VirA and VirG activate the Ti plasmid *repABC* operon, elevating plasmid copy number in response to wound-released chemical signals

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The *vir* genes of *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmids direct the transfer of oncogenic portion of the Ti (tumor-inducing) plasmid that is transferred to plant cells (T-DNA) into plant cells and are coordinately induced by plant-released phenolic chemical signals. We have used DNA microarrays, representing all genes of the octopine- and nopaline-type Ti plasmids, to identify all Ti-plasmid-encoded genes in the *vir* regulons of both plasmids. Acetosyringone (AS) induced the expression of all known members of the *vir* regulons, as well as a small number of additional genes. Unexpectedly, AS also caused a modest induction of virtually every Ti plasmid gene. This suggested that the copy number of the Ti plasmid might increase in response to AS, a hypothesis confirmed by DNA dot blotting. VirA and VirG were the only Vir proteins required for this copy number increase. Promoter resections and primer extension analysis of the *repABC* promoter region showed that expression of the promoter closest to *repA* (promoter P4) was induced by AS. We also identified a sequence resembling a consensus VirG-binding motif ≈ 70 nucleotides upstream from the P4 transcription start site. Mutating this sequence blocked the AS-induced copy number increase of a *RepABC*-dependent miniplasmid, indicating that phospho-VirG increases copy number solely by enhancing *repABC* expression.

agrobacterium | microarrays | transcriptional profiling

Many microbes that associate with plant or animal hosts coordinately regulate large sets of genes required for these associations and induce expression of these genes in response to host-released chemical signals. For example, *Sinorhizobium meliloti* induces expression of the *nod* regulon in response to flavonoids released from host legumes (1), whereas *Vibrio cholerae* induces expression of several toxins and adhesins in response to bile salts released into the gut of human or animal hosts (2). This phenomenon is also well illustrated by the plant pathogen *Agrobacterium tumefaciens*. The entire set of *vir* genes, whose products transfer fragments of oncogenic portion of the tumor-inducing (Ti) plasmid that is transferred to plant cells (T-DNA) from the large Ti plasmid to the nuclei of host cells, is induced by chemical signals released from host plants (3). The primary signal molecules that stimulate expression of the *vir* regulon are phenolic compounds [including acetosyringone (AS), which is widely used in many laboratories as a *vir* gene inducer], particular monosaccharides, and extracellular acidity (4).

The expression of *vir* genes is controlled by the periplasmic sugar-binding protein ChvE, the transmembrane histidine protein kinase VirA, and the cytoplasmic response regulator VirG (4). Phospho-VirG activates expression of at least 14 *vir* operons, containing a total of 30 genes (3, 5, 6) on the octopine-type Ti plasmid, whereas far fewer VirG-inducible genes have been identified on the nopaline-type Ti plasmid (7). The products of some *vir* genes play direct roles in T-DNA transfer, whereas the products of other *vir* genes are not required for tumorigenesis and probably play other roles during the initial stages of infection (8). To date, all genes known to be in the *vir* regulon are located on Ti plasmids. It is quite plausible that additional members of this regulon remain to

be described. It is also quite possible that VirG-regulated genes may exist on replicons other than the Ti plasmid (9).

Ti plasmids are generally referred to by the opines they can catabolize, and the two best studied are often referred to as the nopaline-type plasmid originally found in strain C58 and the octopine-type Ti plasmid, which is found in virtually identical forms in strains such as A6, B6, 15955, Ach5, and R10. All known Ti plasmids contain (i) one or more T-DNAs, (ii) the *vir* genes, which direct the transfer of T-DNA into plant cells, (iii) a set of *tra* and *trb* genes, which direct the conjugation of the Ti plasmid, (iv) a large number of genes that direct the uptake and catabolism of nutrients called opines that are released from plant tumors, and (v) a *repABC* operon, which directs vegetative plasmid replication and partitioning (8). Similar *repABC* operons are widespread among the plasmids and some chromosomes of α -proteobacteria, but their regulation is largely unexplored (10–12).

In this study, we have used DNA microarrays to identify all members of the *vir* regulons that lie on the two most widely studied Ti plasmids. These microarrays contained all ORFs of the two Ti plasmids as well as ≈ 20 chromosomal genes thought to be constitutively expressed and several members of the putative SOS and heat-shock regulons. It seemed conceivable that either or both of these regulons might be AS-inducible, because phenolics are somewhat toxic at the concentrations used (13), and the T-DNA is processed to a single-stranded form (14) that might possibly cause SOS induction (15). As described below, these transcriptional profiling experiments confirmed the induction of virtually all known members of the *vir* regulons on the Ti plasmids and identified a small number of previously undescribed members. They also led to the discovery that VirA and VirG directly activate expression of the *repABC* operon, causing an increased copy number of this plasmid in response to host-released chemical signals.

Materials and Methods

Design of Oligonucleotides for PCR Amplification. Strains and plasmids used in this study are described in Table 2, which is published as supporting information on the PNAS web site. Oligonucleotides were designed by using the PRIMER3 program (16). We used default settings except for the following parameters: product size range, 501–900 (most preferred) or 300–500 (acceptable) and %GC, 40–60; maximum polyX, 3; and CG clamp, 1. The primers were synthesized by IDT Technologies (Coralville, IA) and are listed in Table 3, which is published as supporting information on the PNAS web site. PCR reactions were carried out by using genomic DNA from derivatives of strains R10 or C58 as templates and Platinum TaqDNA polymerase (Invitrogen). PCR products were purified by using 96-well multiscreen filter plates (Millipore).

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Abbreviations: Ti, tumor-inducing; AS, acetosyringone; T-DNA, portion of the Ti plasmid that is transferred to plant cells.

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Fig. 7. Primer extension assay using mRNA from strain NTL4(pHC090)(pPKRR5) after overnight incubation in the presence or absence of 100 μ M AS. Two radiolabeled oligonucleotide primers were added, one of which hybridizes to *repA* mRNA, and the other of which hybridizes to the *lacZ α* gene (of pHC090), which serves as a constitutive control.

Fig. 8. Increase in copy number of *repABC*-dependent plasmids by phospho-VirG. Strain NTL4(pHC090)(pKP23) was incubated overnight in the presence or absence of 100 μ M AS. Purified plasmid DNA was digested with KpnI, which linearizes the minireplicons (upper band of each lane) and cuts pHC090 at two sites, generating two lower bands of each lane. The two pHC090-derived fragments were used to normalize the band intensities of *repABC*-dependent plasmids. Plasmids pHC121 and pHC122 are derivatives of pKP23 with the indicated alterations in the *vir* box motif.

onstrating that this promoter is AS-inducible. This promoter was previously demonstrated to be autorepressed by the RepA and RepB proteins (11).

Phospho-VirG Enhances the Ti Plasmid Copy Number Solely by Activating Promoter P4. The results described above show that phospho-VirG activates transcription of P4 but do not prove that this activation is the sole cause of the AS-induced increase in Ti plasmid replication. To test this, we reconstituted this system using a minimal *repABC*-dependent replicon (pKP23) and a second plasmid expressing VirA and VirG (pHC090). The copy number of pKP23 increased ≈ 4 -fold upon AS induction (Fig. 8). This increase required VirA and/or VirG, because it was abolished in a strain that lacked pHC090 (data not shown). We identified a DNA sequence, centered 116 nucleotides upstream of *repA*, that resembles the consensus VirG-binding site (Fig. 8). This region is required for AS-inducibility of the *repABC* operon. We disrupted this putative VirG-binding site by altering three conserved nucleotides and found that the copy number of the resulting plasmid, pHC122, was not enhanced by AS (Fig. 8). We also altered five nucleotides in this sequence to create a consensus VirG-binding site. The copy number of the resulting plasmid, pHC121, was enhanced by AS slightly more than the wild type (Fig. 8). These data indicate that phospho-VirG binding to a site upstream of P4 is required for enhanced copy number, which in turn indicates that phospho-VirG enhances copy number solely via its effects on *repABC* transcription.

Discussion

One of the goals of this study was to attempt to identify all Ti plasmid-encoded members of the *vir* regulon of pTiA6NC and pTiC58. The core set of *vir* genes that are essential for tumorigenesis are highly conserved in sequence and order between these plasmids (8, 21, 22), and we confirmed older data that each of these genes is induced by AS. In addition to these genes, both plasmids have a large number of members of this regulon that are not essential for tumorigenesis and are not conserved. For example, in pTiA6NC, *virM*, *-L*, *-K*, *-D3*, *-D5*, *-E3*, *-P*, and *-R* are inducible by AS but not essential for tumorigenesis (8). Of these, *virM*, *-L*, *-P*, and *-R* are not conserved between the two Ti plasmids. We also identified two new members of the pTiA6NC *vir* regulon (*ysf* and *ysg*) that are linked to other *vir* genes. The roles of these genes in tumorigenesis have not been determined, but neither is conserved in pTiC58, suggesting that they are not essential for tumorigenesis.

Fig. 9. Summary of the regulation of the *repABC* operon. According to this model, wound-released signals act through VirA to phosphorylate VirG, which activates promoter P4, whereas 3-oxo-octanoylhomoserine lactone-TrpR complexes activate promoters P1–P3 (10). All four promoters are autorepressed by RepA/RepB complexes, which bind to an operator directly downstream of promoter P4 (11).

A larger number of AS-inducible genes were discovered in the nopaline-type Ti plasmid, partly because such genes had not previously been sought as rigorously as in the octopine-type plasmid. These include *Atu6155*, *Atu6157*, *Atu6158*, *Atu6162*, and *Atu6163R*, in addition to three other operons that were anticipated to be induced, because their orthologs on pTiA6NC are inducible (*virH*, *virF*, and *virK*). That so many members of this regulon are not found in the octopine-type Ti plasmid suggests that wound-released chemical signals elicit slightly different responses from strains carrying these different plasmids, suggesting they have slightly different strategies for colonizing host plants.

Our finding that *traM* is induced by AS was of considerable interest, because TraM interacts with TraR to block expression of the *tra* regulon (26, 27). For several years, we and others have wondered why *tra* regulatory machinery includes such an antiactivator, because very few other quorum-sensing systems are controlled by analogous antiactivator proteins (32). Induction of TraM by AS predicts that *vir* gene induction blocks *tra* gene expression. Although *traM* induction by AS is not high, it may well suffice to block low levels of activated TraR. Such a mechanism to block simultaneous expression of the *vir* and *tra* systems might well be useful, because they are both type IV secretion systems and might well interfere with each other's function. The idea that *vir* gene expression blocks *tra* gene expression rather than the converse could be interpreted to suggest that plant transformation takes precedence over interbacterial conjugation.

Perhaps the most unexpected finding of this study is that the *repABC* operon was induced by phospho-VirG. That we did not detect this induction directly from the microarrays was probably due to their extremely low expression of the intact operon, due to negative autoregulation by RepA and RepB (11). This induction was clearly detected by using nuclease protection assays. A series of *rep-lacZ* fusions and primer extension assays clearly showed that promoter P4 is directly activated by phospho-VirG. Induced expression of *repABC* enhanced Ti plasmid copy number and thereby indirectly enhanced the expression of all Ti plasmid-encoded genes. Increased expression of all *vir* genes, as well as increased copies of the T-DNA, could lead to increased production of T-strands and of the T-strand export machinery, which could lead to enhanced levels of tumorigenesis (10).

We have previously shown (10) that the quorum-sensing regulator TraR and its cognate autoinducer also activate transcription of *repABC*, causing an enhanced copy number of this plasmid. In that case, induction occurs at the P1, P2, and P3 promoters. Taken together with the present study, we believe that when *A. tumefaciens* grows saprophytically, its Ti plasmid replicates at rates just high enough to ensure vertical transmission. In contrast, when the bacterium detects either host-released signals or quorum signals, the plasmid's copy number significantly increases by activation of *repABC* operon (Fig. 9). This seems appropriate, because minimizing the plasmid copy number during saprophytic growth would lessen the metabolic burden to the host bacterium. In contrast, during plant colonization, the Ti plasmid carries out numerous important functions. Increased gene expression (due to increased gene dosage) could well facilitate such activities as tumorigenesis and opine utilization.

At present, there is no convincing evidence that VirG regulates any gene localized on either of the two chromosomes or on the cryptic plasmid. Because many natural isolates of *A. tumefaciens* lack Ti plasmids, it would make sense that chromosomal genes not rely on VirA or VirG for their regulation. However, our finding that the copy number of pAtC58 (also referred to as the cryptic plasmid) may also be slightly enhanced, if confirmed, would suggest that at least these two plasmids share common regulatory elements. The use of whole-genome microarrays will facilitate the search for VirG-regulated genes on other replicons.

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