir* induction and T-DNA transfer in a monocot.

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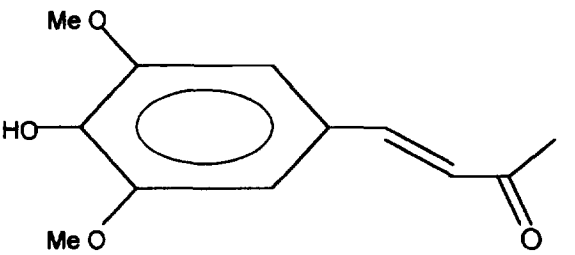
Recent studies of T-DNA transmitted chimeric β-glucuronidase (GUS) gene expression in maize shoots [18] showed that *vir* gene expression is an absolute requirement for the appearance of GUS positive spots. In this paper we demonstrate the beneficial effect of certain efficient *vir* inducing substances such as acetosyringone and benzalacetones for T-DNA transfer in onion.



RESULTS AND DISCUSSION

Action of AS *derivatives on virulence*

If we compare the action of **AS** derivatives on *vir* gene induction (products **1a** to **9a**; **2a** is **AS**) (Fig. 1), **1a**, **3a**, **4a** and **9a** have no action on virulence. The remaining compounds significantly induce *vir* genes by about the same proportion (40% of the **AS** level) but the best compound was **AS**. Thus, the absence of an acetyl group, contrary to results of Melchers *et al.* [6], or of the two *O*-methyl groups gave compounds incapable of *vir* induction. However, if the products conserved one of the two *O*-methyl groups or if acetyl is replaced by an aldehyde or acid group, they kept a part of their *vir* activity.

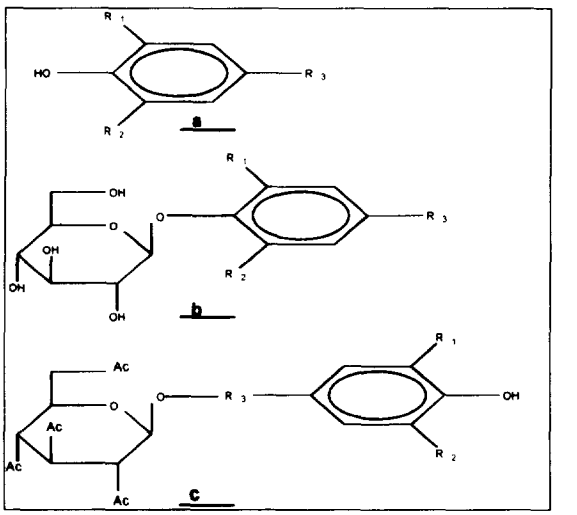


Fig. 3. General structures of tested phenolic compounds. **a**, Simple form; **b**, glucoside; **c**, glucosyl ester. The above chemical structure represents the benzalactone **10a**.

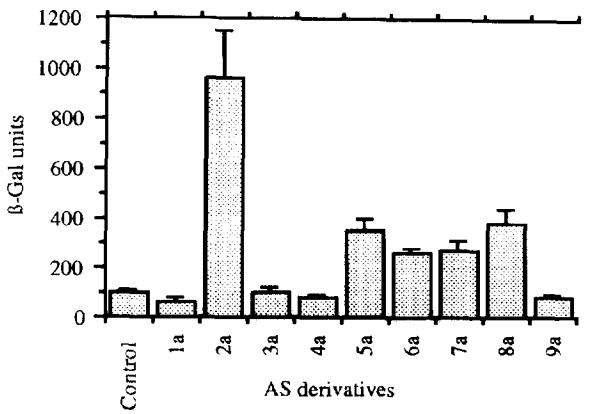


Fig. 1. Action of **AS** derivatives on *virH* gene. All compounds were tested at a concentration of 100 μM. Values are average ± s.e.

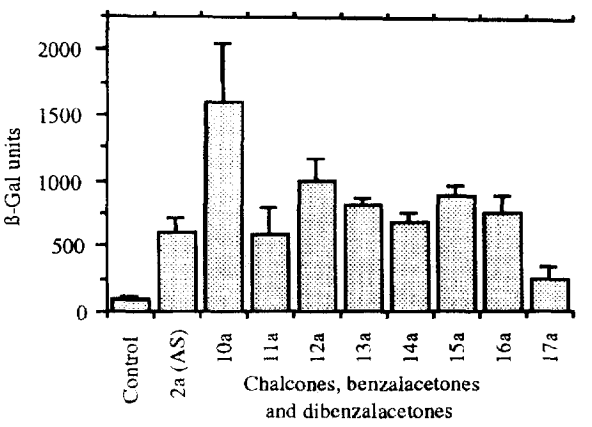


Fig. 2. Action of chalcones, benzalacetones and dibenzalacetones on *virH* gene. All compounds were tested at a concentration of 100 μM. Values are average ± s.e.

Action of chalcones, benzalacetones and dibenzalacetones on virulence

The chalcones, benzalacetones and dibenzalacetones bearing a phenolic function and one or two methoxy groups in the *ortho* position (**10a** to **16a**–Fig. 2) which were tested, increased significantly *vir* gene induction. These compounds have a relatively improved action of between 100 and 120% of the **AS** level. However, one of them, the benzalacetone **10a**, had a much better action on virulence (between 150 and 170% increase with respect to **AS**). In earlier work [9] the corresponding benzalacetone lacking one of the two methoxy groups showed a high *vir* induction, but was not as effective as **AS**. The addition of a second methoxy group (Fig. 2), giving rise to the compound **10a**, led to a very high *vir* induction superior to that of **AS**. The presence of one double bond in the R3 carbon chain (Fig. 3) enhanced the *vir*-inducing activity. But its position in this structure, either between the phenyl and carbonyl (**14a**) functions or after carbonyl (**12a**), seemed to have no effect. Therefore, it is not only the increasing distance between the carbonyl and the phenolic group which is responsible for its enhanced

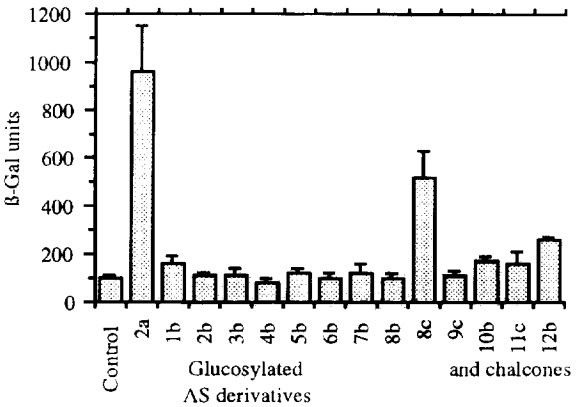


Fig. 4. Action of glucosylated **AS** derivatives and chalcones on *virH* gene. All compounds were tested at a concentration of 100 μM. Values are average $\pm$ s.e.

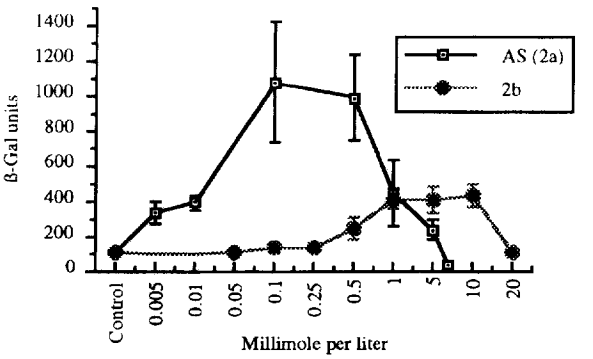


Fig. 5. The effect of different **AS** and glucosylated-**AS** concentrations on *virH* gene. Values are average $\pm$ s.e.

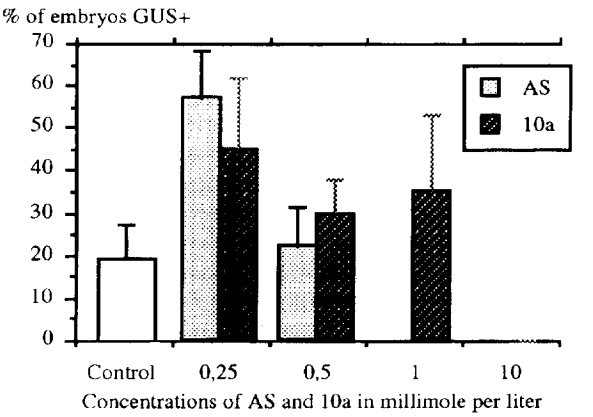


Fig. 6. Influence of acetosyringone and **10a** compounds on embryo *Agrobacterium*-mediated gene transfer. 40–50 embryos are used for each measurement. Values are average $\pm$ s.e.

effect [9]. Moreover, syringic acid (**8a**) and synapic acid (**11a**) give the same effect showing that the role of the double bond is not always clear. In dibenzalacetones (**16a**) the presence of two double bonds does not enhance *vir* gene induction, when compared with the benzalacetone **10a**. Without any *O*-methyl group, the **17a** had nearly lost great part of *vir* activity.

Action of glucosylated AS derivatives and glucosylated chalcones

When compared with the control (Fig. 4), glucosylated **AS** derivatives (**b** form: phenol function involved in glucose-compound linkage) totally lost most of their virulence. Only the **c** form glucosyl ester **8**, in which the phenol function is free, conserved a good virulence induction. It is particularly clear if we compare the **b** and **c** forms. Thus, we already know that acetyl and *O*-methyl functions are necessary for *vir* induction. The phenol function, as we expected, is also very important to maintain this induction.

We have also tested three glucosylated chalcones, **10b**, **11c** and **12b** and they give a *vir* induction two to three times greater than the control, but compared with **AS** or with simple compounds, they seem to have lost much of their *vir* inducing action.

On testing some of the simple compounds (**AS** derivatives and chalcones) in the presence of 100 μM glucose, a poor increase in their *vir* induction was often observed (data not shown). But this difference was not significantly important to conclude that there was a synergistic effect between these phenolic compounds and glucose.

Action of AS derivatives and chalcones on bacterial growth

We observed that the addition of the above organic products, glycosylated or not, always increased bacterial growth by 5–30% when compared with the control (data not shown). Furthermore, it appeared that growth could be correlated with *vir* induction. Indeed all compounds which gave a good *vir* induction also increased bacterial growth by 20–30%. These compounds correspond to most of the **a** forms of each tested product and the glucosyl ester of syringic acid (**c** form). On the other hand, the corresponding glucosylated product (**b** form) led to bacterial growth always slightly lower than the other compounds and was limited to an increase of only 10% with respect to the control.

Influence of phenolic compound concentration on virulence induction and bacterial growth

All preceding experiments were carried out using the same concentration of each different product (100 μM). For some of them, we measured *vir* induction as a function of increasing product concentrations. First, we tested the simple **AS** (**2a**) and its glucosylated form (**2b**) between 5 μM and 20 mM (Fig. 5). With **2a**, *vir* activity increased very quickly up to 100 μM and then it decreased slightly between 100 and 500 μM. It continued to decrease until it reached the control level at 5 mM. With **2b**, the response was completely different and much higher concentrations were required. It began to increase after a plate step and its action was to produce a slight *vir* induction (0.5–10 mM), but at 20 mM the induction fell

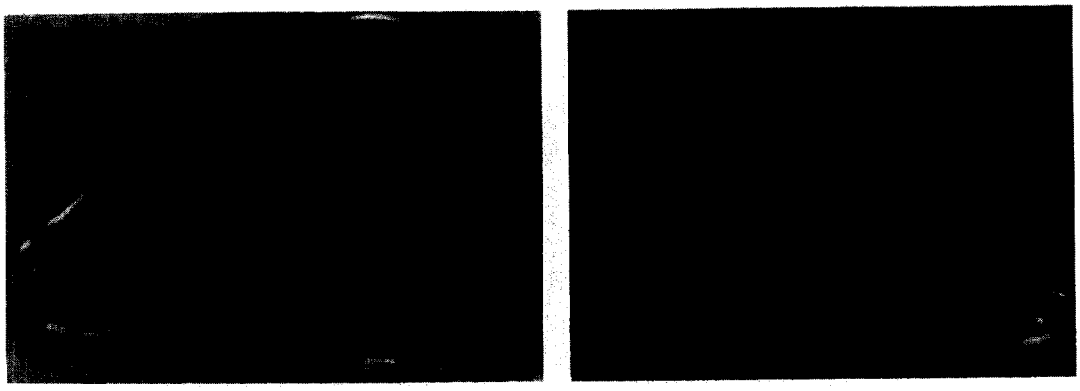


Fig. 7. Representative examples of different amounts of GUS activity on young onion embryo callus with or without the addition of phenolic compounds (**AS** in this case). (a) GUS activity observed in the absence of phenolic compounds was limited to a few small spots. (b) In the presence of **AS** (250 μM), 10–20% of the embryos showed large GUS positive areas.

to the control level (Fig. 5). Furthermore, bacterial growth was correlated with *vir* induction in the two cases. **AS** became toxic to bacteria at relatively low concentrations (100–500 μM). This toxic concentration limit was considerably displaced towards higher concentrations with **2b**. Therefore, the action of glucosylated compounds might be to protect bacteria against the toxicity of phenolic functions. If used in transformation experiments, it would be possible to increase the concentration of these compounds beyond 500 μM (**a** forms) and 10 mM for glucosylated forms.

Action of phenolics on onion Agrobacterium*-mediated GUS gene transfer*

We tested the two phenolic compounds (**AS** and the benzalacetone **10a**), which gave the best *vir*-induction, on onion transformation (Fig. 6). The GUS transient expression in plant embryos showed a control rate of 20%. The addition of 250 μM **AS** or **10a** increased the transformation by at least 20% to give global transformation rates up to 45%. In the presence of higher concentrations of phenolics (250 μM), we observed on the embryos larger areas of GUS activity probably indicating a more efficient gene transfer. Fraction of embryos which showed this kind of large GUS expressing positive areas were 10–20% whereas the control GUS activity was often limited to small single spots and rarely to these types of areas (Fig. 7). Although concentrations above 250 μM gave some GUS positive embryos, these concentrations were toxic for embryos growth and it was found that at 1 mM **AS** or **10a** most explants died.

We show that acetyl (which could be replaced by aldehyde or acid), methoxy and phenol functions are essential to induce the *vir* genes. A group of products including chalcones, benzalacetones and dibenzalacetones also showed a high action on the *vir* genes. In particular the **10a** (Fig. 7) which conserves the four chemical functions mentioned above and also includes an ethylenic function between the phenyl and the acetyl groups has a marked effect on *vir* induction. Its application to onion transformation, as well as that of **AS**, leads to good T-DNA transfer, as measured by transient GUS expression, on onion plants, known, like other monocots plants to be refractory to *Agrobacterium*-mediated DNA transfer.

EXPERIMENTAL

Plant material. The onion (*Allium cepa* L.) genotype used was Matura which was provided by Van Der Have Research (Rilland, The Netherlands). Zygotic mature embryos were cultured on the basal medium containing salts and vitamins of Murashige and Skoog (MS) [19], 3% (w/v) sucrose and 0.8% Difco agar, pH adjusted to 5.6 before autoclaving (120° for 20 min). 15–20 embryos were placed in a Petri dish [20] on MS medium with 4.1 μM Picloram (4-amino-3,5,6-trichloropicolinic acid) during 12 days. The cultures were maintained at 24° under a light regime of 16 hr per day of fluorescent light.

Bacterial strains. Agrobacterium tumefaciens, A348 pSM219 *pinF:lacZ* [21] (*pinF* also referred to as *virH* [22]), was used to test *vir* genes. It was kindly supplied by B. Hohn (FMI, Basel, Switzerland). Strain C58C1Rif pMP90 pVDH65 carrying a binary vector with *NPTII* and *GUS*-intron genes was provided by Van Der Have Research (Rilland, The Netherlands). Bacterial colonies were picked from Petri dishes and grown overnight in Luria Broth (LB) [23] liquid medium supplemented with the appropriate antibiotics at 28° using a rotary shaker (210 rpm).

Determination of vir *gene expression.* To test *vir* gene induction, an overnight colony of *Agrobacterium* A348 pSM219 *pinF:lacZ* was centrifuged and suspended in mannitol–phosphate buffer (0.6 M mannitol, 0.01 M NaPi, pH 5.2). A 100 μl aliquot of this bacterial suspension was added to 900 μl MSPS liquid medium (MS medium supplemented with 62.5 mM NaPi and 3% sucrose, pH 5.25), to which the phenolic compound to be tested was also added, and grown for at least 18 hr at

Table 1. Description of different **AS** derivatives tested, showing the substitutions at R^1, R^2 and R^3

	R^1	R^2	R^3	Usual name of **a** form
1	OH	H	H	Catechol
2	OCH_3	OCH_3	$COCH_3$	Acetosyringone
3	OCH_3	OCH_3	H	—
4	H	H	$COCH_3$	Hydroxy-acetophenone
5	OCH_3	H	$COCH_3$	Aceto-vanillone
6	OCH_3	OCH_3	CHO	Syringaldehide
7	OCH_3	H	CHO	Vanillin
8*	OCH_3	OCH_3	COOH	Syringic acid
9*	H	H	COOH	—

*Some problems with compound stability kept glucosyl esters (c) acetylated.

Table 2. Description of different chalcones tested, showing the substitutions at R^1, R^2 and R^3

	R^1	R^2	R^3	Usual name of **a** form
10	OCH_3	OCH_3	$CHCHCOCH_3$	—
11*	OCH_3	OCH_3	CHCHCOOH	Sinapic acid
12†	OCH_3	OCH_3	COCHCH-Ph-4-OH	—
13	OCH_3	H	COCHCH-Ph-4-OH	—
14	OCH_3	OCH_3	CHCHCO-Ph-4-OH	—
15	OCH_3	H	CHCHCO-Ph-4-OH	—
16	OCH_3	OCH_3	CHCHCOCHCH-Ph-(2,6-OCH_3-4-OH)	—
17	H	H	CHCHCOCHCH-Ph-4-OH	—

*Some problems with compound stability kept glucosyl esters (c) acetylated
†Ph = $-C_6H_4-$.

28–30°. Expression levels of *vir* genes were monitored by measuring the β-galactosidase activity present in the bacteria. Bacterial cells were harvested, β-gal activity [23] was determined as described in Ref. [24]. Bacterial growth was determined by spectrophotometry at 600 nm.

Determination of GUS expression. Histochemical GUS assays were carried out using 10 mg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc; Biomol, Hamburg, Germany) dissolved in 200 μl ethylene glycol monoethyl ether [25].

Inoculation of embryos. Embryo explants were dipped in a bacterial suspension for 20 min, blotted dry on sterile filter paper, and incubated on MS basal medium with the appropriate phenolic compound to be tested during 4 days. Co-cultured embryo explants were washed in liquid MS medium in the presence of 500 mg l^{-1} cefotaxim, blotted dry on filter paper and plated on MS medium containing 4.1 μM picloram and 300 mg l^{-1} cefotaxim.

Phenolic compounds. As shown in Fig. 3 and Table 1, we tested for the first time many derivatives of **AS** (**a**), the corresponding β-glucosides (**b**), and for phenolic acids, their glucosyl esters (**c**). Afterwards we tested (Table 2) many chalcones, benzalacetones, dibenzalacetones and their β-glucosides. Simple phenolic compounds were purchased from Aldrich (Germany). Chalcones and their analogues were synthesized by aldolic condensation catalysed by BF3- etherate [26]. Glucosylation of all compounds were obtained by a Michael reaction from 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide [27] followed by deacetylation under basic conditions.

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