Discrete Regions of the Sensor Protein VirA Determine the Strain-Specific Ability of *Agrobacterium* to Agroinfect Maize

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The ability of *Agrobacterium* strains to infect transformation-recalcitrant maize plants has been shown to be determined mainly by the *virA* locus, implicating *vir* gene induction as the major factor influencing maize infection. In this report, we further explore the roles of *vir* induction-associated bacterial factors in maize infection using the technique of agroinfection. The Ti plasmid and *virA* source are shown to be important in determining the ability of a strain to infect maize, and the monosaccharide binding protein ChvE is absolutely required for maize agroinfection. The linker domain of VirA_{C58} from an agroinfection-competent strain, C58, is sufficient to convert VirA_{A6} of a nonagroinfecting strain, A348, to agroinfection competence. The periplasmic domain of VirA_{C58} is also able to confer a moderate level of agroinfection competence to VirA_{A6}. In addition, the VirA_{A6} protein from A348 is agroinfection competent when removed from its cognate Ti plasmid background and placed in a pTiC58 background. The presence of a pTiA6-encoded, VirA_{A6}-specific inhibitor is hypothesized and examined.

Additional keywords: host range, plant transformation, two-component system.

Agrobacterium is the causal agent of the plant disease crown gall. The mechanism by which *Agrobacterium* causes tumors on plants has been studied and described in great detail (Hooykaas and Beijersbergen 1994; Kado 1991; Zambryski 1992). Briefly, in response to signal compounds present in a

plant wound site, the bacterium induces the expression of a set of virulence (*vir*) genes via the VirA/VirG two-component signal transduction system (Heath et al. 1995). *vir* gene products excise and transfer a piece of the bacterial Ti plasmid DNA (T-DNA) into a susceptible plant cell, where it is targeted to the nucleus and integrated into the plant genome. The T-DNA encodes genes that, when expressed in the plant cell, direct the synthesis of plant growth regulators, resulting in neoplastic growth of the infected cells. In addition, the T-DNA encodes genes for the synthesis of compounds, termed opines, which the infecting bacterium can utilize as carbon and nitrogen sources. This singular ability of *Agrobacterium* to transform a wide variety of plants with high efficiency has become a cornerstone of genetic manipulation of plants for both commercial and scientific purposes.

Major exceptions to the natural broad host range of *Agrobacterium* are the plants in the family Graminae (Chilton 1993). This group of monocotyledonous grasses, which includes the economically important crop plants maize, wheat, and rice, has proven to be difficult to routinely transform by *Agrobacterium*-mediated methods. Recently, however, rice and maize have been transformed by using superior binary vectors and cocultivation conditions that include *vir* gene inducers (Hiei et al. 1994; Ishida et al. 1996). In earlier attempts to identify the barrier to transformation of maize, investigators using the method of agroinfection (Grimsley et al. 1986, 1987) demonstrated that certain *Agrobacterium* strains could transfer T-DNA into maize plants, although tumors were not formed. Agroinfection is an extremely sensitive assay for T-DNA transfer. Any successful transfer event has the potential to be amplified exponentially by replication of the transferred viral genome resident on the T-DNA and systemic spread of the virus. Additionally, agroinfection measures T-DNA transfer independently of T-DNA integration or expression. Therefore, investigators are able to use agroinfection to study T-DNA transfer into the transformation-resistant members of Graminae.

Boulton et al. (1989) showed that the ability of certain *Agrobacterium* strains to agroinfect maize is determined by the opine-utilization profile of the bacterium, which is in turn determined by factors resident on the Ti plasmid. Specifically, most *Agrobacterium* strains that utilize octopine and cucumopine are unable to infect maize, while those that utilize

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nopaline, agropine, and mannopine can. That study was followed by studies that determined that *virA* from C58, an agroinfection-competent strain, was sufficient to confer agroinfection competence on a nonagroinfecting strain, A348 (Raineri et al. 1993). In addition, Hansen et al. (1994) demonstrated that constitutive expression of the *vir* genes was sufficient to convert A348 to agroinfection competence. These results emphasized the importance of *vir* gene induction in determining the ability of certain strains to infect maize.

The relationship between *vir* gene induction and host range has long been recognized. One example is the limited host range of *A. vitis* strain Ag162. The *vir* genes of this strain are not induced by acetosyringone, the prototypical *vir*-inducing phenolic compound, and this strain exhibits a very limited host range of plant cell transformation. Additionally, mutant derivatives of *Agrobacterium* that lack the *chvE* gene product, which is required for monosaccharide-enhanced induction of *vir* expression (Cangelosi et al. 1990), exhibit a reduced host range compared with wild-type strains (Garfinkel and Nester 1980; Huang et al. 1990). Recently, an *Agrobacterium* strain isolated from the soil of South Korea has been shown to possess a vastly different phenolic-recognition profile compared with the extensively studied strains C58 and A6 (Lee et al. 1995). This strain also has an altered host range with respect to C58 and A6, which correlates with its altered phenolic-recognition properties. In this study, we analyze the bacterial factors important in *vir* gene induction, focusing on the sensor protein VirA and exploring further its role in maize agroinfection.

RESULTS AND DISCUSSION

VirA_{A6} is competent for maize agroinfection in a pTiC58 background but not in a pTiA6 background.

It has been shown that the strain-specific ability of *Agrobacterium* to infect maize is largely determined by the *virA* locus (Raineri et al. 1993). A clone harboring *virA* from the nopaline-type Ti plasmid pTiC58 was shown to restore agroinfection ability to the octopine-type strain A348 harboring a *virA* mutation (Raineri et al. 1993). Subsequently, we made the unexpected observation that a *virA* clone from the octopine-type Ti plasmid pTiA6 was able to restore agroinfection in a C58 strain harboring a deletion of the C58 *virA* gene (At13000) (Table 1). The same *virA*_{A6} clone was unable to restore agroinfection by At11068, thereby excluding copy number of the complementing *virA* clone as an explanation. Therefore, VirA_{A6} is fully functional for maize agroinfection

in a pTiC58 background but not in a pTiA6 background. However, VirA_{C58} is fully functional in either background. These observations suggest that pTiA6 encodes a factor that limits or inhibits the activity of VirA_{A6} but not of VirA_{C58} in agroinfection of maize. To determine whether the pTiA6-encoded "inhibitory" activity could be overcome by overexpressing VirA_{A6} prior to inoculating the maize plants, we placed the *virA*_{A6} coding sequence under the control of an inducible *lac* promoter. This clone restored agroinfection of maize in octopine-type strain A1030 when P_{lac}-*virA*_{A6} expression was preinduced by treatment with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Table 1). This result suggests that the VirA_{A6}-specific inhibitor encoded by pTiA6 is limiting and can be overcome by overexpression of VirA_{A6}. These data cannot be explained by invoking a superior interaction of VirA_{A6} with VirG_{C58}, since it was previously reported that a *virG*_{C58} clone cannot complement a *virG*_{A6} mutant strain for agroinfection (Raineri et al. 1993). The explanation most consistent with the data is that pTiA6 encodes an inhibitor specific for VirA_{A6} that restricts its ability to induce the *vir* genes in a maize-wound environment.

We attempted to identify the gene or genes encoding the VirA_{A6}-specific inhibitory activity by testing the maize agroinfection ability of a *virA*_{A6}-containing derivative of At13000 (C58/pTiC58Δ*virA*::Ω) complemented with an ordered set of cosmid clones made from pTiA6 (data not shown). No VirA_{A6}-specific inhibition by a series of clones covering nearly the entire pTiA6 plasmid was observed. The only region that could not be tested was that immediately surrounding the pTiA6 origin of replication because of incompatibility with the pTiC58 origin.

A discrete region of VirA is responsible for the strain specificity of maize agroinfection.

Since the *virA* locus is the determining factor in conferring maize agroinfection ability, we set out to determine what part of VirA_{C58} is responsible for maize infection. It has been suggested that the sensor components of two-component signal transduction systems are modular and composed of domains that perform independent functions, which when combined in one molecule result in the desired function (Parkinson and Kofoed 1992). These domains are broadly defined as input, kinase, and receiver. The input domain of the VirA sensor molecule can be further subdivided into a periplasmic domain that interacts with ChvE (Cangelosi et al. 1990; Shimoda et al. 1993) and a linker domain that may be responsible for sensing phenolic compounds (Chang and

Table 1. Determination of ability to agroinfect maize by *virA* and Ti plasmid source

Strain name	<i>Agrobacterium</i> genotype			<i>virA</i> clone	Inducing condition	Maize infected (%)
	Chromosome	Ti plasmid	Source			
At11068	C58	pTiA6 Δ <i>virA</i> ::Ω	This work	<i>virA</i> _{C58}	...	88
At11068	C58	pTiA6 Δ <i>virA</i> ::Ω	This work	<i>virA</i> _{A6}	...	5
At13000	C58	pTiC58 Δ <i>virA</i> ::Ω	Doty et al. 1996	<i>virA</i> _{C58}	...	100
At13000	C58	pTiC58 Δ <i>virA</i> ::Ω		<i>virA</i> _{A6}	...	100
A1030	C58	pTiB ₆₈₀₆ <i>virA</i> ::Tn5	Garfinkel and Nester 1980	P _{lac} - <i>virA</i> _{A6}	...	2
A1030	C58	pTiB ₆₈₀₆ <i>virA</i> ::Tn5		P _{lac} - <i>virA</i> _{A6}	1 mM IPTG ^a	46
C58	C58	pTiC58	Hamilton and Fall 1971	...	...	100
At11054	C58 <i>chvE</i> ::Tn5	pTiC58	Charles et al. 1994	...	...	0

^a Isopropyl-β-D-thiogalactopyranoside.

Winans 1992; Doty et al. 1996). A series of chimeric VirA proteins composed of both VirA_{C58} and VirA_{A6} were tested for their ability to restore maize agroinfection in an At11068 (C58/pTiA6Δ*virA*::Ω) background. These hybrid proteins are represented in Figure 1 along with their corresponding maize agroinfection frequencies. Replacement of either the VirA_{C58} periplasmic domain or a region composed largely of the linker domain with the corresponding region of VirA_{A6} (pJD105W and pJD129W, respectively) resulted in reduced maize agroinfection in an At11068 (C58/pTiA6Δ*virA*::Ω) background, although these were not reduced to the level of wild-type VirA_{A6}. The reverse hybrids (pJD113W and pJD132W) exhibited an increase in agroinfection frequency; pJD132W restored maize agroinfection to wild-type VirA_{C58} levels. Chimeric VirA molecules composed of mainly VirA_{C58} with residues 525 to 830 from VirA_{A6} (the C-terminal half of the kinase domain and receiver domain) or the promoter and residues 1 to 63 from VirA_{A6} (the N-terminal and first transmembrane domain) exhibited agroinfection frequencies similar to that of wild-type VirA_{C58} (data not shown). These results indicate that a region composed of the C-terminal 20 amino acids of the periplasmic domain, the second transmembrane helix, and the linker domain of VirA_{C58} (residues 241 to 418) is sufficient to confer maize agroinfection competence on VirA_{A6} in a pTiA6 background.

It is interesting to note that the VirA hybrids encoded on plasmids pJD128W, pJD131W, and pJD130W exhibited agroinfection frequencies below those conferred by either of the parent VirA molecules. These hybrids also showed reduced responsiveness in *vir* induction measurements (data not shown), suggesting that they are composed of interacting domain pairs less able to communicate with one another than the wild type.

In addition, these data suggest a role for the periplasmic domain of VirA in maize agroinfection. The periplasmic do-

main of VirA_{C58} is able to partially restore maize agroinfection competence to VirA_{A6} (pJD113W), and the reverse hybrid (pJD105W) is reduced in maize agroinfection competence versus wild-type VirA_{C58}. However, while it is clear that the periplasmic domain does play a role in determining the competence for maize agroinfection, the region composed of residues 241 to 418 harbors the major determining factor or factors.

The DNA segment of *virA* that encodes the major strain-specific determinant of maize agroinfection codes for a portion of the periplasmic domain, the second transmembrane helix, and the cytoplasmic linker domain (Parkinson and Kofoed 1992). Database searches with these segments of different VirA proteins did not reveal any statistically significant matches with other proteins, except for the VirA proteins of other *Agrobacterium* strains. Multiple alignment of the VirA proteins reveals that the agroinfection-determinant domain of VirA is highly conserved. We compared protein sequences from agroinfection-competent and agroinfection-impaired strains in search of the amino acid changes that would discriminate between these two groups of strains. Twenty residues were found to be group specific (Fig. 2). These changes are concentrated at the end of the periplasmic domain and in the C-terminal half of the linker domain. Moreover, the impact of these substitutions might also be different. For example, of the four closely spaced, group-specific substitutions in the periplasmic domain (specifically, the tip of the periplasmic domain), three (K243E/A, Q251K/A, and R254E/V) change the residue polarity and the fourth (S252N/R) significantly alters the size of the side chain. However, only three changes in the linker domain are associated with a group-specific difference in side chain polarity or size, and these substitutions are widely spaced. The variable patch of amino acids preceding the second transmembrane helix is a

Fig. 1. Various VirA chimeras and corresponding maize agroinfection frequencies in two separate pTi plasmid backgrounds. The input (periplasmic and linker), kinase, and receiver domains are indicated by horizontal arrows above VirA_{C58}. Residues derived from VirA_{C58} are shaded. Residue number at chimeric fusion points is indicated above VirA_{C58} and VirA_{A6}. %ID = percentage of identity between peptide sequences of VirA_{C58} and VirA_{A6}. Plasmid designations for the various chimeras are listed to the left of their respective schematic representations.

plausible target for the further analysis of maize agroinfection capacity of VirA proteins.

Given the likely role of the linker domain in phenolic recognition (Chang and Winans 1992; Doty et al. 1996), these data imply that the ability of the sensor molecule VirA to recognize a particular inducing compound or compounds is key in determining which strains will infect maize. These data, in conjunction with the evidence presented above for a pTiA6-encoded, VirA_{A6}-specific inhibitor, suggest that a region of VirA_{A6} including the C-terminal end of the periplasmic domain, second transmembrane domain, and linker domain may interact with the inhibitor, blocking a crucial interaction with a maize-derived inducer. Therefore, the ability to agroinfect maize may be determined by the ability of VirA to escape inhibition and respond to a particular inducing environment.

The chromosomally encoded sugar-binding protein ChvE is absolutely required for agroinfection of maize.

It has long been recognized that monosaccharides play a significant role in potentiating the *vir* induction response to phenolic compounds (Cangelosi et al. 1990; Shimoda et al. 1990). *Agrobacterium* strains in which *chvE* is nonfunctional are defective in *vir* gene induction and exhibit a limited host range compared with wild-type strains (Garfinkel and Nester 1980; Huang et al. 1990). We tested the ability of a C58 strain harboring a Tn5 insertion in *chvE* to agroinfect maize. The agroinfection competence of this mutant strain is completely abolished (Table 1). Again, this result emphasizes the crucial role of *vir* induction in determining host range.

ChvE is known to interact directly with the periplasmic domain of VirA (Shimoda et al. 1993), potentiating the response of VirA to inducing phenolic compounds (Cangelosi et al. 1990). Additionally, a mutant derivative of VirA_{A6}, in which the periplasmic domain is deleted, has been shown to exhibit the same reduced *vir* induction and host range as a *chvE* mutant (Cangelosi et al. 1990). We deleted various regions of the periplasmic domain of VirA_{C58} in an attempt to disrupt the VirA-ChvE interaction and tested those deletion

Fig. 2. Comparison of residues 241 to 418 of VirA proteins from strains competent for maize agroinfection (C58 and A4) with those not able to agroinfect maize (A6 and Ag162). X (single or double) = group-specific conserved residues; XX = those residues that are significantly altered between the agroinfection-competent and agroinfection-incompetent strains; === = the second transmembrane domain (residues 260 to 280); and ~~~ = the amphipathic helix region (residues 278 to 288). The linker domain is represented by residues 278 to 418. A6^a = the sequence of VirA₁₅₉₅₅ is identical to that of VirA_{A6} in this region.

Implications for *Agrobacterium*-mediated transformation of recalcitrant plants.

The bacterial factors demonstrated to be important in determining maize agroinfecting ability are all concerned with sensing environmental conditions favorable for *vir* gene induction. Raineri et al. (1993) reported that a *virA* clone from a C58 Ti plasmid was sufficient to confer maize agroinfection ability on a nonagroinfecting strain. Additionally, they showed that a mutant *VirA*_{Δ6} derivative that induces *vir* gene expression in the absence of signal molecules can restore agroinfection ability to a nonagroinfecting strain. These data implicate a step upstream of VirG activation by VirA as the limiting step in determining the ability of particular strains to agroinfect maize. The steps upstream of VirG activation by VirA are those involving signal recognition by VirA. Since perturbation of the *chvE* locus eliminates monosaccharide-mediated enhancement of *vir* gene induction and also severely limits host range, it is not surprising that a *chvE* mutant failed to agroinfect maize.

While *Agrobacterium* strains have proved to be invaluable tools in the genetic manipulation of many plants, several economically important members of Graminae cannot be routinely transformed. This study underscores the importance of signal recognition by the *vir* gene-induction machinery in the transformation of recalcitrant crop plants. Cells from various plants likely produce a signature complement of *vir* gene inducers and inhibitors in response to wounding. Additionally, a strain of *Agrobacterium* that is very efficient in transforming one plant will not necessarily be effective when used to transform a different species or cultivar of the same species. Therefore, it becomes important to ascertain the effectiveness of various strains in *Agrobacterium*-mediated transformation for each plant species.

While mutant derivatives of *Agrobacterium* that constitutively express *vir* genes may be useful in many cases with plants that are difficult to transform, these constitutive strains express their *vir* genes at a level lower than that of fully induced wild-type strains (Ankenbauer et al. 1991; McLean et al. 1994; Pazour et al. 1991). In fact, in the presence of the pTi plasmid, constitutive mutants of *virA* exhibit severely reduced constitutive *vir* expression (Pazour et al. 1991; J. D. Heath and E. W. Nester, *unpublished data*). Additionally, the exper-

ience of this laboratory is that the constitutive *virG*_{N54D} is unstable and reverts at a high frequency (D. M. Raineri, K. M. Stephens, and E. W. Nester, *unpublished data*). However, other groups have not observed this instability and have shown that constitutive *VirG*_{N54D} can increase T-DNA transfer to maize (Hansen et al. 1994). Nevertheless, *vir*-constitutive strains may not always work as well as wild-type strains or recombinant strains with novel chromosome-pTi combinations. For example, the so-called super-*vir* strain A281 is composed of the Ti plasmid from strain Bo542 and the chromosome of C58 (Hood et al. 1986; Jin et al. 1987). A281 is more virulent and has a broader host range than either parent strain. It is clear that a single bacterial vector will not work in every case, and investigators must be ready to employ a variety of *Agrobacterium* strains in difficult transformation situations.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Strains used in this study are listed in Table 1. Genetic manipulation and growth of *Agrobacterium* strains were essentially as described (Cangelosi et al. 1991) except where noted. Strain At11068 was constructed by replacing the 4.4-kb *KpnI* fragment of A348 pTiA6 containing *virA* with the omega fragment. The omega fragment consists of the spectinomycin and streptomycin resistance gene, *aadA*, flanked by short, inverted repeats containing transcriptional and translational termination signals (Prentki and Krisch 1984). Specifically, a 7.5-kb *HpaI* fragment from the cosmid pVK219 (Knauf and Nester 1982) was cloned into the *SmaI* site on pUC9 (Vieira and Messing 1982), creating pTC284. pTC284 was digested with *KpnI*, releasing a 4.4-kb fragment harboring the *virA* locus. The remaining large fragment of pTC284 was treated with the T4 DNA polymerase to create blunt ends. A *SmaI* fragment containing the omega cassette (Ω) was ligated with the blunt end *KpnI* fragment of pTC284, resulting in the plasmid pTC291. pTC291 was cleaved with *EcoRI*, filled in with Klenow, and ligated with *BamHI*-cleaved, Klenow-treated pMH1801 (which carries the *sacB* gene) (Hynes et al. 1989), resulting in pTC299. pTC299 was introduced into strain A348 by electroporation, streak purified once, and then

Fig. 3. Various *VirA*_{C58} periplasmic domain deletion derivatives and corresponding maize agroinfection frequencies in either a *chvE*⁺ or *chvE*::Tn5 background. The input (periplasmic and linker), kinase, and receiver domains are indicated. Plasmid pJD103W encodes a *VirA*_{C58} derivative with an in-frame deletion of residues 63 to 172; pJD108W encodes an in-frame deletion of residues 173 to 240; and pJD104W encodes an in-frame deletion of residues 63 to 240.

streaked out on AB glucose (Cangelosi et al. 1991), 5% sucrose and 100 µg of spectinomycin per milliliter, to select for double crossover and loss of the vector sequences. The relocation of the $\Delta virA::\Omega$ recombinant locus onto the pTiA6 plasmid was confirmed by Southern hybridization and phenotypic assays (virulence). Thus, At11068 contains a precise deletion of the 4.4-kb *KpnI* fragment.

Plasmids pJD104 and pJD110, harboring identical deletion derivatives of *virA*_{C58} and *virA*_{A6}, respectively, were constructed to aid in the assembly of the chimeric *virA* clones. A 5.6-kb fragment of pTIC58 harboring the *virA* gene was released by digestion with *Bam*HI and *Hind*III and cloned into the pTZ19R cloning vector (United States Biochemical, Cleveland, OH) resulting in plasmid pJD101. The *virA* gene encoded by pJD101 has two *Pst*I sites, at positions 184 and 514, within the region encoding the periplasmic domain. Plasmid pJD103 was created by digesting pJD101 with *Pst*I, releasing a 330-bp fragment. The vector was religated, resulting in a deletion derivative of *virA*_{C58} encoding an in-frame deletion of 110 amino acids of the periplasmic domain (residues 63 to 172). A new *Pst*I site corresponding to the *Pst*I site at position 718 of the *virA*_{A6} coding sequence was introduced at position 718 of the *virA*_{C58} coding sequence in clone pJD101 by PCR (polymerase chain reaction), resulting in a single amino acid change of a glutamic acid to the corresponding *VirA*_{A6} residue glutamine at amino acid 241. The alteration was confirmed by sequence analysis, and the resultant plasmid was designated pJD102. When pJD102 is digested with *Pst*I, two small fragments (330 and 204 bp) are released corresponding to the N- terminal and C-terminal halves, respectively, of the periplasmic domain-encoding region of *virA*_{C58}. The native *Pst*I site at position 514 of the *virA*_{C58}-coding sequence in clone pJD102 was destroyed by PCR with no resulting residue changes. This plasmid was designated pJD111. Plasmid pJD104 was created by digesting plasmid pJD111 with *Pst*I, releasing a 534-bp fragment. The vector was religated, resulting in a deletion derivative of *virA*_{C58} encoding an in-frame deletion of 178 residues of the periplasmic domain. Plasmid pJD108 was created by digesting pJD104 with *Pst*I and reintroducing the 330-bp *Pst*I fragment encoding the N-terminal half of the periplasmic domain. Orientation was confirmed by restriction analysis. pJD110, the deletion derivative of *virA*_{A6} corresponding to pJD104, was made as follows. pIB400 (Cangelosi et al. 1990) harbors a clone of *virA*_{A6} with the same sequences deleted as described for pJD104. pIB400 was digested with the restriction enzyme *Afl*III, releasing a 2.7-kb fragment whose ends were subsequently filled in by the Klenow fragment of the DNA polymerase holoenzyme. pUC7 (Vieira and Messing 1982) was digested with *Bam*HI, and the ends were filled in by the action of the Klenow fragment. The 2.7-kb fragment from pIB400 and the *Bam*HI-digested pUC7 were ligated together, resulting in plasmid pJD110. Orientation was determined by restriction analysis.

pJD134, containing the wild-type *virA*_{A6} sequence, was constructed by digesting pJD110 and a plasmid containing the complete *virA*_{A6} sequence, pSW169 (Winans et al. 1989), with the restriction enzyme *Pst*I. *Pst*I-linearized pJD110 and a 534-bp fragment released from pSW169 upon *Pst*I digestion were ligated together by the action of T4 DNA ligase to create plasmid pJD134. Correct orientation of the 534-bp fragment

was confirmed by restriction analysis in this and all cases. Plasmid pJD105 was created by ligating together *Pst*I-linearized pJD104 and the 534-bp *Pst*I fragment from pSW169. The reciprocal hybrid, pJD113, was constructed by ligating together *Pst*I-linearized pJD110 and the 534-bp *Pst*I fragment from pJD111. Plasmids pJD128 and pJD129 were constructed in the following way. First, pJD104 and pJD110 were digested with *Pst*I and *Bst*EII. Second, the 519-bp *Pst*I-*Bst*EII fragment from pJD110 was ligated with the 657-bp and 6.7-kb *Pst*I-*Bst*EII fragments from pJD104. Since the *Bst*EII sites on pJD104 have different overhangs, the triple ligation can proceed via only one pathway. The resulting plasmid, pJD124, was digested with *Pst*I, and the 534-bp *Pst*I fragments from pSW169 and pJD111 were ligated with linearized pJD124 to create plasmids pJD128 and pJD129, respectively. Plasmid pJD132 is the reciprocal of pJD129 and was constructed just as pJD129, exchanging pJD104- and pJD111-derived fragments for pJD110- and pSW169-derived fragments, respectively. Plasmids pJD130 and pJD131 were constructed as follows: pJD104 and pJD110 were digested with *Pst*I and *Bgl*II. The 841-bp *Pst*I-*Bgl*II fragment from pJD110 was ligated with the 7.1-kb *Pst*I-*Bgl*II fragment from pJD104, creating plasmid pJD125. pJD125 was linearized with *Pst*I, and the 534-bp *Pst*I fragments from pSW169 and pJD111 were ligated to the linearized pJD125, resulting in plasmids pJD130 and pJD131, respectively. Plasmids pJD135 and pJD136 are the reciprocals of pJD130 and pJD131 and were constructed in the same way, exchanging pJD104- and pJD111-derived fragments for pJD110- and pSW169-derived fragments and vice versa.

Each of the above *virA* hybrids and deletion derivatives was moved into a broad host range vector to allow plasmid maintenance in *A. tumefaciens*. The *virA* constructs on plasmids pJD102, pJD103, pJD104, pJD105, pJD108, pJD111, pJD128, pJD129, pJD130, and pJD131 were moved into pDMD2 (Raineri et al. 1993), a derivative of the IncW replicon pUCD2. Each *virA*-containing plasmid was digested with *Bam*HI and *Hind*III, and the large, 5.6-kb fragment was isolated. The IncW plasmid pDMD2 was digested with *Bam*HI and *Hind*III, and the large fragment was isolated. Each of the *virA*-containing 5.6-kb *Bam*HI-*Hind*III fragments was ligated with the large *Bam*HI-*Hind*III fragment from pDMD2. The *virA* constructs on plasmids pJD110, pJD113, pJD132, pJD134, pJD135, and pJD136 were moved into pDMD2 by digesting each *virA*-containing plasmid and pDMD2 with *Bam*HI. The 3.3-kb, *virA*-containing *Bam*HI fragments were isolated and ligated to the large *Bam*HI fragment from pDMD2. All of the resultant IncW plasmids were named by adding "W" to the end of the original *virA* plasmid name. For example, pJD105 became pJD105W.

The maize streak virus (MSV) binary vector pMSV-Ns(G) is an IncP vector conferring gentamycin resistance and harboring an MSV dimer between T-DNA border sequences (Raineri et al. 1993).

Plant growth and inoculation conditions.

Growth of *Zea mays* L. (extra early and eight-row) and preparation of bacterial inocula were essentially as described (Boulton et al. 1989). Except where noted, bacterial strains were not preinduced and were used at an inoculum density of 10⁸ colony-forming units per plant. Plants were examined for

symptoms from 5 days postinoculation (dpi); those showing no symptoms 28 dpi were scored as uninfected. Typically, MSV symptoms are long, chlorotic streaks, abundant on leaves, that emerge 6 to 9 dpi (Boulton et al. 1991).

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