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Molecular and genetic analysis of factors controlling host range in *Agrobacterium tumefaciens*

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Summary. We have investigated the factors which contribute to the host specificity of a tumor inducing plasmid of *Agrobacterium*, pTiAg162, which confers a narrow host range. Determinants both within the T-DNA and virulence regions contribute to host specificity. Within the T-DNA a defective cytokinin biosynthetic gene limits host range. Nucleotide sequence analysis revealed a large deletion in the 5′ coding region of this gene when compared with the homologous gene from the wide host range tumor inducing plasmid, pTiA6. Introduction of the wide host range cytokinin biosynthetic gene into the T-DNA of the limited host range strain expanded the host range and suppressed the rooty morphology of tumors incited by the limited host range strain. Two genes from the virulence region of the wide host range plasmid, designated *vir*A and *vir*C, must also be introduced into the limited host range strain in order to restore a wide host range phenotype. The wide host range strain is avirulent on some cultivars of *Vitis* plants on which the limited host range strain induces tumors. This avirulence is apparently due to a hypersensitive response in which infected plant cells are killed at the site of inoculation. Mutations within the *vir*C locus of the wide host range plasmid prevented the hypersensitive response and allowed the formation of tumors by the wide host range strain.

Introduction

Virulent strains of *Agrobacterium tumefaciens* are capable of inducing tumors on a wide variety of dicotyledonous and on some monocotyledonous plants (DeCleene and DeLay 1976). These strains contain a large, tumor inducing plasmid (Ti plasmid), a part of which (T-DNA) is transferred, integrated, and stably maintained within the nucleus of tumor cells (For reviews see Bevan and Chilton 1982; Depicker et al. 1983; Hooykaas and Schilperoort 1984; Nester et al. 1984). Another region of the Ti plasmid, designated the virulence region (*vir*), is necessary for tumor formation but is not found integrated into plant tumor DNA (Klee et al. 1982; Klee et al. 1983). This region is probably required in the early stages of tumorigenesis (eg. T-DNA transfer and possibly integration), whereas the T-DNA is important in the later stages (eg. tumor formation, maintenance and morphology). A number of studies have demonstrated that the genes responsible for oncogenesis reside in the T-DNA region and are directly responsible for the synthesis of cytokinin (*tmr*) and auxin (*tms*) (Akiyoshi et al. 1984; Barry et al. 1984; Inzé et al. 1984; Joos et al. 1983; Schröder et al. 1983; Thomashow et al. 1984). Recent studies have demonstrated that the *vir* region can function in a *trans* configuration to the T-DNA (i.e., on a separate replicon) (Hoekema et al. 1983; DeFramond et al. 1983). This has allowed the development of a binary vector system for introducing foreign DNA into plant cells (An et al. 1985; Bevan 1984; Klee et al. 1985).

Most *Agrobacterium* research has focused on the wide host range (WHR) strains which are capable of tumor formation on a wide variety of plants. However, some *Agrobacterium* strains in nature are tumorigenic on only a limited number of host plants, particularly *Vitis* (grapevine) cultivars. The limited host range of these strains is primarily due to the particular Ti plasmid harbored by the infecting strain (Loper and Kado 1979; Thomashow et al. 1980a). We have analyzed a limited host range (LHR) Ti plasmid in order to identify some of the factors which contribute to host range. Previous studies have demonstrated that two regions of the LHR Ti-plasmid, T_A-DNA and T_B-DNA, become stably integrated into the genome of transformed tissue of *N. glauca* and *Vitis vinifera* (Buchholz and Thomashow 1984a; Yanofsky et al. 1985). Whereas homology to the WHR *tmr* gene is localized to the T_A-DNA region, homology to the two *tms* loci maps within the region defined as T_B-DNA. This contrasts with the functional organization of the WHR plasmid where the *tmr* and *tms* loci are localized immediately adjacent to one another within a single T-DNA region. The addition of the WHR T_L-DNA (Knauf et al. 1983), and specifically the addition of the WHR cytokinin biosynthetic gene (Buchholz and Thomasho 1984b; Hoekema et al. 1984), leads to the expansion of the host range of the LHR strain. However, the resulting host range is substantially less than that conferred by the WHR plasmid. Interestingly, certain *Vitis* cultivars, which are susceptible to LHR *Agrobacterium* strains, are resistant to tumor formation by WHR strains (Thomashow et al. 1980a; Knauf et al. 1982b). This emphasizes that a more

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detailed analysis is necessary before a complete understanding of the factors controlling host range and specificity can be achieved. In this report we have investigated the contributions of the LHR and WHR T-DNA and *vir* genes to the host range of tumorigenesis by *Agrobacterium*.

Materials and methods

Enzymes and chemicals. Restriction endonucleases and DNA modification enzymes were obtained from Bethesda Research Laboratories or New England Biolabs and were used as recomended by the manufacturer. DNAse I and DNA polymerase I were purchased from Worthington Diagnostics. ^{32}P labeled deoxynucleotides were purchased from New England Nuclear. Other chemicals were purchased from Sigma Chemical Corporation.

Bacterial strains, media, and plasmids. The relevent characteristics of all strains described in Tables 2, 3, and 4 are shown in Table 1. The LHR (pTiAg162) and WHR (pTiA6) plasmids and the strains containing them have been described previously (Thomashow et al. 1981) and these strains differ only in Ti plasmid content. Cosmid clones of the LHR and WHR plasmid have also been described (Knauf et al. 1984; Knauf and Nester 1982a). Mutants in the WHR *vir* region were provided by S. Stachel (manuscript in preparation). *Agrobacterium* strains were maintained on AB minimal medium (Chilton et al. 1974) supplemented with the appropriate antibiotics (200 µg/ml carbenicillin; 100 µg/ml kanamycin; 100 µg/ml gentamicin), and *Escherichia coli* strains were maintained on L-agar medium (Miller 1972) supplemented with the appropriate antibiotics (15 µg/ml tetracycline; 50 µg/ml kanamycin). Bacterial conjugation was performed on nutrient agar by the triparental mating procedure of Ditta et al. (1980). The plasmid pPH1JI, used in the marker exchange procedure, has been described (Garfinkel et al. 1981).

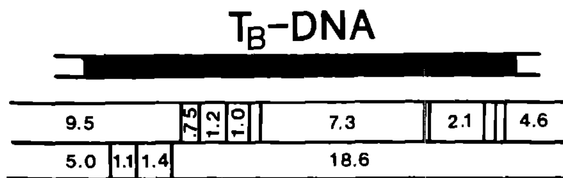
Plant inoculations and tumor lines. Plant inoculations were performed as previously described (Yanofsky et al. 1985). The host range of strains was determined by inoculation of wound sites on stems and/or leaves of *Nicotiana glauca, Nicotiana rustica, Nicotiana tabacum* var. *xanthi, Lycopersicum escalentum* cv. Early Girl (tomato), *Petunia hybrida,* and *Kalanchoë diagremontiana. Vitis* inoculations were performed on stem cultures as described previously (Lowe 1985; Yanofsky et al. 1985) on *V.* sp. cv. Seyval and *V. labruscana* vc. Steuben. The two tumor lines described in this manuscript were derived from the inoculation of decapitated *N. rustica* plants. Tumors were cultured axenically by methods previously described (Johnson et al. 1974).

Nucleic acid sequencing, Southern and Northern blot hybridization. The nucleotide sequence of the *tmr* gene was determined by the dideoxy method of Sanger et al. (1977) as previously described (Lichtenstein et al. 1984). Restriction digests of plant or bacterial DNA were resolved by agarose gel electrophoresis before transfer to nitrocellulose by the procedure of Southern (1975). DNA was isolated (Lichtenstein et al. 1984) and Southern blots were hybridized with nick-translated probes (Maniatis et al. 1975) as previously described (Thomashow et al. 1980b; Thomashow et al. 1981). RNA was isolated by published procedures (Gelvin et al. 1982) and purified by centrifugation through cesium chloride as described by Glisin et al. (1974). Isolation and gel electrophoresis of polyA^{+} RNA and Northern blotting procedures were described previously (Gelvin et al. 1982).

Table 1. The relevent characteristics of all strains described in Tables 2, 3, and 4 are shown

Strains or plasmids	Relevant phenotype or genotype	Source
A856 (pTiAg162)	LHR strain	Thomashow et al. (1981)
A348 (pTiA6NC)	WHR strain	Thomashow et al. (1981)
LHR: : tmr	LHR strain with *tmr* gene from pTiA6NC	This study
A856 (pVCK232)	LHR strain with T_L-DNA from pTiA6NC	This study
A856 (pVCK219)	LHR strain with *vir*A from pTiA6NC	This study
A856 (pVCK225)	LHR strain with *vir*G, C, D, and E from pTiA6NC	This study
A856 (pVCK257)	LHR strain with *vir*A, B, G, and C from pTiA6NC	This study
LHR: : tmr (pVCK257)	LHR strain with *tmr* gene and viriA, B, G, and C from pTiA6NC	This study
226 MX	*vir*A mutant of pTiA6NC	S. Stachel (in preparation)
365 MX	*vir*C mutant of pTiA6NC	S. Stachel (in preparation)
226 MX (pVCK305)	*vir*A mutant of pTiA6NC with LHR cosmid clone	This study
LBA4404	WHR *vir* helper strain with no T-DNA	Ooms et al. (1981)
LBA4404 (pVCK313)	WHR *vir* helper strain with LHR T-DNA	This study
LBA4404 (pVCK232)	WHR *vir* helper strain with WHR T-DNA clone	This study
pVCK418	Cosmid clone of LHR T_B-DNa region	Knauf et al. (1984)
pEND4K	Plant transformation vector	Klee et al. (1985)
pEND4K: LHR-tms	pEND4K with LHR *tms* loci	Klee et al. (1985)

Insertion of WHR tmr gene into LHR T-DNA. The 7.3 kb *Bam*HI fragment from the LHR T_B-DNA region (Yanofsky et al. 1985) was inserted into the vector pRK404 (Ditta et al. in press). This provided a unique *Xho*I site approximately in the middle of the cloned *Bam*HI fragment. A 6.1 kb *Sal*I fragment carrying only the WHR *tmr* gene and the left half of Tn5 (isolated from a Tn5 insertion



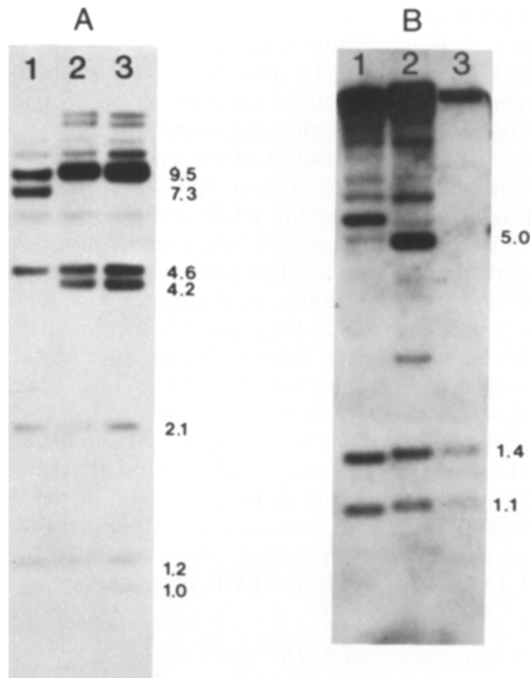


Fig. 1 A, B. Analysis of LHR::tmr construct. The T_B-DNA region, encompassed by the cosmid clone pVCK431, of the LHR plasmid is shown. *Bam*HI (upper) and *Eco*RI (lower) restriction maps are also designated. The verification of the LHR::tmr construct is shown in (**A**). Total *Agrobacterium* DNA was digested with *Bam*HI, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose. Southern blots were then hybridized with nick translated pVCK431 probe and visualized by autoradiography. Lane 1 represents DNA from the LHR strain, and lanes 2 and 3 represent DNA from two independent marker exchange events. Although the 7.3 kb *Bam*HI fragment is clearly present in lane 1, this fragment is absent in lanes 2 and 3 and is replaced by two new fragments of 9.5 and 4.2 kb. **B** shows the analysis of the resulting tumor lines as described in results. Tumor lines are from the LHR strain inoculated onto *V. vinifera* (lane 1) and *N. rustica* (lane 2). Also shown is the LHR::tmr strain inoculated onto *N. rustica* (lane 3)

(pNW34D-7-1::Tn5-342) into transcript 6a of pTiA6, (Garfinkel et al. 1981)), including the kanamycin resistance determinant, was then inserted into this unique *Xho*I site. Introduction of this construct into *Agrobacterium* by conjugation (Ditta et al. 1980) and subsequent marker exchange (Ruvkun and Ausubel 1981) resulted in the insertion of the *tmr* gene into the LHR T-DNA. The fidelity of the marker exhange was verified by Southern blot hybridization of total *Agrobacterium* DNA (Fig. 1A); the resulting strain was designated LHR::tmr.

Construction of pEND4K:LHR-tms. A series of vectors have been constructed which facilitate the cloning of foreign DNA into plant cells. One such vector, pEND4K, stably replicates in *Agrobacterium* and contains the left and right borders of the WHR T-DNA (Klee et al. 1985). Thus, DNA inserted between these borders is transferred into the plant genome with the aid of a helper plasmid containing the *vir* region. A *Sal*I-*Kpn*I fragment containing the LHR *tms* genes (Yanofsky et al. 1985) was inserted into the vector pEND4K and the resulting construct was designated pEND4K:LHR-tms.

Table 2. The role of the *tmr* locus in host range. A comparison of the relative host ranges of the LHR and WHR strains is shown. Also shown is the result of introducing the WHR *tmr* gene into the LHR strain (LHR::tmr), and the result of introducing the WHR T-DNA (pVCK232) into the LHR strain. + = tumor formation; − = no tumor formation. Further details are provided in the text

Plants	Strains			
	WHR	LHR	LHR::tmr	LHR (T_L-DNA)
Vitis[b]	+	+	+	+
N. glauca	+	+	+	+
N. rustica	+	+[a]	+	+
N. tabacum	+	−	+	+
Tomato	+	−	+	+
Sunflower	+	−	+	+
Kalanchoë stems	+	−	−	−
Kalanchoë leaves	+	−	−	−

[a] Roots from tumor
[b] *Vitis sp.* cv. Seyval

Results

Role of tmr locus in host range

Tumors induced by the LHR strain on *N. rustica* proliferated roots extensively analogous to *tmr* mutants of the WHR plasmid. This phenotype suggested that the LHR plasmid lacks a functional *tmr* gene. In order to test this possibility, we inserted the WHR *tmr* gene into the T_B-DNA region of the LHR plasmid. This allowed us to test if the WHR *tmr* gene could complement the lack of a functional *tmr* gene on the LHR plasmid. After inserting the WHR *tmr* gene into the LHR T_B-DNA region, the expected T_B-DNA pattern for this modified Ti-plasmid was verified for tumors induced on *N. rustica*. For this purpose, Southern blots of *Eco*RI digested tumor DNA were probed with nick-translated pVCK431 (Knauf et al. 1984), a cosmid clone which spans the T_B-DNA region is the LHR plasmid. Figure 1B shows the analysis of tumor lines induced by the LHR strain on *Vitis vinifera* (lane 1) and *N. rustica* (lane 2), and by the LHR::tmr strain on *N. rustica* (lane 3). The 1.4 and 1.1 kb *Eco*RI fragments are present as internal fragments in these tumor lines. An additional fragment of approximately 18 kb is also present in all three tumor lines. This large fragment, as well as the additional fragments seen in lanes 1 and 2, probably represent junction fragments of T-DNA sequences joined to plant DNA. We have also shown that the T_A-DNA region is present in these *N. rustica* transformed tumor lines (data not shown).

We next determined if the introduction of the WHR *tmr* gene affected tumor formation by the LHR strain (Table 2). On *N. rustica*, the wild-type LHR strain induced

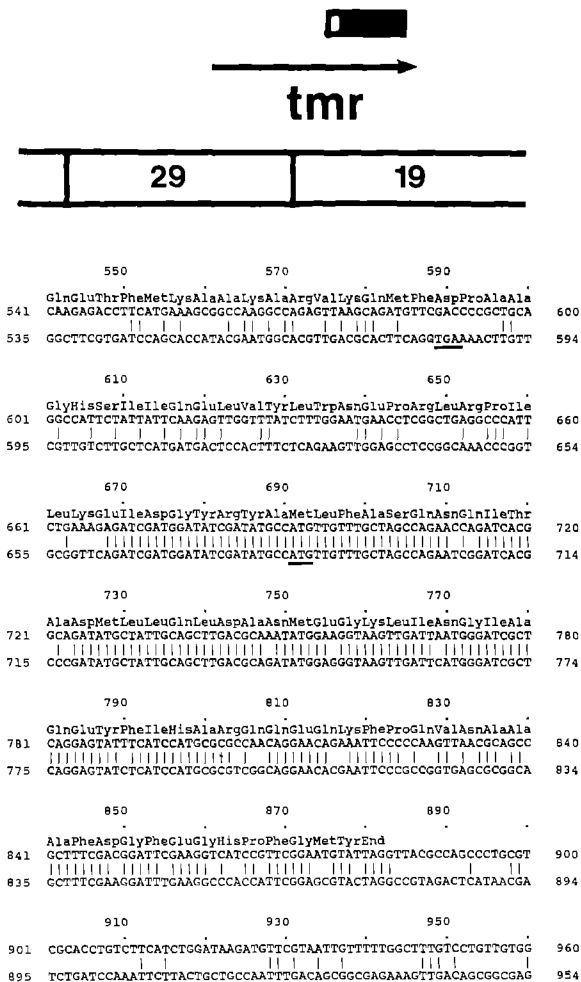


Fig. 2. Nucleotide sequence of the LHR "tmr" region. The WHR *tmr* locus is shown above a *Bam*HI restriction map of the corresponding region of the WHR plasmid. The *black bar* indicates the region of the WHR *tmr* sequence which is conserved on the LHR plasmid. The *open bar* designates the divergence point between the LHR and WHR *tmr* regions as discussed in the text. The nucleotide sequence of the LHR "tmr" region is compared with the corresponding region of the WHR *tmr* gene. The *upper line* shown the WHR *tmr* sequence and the *lower line* indicates the homologous LHR sequence. Because only the 3′ end of the gene is conserved on the LHR plasmid, we have only indicated this region of the sequence. For reference, nucleotide 160 represents the translational start site of the WHR *tmr* gene, and we have only shown nucleotides 541 through 960. *Vertical bars* indicate conserved sequences between the LHR and WHR plasmids. Because the LHR "tmr" open reading frame could continue upstream from the divergence point, we have *underlined* the first termination codon consistent with the expected reading frame. Such a hypothetical protein should begin downstream from this codon. The first initiation codon within this reading frame is also *underlined*

tumors which proliferated roots extensively. In contrast, the LHR: :tmr strain induced unorganized tumors on *N. rustica,* indicating that the introduced WHR *tmr* gene suppressed root formation. The WHR *tmr* gene also allowed tumor formation on *N. tabacum*, sunflower, and tomato, and attenuated tumors on carrot discs. The wild-type LHR strain is avirulent on all of these plants. In fact, the WHR *tmr* gene appeared to expand the host range of the LHR strain to the same extent as did the entire WHR T-DNA (Table 2). We conclude that, within the T-DNA, the *tmr* locus largely limits the host range of the LHR strain. However, the *tmr* locus was unable to fully restore a WHR phenotype as evidenced by the inability to allow tumor formation on plants such as *Kalanchoë* (Table 2).

Nucleotide sequence of the LHR tmr region

The preceding observations suggested that the LHR plasmid, while containing homology to the WHR *tmr* gene, indeed lacks a functional *tmr* gene. The LHR *tmr* homolog was cloned, sequenced, and compared to the WHR *tmr* locus (Fig. 2). The LHR plasmid retains homology only to the terminal 214 nucleotides of the open reading frame of the WHR *tmr* gene, or 30% of the WHR gene. These data strongly suggest that the LHR *tmr* gene is weak or inactive. Although there are 29 base pair substitutions in this conserved region of the LHR gene, there are no frameshift alterations and the translational stop signal is retained. The two sequences diverge from one another immediately after the end of the open reading frame. Just preceeding the 5′divergence point (position 667 of the WHR sequence), the LHR sequence contains a 26 bp sequence (bp 635–661 of the LHR sequence) which is identical to a sequence within *Eco*RI fragment 24 of the WHR plasmid (Barker et al. 1983). This sequence occurs within the region of the WHR plasmid which is between the two T-DNA regions and may indicate a point of rearrangement which generated the observed T-DNA structure of the LHR plasmid.

Northern blot analysis of LHR "tmr" region

We used Northern blot analysis to determine if the LHR *tmr* region was expressed in *N. rustica* transformed tissue (Fig. 3). The probe, specific for the lHR *tmr* region, would detect the possible expression fo the LHR *tmr* region. The expression of the WHR *tmr* gene in the LHR: :tmr tumor line was used as a control. Although no RNA homologus to this probe was detected in the LHR tumor line (lane A, Fig. 3), a 1.2 kb transcript was detected in the LHR: :tmr tumor line (lane B, Fig. 3). A 1.2 kb transcript was also seen in the WHR tumor line (lane C, Fig. 3) and corresponds to the previously reported size for the *tmr* transcript (Lichtenstein et al. 1984). These data indicate that the LHR *tmr* region is not significantly expressed in *N. rustica* tumors. Furthermore, the expression fo the WHR *tmr* gene in the LHR: :tmr tumor line can be correlated with a corresponding alteration in tissue morphology (see above). Although we did not detect expression of the LHR *tmr* region in transformed *N. rustica* tissue, this region may be expressed in other plant hosts.

Role of LHR tms loci in host range

We used the binary vector system to determine whether or not the LHR *tms* loci contribute to the narrow host range conferred by the LHR plasmid. pEND4K:LHR-tms contains the LHR *tms* loci inserted into the plant transformation vector pEND4K (Klee et al. 1985). This plasmid was mobilized into the avirulent strain, LBA4404 (Ooms et al. 1982), which harbors a WHR Ti-plasmid containing the *vir* region but none of the T-DNA region. The resulting strain, LBA4404 (pEND4K:LHR-tms) induced unorganized tumors on *N. glauca*, and root-proliferating tumors

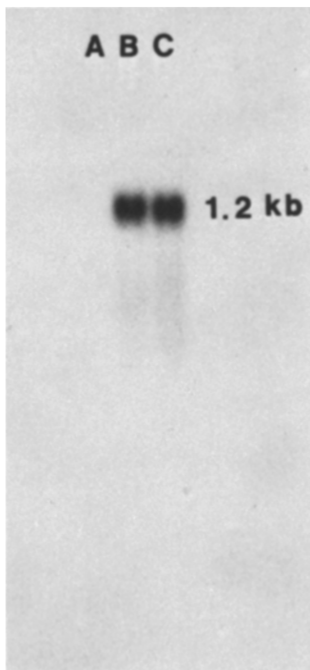


Fig. 3A–C. Northern blot analysis fo the LHR "tmr" region. Northern blots were hybridized with nick translated probe purified from an acrylamide gel. This probe consisted of a 137 bp *Eco*RV–*Eco*RI fragment (nucleotides 673–810 of LHR sequence, Fig. 2), derived from the LHR plasmid region which shares homology to the WHR *tmr* gene. Tumor lines were derived from strains carrying the following plasmids: LHR (**A**); LHR: :tmr (**B**); and WHR (**C**)

on both *N. rustica* and on *Kalanchoë* stems (Klee et al. 1985). Furthermore, LBA4404(pEND4K: LHR-tms) was also capable of complementing *tms* mutants of the WHR plasmid on a wide range of plants, including *N. tabacum* var. *xanthi*, tomato, carrot slices, and on *Kalanchoë* leaves and stems (data not shown). Because the LHR *tms* loci functioned on plants in which the LHR strain is avirulent, these loci are probably not responsible for the narrow host range phenotype of the LHR plasmid.

Role of Vir loci in host range

Other factors must also contribute to host range since the introduction of the WHR *tmr* gene into the LHR T-DNA was unable to fully restore a WHR phenotype. The *vir* genes, which are involved in the early stages of tumorigenesis, are likely candidates for contributing to host specificity. Cosmid clones from the WHR *vir* region (Knauf and Nester 1982) were mobilized into the LHR strain and assayed for their ability to allow tumor formation on *Kalanchoë* and petunia, two plants which were not susceptible to tumor formation with the LHR: :tmr strain (Fig. 4 and Table 3). pVCK257 contains the *vir*A, *vir*B, *vir*G, and *vir*C loci, and when present in the LHR strain, expanded host range to include these plants. pVCK225, which contains the WHR *vir*C, *vir*D, and *vir*E loci, was unable to expand host range. These data suggest that one or more of the *vir* loci present on pVCK257 is needed to expand the host range of the LHR strain, and that the *virD* and *virE* loci are not needed.

Additional experiments were performed in order to specifically identify which loci were needed to expand host range. Derivatives of pVCK257 which carried transposon insertions into the *vir*A and *vir*B loci were assayed for host range expansion (Fig. 4). The insertion into the *vir*A locus (pSM409) eliminated the expansion of host range, while the insertion into the *vir*B locus (pSM402) had no effect on the expanded host range. Therefore, the WHR *vir*A locus and not *vir*B was necessary to expand host range. Since no *vir*G or *vir*C mutants were available on pVCK257, we determined whether any cosmid clone from the LHR plasmid was capable of complementing *vir*G (363MX) and *vir*C (364MX, 365MX) mutations in a WHR strain. A LHR cosmid clone was found that complemented WHR *vir*G mutants on *Kalanchoë*. Therefore, this locus probably does not contribute significantly to host specificity. A clone of the LHR Ti plasmid capable of complementing WHR *vir*C mutations was not obtained. One or more of the other *vir* loci present on pVCK257, in addition to the *vir*A locus, is needed to expand host range since a clone containing the *vir*A locus alone (pVCK419) was not sufficient to allow tumor formation (Table 3) (delayed tumors did form on petunia after six weeks). Since *vir*C is the only remaining *vir* locus on pVCK257, by a process of elimination, the data strongly suggest that this locus is also needed to expand host range to *Kalanchoë* and petunia. Figure 5 shows an example of the expansion of host range observed on Petunia when various clones from the WHR *vir* region were introduced into the LHR strain. It can be seen that host range is expanded when *vir*A and *vir*C, carried on the cosmid pVCK257, are introduced into the LHR strain. Tumorigenesis is only weakly evident when *vir*A alone, carried on cosmid pVCK219, is introduced into the LHR strain.

Although the WHR *vir*A and *vir*C loci expanded the host range of the LHR strain to include pentunia and *Kalanchoë*, they allowed tumor formation only on *Kalanchöe* stems, but not *Kalanchoë* leaves. This lack of virulence on leaves is presumably due to the lack of a functional *tmr* locus since *tmr* mutations in the WHR plasmid also resulted in avirulence on *Kalanchoë* leaves. We have found that the WHR *tmr* locus as well as the WHR *vir*A and *vir*C loci, must be present within the LHR strain for tumor formation on *Kalanchoë* leaves (Table 3).

Mutations in the WHR *vir*A locus caused avirulence on *N. glauca* and *N. rustica* (Table 3), suggesting that the LHR plasmid, which confers virulence on these plants, must also contain a functional equivalent of the *vir*A locus. In order to identify such a locus, cosmid clones encompassing the entire LHR plasmid were mobilized into a *vir*A mutant of the WHR plasmid (strain 226MX). Two overlapping cosmid clones, pVCK301 and pVCK305, restored virulence on *N. glauca* and *N. rustica*. These clones were, however, unable to complement WHR *vir*A mutants on *Kalanchoë* leaves or stems (Table 3). This indicates that the LHR plasmid contains a functional equivalent to the WHR *vir*A locus, but its activity is inadequate for tumorigenesis on certain plants. As mentioned previusly, no cosmid clone of the LHR plasmid was able to complement a *vir*C mutation of the WHR plasmid.

Homology of LHR plasmid to probes specific for WHR vir loci

To further characterize the LHR *vir* loci, probes for each of the WHR *vir* loci were used in Southern blot hybridizations in order to identify and map homologous regions on

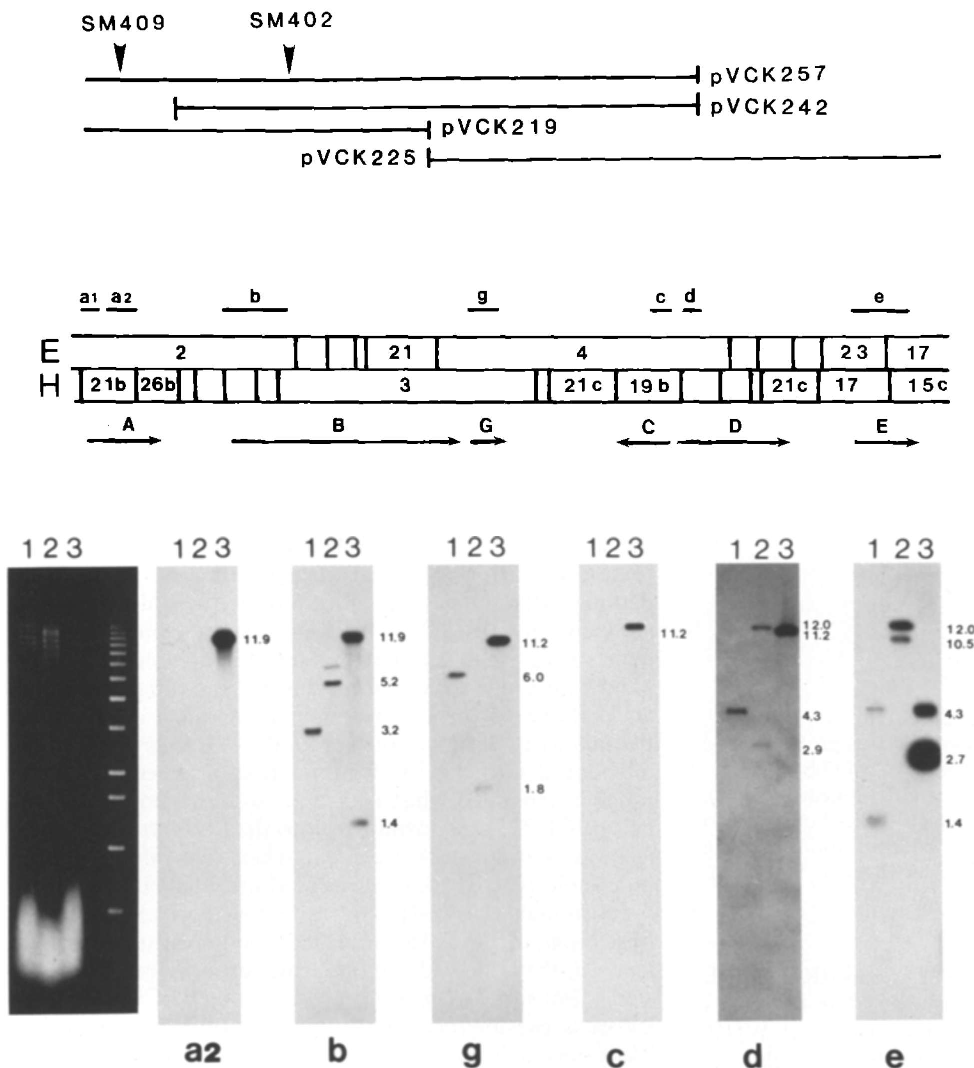


Fig. 4a–e. Southern blot analysis of the LHR *vir* region. Cosmid clones (Knauf and Nester 1982) are designated from the WHR *vir* region. SM402 and SM409 are transposon insertions into the cosmid clone pVCK257. *Eco*RI (E) and *Hin*dIII (H) restriction maps of this region are also given. The genetic (Klee et al. 1982; Klee et al. 1983) and transcriptional (Stachel et al., in preparation) organization of the WHR *vir* region is also shown. Total *A. tumefaciens* DNA was prepared and hybridized, under conditions of low stringency, as described previously (Thomashow et al. 1981). Lanes 1 and 2 represent *Eco*RI and *Sal*I digests respectively of DNA isolated from the LHR strain, and lane 3 represents *Eco*RI digested DNA from the WHR strain. An ethidium bromide stained gel, representative of the gels used in this analysis is shown adjacent to the autoradiographs of Southern blots. Probes, designated above the restriction map, were obtained from specific restriction fragments of the WHR *vir* region: *Hin*dIII-*Sst*I (**a1**); *Sst*I–*Sst*I (**a2**); *Sal*I–*Sal*I (**b**); *Bgl*II–*Pst*I, (**g**); *Bam*HI–*Cla*I (**c**); *Sma*I–*Bam*HI (**d**); *Sal*I–*Sal*I (**e**)

Table 3. The role of *vir*A and *vir*C in host range. Various clones from the WHR *vir* region (see Fig. 4) were assayed for their ability to expand the host range of the LHR strain. + = tumor formation; +/− = attenuated tumor formation; − = no tumor formation. 226MX and 365MX correspond to *vir*A and *vir*C mutants of the WHR plasmid respectively. pVCK305 is a cosmid clone from the LHR *vir* region. Further details are provided in the text

Plants	Strains								
	LHR	LHR (pVCK219)	LHR (pVCK225)	LHR (pVCK257)	LHR::tmr (pVCK257)	WHR	365MX	226MX	226MS (pVCK305)
N. glauca	+	+	+	+	+	+	+	−	+
N. rustica	+ [a]	+ [a]	+ [a]	+ [a]	+	+	+	−	+
Kalanchoë stems	−	−	−	+ [a]	+	+	+	−	−
Kalanchoë leaves	−	−	−	−	+	+	+ [b]	−	−
Petunia	−	+/−	−	+ [a]	+	+	+/−	−	N.D.

[a] Roots from tumor
[b] Very attenuated

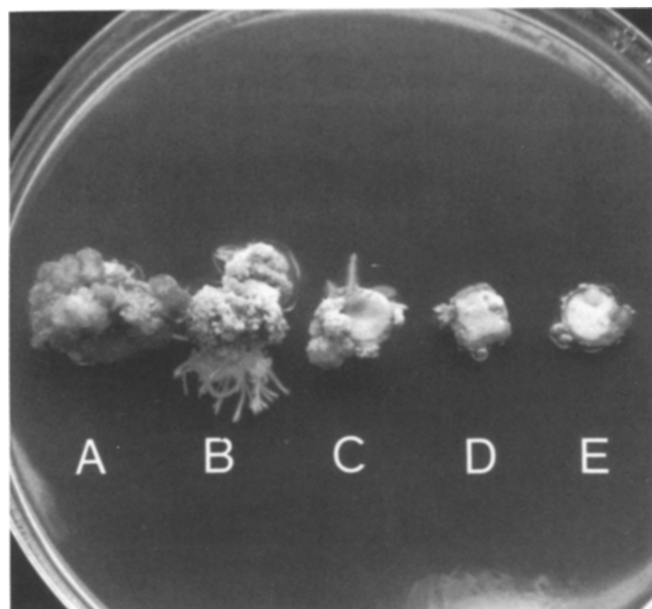


Fig. 5A–E. Petunia leaf disc assay. Leaf discs were prepared (Horsch et al. 1985) and inoculated with the following strains: WHR (**A**); LHR(pVCK257) (**B**); LHR(pVCK219) (**C**); LHR(pVCK225) (**D**); and the LHR strain (**E**). After inoculation, leaf discs were transferred onto MS$^-$ medium (Murashige and Skoog 1962) to select for phytohormone autonomous callus growth. Carbenicillin (500 µg/ml) was also added to inhibit bacterial growth. Photographs were taken six weeks after inoculation

the LHR plasmid. Hybridization was observed at high stringency (approx. T_m, $-17°$ C) to probes specific for the WHR *vir*B, *vir*G, *vir*D, and *vir*E loci (Fig. 4), suggesting that these loci are highly conserved on the LHR plasmid. In contrast, no significant hybridization was detected when probes specific for either the WHR *vir*A or *vir*C loci were used, even at low stringency (approx. T_m, $-42°$ C) (Fig. 4). These data further suggest that homologous counterparts to the WHR *vir*A and *vir*C loci are either not present on the LHR plasmid, or have diverged significantly at the nucleotide sequence level.

Table 4. Analysis of hypersensitive response on *Vitis labruscana* cv. Steuben. The relevent genotypes of various strains are shown and are discussed in detail in Results. The presence or absence of the hypersensitive response is indicated as a " + " or " – " respectively. Similarly, tumor formation was scored as " + " and a lack of tumor formation as " – "

Strains	Hypersensitive response	Tumor formation
WHR strain	+	–
LHR strain	–	+
LHR *vir* and WHR T-DNA	–	+
WHR *vir* and no T-DNA	–	–
WHR *vir* and LHR T-DNA	–	+
WHR *vir* and WHR T-DNA	+	–
WHR *vir*C mutant strain	–	+

Role of virC locus inhypersensitive response

It has previously been reported that the WHR strain is unable to induce tumors on certain cultivars of *Vitis* (i.e., *Vitis labruscana* cv. Steuben) on which the LHR strain is tumorigenic. Avirulence of the WHR strain is apparently due to a hypersensitive response (Lowe 1985; Yanofsky et al. 1985) in which the plant cells at the wound site turn dark brown and die (Fig. 6C). We investigated the role of the WHR T-DNA and *vir* regions in inducing this hypersensitive response (Table 4). We found that an avirulent strain containing the WHR *vir* region (LBA4404) (Ooms et al. 1981), but lacking the T-DNA sequences, did not induce this hypersensitive response (Fig. 6A). Also, the WHR T-DNA, introduced into the LHR strain on the plasmid pVCK232 (Knauf et al. 1982a), did not elicit the response. Additionally, when cosmid clones covering either LHR T-DNA region (pVCK311 and pVCK313) (Knauf et al. 1984) were present in LBA4404, the hypersensitive response was not observed. However, when the cosmid clone carrying the WHR T-DNA (pVCK232) was present in the strain carrying the WHR *vir* region (LBA4404), a hypersensitive response was observed (Fig. 6B). Thus, only when the WHR *vir* and T-DNA regions were both present within the same cell was the hypersensitive response elicited. Be-

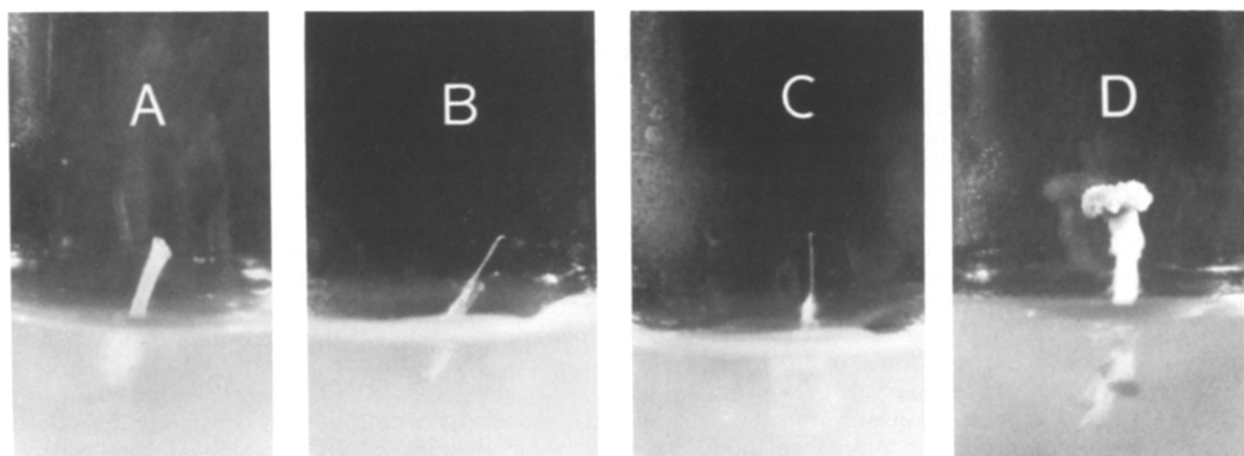


Fig. 6A–D. Analysis of hypersensitive response. Stem cultures of *Vitis labruscana* cv. Steuben were inoculated with LBA4404 (**A**); LBA4404(pVCK232) (**B**); and the WHR strain (**C**). For comparison, the WHR strain was inoculated onto *Vitis sp.* cv. Seyval (**D**). The avirulent response in figure A is clearly distinguished from the hypersensitive response (**B** and **C**) where there is a necrosis at the wound site and the stem turns dark brown and narrows

cause the LHR plasmid does not appear to contain a functional equivalent to the WHR *vir*C locus, we determined whether or not this locus played a role in the induction of the hypersensitive response. Two *vir*C mutants of the WHR strain, 364MX and 365MX, were tested for their influence on tumor formation on *Vitis labruscana* cv. Steuben. Both of these mutations prevented the hypersensitive response. Furthermore, whereas the WHR strain never induced tumors on *V. labruscana* cv. Steuben, the plants inoculated with WHR *vir*C mutants gave rise to tumors that could be cultured in the absence of phytohormones.

Discussion

The host range of any pathogen reflects a complex interaction between the invading pathogen and the host plant. *Agrobacterium* is the most extensively characterized plant pathogen at the molecular level and is thus an attractive organism for studying the factors which contribute to host range. Previous work demonstrated that the Ti plasmid is primarily responsible for host range differences in *Agrobacterium* (Loper and Kado 1979; Thomashow et al. 1980a). We have studied a limited host range (LHR) Ti plasmid of *Agrobacterium* in order to compare this plasmid with the well characterized wide host range (WHR) Ti plasmids. We have found that determinants within the T-DNA and *vir* regions contribute to the natural host range variation among *Agrobacterium* strains. This is consistent with the results of other researchers who have shown that mutations in the WHR Ti plasmid can alter host range (Inzé et al. 1984; Klee et al. 1985; Lundquist et al. 1984; Hooykaas et al. 1984).

Host specificity is intimately tied to the physiology of the particular host plant as well as the invading pathogen. Some plants require the introduction of both the auxin (*tms*) and cytokinin (*tmr*) biosynthetic loci for tumor formation, and other plants require only one of these loci (Inzé et al. 1984; Klee et al. 1985). Therefore, the Ti plasmid encoded oncogenes can play an important role in host specificity, depending on the oncogene requirements of the host. Wide host range strains of *Agrobacterium* are capable of inducing tumors on a wide variety of dicotyledonous plants. The effectiveness of the WHR Ti plasmid appears to be due, in part, to the transmission of both auxin and cytokinin biosynthetic loci into plant cells. The limited host range (LHR) Ti plasmid, pTiAg162, is responsible for the formation of tumors on a very narrow range of host plants. Our results and those of others suggest that the LHR Ti plasmid contains a defective T-DNA oncogene complement compared to the WHR plasmid. Specifically, the LHR T-DNA appears to possess a weak or inactive cytokinin biosynthetic gene(s). Nucleotide sequence analysis has shown that only the 3′ end of the WHR *tmr* gene is conserved on the LHR plasmid and indicates that a deletion has removed the entire 5′ end of this gene. Northern blot hybridization experiments demonstrated that expression of the LHR *tmr* region is not detectible in *N. rustica* tissue transformed by the LHR strain, further indicating that the gene is inactive.

The WHR *tmr* gene is capable of greatly expanding the range of hosts which are susceptible to tumor formation by the LHR strain (this study; Buchholz and Thomashow 1984b; Hoekema et al. 1984). Furthermore, the WHR *tmr* gene expanded host range to the same extent as did the entire WHR T-DNA for the plant hosts tested. In addition, the LHR *tms* loci are functional on a wide range of host plants, indicating that these loci do not contribute significantly to the narrow host range phenotype conferred by the LHR plasmid. We conclude that, within the T-DNA, it is the lack of an effective *tmr* locus which is primarily responsible for the narrow host range phenotype of the LHR plasmid. This suggests that plants which are susceptible to tumor formation by the LHR strain can complement the apparrent lack of a cytokinin biosynthetic gene on the LHR plasmid. Perhaps the endogenous cytokinin levels in these plants is sufficient to complement this deficiency.

The WHR *tmr* gene (or the entire WHR T-DNA) was unable to fully restore a WHR phenotype as evidenced by its inability to allow tumor formation on plants such as *Kalanchoë*. We suspected that the LHR strain was blocked at a stage in transformation prior to the expression of the T-DNA genes. This suggested that the *vir* region may also play an important role in determining host specificity. A clone containing the WHR *vir*A and *vir*C loci, when present in the LHR strain, allowed tumor formation on *Kalanchoë*. Neither *vir*A or *vir*C alone expanded host range, suggesting that both of these loci must be present in the LHR strain to allow tumor formation on *Kalanchoë*. Furthermore, the LHR plasmid shares homology to the WHR *vir*B, *vir*G, *vir*D, and *vir*E loci, but even at low stringency, no hybridization was observed for probes specific for the WHR *vir*A and *vir*C loci. Thus, within the *vir* region it appears that the *vir*A and *vir*C loci limit the overall host range of the LHR plasmid.

Mutations in the WHR *vir*A locus resulted in avirulence on *N. glauca* and *N. rustica,* suggesting that the LHR plasmid, which confers virulence on these plants, must contain the functional equivalent of the *vir*A locus. A region of the LHR plasmid, capable of complementing *vir*A lesions of the WHR plasmid on *N. glauca* and *N. rustica,* maps immediately to the left of the other LHR *vir* loci (Knauf et al. 1984). However, the LHR *vir*A locus is unable to complement WHR *vir*A mutants on *Kalanchoë*, and apparently only functions on a limited number of host plants. Perhaps its activity or expression is decreased in comparison to its WHR counterpart. A decrease in function could cause a decrease in T-DNA transfer efficiency and hence limit host range. We are now determining whether the LHR *vir*A counterpart has simply diverged to the point where hybridization can no longer be detected, or if this represents an entirely different gene with a similar function. THe *vir*C mutants of the WHR strains still incited tumors on all plants which are susceptible to tumor induction by the LHR strain (i.e., *Vitis* sp. cv. Seyval, *N. glauca,* and *N. rustica*). There does not appear to be an equivalent *vir*C locus on the LHR plasmid although there may be a gene with a related function.

Interestingly, the WHR strain is avirulent on some cultivars of *Vitis*. Avirulence apparently results from a hypersensitive response, which indcued in the host, kills the plant cells (and/or the bacteria) at the site of inoculation (Lowe 1985; Yanofsky et al. 1985). In contrast to the WHR strain, the hypersensitive response has never been observed for the LHR strain. An avirulent strain containing the WHR *vir* region, but lacking T-DNA sequences, did not induce the hypersensitive response, and the response was not observed when the WHR T-DNA was present in the LHR strain. The T-DNA and *vir* regions of the WHR plasmid must both be present in the same strain to induce a hyper-

sensitive response. Specifically, the WHR *vir*C locus, in the presence of the WHR T-DNA, was involved in the induction of the hypersensitive response since mutations in the WHR *vir*C locus prevented the hypersensitive response and allowed tumor formation. Therefore, the hypersensitive response does appear to be the cause of avirulence on these plants. This locus is also similar in certain aspects to the dominant avirulence gene recently identified by Staskawicz et al. (1984) from *Pseudomonas syringae* pv. glycinea. However, the *vir*C locus must act in concert with the T-DNA because the WHR *vir* region alone did not induce hypersensitivity. We are currently determining which factor(s) within the T-DNA region is involved in eliciting the hypersensitive response.

One model consistent with the above observations is that the *vir*C locus acts to increase the frequency of T-DNA transfer into the plant genome. The WHR strain may induce the hypersensitive response on certain hosts because too many cells receive T-DNA sequences, perhaps leading to the overproduction of phytohormones. There does not appear to be a strict requirement for the *vir*C locus as evidenced by the fact that *vir*C is only required for efficient tumor formation on certain host plants. Furthermore, on plants in which *virC* mutant strains induce tumors, the tumor response is delayed. If the *vir*C locus does act to increase the frequency of T-DNA transfer then this would also be consistent with the host range properties of this locus. Certain susceptible host plants (i.e., *N. glauca, Vitis*) may require the incorporation of T-DNA sequences into only a few cells at the site of inoculation to induce rapid tumor proliferation, thereby obviating the need for the *vir*C locus. Other plants (i.e. *Kalanchoë*), however, may require the incorporation of T-DNA sequences into many cells at the wound site in order for tumor symptoms to result. These observations are consistent with *vir*C mutants leading to the incorporation on T-DNA sequences into fewer cells at the site of inoculation. Further studies are needed to determine if *vir*C does in fact act to increase the frequency of T-DNA transfer, and if so, at which step in the T-DNA transfer process does *vir*C function.

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