HYDROXYACETOSYRINGONE IS THE MAJOR VIRULENCE GENE ACTIVATING FACTOR IN BELLADONNA HAIRY ROOT CULTURES, AND INOSITOL ENHANCES ITS ACTIVITY

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α-Hydroxyacetosyringone (HOAS) and acetosyringone (AS) were isolated as virulence gene inducing compounds of *Agrobatcerium* from the hairy root cultures of belladonna. α-Hydroxyacetosyringone was the major signal compound and was markedly accumulated in the culture medium by wounding. A potentiating factor of virulence inducing activity in the culture medium was identified as inositol. Among the sugars tested D-glucose, L-rhamnose and D-xylose, the main constituents of cell walls enhanced the activity of acetosyringone.

KEYWORDS *Agrobacterium*; α-hydroxyacetosyringone; acetosyringone; vir gene; induction; signal compound; inositol; glucose; potentiation

Ti-Plasmid of *Agrobacterium tumefaciens* has been extensively used as vectors to transfer foreign genes into plants. T-DNA and virulence inducing genes (vir gene) are two distinct regions of Ti-plasmid and play different roles in gene transfer. The T-DNA is transferred to plant genomes by a process not completely clarified. Vir gene which is coded in a left region of the T-DNA is expressed by a chemical signal from the plant. The translation products of vir gene take part in the follwing gene transfer process.[1] Two phenolic compounds, acetosyringone (AS) and α-hydroxyacetosyringone (HOAS), were isolated as vir gene inducing compounds from tobacco hairy root cultures.[2] Following this a variety of phenolic compounds including rather common plant constituents were tested for their vir gene inducing activity. Several compounds possess somewhat higher vir inducing activity than AS, however they rarely occur in plants.[3] To identify the intrinsic signal compounds of plants other than tobacco, a variety of materials such as the extracts of medicinal plants,[4] cultured plant cells with or without elicitor induction,[5] and hairy root cultures[6] were tested for their vir gene inducing activity. *A. tumefaciens* A384 pSM 234cd carring a lac Z fused vir B gene plasmid was used to monitor vir gene inducing activity and β-galactosidase activity was measured photometrically using *o*-nitrophenyl-β-galacotoside as a substrate.[7] Among the samples tested, the culture medium of belladonna hairy roots induced vir gene expression most significantly. During preliminary fractionation experiments of the culture medium, vir inducing activity was found in rather non-polar fractions, whereas polar fractions having no vir inducing activity significantly enhanced the activity of non-polar fractions in a synergistic manner. It was of interest to clarify this synergism at the chemical level, and we experimented with the isolation and identification of the signal compounds as well as the potentiating compounds from the hairy root cultures of belladonna.

Starting from a 2.5 l culture medium of 20 days after subculturing, two active compounds with vir gene inducing activity were obtained by fractionation on XAD-2 and reversephase Lobar columns followed by the separation with HPLC. They were identified as AS and HOAS with MASS and ^{1}H-NMR spectroscopies. To our surprize HOAS itself had not been synthesized before, so we synthesized HOAS from commercially available AS to prepare a standard sample for the quantitative evaluation of vir gene inducing activity. Diacetyl-HOAS was synthesized from AS via acetyl-α-bromoacetosyringone by a known method.[8] Upon hydrolysis with dil. hydrochloric acid it gave HOAS which was identical to the sample obtained from belladonna hairy root cultures. The dose-response curves of the vir gene inducing activity of AS and HOAS indicate AS is more potent than HOAS. (Fig. 1) However the content of HOAS in belladonna hairy root cultures is higher than that of AS. (Table I) This is unlike tobacco hairy root cultures where AS is the major signal compound.[2] *De novo* production of the signal compounds were

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induced by wounding or by the elicitor treatment of plant tissue cultures.[9] To clarify the time course change of the signal compounds after wounding, belladonna hairy roots were cut into small pieces and reincubated in the same medium for 24 to 72 h. The contents of AS and HOAS in the incubated medium and hairy roots were quantified by HPLC and the results are summarized on the dry weight basis of hairy roots. (Table I) The content of HOAS greatly increased after wounding and reached 6 times of that of the control value after 72 h, while the change of AS content was not so significant as HOAS. It is quite clear from the results that HOAS was synthesized *de novo* after wounding and rapidly excreted to the incubation medium, since 89% of HOAS was present in the medium. This may reflects the intrinsic function of signal compounds which activate the *Agrobacteria* near the wounded tissue.

Table I. Time Course Change of α-Hydroxyacetosyringone and Acetosyringone after Wounding (μg/g Dry Weight of Roots)

	α-Hydroxyacetosyringone(OHAS)		Acetosyringone(AS)	
	Culture medium	Hairy roots	Culture medium	Hairy roots
Control	72.2	27.9	13.0	7.0
Wounded 24h	80.5	28.5	20.4	13.6
Wounded 48h	302.0	44.0	11.1	9.2
Wounded 72h	551.0	69.4	12.7	10.4

The compound that potentiates the signal compounds was not absorbed on the XAD-2 column. A preliminary gel filtration experiment showed that the compound in question has a low molecular weight. Similar synergistic effects was detected in the fresh Murashige-Skoog's (MS) medium which had been treated as the culture medium. The stock solutions of MS basal medium were then directly tested for their enhancing activity against AS. The MS organic stock solution at 200 times concentration, which is routinely used in our laboratory, significantly enhanced the vir gene inducing activity of AS, though the solution itself exhibited no vir gene inducing activity. All the components of the organic stock solution were then tested separately and the enhancing compound was finally identified as inositol. A dose-response curve of inositol showed that its optimum concentration is 50 mM with 25 μM of AS. (data not shown) There was no practical difference between AS and HOAS in the potentiating effect of inositol. Inositol seems to elicit the maximum activity of AS and HOAS, since the dose response curves of AS and HOAS shifted to lower concentration in parallel manner. Following this finding, various aldoses, ketoses and sugar alcohols were tested for their enhancing activity and the results are summarized in Table II. D-Glucose, D-xylose and L-rhamnose had a potentiating activity comparable to inositol. In studies of vir

Table II Potantiating Effect of Carbohydrates to Vir Inducing Activity of Acetosyringone (AS)

	β-Galactosidase activity		
AS	25 μM	50 μM	0
Carbohydrate	25mM	50mM	50mM
No carbohydrate	254	254	0
Inositol	1290	1327	0
D-Glucose	1357	1274	0
D-Fructose	264	249	1.5
D-Xylose	1533	1249	0
L-Rhamnose	1597	1359	0
L-Fucose	226	246	0
L-Sorbose	110	121	0
Ducitol	586	754	2.1
D-Mannitol	224	186	0
D-Sorbitol	244	200	0

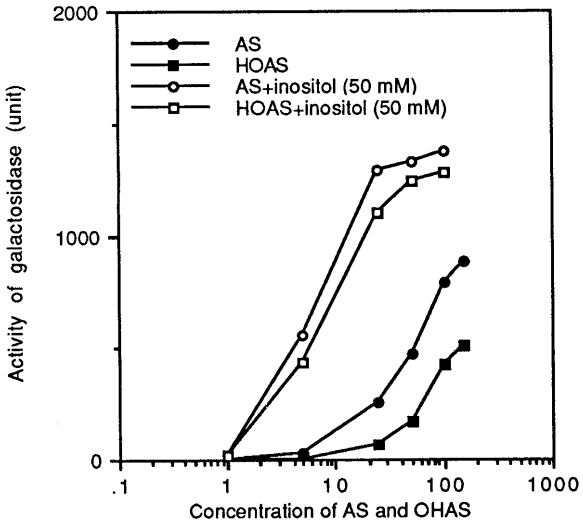

Fig. 1 Dose Response Curves of AS and HOAS

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gene inducing factors in monocotyledonous plants, Machida *et al.* found that D-glucose, D-galactose and L-arabinose markedly increase the induction of the vir gene expression by AS.[10] They also found that 2-deoxyglucose, nonmetabolizable sugar, had the enhancing effect. The results so far obtained by us and by Machida *et al.* indicate that the synergistic effect of carbohydrates and sugar alcohols is not caused by indirect action as bacterial metabolism, but by direct binding to a protein such as vir A product which acts as a receptor in a signal transduction process.[11] It is worth to note that almost all of the sugars of higher activity are the major constituents of plant cell walls.[12] Inositol phosphates, phytates, are also known to be contained as storage compounds in seeds and rapidly hydrolyzed at the onset of germination.[13] It is highly probable that these sugars are released from cell wall together with signal compounds such as AS and HOAS by the action of induced enzymes when plant tissues are wounded, and synergistically activate the bacteria present close to the wounded cells. The sugars would be particularly effective when the concentration of signal compounds are rather low or the activity of signal compounds is not so high as AS and HOAS.[14] It is of interest to consider that the cooperative effect has been acquired by the bacteria during the process of coevolution with the plants.

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