MPMI Vol. 8, No. 4, 1995, pp. 538–548. © 1995 The American Phytopathological Society

T-DNA Genes Responsible for Inducing a Necrotic Response on Grape Vines

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Received 12 October 1994. Accepted 13 March 1995.

***Agrobacterium tumefaciens* supervirulent strain A281 induces a progressive necrotic response, rather than tumor formation, when inoculated on stems of several grape cultivars. The Ti plasmid, and specifically its T-DNA, is required for the process. In the present study, 40 T-DNA insertion mutants of A281 were generated via transposon mutagenesis and tested for their necrosis-inducing ability on grape stems in vitro. Ten mutants were attenuated in inducing necrogenesis. Restriction mapping and DNA sequencing revealed that at least two genes, *tms*1 and 6b, whose gene products are involved in the synthesis and activity modulation of auxin, are responsible for inducing necrogenesis. Double mutants of *tms*1 and 6b were totally non-necrogenic. The orientation of grapevine stem explants showed strong effects on the occurrence and progress of necrogenesis. Inoculation of *Agrobacterium* on physiological basal ends resulted in the greatest degree of necrogenesis. In addition, gene 5 of T-DNA, which modulates auxin responses in plants by the autoregulated synthesis of an auxin antagonist, was found to be separated from other TL-DNA genes by a novel insertion sequence, IS1312. Since a T-DNA borderlike sequence occurs in IS1312, gene 5 might not always be transferred into plants. Based on the accumulated data, we propose that the necrogenesis induced by *Agrobacterium* results from the sensitivity of grapevine cells to elevated levels of auxin or a precursor of auxin.**

Additional keywords: pTiBo542, *Vitis*, host-pathogen interaction, host range.

Agrobacterium tumefaciens can induce crown gall tumors on a wide variety of plant species. It accomplishes this by transferring a piece of DNA, the T-DNA, located on its tumor-inducing (Ti) plasmid into the plant cell through a process analogous to bacterial conjugation (for reviews see: Kado 1991; Zambryski 1992; Lessl and Lanka 1994). The processing and transfer of T-DNA to the plant cell nucleus requires the action of the virulence (*vir*) genes, which are also located on the Ti plasmid. The expression of these genes is induced by compounds released from wounded plant cells. The T-DNA, which encodes functions for the biosynthesis and modulation of activity of two plant growth regulators, auxin and cytokinin, is integrated into the plant nuclear genome and expressed, leading to tumor formation.

The roles of several T-DNA genes in tumorigenesis and the functions of their encoded proteins have been demonstrated (for reviews see: Thomashow et al. 1986; Kado 1991; Winans 1992). The pathway of auxin biosynthesis encoded by the T-DNA is a two-step process; the *tms*1 and *tms*2 genes act sequentially to convert tryptophan first to indole-3-acetamide (IAM) and then to indole-3-acetic acid (IAA, auxin) (Schröder et al. 1984; Thomashow et al. 1984; Thomashow et al. 1986). The *tmr* gene is responsible for cytokinin synthesis (Akiyoshi et al. 1984; Barry et al. 1984; Buchmann et al. 1985). While these genes are involved in the biosynthesis of auxin and cytokinin, two other T-DNA genes, 5 and 6b, modulate the activities of auxin and/or cytokinin. Gene 5 modulates auxin responses in plants by the autoregulated synthesis of an auxin antagonist (Körber et al. 1991). Gene 6b is an *onc* gene (Hooykaas et al. 1988), but its exact biochemical function remains unknown. It can affect the activity of auxin as well as cytokinin genes. Some studies have suggested that it has an auxinlike effect (Tinland et al. 1989, 1990), whereas other studies have indicated that it reduces cytokinin activity (Spanier et al. 1989). Both results on the function of the 6b gene are consistent, since it is widely accepted that auxins antagonize cytokinins in many plant systems.

Although the molecular mechanism of tumor induction has been studied in considerable detail, little is known about the response of the plant cell to infection by *A. tumefaciens* and to the expression of T-DNA genes. It is well documented that a great number of dicotyledonous plants, especially fruit trees and grapevines, can be affected by the crown gall disease, but some plants fail to respond to the inoculation of *A. tumefaciens*, giving rise to a null phenotype (DeCleene and DeLey 1976). Earlier studies demonstrated that the host range of *A. tumefaciens* is determined by the Ti plasmid (Loper and Kado 1979; Thomashow et al. 1980; Knauf et al. 1982) and that genes in the T-region as well as in the *vir* region are involved (Hoekema et al. 1984; Yanofsky et al. 1985a; Leroux et al. 1987; Turk et al. 1993). The observation that different plant species, or even different cultivars of the same species, respond differently to the same *Agrobacterium* strain (Anderson and Moore 1979) strongly suggests that the host range of any *A. tumefaciens* strain is determined by both bacterial and

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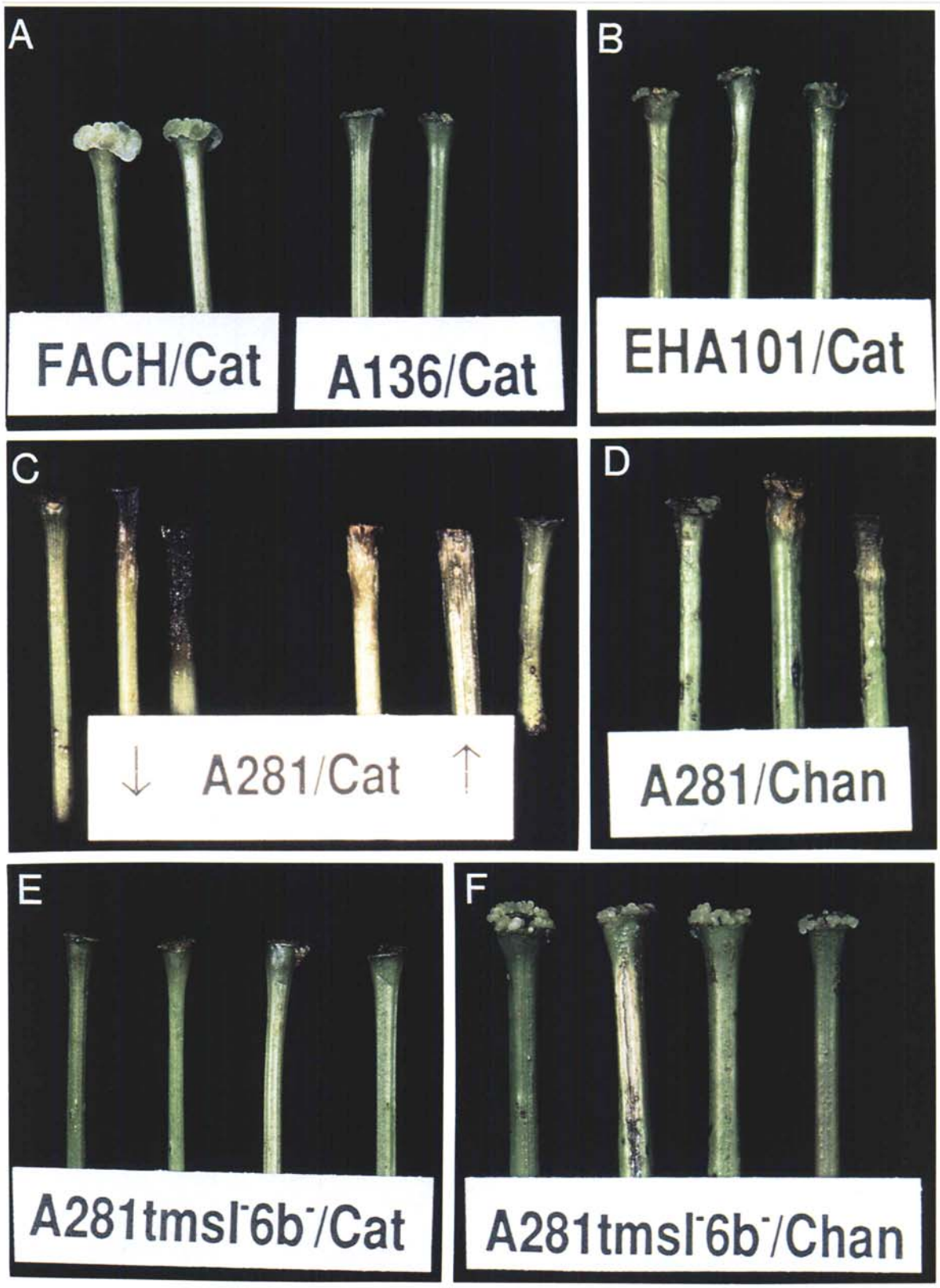

Fig. 1. Responses of *Vitis* cultivars, Catawba and Chancellor, to inoculations of various *Agrobacterium tumefaciens* strains. Bacteria were inoculated on the basal surfaces of grapevine explants except when indicated otherwise. Pictures were taken 2 weeks after inoculation. See Table 3 for relevant characteristics of the strains. **A,** Grape isolate, biovar 1 strain FACH (left) and Ti-plasmidless strain A136 (right) on cultivar Catawba. **B,** EHA101, a T-DNA deletion mutant of A281, on Catawba. **C,** A281 on Catawba with inoculations on the basal (left) or apical (right) surfaces of the explants. **D,** A281 on cultivar Chancellor. **E,** *tms*1 and 6b double mutant of A281 on Catawba. **F,** *tms*1 and 6b double mutant of A281 on Chancellor.

plant factors. It also suggests that plant responses to infection by *Agrobacterium* and subsequent T-DNA integration and expression may play a significant role in determining the outcome of the plant–*Agrobacterium* interaction. In the case of grapevine (*Vitis*)-*Agrobacterium* interactions, some *A. tumefaciens* strains induce a necrotic response on some *Vitis* cultivars, while they induce tumor formation on other plants or even different cultivars of *Vitis* (Yanofsky et al. 1985a, 1985b; Lowe and Krul 1991; Pu and Goodman 1992). Therefore the response of a plant to infection by a specific strain of *A. tumefaciens* can be tumor formation, resistance, or necrosis, depending on the properties of the *Agrobacterium* strain and the particular plant species infected.

The necrotic response induced by wide-host-range (WHR) strains of *A. tumefaciens* on grapevines was defined as plant cell death at the site of *Agrobacterium* inoculation (Yanofsky et al. 1985b). It is an *Agrobacterium* strain- and *Vitis* cultivar-specific process (Yanofsky et al. 1985b; Lowe and Krul 1991; Pu and Goodman 1992). Earlier studies demonstrated that although both the T-DNA and the *vir* region are required, the T-DNA is directly responsible for inducing the response (Yanofsky et al. 1985b; Pu and Goodman 1992). Mutations within the *virC* locus of a WHR strain prevent the necrotic response and allow the formation of tumors by the strain, implying that high T-DNA transfer efficiency is important for inducing the necrotic response (Yanofsky et al. 1985b; Toro et al. 1989). Previous attempts to identify the T-DNA genes of A348, a WHR strain, responsible for inducing necrogenesis were inconclusive (Lowe and Krul 1991).

A. tumefaciens A281, having the pTiBo542 of strain Bo542 in the chromosomal background of strain C58, is highly virulent (Hood et al. 1986b; Jin et al. 1987). While it induces large, fast-appearing tumors and infects more plant species than many *Agrobacterium* strains (Hood et al. 1986b; Jin et al. 1987), it consistently induces necrosis on several cultivars of *Vitis* (Lowe and Krul 1991; Pu and Goodman 1992). Other WHR strains are not nearly as efficient in inducing the necrotic response on these cultivars. It has been shown that A281-induced necrogenesis follows T-DNA transformation and expression (Pu and Goodman 1992).

The characterization of the T-DNA genes involved in inducing necrogenesis should contribute to a better understanding of how *Agrobacterium* and plants interact and how plant cells respond to the integration and expression of T-DNA genes. In this paper, we report the identification of the T-DNA genes of A281 responsible for inducing necrogenesis and their role in the process. In addition, we provide a detailed TL-DNA gene map for pTiBo542, a succinamopine-type Ti plasmid (Hood et al. 1986a).

Table. 1. Responses of *Vitis* cultivars, Catawba and Chancellor, to various *Agrobacterium tumefaciens* strains[a]

	Percentage of explants with			
	Catawba		Chancellor	
Strain[b]	Necrosis	Tumor	Necrosis	Tumor
A281	100	0	100	0
A136	0	0	0	0
EHA101	0	0	0	0
A348	0	100	0	100
FACH	0	100	0	100
EHA101(pTVK25)	20	80	30	70

[a] Data are averages of three independent experiments. For each treatment, eight to 10 stem explants were inoculated at their basal surfaces with each strain. The responses were recorded 2 weeks after inoculation.

[b] For relevant characteristics of the strains, see Table 3.

RESULTS

A281 induces a necrotic response on grape cultivars Catawba and Chancellor.

Supervirulent *Agrobacterium* strain A281 induced a progressive necrotic response on *Vitis* cultivars Catawba and Chancellor, whereas strain FACH, a biovar 1 strain isolated from grapevine, caused tumors (Figs. 1A, 1C and 1D). A281 induced necrogenesis with high efficiency on the two grape cultivars, while another WHR strain A348 was not effective in this respect (Table 1). The degree of necrosis induced by A281 on different explants can vary (Fig. 1C), probably due to variations in the physiological state of the explants. Necrosis was usually visible at the inoculated surface 1 week after inoculation, and it progressed down the axes of the explants. It has been reported that A281 induces tumors on many grape cultivars including Belandais and Chardonnay, although the tumors incited are small (Pu and Goodman 1992). These results suggest that the necrotic response is both *Agrobacterium* strain and *Vitis* cultivar specific. When A281 was cured of its Ti plasmid or its T-DNA deleted, it could induce neither necrosis nor tumors (Figs. 1A and B; Table 1). This indicates that T-DNA is required for both reactions and is directly responsible for inducing the necrotic response. It further suggests that T-DNA transfer into grape cells precedes necrogenesis, in agreement with other reports on necrosis induced by A281 or other WHR strains (Yanofsky et al. 1985b; Lowe and Krul 1991; Pu and Goodman 1992).

We were interested in identifying the specific T-DNA genes responsible for inducing the necrotic response. Previous studies suggested that plant growth regulators mediate its induction (Lowe and Krul 1991; Pu and Goodman 1992). The T-DNA region of pTiBo542 is composed of two regions, namely TL-DNA and TR-DNA, very similar to that of octopine strains (Hood et al. 1986a). The oncogenic genes are located in the TL-DNA, whereas the opine synthetic loci are present in the TR-DNA (Hood et al. 1986a; Komari et al. 1986). It was known that the T-DNA genes related to hormone biosynthesis and modulation are located in the TL-region (Komari et al. 1986; Strabala et al. 1989). Since A281 T-DNA mutants were unavailable and the T-DNA genes of pTiBo542 had not been mapped, we decided to mutate the TL-DNA region of pTiBo542 to identify the T-DNA genes responsible for inducing necrogenesis.

Plasmid pTVK25, a cosmid clone which contains most of the TL-DNA of pTiBo542 and sufficient information for tumorigenesis (Komari et al. 1986), was introduced into EHA101, a disarmed strain of A281, to determine whether the resultant composite strain could induce necrosis on grapevines. As shown in Table 1, the bipartite strain did not mimic the necrosis-inducing ability of A281. We later showed that pTVK25 lacks the left part of pTiBo542 TL-DNA beyond the insertion sequence IS1312, including the left border region (See Fig. 2 and following sections; Komari et al. 1986; Hood et al. 1986a). It is therefore possible that the part of TL-

DNA missing in pTVK25 is also involved in inducing necrosis, or contributes to the T-DNA transfer process, thus affecting the necrosis-inducing ability of the bipartite strain. It is also conceivable that a bipartite system works less efficiently than the original Ti plasmid which carries both the *vir* region and the T-DNA region on the same plasmid. This can be especially true in the case of strain EHA101(pTVK25) since pTVK25 does not contain the left border region of the TL-DNA. This was supported by our observation that tumors induced by the bipartite strain on *Kalanchoe daigremontiana* were smaller than those induced by A281 (data not shown). The same phenomenon was also noticed by Hood and associates in their tumor induction assay using EHA101-based binary vector systems (Hood et al. 1986b).

The reason we first tested pTVK25 was that it would be much easier and more convenient to mutate the TL-DNA and screen for mutations which affect the necrosis inducing ability in a binary system than in A281, because we could avoid the labor-intensive process of marker-exchanging all the mutations back into the A281 background (Ruvkun and Ausubel 1981; Garfinkel et al. 1981; Stachel et al. 1985). However, the bipartite strain EHA101(pTVK25) did not work as well as A281 in inducing the necrotic response on grapevines. To bypass the problems mentioned above, we randomly mutagenized pTVK12 and pTVK25, two cosmid clones which would surely cover all of the TL-DNA region of pTiBo542 (Fig. 2; Hood et al. 1986a; Komari et al. 1986), with transposon Tn3HoHo1. Those insertion mutations in the TL-DNA region were mapped with restriction enzymes. They were then reintroduced into A281 via the marker exchange (homologous recombination) technique (Ruvkun and Ausubel 1981; Garfinkel et al. 1981; Stachel et al. 1985), and screened for necrogenesis-inducing activity.

Some A281 T-DNA mutants induce an attenuated necrotic response.

A total of 40 T-DNA mutants were tested on grape stems using a method described by Pu and Goodman (1992). The locations of the mutations are shown in Figure 2. Ten of the mutants induced necrogenesis only weakly, with the frequency of necrogenesis ranging from 20 to 55% of that of A281 (Table 2 and Fig. 2). However, none were completely non-necrogenic. This suggests that more than one locus is responsible for inducing necrosis and that these loci may have additive or synergistic effects on necrogenesis. We then tested the tumor-inducing ability of these 10 mutants. When inoculated onto either leaves or stems of *Kalanchoe* plants, all mutants still caused tumors, although in many cases tumor formation was delayed (Table 2; data not shown).

Identification of T-DNA genes responsible for the induction of necrogenesis.

The ten Tn3HoHo1-generated mutations which attenuated necrogenesis were mapped with restriction enzymes. They formed two clusters (Fig. 2). To identify the T-DNA genes mutated, we subcloned and partially sequenced the junction fragments between the Tn3HoHo1 transposon and the T-DNA in the 10 mutants. Our identification of the mutated T-DNA genes was facilitated by the facts that several T-DNA regions of other Ti plasmids have been sequenced and that T-DNA genes from different Ti plasmids share high homology (Barker et al. 1983; Gielen et al. 1984; Klee et al. 1984).

Homology searches in the database and DNA sequence analysis (Fig. 2 and data not shown) revealed that: i) Five Tn3HoHo1 insertions were located in gene *tms*1 with insertions 150, 153, and 179 in the 5′ and insertions 99 and 120 in the 3′ end of its coding region; ii) Two insertions mapped to gene 6b with insertion 41 in the middle of its coding region and insertion 71 in the TATA box of its promoter; iii) Three insertions, 46, 82, and 213, did not map to any known T-DNA genes, but were very close to the region where the right border sequence of TL-DNA is located (Hood et al. 1986a), where they might interfere with the function of the right border sequence, thus reducing T-DNA transfer. As shown in Figure 2 and below, insertions in *tms*2, *tmr*, and genes 5 and 7 did not affect A281's ability to induce necrosis.

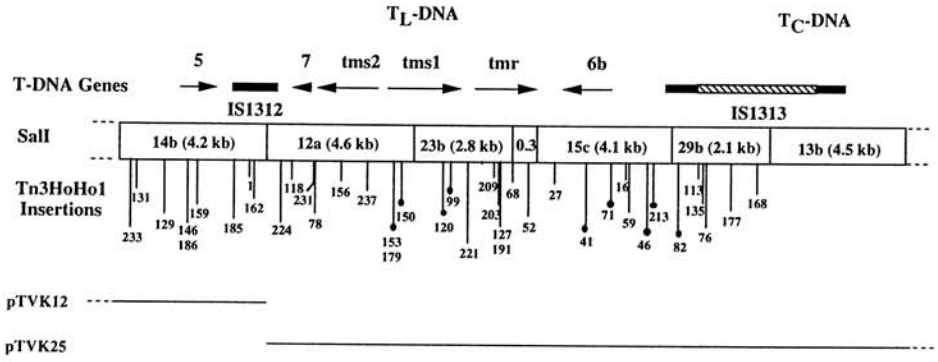

Fig. 2. TL-DNA gene map of pTiBo542 and localization of Tn3HoHo1-generated mutations. The TL-DNA region is presented as the *Sal*I restriction map described by Komari et al. (1986) and modified by us based on our sequencing data (data not shown). Putative TL-DNA genes were localized by us and by others (Komari et al. 1986; Strabala et al. 1989) by sequencing and by hybridization with known TL-DNA gene probes from pTiA6 and pTiAch5. Locations of the 40 Tn3HoHo1 insertions in the TL-DNA region were determined by using restriction enzymes *Sal*I, *Eco*RI, *Bam*HI, and *Hin*dIII. These insertions were first generated in cosmid clones pTVK12 and pTVK25 (Komari et al. 1986), and then marker-exchanged back into pTiBo542 in A281 (Ruvkun and Ausubel 1981). Insertions causing attenuation in A281 in inducing necrosis are marked with closed circles (also see Table 2). Those unmarked insertions did not affect the induction of necrosis by A281. Two copies of a novel insertion sequence, IS1312, were found in the T-region, with the copy in the TC-region disrupted by another putative insertion element IS1313 (Deng et al. 1995). The two copies of IS1312, represented as solid boxes, are arranged as inverted repeats. IS1313 is indicated as a cross-hatched box. Their positions were determined by DNA hybridization and sequencing. Sizes of restriction fragments are indicated in kilobases (kb).

Genetic Map of TL-DNA of pTiBo542.

A detailed gene map for the TL-DNA of pTiBo542 is presented in Fig. 2. Strabala et al. (1989) have sequenced the *tmr* gene in the Ti plasmid. By using Southern hybridization and DNA sequencing techniques, we identified and characterized the other TL-DNA genes. Genes *tms*1, *tms*2, 7, and 6b were identified by DNA sequencing, but only gene 6b was sequenced in both strands. Its DNA sequence is available under GenBank accession number U19150. The presence of gene 5 in pTiBo542 was detected by using pTiAch5 gene 5 as a probe (see Fig. 3 and the following sections). Its putative direction of transcription was determined by partial DNA sequencing (data not shown). All of these genes were highly homologous to the T-DNA genes of other Ti plasmids. No homolog of gene 6a or opine synthase genes was found in the TL-DNA region. This was expected since Hood et al. (1986a) showed that the opine synthetic loci in pTiBo542 are located in the TR-DNA. In addition, two novel putative insertion sequences, IS1312 and IS1313, were identified by DNA sequencing in the T-region of pTiBo542 (Deng et al. 1995). The right border region, as determined by Hood et al. (1986a and 1986b), was also sequenced, but we could not find the right border sequence by comparing the DNA sequence to the 25 bp border sequences of octopine- and nopaline-type Ti plasmids (for a review see: Kado 1991). However, a sequence in the putative insertion sequence IS1313 (GenBank accession number U19149) showed similarity to the octopine T-DNA border sequence (data not shown). As shown in Fig. 2, the gene organization of the pTiBo542 TL-DNA is very similar to that of the TL-DNA of octopine-type Ti plasmids and the T-DNA of nopaline-type Ti plasmids (for reviews see: Winans 1992; Otten et al. 1992).

Double mutants of *tms*1 and 6b are non-necrogenic.

Our genetic studies indicate that *tms*1 and 6b genes of the T-DNA are necessary for A281 to fully induce necrosis on grapevines and that both genes are partially responsible. In order to test whether *tms*1 and 6b are the only two T-DNA genes responsible for inducing necrosis on grapevines, double mutants of *tms*1 and 6b in A281 (A281 *tms*1::Tn*pho*A 6b::Tn3HoHo1) were created. When tested on grapevines, the mutants were non-necrogenic on both Catawba and Chancellor (Figs. 1E and 1F; Table 2). These double mutants were non-tumorigenic on Catawba explants and *Kalanchoe* plants, but induced small tumors on Chancellor (Fig. 1F and Table 2). This supports previous observations that Chancellor is more sensitive than Catawba to tumor induction by a variety of *A. tumefaciens* strains tested (Haque 1990; Pu and Goodman 1992). These results indicate that *tms*1 and 6b are the only two T-DNA genes of A281 responsible for inducing the necrotic response on grapevines.

Table 2. Plant responses to *Agrobacterium tumefaciens* A281 and its T-DNA mutants

Strain[a]	Necrotic response on Catawba (%)[b]	Tumor induction on *Kalanchoe* plants[c]
A281 (parent)	100	+
EHA101	0	–
Single mutants		
A281mx41	55	+[d]
A281mx46	35	+
A281mx71	35	+[d]
A281mx82	30	+
A281mx99	50	+[d]
A281mx120	55	+[d]
A281mx150	20	+[d]
A281mx153	20	+[d]
A281mx179	20	+[d]
A281mx213	45	+
Double mutants		
A281*tms*1::Tn*pho*A 6b::Tn3HoHo1	0	–

[a] For relevant characteristics of the strains, see Table 3.

[b] Percentage of explants with necrosis. The necrotic response was assayed as described in detail in the legend to Table 1. The data are averages of three indepenent experiments.

[c] Stems and leaves of greenhouse-grown *Kalanchoe* plants were inoculated with each strain and tumor formation was scored 3 weeks later. +, tumor; –, no tumor.

[d] Delayed tumor formation.

Stem polarity strongly affects necrogenesis

Explant polarity has been shown to be very important in *Agrobacterium*-induced tumorigenesis (Ryder et al. 1985; Haque 1990), probably because of the unidirectional movement of auxin (from shoot tip to root tip) in plants (Goldsmith 1977). Since earlier studies and our current work suggested that plant growth regulators mediate the induction of necrosis by *A. tumefaciens* (Lowe and Krul 1991; Pu and Goodman 1992; Fig. 2), we tested whether orientation of the explant affects necrogenesis. Strain A281 induced necrosis in 100% of the stems inoculated when grape stem explants were cultured apical ends down and bacteria were inoculated onto their physiological basal ends, where auxin accumulates. However, when the stem explants were cultured in their natural position and their apical ends inoculated, necrosis was

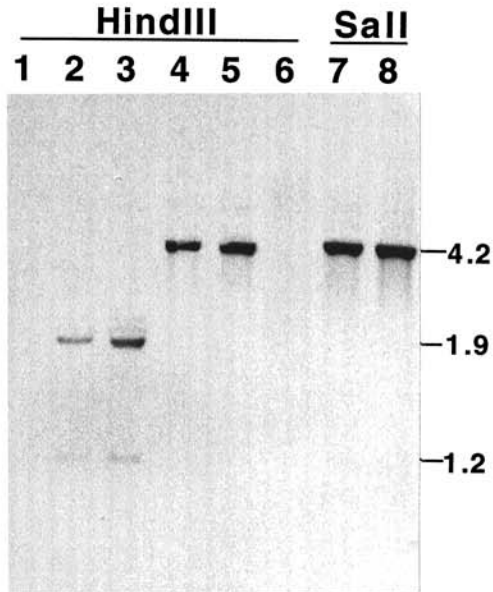

Fig. 3. Hybridization of total *Agrobacterium* DNA using gene 5 of Ach5 as a probe. The DNAs in lanes 1 to 6 were digested with *Hin*dIII; the DNAs in lanes 7 and 8 were digested with *Sal*I. See Table 3 for relevant characteristics of the strains. Lane 1, A136; lanes 2 and 7; Bo542; lanes 3 and 8, A281; lane 4, C58; lane 5, A6; lane 6, *A. vitis* strain Ag57. The positive hybridization band in lane 8 (A281) is *Sal*I fragment 14b shown by screening the *Sal*I clone bank of pTiBo542 (Komari et al. 1986) with the same probe (data not shown). The numbers on the right side represent the sizes of fragments in kilobase pairs.

reduced to 20% of the inoculated explants. Furthermore, even in those explants showing a response, necrosis was greatly delayed and attenuated (Fig. 1C).

Gene 5 of pTiBo542 in A281 is likely not transferred into grapevine cells.

Gene 5 of T-DNA encodes a protein which antagonizes auxin activity (Körber et al. 1991). We mapped gene 5 in the TL-DNA region of pTiBo542. By using the coding region of gene 5 from *A. tumefaciens* Ach5 as a probe, we found a homolog in the *SalI* fragment 14b of pTiBo542 (Fig. 3; Komari et al. 1986). DNA sequencing and restriction mapping showed that gene 5 of pTiBo542 was separated from the other TL-DNA genes by a novel insertion sequence, IS1312 (GenBank accession number U19148; Figs. 2 and 3). Whether this A281 homolog of gene 5 is functional or not remains to be demonstrated. No sequence similar to gene 5 was found in *A. vitis* strain Ag57, confirming a previous report (Paulus et al. 1991), whereas similar sequences were present in A348 and C58 (Fig. 3).

Hood et al. (1986a) have shown that in A281 the portion of the T-DNA beyond the insertion sequence is transferred into some plants (e.g. alfalfa) but not others (e.g. soybean). Interestingly, the right inverted terminal repeat of IS1312, which separates gene 5 from other TL-DNA genes, contains a sequence which shows high homology to the 25 base-pair T-DNA border sequences (data not shown; Deng et al. 1995). The similarity is even greater in the consensus nick sequence shared between T-DNA borders of *Agrobacterium* and the transfer origins of IncP, IncQ, and Ti plasmids, all of which are similar (Waters et al. 1991; Cook and Farrand 1992). Therefore, it is likely that gene 5 of pTiBo542 is not transferred into plants as part of the TL-DNA if this pseudo-border sequence is processed by the VirD2 endonuclease.

DISCUSSION

Agrobacterium can affect plants in a variety of ways. Although some plant species are recalcitrant or even resistant to tumor formation, many dicot plants and gymnosperms, as well as a few monocot plants, are susceptible to infection by *A. tumefaciens* (DeCleene and DeLey 1976). While the formation of a gall is the outcome for most plant–*A. tumefaciens* interactions, some *Agrobacterium* strains cause other kinds of diseases or symptoms on specific plants. For example, *A. vitis* strains, which specifically infect grapevines, not only cause galls but also root decay specifically on grape plants (Burr et al. 1988). The virulence factor responsible for the root decay is polygalacturonase, a pectic enzyme that is chromosomally encoded and is not present in *A. tumefaciens* strains (Rodriguez-Palenzuela et al. 1991). Thus, root decay is totally different from the necrotic response induced by WHR strains of *A. tumefaciens* on grapevines, which is Ti plasmid-related (Yanofsky et al. 1985a, 1985b; Lowe and Krul 1991; Pu and Goodman 1992).

It has been known for some time that different plants respond differently to the same *A. tumefaciens* strain and that tumorigenesis is a plant species- and *A. tumefaciens* strain-specific process (DeCleene and DeLey 1976; Anderson and Moore 1979). The formation of a crown gall results from the active production of plant growth factors, auxin and cytokinin, encoded by T-DNA genes (Schröder et al. 1984; Thomashow et al. 1984; Thomashow et al. 1986). Auxin and cytokinin must be at appropriate levels and ratios to each other for *A. tumefaciens* to induce tumors on its host plant, since accumulation of the growth factors at high levels could cause cell death. Because tumor formation is the desired end result for *A. tumefaciens*, its WHR strains have evolved mechanisms to balance hormone production in transformed plant cells. Their T-DNA consists of not only oncogenes responsible for the biosynthesis of auxin and cytokinin, but also genes, namely genes 5 and 6b, that either regulate the hormone levels or influence the effects of the hormones (for reviews see: Kado 1991; Winans 1992). The lack of genes for hormone biosynthesis or genes responsible for regulating hormone levels and/or effects likely contributes to the host range of *A. vitis* strains and some LHR strains of *A. tumefaciens* (Hoekema et al. 1984; Yanofsky et al. 1985a; for a review see: Otten et al. 1992).

Although the gene organization in the T-DNA of WHR strains is geared to ensure tumorigenesis, these strains do not induce tumors on all plants either. The apparent resistance of certain plants to tumor induction by *A. tumefaciens* can be explained in a number of ways. The specific *A. tumefaciens* strain may not be able to transform the specific plant because any one of the steps leading to tumorigenesis is blocked or the plant may fail to respond to the levels of growth regulators resulting from T-DNA gene expression. In the latter case, the sensitivity of a plant to changes in hormone levels resulting from T-DNA gene expression is critical in determining whether tumors will form. Some plants may have evolved mechanisms that overcome drastic fluctuations in growth regulator levels, and thus have become insensitive to tumor formation. On the other hand, some plants may be extremely sensitive to such changes and respond to such alterations in a way other than tumorigenesis.

The necrotic response induced by WHR *A. tumefaciens* strains on grapevines might represent such a sensitive system. Both the virulence and the T-DNA regions in the Ti plasmid must be present in order for *A. tumefaciens* to induce the necrotic response (Yanofsky et al. 1985a, 1985b; Lowe and Krul 1991; Pu and Goodman 1992). Our genetic studies indicate that *tms*1 and 6b genes of the T-DNA are necessary for A281 to fully induce necrosis on grapevines and that both genes are partially responsible. Since the *tms*1 gene is involved in auxin biosynthesis (Schröder et al. 1984; Thomashow et al. 1984), and gene 6b has an auxinlike effect on plants (Tinland et al. 1989; Spanier et al. 1989; Tinland et al. 1990), they are likely to have additive or synergistic effects on the auxin activity in transformed plant cells. Synergistic effects have been reported in tumor induction between the auxin biosynthetic genes and the 6b gene of the limited-host-range (LHR) *A. vitis* strain AB3 (Paulus et al. 1991). This could explain why non-necrogenic mutants did not result from single insertions. It is worth noting that no single transposon insertion caused a complete loss of oncogenicity by WHR *A. tumefaciens* strains either (Garfinkel et al. 1981; Ooms et al. 1981). Based on these genetic studies, we propose that auxin is the inducing agent for necrosis and that the necrogenesis induced by *Agrobacterium* on grapevines results from auxin toxicity.

Our hypothesis is supported by the following findings. Gene 5, which modulates auxin responses in plants by au-

toregulating the synthesis of an auxin antagonist (Körber et al. 1991), can suppress any possible auxin toxicity conferred by the auxin biosynthetic genes *tms*1 and *tms*2 in the T-DNA. Interestingly, in strain A281 this locus is separated from other TL-DNA genes by a novel insertion sequence (IS1312), part of which is similar to the T-DNA border sequence (Figs. 2 and 3). It is possible that this locus is not transferred to grape cells. In addition, A281 has been reported to induce tumors even on plants like legumes which are extremely difficult to be transformed by other *Agrobacterium* strains (Hood et al. 1986b; Jin et al. 1987). The supervirulence and high transformation frequency of A281 has been attributed to its *vir* genes (Hood et al. 1986b; Jin et al. 1987). By using the β-glucuronidase gene as a reporter, A281 has been shown to transform grape cultivars Catawba and Chancellor at a higher frequency than other tumor-inducing *Agrobacterium* strains (Pu and Goodman 1992; data not shown). Therefore, the highly efficient T-DNA transfer system of A281 (Hood et al. 1986b; Jin et al. 1987), in combination with its unique T-DNA gene organization, apparently results in a high auxin level following expression of T-DNA genes which the grape cultivars Catawba and Chancellor cannot tolerate. The resulting necrosis at the site of inoculation then blocks uncontrolled cell division which would have led to tumor formation. If auxin is responsible for inducing necrosis on grapevines, then the orientation of the explant should affect necrogenesis. This is indeed the case. Inoculation of *Agrobacterium* on physiological basal ends, where auxin accumulates, resulted in the greatest degree of necrogenesis. Our hypothesis that auxin is the responsible agent is further supported by the observation that exogenously added auxin accelerates necrogenesis by A281, while kinetin and abscisic acid tend to delay the process (Pu and Goodman 1992).

It should be noted that although both *tms*1 and *tms*2 are required for auxin biosynthesis in T-DNA-transformed plant cells, mutations in *tms*2 did not affect the ability of A281 to induce necrogenesis (data not shown). This raises the possibility that IAM, rather than IAA, is the necrogenic factor. Although our data do not rule out this possibility, it seems unlikely for a number of reasons. First, IAM has been shown to be an inactive compound and does not have any biological activity when applied to a variety of plants in physiological concentrations (Wightman 1962; Budar et al. 1986; Klee et al. 1987; Karlin-Neumann et al. 1991). A toxic effect is seen only when very high concentrations of IAM are used. Second, the growth of plants transformed with the *tms*2 gene, which converts IAM into IAA, is strongly inhibited by concentrations of IAM that do not significantly affect wild-type plants (Budar et al. 1986; Klee et al. 1987; Karlin-Neumann et al. 1991). These results all suggest that IAM exerts its biological effect only after it is converted into IAA. Our observation that *tms*2 mutations did not influence the ability of A281 to induce necrogenesis can be readily explained if *Vitis* cells can hydrolyze IAM into IAA. Such IAM hydrolyzing activities have been reported in plants (Wightman 1962; Klee et al. 1987; Kawaguchi et al. 1991).

Lowe and Krul (1991) studied the effects of a selected group of mutants of A348 T-DNA on stem necrosis in the grapevine cultivar Steuben, another cultivar that is highly sensitive to necrosis. They showed that a mutation in *tms*1 reduced necrogenesis slightly, whereas a Tn5 insertion in *tms*2 resulted in a significant reduction in the necrotic reaction. This observation is also consistent with our conclusion that auxin is responsible for inducing necrogenesis. However, it contrasts with our finding that *tms*1 mutations profoundly affected necrogenesis induced by A281 on the cultivar Catawba, while mutations in *tms*2 had no effect. This discrepancy in the data could be due to differences in the *Agrobacterium* strains and/or *Vitis* cultivars studied. Since *tms*2 codes for the enzyme that carries out the second reaction of the two-step process of auxin biosynthesis encoded by T-DNA and plants do not normally contain IAM (Klee et al. 1987), it is reasonable to expect that *tms*1 mutations would have a more drastic effect on the induction of necrogenesis than *tms*2 mutations. Mutations in the *tmr* locus had no effect on the necrosis either in the A281-Catawba or the A348-Steuben interactions (data not shown; Lowe and Krul 1991). The effect of mutations in the 6b gene on necrogenesis was not studied by Lowe and Krul.

A. tumefaciens is not the only bacterium that utilizes auxin and other plant growth regulators as virulence factors. Other soil and plant-associated microorganisms, including species of *Pseudomonas*, *Xanthomonas*, *Erwinia*, and *Rhizobium*, also produce auxins (Fett et al. 1987; Clark et al. 1993; Mazzola and White 1994; for a review see: Morris 1986). *P. savastanoi* induces tumorous overgrowths, known as olive knot disease, on olive and oleander plants by secreting high levels of auxin (Comai and Kosuge 1982). The importance of auxin production in the virulence and pathogenicity of *Erwinia herbicola* pv. *gypsophilae* and *Pseudomonas syringae* pv. *syringae* has been demonstrated recently. While mutations of the auxin biosynthetic genes in *E. herbicola* pv. *gypsophilae* reduce virulence (Clark et al. 1993), a similar alteration in *P. syringae* pv. *syringae* affects growth of the bacteria in *Phaseolus vulgaris* and also syringomycin production (Mazzola and White 1994). The significance of auxin production by *Rhizobium* species and most plant-pathogenic pseudomonads and xanthomonads, which do not cause obvious hypertrophy of host tissues, is not yet understood. It is conceivable that during interactions with these phytopathogenic bacteria, certain plants, like some grapevine cultivars, may have adopted the necrotic response as a defense mechanism to prevent *A. tumefaciens* or other bacterial pathogens from causing even more severe damage to the plant from the disease they normally cause.

However, it is important to note that the necrotic response is not the typical hypersensitive response (HR), the well-known plant defense mechanism which results from an incompatible interaction with a plant pathogen. This reaction is characterized by the rapid, localized death of plant cells at the site of pathogen invasion (for reviews see: Klement 1982; Keen 1990; Goodman and Novacky 1994). As Pu and Goodman (1992) have pointed out, the necrosis induced by *A. tumefaciens* on grapevines occurs much more slowly than the HR. In addition, *Agrobacterium*-induced necrosis develops progressively beyond the inoculation site (Pu and Goodman 1992). The notion that the necrotic response is different from the hypersensitive response is further supported by the report that some *A. tumefaciens* strains can in fact inhibit the development of the HR elicited by *Pseudomonas syringae* pv. *phaseolicola* on tobacco (Robinette and Matthysse 1990).

Although it is quite clear that T-DNA genes related to the

biosynthesis and modulation of activity of auxin are responsible for inducing the necrotic response on grapevines, little is known about the mechanism by which the response is initiated in plant cells. Since the necrotic response is both *A. tumefaciens* strain and plant cultivar specific, further studies should be directed at elucidating the plant chemistry during the necrotic response and identifying and characterizing the plant genes involved in this interaction. Such studies will, no doubt, help us better understand the pathogenesis of *Agrobacterium* and the reaction of a plant cell to the expression of T-DNA genes.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The strains and plasmids used in this study are listed in Table 3. Media, use of antibiotics, and growth conditions were as previously described (Cangelosi et al. 1991; Charles and Nester 1993).

DNA manipulations.

Standard techniques for DNA cloning and mapping were conducted according to Sambrook et al. (1989). Total DNA of *Agrobacterium* strains was prepared as described by Charles and Nester (1993). Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Maryland) or New England Biolabs, Inc. (Beverly, Mass.) and used according to the supplier's recommendations.

For Southern hybridization, DNA digests were transferred to nylon membrane (Nytran; Schleicher & Schuell, Keene, New Hampshire) by using the vacuum blotting system (Pharmacia, Uppsala, Sweden). Probe DNA was labeled with fluorescein. Hybridization and signal detection were carried out by using the Amersham ECL random prime labeling and detection system (Amersham Corp., Arlington Heights, Ill.) according to the recommended conditions.

Transposon mutagenesis of the TL-DNA region of pTiBo542.

Cosmid clones pTVK12 and pTVK25, which cover the TL-DNA region of pTiBo542 (Komari et al. 1986), were mutagenized with Tn3HoHo1 by the method described by Stachel et al. (1985). Insertions were mapped with restriction enzymes *Sal*I, *Eco*RI, *Bam*HI, and *Hin*dIII. When needed, the precise insertion sites of Tn3HoHo1 in the T-DNA were determined by sequencing with an oligonucleotide primer complementary to the fusion-generating end of Tn3HoHo1 (5′-CCATTAAAAGAGGCGTCAGAGGCAG-3′) after subcloning the junction fragments between the transposon and the TL-DNA sequences into pTZ18R. The junction sequences were then compared to similar sequences in the database. Those cosmids having insertion mutations in the TL-DNA region, which were defined by Hood et al. (1986a) and Komari et al. (1986), were introduced individually into A281 by electroporation (Cangelosi et al. 1991). Double crossover (homologous recombination) between the mutated cosmids and pTiBo542 was achieved by using the marker exchange technique (Ruvkun and Ausubel 1981; Garfinkel et al. 1981). After colonies were purified, clones resulting from the desired recombination events were verified by Southern hybridiza-

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Agrobacterium tumefaciens		
A136	C58 cured of pTiC58	Watson et al. 1975
A348	A136(pTiA6NC) (octopine-type)	Garfinkel et al. 1981
C58	Wild type, nopaline strain	Laboratory collection
A6NC	Wild type, octopine strain	Laboratory collection
FACH	Wild type, grape isolate	Laboratory collection
A281	A136(pTiBo542) (succinamopine-type)	Sciaky et al. 1978
Bo542	Wild type, succinamopine strain	Sciaky et al. 1978
EHA101	Disarmed A281 (T-DNA deleted)	Hood et al. 1986b
A281mx41	A281 $6b_{41}$::Tn3HoHo1	This study
A281mx46	A281 T-$region_{46}$::Tn3HoHo1	This study
A281mx71	A281 $6b_{71}$::Tn3HoHo1	This study
A281mx82	A281 T-$region_{82}$::Tn3HoHo1	This study
A281mx99	A281 $tms1_{99}$::Tn3HoHo1	This study
A281mx120	A281 $tms1_{120}$::Tn3HoHo1	This study
A281mx150	A281 $tms1_{150}$::Tn3HoHo1	This study
A281mx153	A281 $tms1_{153}$::Tn3HoHo1	This study
A281mx179	A281 $tms1_{179}$::Tn3HoHo1	This study
A281mx213	A281 T-$region_{213}$::Tn3HoHo1	This study
A281 *tms*1 6b double mutant	A281 $tms1_{179}$::Tn*pho*A $6b_{41}$::Tn3HoHo1	This study
Escherichia coli		
C2110	*polA rha his*	Prince and Barlam 1985
MT616	MT607(pRK600), mobilizer	T. Charles
HB101(pHoHo1, pSShe)	Tn3HoHo1 mutagenesis strain	Stachel et al. 1985
HB101(pPH1JI)	pPH1JI, IncP plasmid	J. Beringer
MT621	*pro-82 thi-1 hsdR17 supE44 endA1 malF*::Tn*phoA*	T. Charles
Plasmids		
pUC19g5SalI	Ach5 gene 5 cloned in pUC19	C. Koncz
pTVK25	Cosmid clone of pTiBo542 T-region	Komari et al. 1986
pTVK12	Cosmid clone of pTiBo542 T-region	Komari et al. 1986
pTZ18R	Cloning vector, ColE1 *oriV*	U. S. Biochemical Corp.
pSP329	Broad host range cloning vector, IncP	S. Porter
pSP329S12a	*Sal*I fragment 12a from pTVK25 cloned in pSP329	This study

tion. The T-DNA mutants which resulted from marker exchange are designated A281mx followed by the number of the corresponding Tn3HoHo1 insertion as shown in Fig. 2 and Table 2.

Generation of A281 *tms*1::Tn*pho*A 6b ::Tn3HoHo1 double mutants.

To make a *tms*1 and 6b double mutant of A281, the SalI fragment 12a of pTVK25 (Komari et al. 1986), which contains most part of the *tms*1 gene of pTiBo542, was subcloned into an IncP broad host range vector pSP329, creating pSP329S12a. Tn*pho*A insertions in pSP329S12a were generated by passing the plasmid through the *E. coli* chromosomal Tn*pho*A strain MT621 as described by Charles and Nester (1993). Transposition events onto the plasmid were identified by mobilizing the resulting kanamycin-resistant plasmids into the *E. coli polA* strain C2110 using MT616 as a mobilizer. The position and orientation of Tn*pho*A insertions in the *tms*1 region were determined by mapping with restriction enzymes and sequencing with a primer complementary to part of the *pho*A sequences (5′-ACCCGTTAAACGGCGAGCACCGCCGGG-3′). One plasmid, pSP329S12a ::Tn*pho*A#179, which has a Tn*pho*A insertion in the 5′ coding region of the *tms*1 gene (126 base pairs away from the start codon), was introduced into two pPH1JI-cured A281 6b::Tn3HoHo1 mutants (A281mx41 and A281mx71) by electroporation. Plasmid pPH1JI was then reintroduced into the resulting strains and double mutants were selected using the marker exchange technique (Ruvkun and Ausubel 1981; Garfinkel et al. 1981; Cangelosi et al. 1991). Putative A281 *tms*1::Tn*pho*A 6b::Tn3HoHo1 double mutants were confirmed by Southern analysis.

DNA sequencing and analysis.

Double-stranded plasmid DNA was sequenced by the chain termination method with Sequenase version 2.0 according to the protocol recommended by the supplier (U.S. Biochemical Corp., Cleveland, Ohio). When long pieces of DNA needed to be sequenced, exonuclease III was used to generate nested sets of deletions according to the standard method (Sambrook et al. 1989). DNA sequences were analyzed by using Genetics Computer Group software from the University of Wisconsin, and similarity searches were conducted at the National Center for Biotechnology Information (Bethesda, Md.) with the BLAST network service (Altschul et al. 1990).

Plant assays.

Tests of various *A. tumefaciens* strains on *Vitis* cultivars Catawba and Chancellor were conducted as described before (Pu and Goodman 1992). Briefly, internodes 3, 4, 5, 6, and 7 from the apex were cut from grape plants grown in the greenhouse and surface sterilized in 75% ethanol for 2 min, soaked in 2.5% sodium hypochlorite for 20 min, and washed three times in sterile distilled water. The stem pieces were then trimmed aseptically at both ends and placed apex down, except when indicated otherwise as in the polarity tests (see text), in half-strength B5 culture medium without hormones (Gamborg et al. 1968). Bacteria were grown overnight in MG/L broth (Cangelosi et al. 1991) supplemented with appropriate antibiotics at 28°C, centrifuged and washed in 0.1 M phosphate-buffered saline (pH7.0). Cell concentration was adjusted to about 10^9 cells per milliliter in sterile distilled water. Droplets of inocula, 5 µl each, were applied onto grape stem explants. Eight to 10 internode explants were inoculated for each treatment per strain, and each treatment consisted of representatives of all internodes, so that any variation due to physiological age was minimized. The inoculated explants were incubated in a growth chamber with a 14-h photoperiod at 25°C. Each experiment was repeated at least once.

The responses, necrotic or tumorigenic, were evaluated and recorded 1, 2, and 3 weeks after inoculation. Necrosis was usually visible 1 week after inoculation, appearing brown on the inoculated surface of the explants. It developed progressively down the axes of the explants and, 2 weeks after inoculation, the upper part of some necrosed shoots turned black. The degree of necrosis on different explants can vary, probably due to variations in the physiological state of the explants. Therefore, in scoring necrosis, samples were always contrasted to negative controls: uninoculated explants and explants inoculated with buffer only or *A. tumefaciens* strain A136 which was already shown not to induce necrosis. The explants showing more severe browning or blackening than the negative controls were considered necrosis positive. Since both tumor and necrosis were fully developed 2 weeks after inoculation, all the data presented in this paper were collected at that time.

Oncogenicity tests of *Agrobacterium* strains on *Kalanchoe daigremontiana* leaves or stems were performed as previously described (Garfinkel et al. 1981).

Insertion sequence numbers and nucleotide sequence accession numbers.

IS numbers were assigned to the two insertion sequences mentioned in this study by the Plasmid Reference Center (E. Lederberg, Plasmid Reference Center, Department of Microbiology and Immunology, 5402, Stanford University School of Medicine, Stanford, CA 94305-2499). The two IS elements in pTiBo542 are designated IS1312 and IS1313. The DNA sequences of IS1312, IS1313, and gene 6b of pTiBo542 are available from GenBank database under accession numbers U19148, U19149, and U19150, respectively.

ACKNOWLEDGMENTS

We thank C. Koncz for providing plasmid pUC19g5SalI and T. Charles for providing bacterial strains. We also thank all of the members of our group for helpful discussions. We especially acknowledge the help of S. Jin in the computer analysis of DNA sequences and his critical reading of the manuscript. This work was supported in part by National Science Foundation grant DMB8704292 and the Missouri Grape and Wine Program, Jefferson City. W. Deng was a recipient of a predoctoral fellowship from the Rockefeller Foundation.

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