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Factor inducing *Agrobacterium tumefaciens vir* gene expression is present in monocotyledonous plants

(crown gall tumors/transferred DNA/tumor-inducing Ti plasmid/hydrophilic and high molecular weight inducer)

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ABSTRACT **_Agrobacterium tumefaciens_ harboring the tumor-inducing Ti plasmid incites crown gall tumor on dicotyledonous species. Upon infection of these plants, Ti plasmid DNA sequence is stably transferred (T-DNA) by unknown mechanisms to plant cells to be integrated into nuclear DNA. The T-DNA processing and transfer require the expression of _vir_ (virulence) genes on the Ti plasmid, which are known to be induced by certain phenolic compounds released from cells at the wounded inoculation site. The results of the present study demonstrate that wheat and oats contain a substance(s) that induces _vir_ gene expression, yet the inducing substance of wheat differs from the phenolic inducers in that it is hydrophilic and has a molecular weight of several thousand. The novel inducer was not detectable in the exudates of seedlings of these plants but was found in an extract from the transition region between shoot and root of the seedlings and also in extracts from the seeds, bran, and germ. This finding suggests that T-DNA processing and possibly its transfer should take place when _Agrobacterium_ invades suitable tissues of monocotyledonous plants.**

Agrobacterium tumefaciens harboring a tumor-inducing Ti plasmid is a pathogenic agent, inducing crown gall tumor in a wide variety of dicotyledonous plants (1). Formation of the crown gall tumor involves two successive steps: (*i*) the stable transfer of Ti plasmid DNA sequence (T-DNA) of *Agrobacterium* to plant cells and its integration into plant chromosomes and (*ii*) the unorganized growth of transformed cells as the result of expression of the phytohormone genes in T-DNA. The T-DNA transfer requires a cis-acting function of the 25 base-pair (bp) terminal repeats of T-DNA and trans-acting functions encoded by two separate genetic regions in *Agrobacterium*—the *vir* loci (*A, B, C, D, E,* and *G*) of the Ti plasmid (2, 3) and the *chv* (chromosomal virulence) region (4). The expression of the virulence genes (*vir*) except for *virA* is specifically induced by phenolic compounds, including acetosyringone, which are released from the wounded plant cells (5, 6). Since *virA* is absolutely required for the expression of other *vir* genes (7) and its product (VirA) is localized in bacterial membrane (8), the VirA product is thought to function as a sensor protein for the inducer (8). The products expressed by the *virD* locus promote the border-repeat-associated T-DNA processings, such as circularization of T-DNA (9, 10), formation of single-stranded T-DNA (11), and single- (12) and double-strand (13) cleavages in the border repeats, some of which are thought to be involved in the transfer of T-DNA.

In contrast to dicotyledonous plants, monocotyledonous plants have been generally thought to be resistant to *Agrobacterium* (14). We previously showed that the inducible molecular events described above take place in the presence of seedlings of dicotyledonous plants but not in the presence of seedlings of monocotyledonous plants (15). Therefore, it was concluded that monocotyledonous plants have no detectable amounts of diffusible inducers and that the *vir* genes should be minimally expressed in monocotyledonous plants even when *Agrobacterium* is inoculated. Several lines of evidence suggest, however, that the T-DNA can be transferred to some monocotyledonous plants by using the *Agrobacterium*-encoded functions (16–21). These observations led to the hypothesis that monocotyledonous plants have a different type of inducing factor. To test this idea, we prepared extracts from various parts of wheat and oat seedlings and seeds and examined these extracts for activities to induce T-DNA circularization and *vir* gene expression. The results of these analyses demonstrated that they contain a substance that induces these molecular events and yet differs from acetosyringone.

MATERIALS AND METHODS

Plants, Bacteria, and Plasmids. Seeds of *Triticum aestivum, Avena sativa,* and *Nicotiana tabacum* were germinated, and seedlings were cultured at 28°C with 16:8 hr light (3000 lux)/dark cycle. *A. tumefaciens* C58C1Cm harboring pTiB6S3 and the pTMA model plasmid (22) and *A. tumefaciens* A348 harboring pTiA6, pTi226 (*virA*), pTi363 (*virG*), pTi304 (*virD*), or pTi358 (*virE*) (3) have been described. pS0304 plasmid was constructed by inserting the *Eco*RI DNA fragment containing the *virD/lacZ* fusion gene of pTi304 (*virD*) into the pHK17 cosmid vector (23).

Preparation of Extracts for the Induction of the *vir* Gene Expression. To prepare the seedling extracts, 5.0 g fresh weight of 5-day-old tobacco seedlings (≈1000 pieces) and 5.0 g fresh weight of 3-day-old seedlings of wheat and oats were frozen at −70°C and homogenized by using a mortar and a pestle. Distilled water (10 ml) was added to the homogenates, and the materials were ground again. To prepare extracts from different parts of seedlings, 3-day-old wheat seedlings (10 cm) were divided into three parts: the shoot comprising leaves, leaf sheaths, and stem; the transition region between shoot and root—i.e., a 1- to 2-cm region where the remnant of seeds is attached; and the root. Five grams each of these parts (weight ratio of these parts in the seedlings was 1:3:1) was homogenized and suspended in 10 ml of distilled water. The seed extracts were prepared by homogenizing 5 g of seeds in 10 ml of distilled water. Mannitol and phosphate buffer were added to concentrations of 0.6 M and 10 mM, respectively, and the pH of the extract was adjusted to 5.2. After the extract was filter-sterilized, it was used for culturing agrobacterial cells as described below.

To prepare the extracts of wheat bran (Yakult Institutes, Tokyo) and wheat germ (General Mills), 20 g each of wheat

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Abbreviations: T-DNA, Ti plasmid DNA sequence stably transferred to plant; *vir*, virulence gene.
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bran and wheat germ was dissolved in 100 ml of distilled water and homogenized with a mortar and a pestle. After being stirred for 12 hr at 4°C, the homogenate was centrifuged to remove insoluble materials, and the supernatant was filtered with Whatman 3MM paper. Heated extracts were prepared by autoclaving the extracts at 120°C for 10 min, followed by centrifugation to recover the soluble extracts. Before use, sodium phosphate buffer and mannitol were added to concentrations of 10 mM and 0.6 M, respectively. After the pH was adjusted to 5.2 with HCl, the samples were filter-sterilized.

Assay of the Frequency of T-DNA Circularization. *A. tumefaciens* C58C1Cm, harboring pTiB6S3 and the pTMA model plasmid, was cultured in the extracts for 48 or 96 hr, and the frequency of T-DNA circularization was measured as described (22). We used the pTMA model plasmid having the promoter and the coding sequences of the kanamycin resistance gene, which were separately placed close to the right and left T-DNA borders, respectively. Circularization of T-DNA by recombination or cleavage and rejoining at the borders thus results in a plasmid containing the promoter and the coding sequences adjacent to each other, allowing the expression of kanamycin resistance. The frequency of the T-DNA circularization was estimated by counting a number of kanamycin-resistant agrobacterial cells after culturing the bacterial cells in the presence of plant inducers. The pH of the culture did not change during bacterial incubation under the conditions we used.

Induction of *vir* Gene Expression and Assay of β-Galactosidase Activity in Agrobacterial Cells. Cells of *A. tumefaciens* A348 carrying various Ti plasmids cultured overnight in L broth were washed once with 10 mM sodium phosphate buffer (pH 5.2) containing 0.6 M mannitol and suspended in the original volume of the same buffer. The bacterial suspension (0.2 ml) was added to 2 ml of the extract, and the culture was incubated at 30°C for 24 hr. For the induction by acetosyringone, the washed bacteria (0.2 ml) were added to 2 ml of MSSP medium (6) containing 100 μM acetosyringone and incubated at 30°C for 24 hr. After harvesting the bacteria, β-galactosidase activity was determined essentially as described by Miller (24). Bacteria were suspended in 0.5–1.5 ml of Z buffer (24). Bacterial density at 600 nm (1 cm) was measured by using an aliquot of the cell suspension. The cell suspension (0.5 ml) was vigorously mixed with 25 μl of toluene. The reaction was started by adding 0.1 ml of *o*-nitrophenyl-β-D-galactopyranoside at 4 mg/ml. The reaction mixtures were incubated at 28°C for 30 min, and the reaction was stopped by adding 0.25 ml of 1 M Na_2CO_3. Insoluble materials were removed by centrifugation, and the absorbance at 420 nm (1 cm) of the supernatant was measured. Specific units of β-galactosidase activity were calculated by using the formula $A_{420} \times 1000/A_{600} \times t$, where t is the incubation time in minutes.

Fractionation with a Sep-Pak C_{18} Cartridge. Wheat-bran and -germ extracts (10 ml each) were applied to a Sep-Pak C_{18} cartridge (Waters Associates), which retains hydrophobic molecules such as acetosyringone. The cartridges were washed with 15 ml of distilled water, and the retained materials were eluted successively with 10 ml of 20%, 40%, and 90% methanol. The fractions were evaporated to dryness, and the residues were dissolved in 5 ml of distilled water. *A. tumefaciens* A348 carrying pTi358 was cultured in 2 ml of each fraction, and the activity of β-galactosidase was measured as described above.

Gel Filtration on Sephadex G-25. Wheat-bran extract (30 ml) prepared as above was mixed with an equal volume of cold ethanol. After being kept at −20°C for 30 min, the sample was centrifuged, and the supernatant was recovered (all activity was recovered in the supernatant, no activity being present in the precipitated fraction; data not shown). The supernatant was evaporated to dryness, and the residue was dissolved in 1.5 ml of distilled water. The sample was fractionated on a Sephadex G-25 column (1.2 cm × 30 cm) equilibrated with 30 mM NaCl.

RESULTS

Soluble extracts of seedlings of tobacco, oats, and wheat were prepared, and the frequency of circularization of T-DNA was measured. Table 1 shows that circularization of T-DNA was induced by the extracts of seedlings of oats and wheat as well as by that of tobacco seedlings (lines 1, 2, and 3). When extracts were separately prepared from the shoot, the root, and the transition region between shoot and root of wheat seedlings, circularization of T-DNA was induced by the extract from the transition region but not by those from shoot and root (lines 4–6). In the shoot extract, more than 90% of bacteria died during culturing and no induction was observed. In the root extract, the viable cell counts also decreased to 30% of the initial cell number. Therefore, shoot and root portions of wheat seedlings may contain inhibitory substance(s) for growth of agrobacterial cells. In contrast, the density of bacteria increased by 10- to 20-fold during incubation in the extract of the transition region. Extracts of wheat and oat seeds also induced T-DNA circularization (lines 7 and 8), whereas extract of tobacco seeds did not (line 9).

The above results indicated that the transition region of the wheat and oats contain the inducing substance. Although the circularization event was not detected when using the extracts of shoot and root portions, the possibility may not be ruled out that these portions also contain the inducer because the viable cell counts decreased during incubation in these extracts.

Table 2 shows that circularization of T-DNA was induced by the extracts of wheat bran and germ (lines 2 and 4). When the heated extracts were used, circularization occurred at higher frequencies (lines 3 and 5). Expression of the *vir* gene was also measured as induced by these extracts. To do this, *A. tumefaciens* A348 harboring pTi358 having a *virE*/*lacZ* fusion gene in the *virE* locus of Ti plasmid was used, so that the expression of the *lacZ* gene is controlled by the inducible *virE* promoter (5). This system enabled us to estimate the induction of *virE* by assaying β-galactosidase activity. When the bacteria were cultured in either heated or nonheated extract of wheat bran, β-galactosidase activity was induced at low but significant levels (lines 2 and 3). However, no detectable enzyme activity was induced by wheat-germ

Table 1. Frequency of T-DNA circularization induced by extracts of seedlings and seeds

Extract	Bacteria containing circular T-DNA, no. per 10^7 bacteria incubated	
	48 hr	96 hr
Seedling		
Tobacco	26.4	25.4
Oats (whole)	0.4	2.3
Wheat (whole)	0.9	34.3
Wheat (shoot)	ND	ND
Wheat (transition region)*	13.4	48.7
Wheat (root)	ND	ND
Seed		
Wheat	1.1	16.4
Oats	1.4	2.0
Tobacco	NE	ND

ND, not detected; NE, not examined.

*Between shoot and root—i.e., a 1- to 2-cm region where the remnant of seeds is attached (see *Materials and Methods*).

Table 2. Induction of T-DNA circularization and *virE* gene expression by extracts of wheat bran and germ

Extract	Bacteria containing circular T-DNA, no. per 10^7 bacteria*	Activity of β-galactosidase, units
No extract†	ND	10
Wheat bran	2.3	36
Wheat bran, heated	7.6	44
Wheat germ	1.0	10
Wheat germ, heated	3.5	29

ND, not detected.
*Bacteria were incubated for 96 hr.
†Bacteria were incubated in 2 ml of MSSP medium (6) containing 0.6 M mannitol.

extract, whereas T-DNA circularization at a significant level was induced by the extract (line 4). When the heated extract of wheat germ was used, a detectable level of enzyme activity was induced. These results show that wheat bran and germ contain a substance(s) that induces both the circularization of T-DNA and the expression of *vir* genes.

Although the crude extracts from wheat bran and germ definitely contained the inducing substance, it was difficult to quantitate it; when the extracts were concentrated, the levels of induction did not increase (data not shown). It is presumed that the crude extracts contain materials that are inhibitory to the action of the inducer. The inducing activity was able to be quantitatively assayed after gel filtration of the putative inducer as described below.

Wheat-bran and -germ extracts were applied to a Sep-Pak C_{18} cartridge. The cartridge was washed with distilled water and then successively with 20% (vol/vol), 40%, and 90% methanol. Induction of the *virE/lacZ* fusion gene was examined with these fractions as described. Table 3 shows β-galactosidase activity induced by these fractions. Practically all of the inducing activity present in the extracts was recovered in the pass-through and water-wash fractions, and no activity was found in the methanol-eluted fractions. When the pass-through and the water-wash fractions were applied to a new cartridge, the activity passed through again (data not shown). Under the same conditions, acetosyringone was retained quantitatively by the cartridge and was eluted in both 20% and 40% methanol fractions (data not shown). Even after chloroform extraction, the inducing activity remained in the water phase (data not shown). These results show that the inducer from wheat is hydrophilic in nature.

Table 3. Fractionation of extracts from wheat bran and germ using a Sep-Pak C_{18} cartridge and their ability to induce *virE* expression

Fraction	Activity of β-galactosidase, units*	
	Wheat bran	Wheat germ
Crude extract (input)	358	147
Fractions eluted		
Pass through	163	144
Water wash	134	146
20% methanol	2	NE
40% methanol	0	0
90% methanol	0	NE

NE, not examined.
*The assays were conducted as described. The background units of the experiments for wheat bran and germ were 9 and 7, respectively, which were subtracted. The units in the crude extracts represent those in 10 ml, and the units in each fraction represent those in 5 ml.

The extract from wheat bran was also fractionated by gel filtration on Sephadex G25, and β-galactosidase activity induced by the fractions was measured. The activity was recovered only in the void volume fractions (fractions 16–25) (Fig. 1). No activity was detected in the included fractions. Acetosyringone was eluted around fraction 55 from the same column. Therefore, the inducer in wheat bran is larger than acetosyringone (196 in molecular weight). The molecular weight of wheat inducer seems to be larger than several thousands, since the exclusion limit of Sephadex G-25 is $\approx$5 kDa (data supplied by Pharmacia). However, it is not precipitated with 50% ethanol (see *Materials and Methods*). When the heated extract was fractionated on Sephadex G-25, the same elution profile of the inducing activity was observed (data not shown), indicating that the size of the inducer in the extract of wheat bran does not markedly decrease by heat treatment (120°C for 10 min).

Throughout the experiments described above, the *virE/lacZ* fusion gene has been used for examining the inducing activity of the extracts of wheat bran and germ. The inducing activity of the extract was also examined by using *virG* and *virD/lacZ* fusion genes. *A. tumefaciens* A348 cells harboring pTi363 (*virG/lacZ* fusion) or pTi304 (*virD/lacZ* fusion) were cultured in the pass-through fraction in Fig. 1, and measured β-galactosidase activity was induced in these cells. The pass-through fraction induced the expression of *virG*, *virD*, and *virE* genes (Table 4, lines 1–3). The induction by the

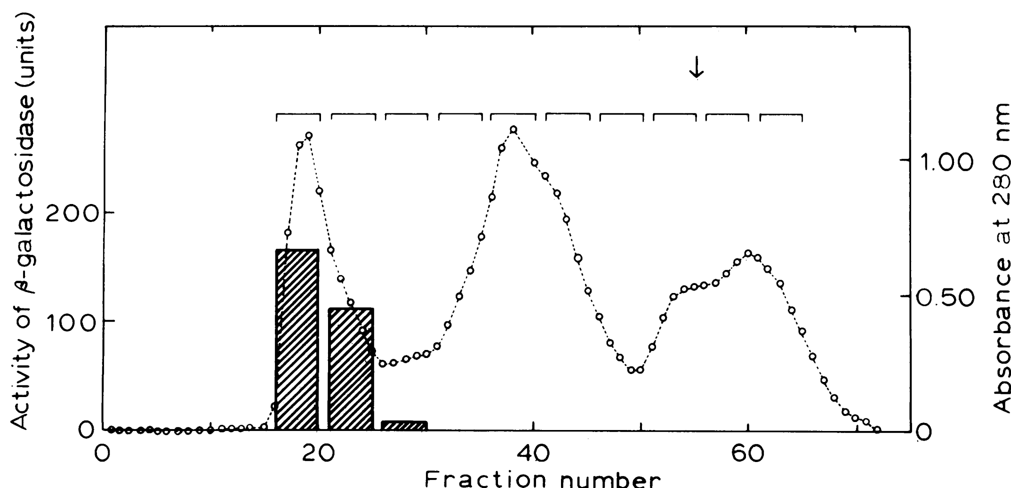

FIG. 1. Gel filtration of the inducing substance on Sephadex G-25. The histogram represents activity of β-galactosidase obtained as described. ○, A_{280} of fractions diluted 1:50; arrow, position where acetosyringone was eluted. Fractions were pooled (5 ml) as bracketed. Two milliliters of the pooled fractions was used for culture of *A. tumefaciens* A348 harboring pTi358, and β-galactosidase activity was measured 24 hr after incubation.

Table 4. Induction of *virG* and *virD* expression by the G-25 pass-through fraction of a wheat-bran extract

Plasmid	Locus	Activity of β-galactosidase, units		
		No extract*	Pass through†	AS‡
pTi363	*virG*	16.3	156	133
pTi304	*virD*	5.0	52	45
pTi358	*virE*	5.3	164	110
pTiA6, pS0304	*virD*	5.1	65	98
pTi226 (*virA*), pS0304	*virD*	3.4	7.5	5.1

*Bacteria were incubated in 2 ml of MSSP medium (6) containing 0.6 M mannitol.
†Two milliliters of the pass-through fraction was used.
‡Bacteria were incubated in 2 ml of MSSP medium containing 0.6 M mannitol and 100 μM acetosyringone (AS).

pass-through fraction we used here was at the same level as that by acetosyringone (100 μM). Subsequently, we tested the requirement of the *virA* gene for the inducible expression of *virD* by the pass-through fraction, since the *virA* gene is known to be absolutely required for the induction of other *vir* gene expression by acetosyringone (7). To do that, we introduced the pS0304 plasmid containing the *virD/lacZ* fusion gene into (*i*) *A. tumefaciens* A348 harboring pTi226 with a mutation in *virA* and (*ii*) A348 harboring wild-type Ti plasmid pTiA6, enabling expression of the *virD/lacZ* fusion gene in pS0304 to be controlled by the mutant or wild-type *virA* gene present trans in each Ti plasmid. The expression of the *virD* gene was not induced in the *virA* mutant by either the pass-through fraction or acetosyringone, whereas it was efficiently induced in the wild-type strain (Table 4, lines 4 and 5).

The induction of the expression of the *vir* genes by the pass-through fraction was pH sensitive: the efficient induction by this fraction was observed only when agrobacterial cells were cultured at the pH that was between 5.0 and 5.5, whereas no induction was detectable at the pH higher than 6.0. Acetosyringone also acts as a strong inducer for the *vir* gene expression between pH 5.0 and pH 5.5 (5).

DISCUSSION

The results presented in this paper demonstrate that wheat and oats contain a substance(s) that induces the expression of the *vir* loci of Ti plasmid and the T-DNA-processing reaction. The putative inducing substance was present in the transition region of wheat seedlings. Since in the extracts of shoots and roots, agrobacterial cells did not grow, the inducing activity might not be detected when the inducer was present (Table 1). The present results show that seeds of wheat and oats contain the inducing substance. They also show that wheat bran and germ, which are parts of the seed, are rich sources of the inducer. The inducer found in the transition region of wheat seedlings probably originates from the seed tissues rather than *de novo* production after germination. The inducing substance could be extracted only after homogenizing these materials. When *Agrobacterium* was incubated with nonhomogenized or nongerminated seeds, T-DNA circularization and the *vir* gene expression were not observed (unpublished results). Therefore, this substance may be a structural component of tissues in the wheat seeds.

The putative inducing substance described here was obviously different from the known phenolic inducer, acetosyringone, since it is larger than several thousands in molecular weight and is relatively hydrophilic (Table 3 and Fig. 1). The inducing activity in the pass-through fraction of the gel filtration (Fig. 1) was resistant to Pronase, DNase I, RNase I, and cellulase. The elution pattern of the inducing activity upon gel filtration was not influenced by treatment with these enzymes (unpublished results).

We examined the effect of the inducing substance in the extract on the expression of *virG, virD,* and *virE* genes. The substance was effective on the induction of the expression of these genes. Furthermore, induction of the expression absolutely required the intact *virA* gene, and it took place only under conditions of acidic pH. Although we have not yet tested the induction of *virB* and *virC* expression by this substance, these observations suggest that the substance from wheat and oats, like acetosyringone, induces the expression of a series of *vir* genes via the VirA product, which could result in activation of agrobacterial cells to transfer T-DNA into plant cells. Our results support the possibility recently suggested by other laboratories (16–21) that T-DNA is transferred to some monocotyledonous plants by the same mechanisms as it is transferred to dicotyledonous plants. Because some monocotyledonous tissues appear to contain inhibitors of the inducing substance (see text), it is not clear how efficiently the *vir* genes are expressed in *Agrobacterium* that invades monocotyledonous plants.

The phenolic inducers previously reported are known to be structurally analogous to acetosyringone (6). Taken together with the involvement of the VirA product in the signal transmission of the wheat inducer, it seems likely that the inducing substance, in part, has a structural relation to these phenolic inducers in such a way that the phenolic structure is conjugated to a hydrophilic molecule.

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