VirA, the plant-signal receptor, is responsible for the Ti plasmid-specific transfer of DNA to maize by Agrobacterium

(agtroinfection/maize streak virus/virA gene)

D. M. Raineri*, M. I. Boulton†, J. W. Davies‡, and E. W. Nester*

*Department of Microbiology, University of Washington, Seattle, WA 98195; and †John Innes Institute, Institute of Plant Science Research, Norwich NR4 7EH, United Kingdom

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ABSTRACT Agrobacteria exhibit marked Ti (tumor-inducing)/Ri (root-inducing) plasmid specificity in their interaction with the Gramineae. In this study, we have used the technique of "agroinfection," in which Agrobacterium-mediated delivery of viral genomes into plants is detected by the development of viral disease symptoms, to identify the region of the Ti plasmid which is responsible for the major differences seen in the ability of nopaline- vs. octopine-type Ti plasmids to transfer maize streak virus (MSV) DNA to maize. Introduction of fragments of the C58 (nopaline-type) Ti plasmid into strains containing an octopine-type Ti plasmid showed that a fragment containing the nopaline-type virA locus was able to complement these normally non-agroinfectious strains to high levels of MSV DNA transfer. Octopine-type virA mutant strains that express vir genes at high levels in the absence of the plant inducing compound acetosyringone also efficiently transferred MSV DNA. These findings imply a functional difference between the virA gene products encoded by octopine- and nopaline-type Ti plasmids which has a profound effect on their ability to mediate DNA transfer to maize.

The Ti plasmids in Agrobacterium tumefaciens strains and the Ri plasmids in Agrobacterium rhizogenes strains cause crown gall tumor and hairy root diseases, respectively. Two regions of the Ti/Ri plasmid are required for pathogenesis: the T-DNA, which is transferred to and stably integrated in the nuclei of transformed plant cells, and the vir region, which encodes the enzymes and structures required for processing, transfer, and integration of the T-DNA. The integrated T-DNA directs the production and metabolism of plant growth hormones as well as classes of compounds termed opines. Most Ti plasmids are classified as octopine- or nopaline-type and most Ri plasmids as agropine-, mannopine-, or cuciumpine-type based on their ability to encode the synthesis and catabolism of these respective opines. Unlike T-DNA, the vir region is not transferred to plant cells but is essential for T-DNA transfer. Six operons (virA, -B, -C, -D, -E, and -F) have been characterized in detail and three other strain-specific regions (virH, virF, and C1s) are less well characterized (for reviews, see refs. 1–3).

The host range of Agrobacterium, as judged by tumor formation, was thought to be limited almost exclusively to dicotyledonous (dicot) plants (4, 5). However, more recent work reported that certain monocotyledonous plants (monocots) are also susceptible (6–9). Furthermore, although maize and other graminaceous monocots have remained refractory to tumor formation, the agroinfection technique used to study the transfer of viral DNA to dicots (10, 11) has confirmed that Agrobacterium is able to transfer DNA to cereal plants (12–17). Agroinfection is mediated through the same vir gene pathway as is required for tumor formation in dicots (18).

This technique provides a very sensitive assay for Agrobacterium-mediated DNA transfer, as the viral sequences escape from the T-DNA and are amplified as the virus replicates and spreads systemically. DNA transfer is thus detected by the formation of disease symptoms.

The ability to agroinfect maize was found to be Ti plasmid-specific (13, 16): octopine-type strains either were unable, or only weakly able (<5%), to transfer maize streak virus (MSV) DNA, while nopaline-type strains infected 80–100% of the inoculated plants. Agropine- and mannopine-type strains of A. rhizogenes also efficiently agroinfect maize. With appropriate viral DNAs as markers, similar results were obtained for other members of the Gramineae (14, 15, 17). In contrast, Ti plasmid specificity is not typical of agroinfection–dicit interactions (10, 11).

A "disarmed" (T-DNA-less) nopaline strain agroinfect maize as efficiently as the wild type (13). Therefore, Ti plasmid specificity cannot be due to differences in phytohormone production specified by the T-DNA. One explanation for the inability of octopine-type strains to agroinfect maize and other graminaceous monocots is that octopine-type Ti plasmids lack an essential function present in nopaline-, agropine-, and mannopine-type Ti/Ri plasmids. Alternatively, octopine-type Ti plasmids might express a counteractive function.

We have used the agroinfection system to characterize the Ti plasmid requirements for transfer of MSV DNA to maize. We observed that a DNA fragment containing the nopaline-type virA locus complemented octopine-type Ti plasmids to high levels of agroinfection. Furthermore, whereas preinduction of octopine-type strains does not promote maize agroinfection, octopine-type virA mutant strains, which express vir genes at high levels in the absence of the inducing compound acetosyringone, efficiently transferred MSV DNA to maize. We discuss here the implications of these observations and possible causes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. The MSV-containing binary vectors and other plasmids used in this study are described in Table 1. Agrobacterium strains are listed in the relevant tables or are described in the text. Escherichia coli was grown at 37°C in Luria broth (LB). Carbenicillin, spectinomycin, and kanamycin were used at 100 μg/mL; gentamicin and tetracycline were used at 10 μg/mL. For selective growth of Agrobacterium strains, the concentrations of gentamicin and tetracycline were modified to 100 μg/mL and 5 μg/mL, respectively. Agrobacterium strains were grown at 28°C in MG/L (27).

Plasmids were introduced into Agrobacterium by tripertontal matings (28), using pRK2073 or pRK2013 as helper plasmid, or by electroporation (29).

Abbreviations: MSV, maize streak virus; cfu, colony-forming units.
Plasmid Constructions. The MSV vector pMSV-Ns-CGN, which contains the origin of replication of the A. rhizogenes plasmid pRIH1, was constructed by inserting a 5.4-kb tandem dimeric copy of MSV isolated from pMSV-Ns-ICR into the EcoRI site of the binary plasmid pCGN1557. The pMSV-Ns-CGN vector was used in combination with the IncP-derived recombinant plasmids (Table 1), which were incompatible with the pMSV-Ns vectors used in previous studies.

The spectinomycin resistance-encoding vector pMSV-Ns(S) contained the EcoRI–XhoI dimeric fragment of MSV, isolated from pMSV-Ns, inserted into the multiple cloning site of pBIN400 (provided by M. Bevan, John Innes Institute, Norwich, U.K.). A gentamicin resistance-encoding vector, pMSV-Ns(G), was generated by inserting the EcoRI MSV fragment from pMSV-Ns-ICR into the single EcoRI site in the T-DNA border fragment of pDR329 (D.M.R., unpublished data), a gentamicin resistance-encoding binary vector derived from pSP329 (29). These IncP-derived, MSV-containing vectors were used in complementation studies in combination with the IncW plasmid pUCD2 or with the derivative plasmid pDM2D, carrying either Ri or Ti fragments of interest. Plasmid pDM2D was generated by deleting the SacII–KpnI I fragment of pUCD2, creating single restriction sites for EcoRI and HindIII. Comparative data were based on studies using identical combinations of recombinant plasmid and MSV vector. Where this was not possible, the efficiency of agroinfection was assessed relative to the nopaline-type strain C58, which was included in all inoculations as a positive control.

Plasmid pDR178 contains a fusion of the octopine-type virA gene from pTIa6 with the pTIC58 virA promoter. A 4.8-kb BamHI–XhoI fragment containing the pTIC58 virA gene was ligated to BamHI/SalI cut pTZ19R (carbendazim resistance; United States Biochemical) to give pDR169. The pTIC58 virA promoter was excised by Xmn I digestion of pDR169 and inserted in front of a promoterless pTIa6 virA gene generated by Xmn I digestion of pSW196. Digestion of the resulting plasmid, pDR172, with Kpn I and Hpa I released a 3.8-kb pTIC58 virA promoter–pTIa6 virA fusion fragment for insertion into Kpn I/Pvu II-cut pUCD2.

RESULTS

All Ti/Ri Plasmid Requirements for Maize Agroinfection Reside Within the pTIC58/pRIa4 vir Regulons. To determine whether portions of the nopaline-type Ti plasmid pTIC58 and the agropine-type Ri plasmid pRIa4 could complement an octopine-type Ti plasmid for maize agroinfection, large regions of these plasmids were mobilized into strain A. tumefaciens C58 chromosome; pTIa6). In agroinfection, clones of the entire pRIa4 and pTIC58 vir regulons were mobilized into strain LA4001 (30) or the LA4011-derived rec-deficient mutant LA4301 (31). Ti plasmid-less derivatives of the octopine-type strain Ach5 (Ach5, octopine-type, chromosome). Finally, the MSV binary vector was inserted into each of the recombinant plasmid-containing strains.

Clone pFW32, containing the entire pRIa4 vir regulon, promoted MSV DNA transfer from LA4001 into 38% of the inoculated plants. Strain LA4301 containing a clone of the pTIC58 vir regulon, pUCD2614 (Fig. 1), mediated disease symptoms in 83% of the inoculated plants, similar to the 76–89% range of infectivity obtained when pUCD2614 was introduced into the cured nopaline-type strain A136 (Table 2). Clones of non-vir regions of the Ti and Ri plasmids did not promote MSV DNA transfer.

Subcloning of the vir regions showed that BamHI fragment 2 from pTIC58 promoted maize agroinfection when introduced into A348 and the octopine-type virA mutant strain MX226 (34). Infectivities were consistently higher for the complemented virA mutant [MX226(pDR106B)] than for the parental strain [A348(pDR106B)] (Table 2). The equivalent region of pRIa4 (subclone pDR237B) promoted MSV DNA transfer from A348 into 62% of the inoculated plants, whereas other regions of the Ti and vir regions failed to promote maize agroinfection (data not shown).
subclones encompassed a common region that included the
virA and tzs loci as well as a flanking, non-vir region of ~5 kb.

Efficiencies of MSV Infection Mediated by pTiC58 and the
Complemented Octopine-Type Ti Plasmids Are Similarly Affected
by Inoculum Dilution, but Those Mediated by a virF
Mutant Are Not. Symptom formation mediated by the
complemented octopine-type Ti plasmids was not markedly
delayed, suggesting that MSV DNA transfer from these strains
was efficient. Titration of C58(pMSV-Ns) showed that 100% agroinfection could be obtained with as few as 10^3 cfu/ml, or
2 x 10^5 cfu per plant (13). The gradual decline in MSV
infectivity seen with lower cell concentrations of the comple-
tmented A348 strain closely paralleled that obtained with C58,
both strains being capable of efficient transfer at a 1:10,000
dilution of the normal inoculum concentration (Fig. 2).

Jarchow et al. (16) proposed that the limitation to maize
groinfection by octopine-type strains lies in the octopine-
type Ti plasmid−specific virF locus (35). We also have ob-
erved that A348−derived virF mutants promote maize
groinfection (36). However, compared with C58, MSV
infectivities of the virF mutants were low (only 0−15% greater
than those mediated by strain A348), streaking was less severe,
and symptom appearance was significantly delayed. We con-
cluded that virF plays some minor role but is not the major
contributor to the limitation of the octopine-type Ti

plasmids (36). To address this discrepancy, inoculum titra-
tion studies were used to quantify the agroinfection effi-
ciency of the octopine-type virF mutant LBA1517 used by
Jarchow et al. (16).

A 100-fold dilution of the LBA1517 inoculum completely
abolished infectivity (Fig. 2), whereas 22 of the 23 C58−
inoculated plants showed symptoms at this dilution.

Insertion of a Fragment Containing the Nopaline-Type virA
Locus into A348 Promotes Maize Agroinfection. To localize
the complementing activity, subclones of BamHI fragment 2
from pTiC58 were generated in the IncW plasmid pUCD2 or
pDMD2 (Table 1).

Digestion of BamHI fragment 2 with HindIII generated a
5.6-kb BamHI−HindIII fragment encompassing the virA and
tzs loci (subclone pDRB56, Fig. 1) and a 5.0-kb HindIII
fragment encompassing the non−vir region. pDRB56 pro-
moted DNA transfer from A348 with MSV infectivities of
38−68%, compared with 40−60% for clone pDR106 (Table 2),
which contains BamHI fragment 2 in the same IncW plasmid
(Fig. 1). Further subcloning localized the complementing
activity to a 3.1-kb Nde I fragment containing the pTiC58 virA
locus (subclone pDRB31; Fig. 1 and Table 2). Other regions of
the 5.6-kb vir fragment failed to promote DNA transfer
(data not shown).

In contrast, pUCD2 containing the octopine-type virA
locus alone (pTB108) or in combination with the octopine-
type virG locus (pIB100) resulted in only slight enhancements
in MSV DNA transfer when introduced into A348 and the
octopine-type virA mutant strain A1030 (Table 3). In both
cases, symptom formation was delayed by up to 12 days
and streaking was initially much less severe than that observed
with C58 or the complemented A348 strains.

To investigate the possibility that differences in the virA
promoter region might be responsible for specificity in maize
groinfection, the pTiC58 virA promoter was cloned in front of

a promoterless pTiA6 virA gene (pDR178) and introduced
into A348 and octopine-type virA mutant backgrounds. The
resulting strains were not complemented for MSV DNA
transfer to maize (Table 3) but were competent for tumor
formation on dicots (data not shown), indicating that func-
tional VirA was being expressed from the pTiC58−pTiA6
virA fusion.

Table 2. Complementation studies

<table>
<thead>
<tr>
<th>Complementing plasmid</th>
<th>Strain</th>
<th>Chromosome/Ti plasmid</th>
<th>Opine family</th>
<th>No. of plants inoculated</th>
<th>Infectivity, %</th>
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</thead>
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<td>C58/−</td>
<td>(nop)</td>
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<td>Nopaline pTiC58 BamHI fragment 2 and subclones</td>
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<td>oct</td>
<td>67</td>
<td>0−3</td>
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<td>111</td>
<td>40−60</td>
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<td>oct</td>
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<td>38−68</td>
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<td>pDRB31</td>
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<td>C58/pTiBb806::virATn5</td>
<td>oct</td>
<td>66</td>
<td>53−73</td>
</tr>
</tbody>
</table>

In addition to the plasmids listed, each strain carried one of the MSV binary vectors. Plasmids and vectors are described in Table 1. Specific infectivities represent data from a single experiment, ranges of infectivities represent data from a minimum of four independent experiments. Whenever possible, pTiA6-derived vir mutants were used for complementation studies. When this was not possible, vir mutants of pTiBa806, an octopine-type plasmid with >87% homology by hybridization analysis to pTiA6 (33), were used. agr, Agropine; oct, octopine; nop, nopaline. Where Ti plasmid-less strains were used the opine designation is given in parentheses.
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**Figure 2.** Effect of inoculum dilution on MSV DNA transfer efficiency from LBA1517 (●), CS8 (●), and A348(pDR106B) (▲). Percent infectivity equals the number of infected plants divided by the number of inoculated plants and multiplied by 100.

**Octopine-Type Acetosyringone-Independent Signaling (Ais)**

virA Mutants Promote Maize Agroinfection at High Frequency.

The octopine-type virA(Ais) locus carried by pEB112 (25) mediates vir gene expression in the absence of inducing stimuli. Introduction of pEB112 into A348 and the octopine-type virA mutant A1030 resulted in MSV infectivities of 75–89% and 75%, respectively, in the absence of acetosyringone (Table 4). Two other virA(Ais) mutant plasmids, pEB129 and pEB137, were also tested. These mutants exhibit 2.5-fold (pEB137) and 7-fold (pEB129) lower levels of acetosyringone-independent vir gene expression than pEB112 (25) but still resulted in significant numbers of MSV-infected plants when introduced into strain A348 (Table 4).

**DISCUSSION**

*Agrobacterium* exhibits marked Ti/Ri plasmid specificity in its interaction with the Gramineae (13–17). In this study we have used the agroinfection technique to identify the region of the Ti plasmid which is responsible for the specificity in the agroinfection–maize interaction.

We identified a region of the nopaline- and agropine-type Ti/Ri plasmids which complemented octopine-type Ti plasmids for maize agroinfection. Since there is no inhibition of MSV DNA transfer from the nopaline-type strain CS8 by octopine-type Ti plasmid sequences (36), we concluded that nopaline- and agropine-type strains contain an essential function for maize agroinfection which is either lacking, nonfunctional, or specifically inhibited in octopine-type strains.

Subcloning of the complementing region of the nopaline-type Ti plasmid pTiC58 localized the complementing activity to the virA locus. By contrast, Jarchow et al. (16) found that the nontransferring octopine-type strain LBA4301(pTiA6) could be complemented with a plasmid containing all of the vir genes of pTiC58 except tiz, virA, and part of the virB operon. The IncW-derived plasmid used by those investigators, pTH60, is estimated to give four to five copies per Agrobacterium cell (37) whereas the Ti plasmid has a copy number of 1–2. Therefore, the differences in the data might be explained by a "multicopy effect" in which the need for virA-mediated induction is bypassed by increased expression of virG (37, 38).

An octopine-type virA gene under control of the nopaline-type virA promoter was not complemented for MSV DNA transfer to maize. This indicated that the VirA coding region accounts for the specificity in maize agroinfection, perhaps reflected in the greater similarity between nopaline- and agropine- than between nopaline- and octopine-type VirA proteins (90% and 73% amino acid identities, respectively; refs. 39 and 40).

Higher levels of maize agroinfection were mediated by the complemented octopine-type virA mutant than by the complemented wild-type strain. Based on recent findings which indicated that VirA is multimeric (Shen Pan, personal communication), the most likely explanation for the decreased MSV DNA transfer when octopine-type VirA was present is that heteromultimers of nopaline- and octopine-type VirA had intermediate phenotypes.

Octopine-type virA(Ais) mutants efficiently agroinfect maize. These findings support our contention that a function or property of the virA locus, which can be altered by a mutation conferring an acetosyringone-independent phenotype, is responsible for the octopine-type Ti plasmid-specific defect in agroinfection. The final level of vir gene expression mediated by the virA(Ais) mutants in the absence of acetosyringone is lower than that mediated by the wild-type virA at saturating levels of acetosyringone (25). The only apparent difference between the wild-type virA and the virA(Ais) mutants is that virA(Ais)-mediated vir gene expression occurs in the absence of acetosyringone, acidic conditions, and inducing monosaccharides. Therefore, the reason for the inability of octopine-type strains to agroinfect maize may be that octopine-type VirA activity is uninduced or inhibited by maize extracts.

Induction of octopine-type vir genes by extracts from wounded maize seedlings has been demonstrated (16, 18). However, those studies were done in vitro after pH adjustment of the extracts and so may not reflect the situation in planta. This is especially relevant since Turk et al. (41) have shown that octopine- and nopaline-type VirA proteins differ in their pH requirements for optimal vir induction. Thus, it may be that cereal sap is more compatible with the functioning of nopaline-type VirA than octopine-type VirA. An increase in copy number of virA and virG can partially relieve the pH dependence of vir gene induction (42), perhaps explaining the increased MSV infectivity mediated by octopine-type Ti plasmids when the octopine-type virA and virG were inserted into a higher-copy-number plasmid.

Boulton et al. (13) and Jarchow et al. (16) demonstrated that preinduction of octopine-type strains does not promote agroinfection, leading them to conclude that the limitation was not due to lack of vir gene induction. However, Alt-Moerbe et al. (43) demonstrated that removal of inducer leads to an immediate stop in the formation of vir products and

<table>
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<th>Complementing plasmid</th>
<th>Strain</th>
<th>Chromosome/Ti plasmid</th>
<th>Opine family</th>
<th>No. of plants inoculated</th>
<th>Infectivity, %</th>
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</table>

For a description of recombinant plasmids, see Table 1. For other details, see legend to Table 2.
T-DNA intermediates. In summary, these observations alone are not sufficient evidence that induction of octopine-type vir genes by maize occurs in planta. It is also possible that inhibitory substances, presumably specific to graminaceous plants, specifically block octopine-type VirA activity. The observation that octopine-type \( \text{vir}^A \text{Algo} \) mutants can promote agroinfection might be explained by the inability of maize inhibitors to block the signal-independent induction of vir gene expression by the mutant sensor proteins.

It has been suggested that the limitation to maize agroinfection by octopine-type Ti plasmids lies in \( \text{vir}F \) (16). Titration of inocula of strains containing octopine-type Ti plasmids and the complementing region from \( \text{pTiC58} \) gave an infectivity gradient analogous to that obtained with wild-type C58. In contrast, we rapidly reached an end-point dilution of the \( \text{vir}F \) mutant LBA1517 (35). Furthermore, VirD did not suppress agroinfection mediated either by nopaline-type Ti plasmids or by the complemented octopine-type Ti plasmids (16, 36). Thus, we concluded that \( \text{vir}F \) does not play the major role in determining the specificity of maize agroinfection as suggested by Jarchow et al. (16).

The simplest interpretation of the data presented above is that the block to maize agroinfection by octopine-type Ti plasmids involves a function or property of the \( \text{vir}A \) locus. VirA acts as an environmental sensor of plant-derived inducer molecules and transmits this information to the level of vir gene expression. We propose that slight variations at the \( \text{vir}A \) locus could have a profound effect on T-DNA transfer efficiency. An understanding of the differences between the octopine- and nopaline-type \( \text{vir}A \) gene products will provide important information on the requirements for efficient DNA transfer into maize. We assume that the observations made on agroinfection of maize will hold for agroinfection of graminaceous monocots in general.

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### Table 4. Transfer efficiencies of octopine-type \( \text{vir}A \) mutants exhibiting acetylsyringone-independent expression of the \( \text{vir} \) region

<table>
<thead>
<tr>
<th>Complementing plasmid</th>
<th>Strain</th>
<th>Chromosome/Ti plasmid</th>
<th>Opine family</th>
<th>No. of plants inoculated</th>
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For description of recombinant plasmids, see Table 1. For other details, see legend to Table 2.