A Second T-Region of the Soybean-Supervirulent Chrysopine-Type Ti Plasmid pTiChry5, and Construction of a Fully Disarmed *vir* Helper Plasmid

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Agrobacterium tumefaciens Chry5, which is particularly virulent on soybeans, induces tumors that produce a family of Amadori-type opines that includes deoxyfructosyl glutamine (Dfg) and its lactone, chrysopine (Chy). Cosmid clones mapping to the right of the known oncogenic T-region of pTiChry5 conferred Amadori opine production on tumors induced by the nopaline strain C58. Sequence analysis of DNA held in common among these cosmids identified two 25-bp, direct repeats flanking an 8.5-kb segment of pTiChry5. These probable border sequences are closely related to those of other known T-regions and define a second T-region of pTiChry5, called T-right (T_R), that confers production of the Amadori opines. The oncogenic T-left region (T_L) was located precisely by identifying and sequencing the likely border repeats defining this segment. The two T-regions are separated by approximately 15 kb of plasmid DNA. Based on these results, we predicted that pKYRT1, a *vir* helper plasmid derived from pTiChry5, still contains all of T_R and the leftmost 9 kb of T_L. Consistent with this hypothesis, transgenic *Arabidopsis thaliana* plants selected for with a marker encoded by a binary plasmid following transformation with KYRT1 co-inherited production of the Amadori opines at high frequency. All opine-positive transgenic plants also contained T_R-DNA, while those plants that lacked T_R-DNA failed to produce the opines. Moreover, *A. thaliana* infected with KYRT1 in which an *nptII* gene driven by the 35S pro-

moter of *Cauliflower mosaic virus* was inserted directly into the *vir* helper plasmid yielded kanamycin-resistant transformants at a low but detectable frequency. These results demonstrate that pKYRT1 is not disarmed, and can transfer Ti plasmid DNA to plants. A new *vir* helper plasmid was constructed from pTiChry5 by two rounds of *sacB*-mediated selection for deletion events. This plasmid, called pKPSF2, lacks both of the known T-regions and their borders. pKPSF2 failed to transfer Ti plasmid DNA to plants, but mobilized the T-region of a binary plasmid at an efficiency indistinguishable from those of pKYRT1 and the nopaline-type *vir* helper plasmid pMP90.

Additional keywords: binary system, disarmed plasmid, *Glycine max*, T-DNA.

Agrobacterium tumefaciens, a phytopathogenic soil bacterium, causes crown gall disease on many dicotyledonous plants. Most of the traits responsible for pathogenesis are borne on a large, extrachromosomal element called the Ti (tumor-inducing) plasmid (Zupan and Zambryski 1995). Upon infection, a portion of the Ti plasmid, called the T-region, is transferred to the plant nucleus, where it can be stably integrated into chromosomal DNA. Functions involved in processing the T-region and transferring the resultant T-DNA to the plant cell are coded for by the *vir* region of the Ti plasmids (de la Cruz and Lanka 1998). Oncogenes on some T-DNAs encode enzymes for synthesis of indole acetic acid and cytokinins, the overproduction of which in the transformed plant cells is responsible for the tumorous phenotype (Binns and Costantino 1998). Other T-DNA genes encode biosynthetic pathways for novel, plant-produced compounds called opines. The opines, in turn, are taken up and specifically utilized by the bacteria that induced the tumors via catabolic systems coded for by genes also located on the Ti plasmid (Dessaix et al. 1998). On the Ti plasmids, the T-regions are delimited by 25-bp, imperfect, direct repeats called border sequences (Yadav et al. 1982; Zambryski et al. 1982; Peralta and Ream 1985; Wang et al. 1984). Although some Ti plasmids contain a single T-region, others contain two such regions, each bounded by border sequences. In such cases each resulting T-DNA can be transferred independently to the plant cell nucleus during infection (Hooykaas and Schilperoort 1992).

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Nucleotide and/or amino acid sequence data have been deposited at the GenBank data base as accession nos. AF229155 and AF229156 for the right and left borders of T_L, respectively, and AF229157 for the left and right borders of T_R.

Agrobacterium tumefaciens and its Ti plasmids have proven useful for the directed genetic transformation of plants. Any foreign DNA can be transferred to the plant nucleus via the *vir* system provided the segment is flanked by T-region border repeats. Because the *vir* functions operate in *trans*, the T-regions and the *vir* genes need not be located on the same replicon (Hoekema et al. 1983; de Framond et al. 1983). Thus, experimentally, the *vir* functions often are provided by a non-tumorigenic Ti plasmid derivative called a disarmed Ti plasmid or a *vir* helper plasmid, in which the native oncogenes and the T-region borders have been removed. Such *vir* helper plasmids have been constructed from several Ti plasmid types (Hood et al. 1986, 1993; Hoekema et al. 1983; Torisky et al. 1997; Zambryski et al. 1983). Genes to be transferred are contained on special binary vectors that code for two border

repeats flanking a multiple cloning site, a plant-selectable marker, and other useful sequences (Jones et al. 1992).

Many isolates of *Agrobacterium tumefaciens* exhibit a broad host range. However, some plants, including soybean (*Glycine max* (L.) Merr.) are only poorly transformed by this bacterium. Although genes have been introduced into soybeans by several methods including microprojectile bombardment (McCabe et al. 1988), sonication-assisted *Agrobacterium* transformation (SAAT) (Trick and Finer 1997; Meurer et al. 1998) and *Agrobacterium*-mediated DNA transfer (Hinchey et al. 1988; Chee et al. 1989; Di et al. 1996), the efficiency of these techniques remains poor. However, recent studies with *Agrobacterium tumefaciens* Chry5 has generated renewed interest in this bacterium as a gene delivery system. Chry5, which is hypervirulent on soybeans, harbors the chrysopine-type Ti plasmid pTiChry5 (Bush and Pueppke

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
<i>Escherichia coli</i>		
DH5a	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA-1</i>	Sambrook et al. 1989
S17-1	Pro ⁻ Res ⁺ Mod ⁺ <i>recA</i> ; integrated RP4 Tc::Mu-Km::Tn 7, Mob ⁺ ; Sm ^r	Simon et al. 1983
<i>Agrobacterium tumefaciens</i>		
Chry5	Chrysopine-type strain, hypervirulent on soybean	Bush and Pueppke 1991
Chry5c	Ti plasmidless derivative of Chry5; Rif ^r	Kovacs and Pueppke 1993
C58	Nopaline-type strain	Our collection
NTL4	Tc ^s , Ti plasmidless derivative of strain C58	Our collection
GV3101	Ti plasmidless derivative of strain C58, Rif ^r	Holsters et al. 1980
LBA4404	Ach5 harboring the <i>vir</i> helper plasmid pAL4404	Hoekema et al. 1983
Plasmids		
pUC19	ColE1 cloning vector; Ap ^r	Sambrook et al. 1989
pSW213	IncP cloning vector; Tc ^r	Chen and Winans 1991
pRK415K	Derivative of pRK415; IncP; Km ^r , Tc ^r	Cook and Farrand 1992
pUM24	Source of the <i>sacB-sacR-nptI</i> cassette, Km ^r	Ried and Collmer 1987
pR29	Source of the erythromycin resistance gene; Em ^r	Morrison et al. 1984
pBIN19	Binary vector; Km ^r	Bevan 1984
pCaMVneo	Binary vector, source of the plant selectable <i>nptII</i> driven by 35S promoter of <i>Cauliflower mosaic virus</i> (CaMV); Km ^r , Ap ^r	Fromm et al. 1986
pBINm-gfp5-ER	Binary vector with <i>gfp</i> reporter gene; Km ^r	Haseloff et al. 1997
pBBRMCS-2	Broad-host-range cloning vector; Km ^r	Kovach et al. 1995
pPH11I	IncP; eviction plasmid; Gm ^r	Hirsch and Berenger 1984
pCH3701	Cosmid clone of pTiChry5; coordinates 163.4–191.6; contains the region to the left of TL-DNA; Tc ^r	Kovacs and Pueppke 1994
pCH230	Cosmid clone of pTiChry5; coordinates 184.2–7.6; contains the entire T _L -DNA; Tc ^r	Kovacs and Pueppke 1994
pCH12	Cosmid clone of pTiChry5; coordinates 186.0–18.6; contains the entire T _L -DNA; Tc ^r	Kovacs and Pueppke 1994
pCH77	Cosmid clone of pTiChry5; coordinates 195.4–22.6; contains the entire T _L -DNA; Tc ^r	Kovacs and Pueppke 1994
pCH235	Cosmid clone of pTiChry5; coordinates 5.8–33.7; contains the entire T _R -DNA; Tc ^r	Kovacs and Pueppke 1994
pCH1755	Cosmid clone of pTiChry5; coordinates 18.6–44.7; contains part of T _R -DNA; Tc ^r	Kovacs and Pueppke 1994
pCH2546	Cosmid clone of pTiChry5; coordinates 38.2–63.3; contains the region to the right of T _R -DNA; Tc ^r	Kovacs and Pueppke 1994
pSWE9	<i>EcoRI</i> fragment 9 of pTiChry5 in pSW213; Tc ^r	This study
pSW9sac	<i>sacB-sacR-nptI</i> cassette cloned in <i>BamHI</i> site of the insert in pSWE9; Tc ^r , Km ^r	This study
pUCE4	<i>EcoRI</i> fragment 4 of pTiChry5 in pUC19; Ap ^r	This study
pUCEd4	pUCE4 deleted for <i>Clal</i> fragments internal to <i>EcoRI</i> fragment 4; Ap ^r	This study
pUCERd4	Em ^r gene from pR29 cloned into the <i>Clal</i> site of pUCEd4; Ap ^r , Em ^r	This study
pRKERd4	<i>EcoRI</i> fragment from pUCERd4 cloned in pRK415K; Km ^r , Em ^r	This study
pPOC5E13	<i>EcoRI</i> fragment 13 of pTiChry5 cloned in pUC19; Ap ^r	This study
pPOC5E5	<i>EcoRI</i> fragment 5 of pTiChry5 cloned in pBBRMCS-2; Km ^r	This study
pPOC5B6	<i>BamHI</i> fragment 6 of pTiChry5 cloned in pBBRMCS-2; Km ^r	This study
pUCBE1	<i>BamHI</i> to <i>EcoRI</i> fragment from coordinates 168 to 178.3 of pTiChry5 in pUC19; Ap ^r	This study
pUCBENP1	<i>nptII</i> driven by the CaMV 35S promoter inserted into the <i>HindIII</i> site in the pTiChry5 insert of pUCBE1; Ap ^r , Km ^r	This study
pMx230	<i>sacB-sacR-nptI</i> cassette marker-exchanged into <i>EcoRI</i> fragment 9 of pTiChry5; Km ^r	This study
pMx106	Erythromycin resistance gene marker-exchanged into <i>EcoRI</i> fragment 4 of pMx230 Km ^r , Em ^r	This study
pKPSF1	<i>sacB</i> -mediated deletion derivative of pMx106; Em ^r	This study
pKPSF2	<i>sacB</i> -mediated deletion derivative of pKPSF1; <i>vir</i> helper plasmid; Em ^r	This study
pKYRT1	pTiChry5 deleted for 16.5 kb of DNA including most of T _L	Torisky et al. 1997
pMP90	Nopaline-type <i>vir</i> helper plasmid	Koncz and Schell 1986
pAL4404	Octopine-type <i>vir</i> helper plasmid	Hoekema et al. 1983
pKYRNP1	Cointegrate of pUCBENP1 with pKYRT1; Km ^r , Cb ^r	This study
pKPSNP2	Cointegrate of pUCBENP1 with pKPSF2; Em ^r , Km ^r , Cb ^r	This study

1991; Kovacs and Pueppke 1993). Tumors induced by strains harboring this plasmid produce a set of Amadori-type opines (Chilton et al. 1995; Vaudequin-Dransart et al. 1995) that are conjugates of fructose and glutamine or glutamic acid. pTiChry5 is closely related to the agropine-type Ti plasmid pTiBo542 (Kovacs and Pueppke 1994). However, instead of the Amadori opines, this latter plasmid induces in tumor cells the production of the related mannose conjugates, the mannityl opines (Dessaux et al. 1998).

Given the high degree of tumorigenicity on soybean established by Chry5, Torisky et al. (1997) constructed a disabled derivative of pTiChry5 called pKYRT1 for use as a *vir* helper plasmid. However, the *vir* helper strain KYRT1 maintains very weak oncogenic activity, suggesting that pKYRT1 still harbors one or more T-region border repeats and at least one *onc* function. In this report, we locate the genes of pTiChry5 required for the synthesis of the Amadori opines to a second, previously unidentified T-region. In the process, we determined the precise locations of both T-regions and their probable flanking border sequences. We show that the *vir* helper plasmid pKYRT1 harbors one complete T-region as well as the left border repeat of the second and that, when used as a *vir* helper plasmid in a binary system, this second T-DNA of pKYRT1 is cotransferred to the plant genome at high frequency. Finally, we describe the construction and properties of pKPSF2, an improved *vir* helper derivative of pTiChry5 that is deleted for all known T-regions and border sequences.

RESULTS AND DISCUSSION

Mapping a second T-region of pTiChry5 responsible for synthesis of the Amadori opines.

Tumors induced by *Agrobacterium tumefaciens* strains harboring pTiChry5 accumulate the Amadori-type opines deoxy-

fructosyl glutamine (Dfg) and its lactone, chrysopine (Chy) (Chilton et al. 1995). We localized the genes responsible for the synthesis of these compounds by testing the nopaline-type strain C58 containing a series of overlapping clones from a cosmid library of pTiChry5 for the ability to induce tumors that produce both the arginyl- and Amadori-families of opines. Strains harboring two such clones, pCH235 and pCH1755 (Table 1), yielded tumors that produced nopaline and one or more of the Amadori opines (Fig. 1; data not shown). Tumors induced by C58(pCH1755) produced both Dfg and Chy, while tumors induced by C58(pCH235) contained only Dfg. The inserts in the two clones overlap with each other and cover a region located 25 kb to the right of the proposed oncogenic T-region (Fig. 1). Consistent with the report of Kovacs and Pueppke (1994), LBA4404 (Table 1) harboring cosmid clones overlapping the putative oncogenic T-region of pTiChry5 induced tumors (Fig. 1). However, the resulting transformed tissues did not contain detectable amounts of the Amadori opines (Fig. 1; data not shown), which is consistent with our observation that the region of pTiChry5 responsible for synthesis of these opines maps well to the right of the known oncogenic T-region.

To localize this putative second T-region, we subcloned the segment of DNA corresponding to the region of overlap between cosmid clones pCH1755 and pCH235 into a broad-host-range vector as *Bam*HI or *Eco*RI fragments and introduced these plasmids into strain C58. Similar to what we observed with pCH235 and pCH1755, *Eco*RI fragment 5 present in pPOC5E5 conferred synthesis of Dfg but not chrysopine on tumors induced by C58, while *Bam*HI fragment 6 present in pPOC5B6 resulted in the synthesis of both Dfg and its lactone (Fig. 1; data not shown). We conclude from these observations that pCH1755 and pPOC5B6 contain the entire locus for syn-

Fig. 1. Organization of the T-regions of pTiChry5. Region of pTiChry5 shown corresponds to coordinates 175 to 47 from the map of Kovacs and Pueppke (1994). Numbered boxes show order of *Eco*RI fragments in this region. T-regions as identified in this study are represented by open bars, at the ends of which black squares indicate positions of the likely border sequences. Representative cosmid clones and plasmids used to locate the T_R-DNA are shown above map. Thicker portions of black bars underneath the map represent the extent of DNA deleted in pKYRT1, pKPSF1, and pKPSF2. Vertical arrowheads indicate sites of coinfection of pUCBENP1, which codes for a plant-expressible *nptII* gene, in pKYRT1-K and pKPSF2-K. Tumorigenicity was assayed for each cosmid clone in LBA4404 that contains the *vir* helper plasmid pAL4404 (Hoekema et al. 1983). Opine production was determined in tumors on tomato plants induced by Chry5, or by the nopaline strain C58 containing one of the appropriate cosmid clones of pTiChry5. Kanamycin-resistant T₁ seedlings of transgenic *Arabidopsis thaliana* plants transformed with binary vectors pBIN19 or pBINm-gfp5-ER were analyzed for cotransfer of opine production by the *vir* helper plasmids pKYRT1 or pKPSF2.

Fig. 2. Opine production and T-DNA presence in *Arabidopsis* plants transformed by *Agrobacterium tumefaciens* harboring pKYRT1 as the *vir* helper plasmid. **A.** *A. thaliana* plants were transformed with *Agrobacterium tumefaciens* NTL4(pKYRT1) containing binary vector pBINm-gfp5-ER. Twenty-one Km^r T_1 plants were selected and 18 T_1 plants grew large enough to be assayed for opines. Fourteen lines survived to the T_3 generation and were assessed by Southern analysis for the presence of T_R DNA from pKYRT1. **A.** Opine production. Extracts of plants were prepared and subjected to high-voltage paper electrophoresis. The developed electrophoretogram was stained with alkaline silver nitrate to visualize the Amadori opines. Extracts from lanes 1 and 22, wild-type *A. thaliana* supplemented with 3 mM mannopine; lanes 2 and 21, wild-type *A. thaliana*; and lanes 3 through 20, Km^r transgenic T_1 *Arabidopsis* lines A–I, K–O, Q–S, and U isolated following transformation by NTL4(pKYRT1, pBINm-gfp5-ER). **B.** Southern analysis of genomic DNA from transgenic *A. thaliana* lines. Total genomic DNA from T_3 plants, digested with *Hind*III, was subjected to Southern analysis with as probe a DNA fragment corresponding to the right-hand side of the T_R -DNA of pTiChry5. Lane 1, pPOC5E13; lane 2, pBINm-gfp5-ER + pKYRT1; lane 3, MW marker; lanes 4–16, DNA from lines A–C, E–G, I, L, M, O, Q, R, and S; and lanes 17 through 19, three segregating lines of the original line U. Lines D, H, K, and N for which opine content is shown in **A** did not survive to the T_3 generation.

thesis of the two Amadori opines, while pCH235 and its subclone pPOC5E5 lack the gene or genes necessary for the synthesis of Chy. Furthermore, these results suggest the possibility that chrysopine is synthesized from Dfg. The former opine could be synthesized from Dfg by a pathway similar to the lactonization of mannopine to agropine catalyzed by agropine cyclase. This enzyme is coded for by the T_R -DNA of octopine-type Ti plasmids (Hong et al. 1997). Consistent with this hypothesis, strain Chry5 produces an enzyme that lactonizes Dfg to Chy (Chilton et al. 1995). Furthermore, since the vectors used in this study do not provide T-region border sequences, at least one border repeat is present on *EcoRI* fragment 5 of pPOC5E5, while at least one other border repeat must be present on the *BamHI* fragment of pPOC5B6. Because the region defined by these clones lies well to the right of the known *onc* T-region (Fig. 1), we conclude from these results that pTiChry5 contains a second T-region harboring at the least the genes for synthesis of the Amadori opines. In this respect, the structure of the T-region of pTiChry5 could be similar to that of pTiBo542. This latter element also contains two T-regions (Hood et al. 1986). The first, T_L , harbors a set of oncogenes as well as genes for the synthesis of two opine families, succinamopine and agropinopines C and D. The second, T_R , harbors a second set of oncogenes as well as the genes for synthesis of the mannityl opines (Hood et al. 1986; P. Oger, V. Vaudequin-Dransart, Y. Dessaux, and S. K. Farrand, *in preparation*).

The *vir* helper plasmid pKYRT1 transfers Ti plasmid DNA to plants.

Given its supervirulent characteristics on soybean, *Agrobacterium tumefaciens* Chry5 has drawn interest as a tool for genetically engineering recalcitrant plant species. Torisky et al. (1997) reported the construction of a disarmed derivative of pTiChry5 called pKYRT1 that is deleted for a 16.5-kb segment overlapping the previously described oncogenic T-region (Fig. 1). However, our genetic analysis suggested that pKYRT1 still harbors at least one complete T-region, the T_R . To determine whether pKYRT1 transfers this second T-DNA to plant cells, we examined derivatives of *Arabidopsis thaliana* transformed by the binary strain KYRT1(pBINm-gfp5-ER) (Table 1). Twenty-one T1 *Arabidopsis* transformants selected for resistance to kanamycin encoded by the binary plasmid were examined for coinheritance of other traits. As assessed by fluorescence microscopy, all expressed green fluorescent protein (GFP) positive, consistent with the linkage of the *gfp* gene with the *nptII* gene on the binary T-DNA. Of the 18 T1 transformants that grew large enough to be assayed, eight (45%) produced the Amadori opines (Fig. 2). Furthermore, Southern analysis of genomic DNA from 14 plants that survived to the T3 generation revealed that all clones that tested positive for opine production also contained DNA homologous to the T_R -DNA of pTiChry5. Most of the lines that failed to produce the opines lacked sequences homologous to this T-DNA (Fig. 2). However, two lines, E and G, contain T-DNA but did not produce detectable levels of opines (Fig. 2). No lines were identified that produced opines and lacked sequences homologous to the T-DNA probe. In all cases examined, expression of *gfp* cosegregated with resistance to Km. However, these two traits segregated independently from opine production (data not shown). In an analysis of opine/T-DNA-positive line U, opine production cosegregated with the presence of DNA sequences

homologous with the T_R -DNA probe; segregants that produced opines retained the homologous DNA, while those that had lost the T_R -DNA failed to produce opines (Fig. 2B, lanes 17–19; data not shown). Thus, pKYRT1 transfers T_R -DNA to plants, and this DNA, which is stably integrated into the plant nuclear genome, confers production of the Amadori opines. Moreover, T_R is cotransferred with the engineered T-DNA on the binary vectors at high frequency. Given this observation, we do not recommend the use of pKYRT1 as a *vir* helper plasmid. Furthermore, this set of experiments focused only on the transfer of the T_R -DNA. Because KYRT1 produces a few, very small, gall-like growths on infected plants (Fig. 1) (Torisky et al. 1997) it remained possible that the transgenic plants also received and integrated a portion of T_L -DNA from borders still retained in pKYRT1. This possibility prompted us to identify and map the border sequences present on pTiChry5.

Location and nucleotide sequence of the border repeats defining the two T-regions of pTiChry5.

We located the likely borders of T_R -DNA by sequencing the two fragments contained in pPOC5E13 and pPOC5E5 (Fig. 1). Two sequences we propose as T_R LB and T_R RB were identified that showed strong similarities to the known T-region borders of other Ti plasmids (Fig. 3). The presence of a known T_R -DNA gene, *orf18*, just to the right of the left border repeat, and *chs*, a homolog of the agropine synthase gene, *ags* (Hong et al. 1997), which could code for chrysopine synthase (P. Oger, V. Vaudequin-Dransart, Y. Dessaux, and S. K. Farrand, *in preparation*), located just to the left of the right border repeat (Fig. 4), is consistent with our hypothesis that these repeats are the true borders of T_R . These two border repeats are located at coordinates 26.4 and 34.5 on the standard map of pTiChry5 (Fig. 4) (Kovacs and Pueppke 1994) and delimit an approximately 8-kb T-region that contains the genes required for synthesis of the Amadori opines (Fig. 1).

Chry5 T_L LB	c GGCAGGATATAT Tcaaa TGT AAAt
Chry5 T_L RB	TGGCAGGATATAT Tggga TGT cAAa
Chry5 T_R LB	TGGCAGGATATAT cgagg TGT AAAA
Chry5 T_R RB	TGGCAGGATATAT gcagg TGT cgtg
15955 LB-L	TGGCAGGATATAT Tgtgg TGT AAAg
15955 RB-L	c GGCAGGATATAT Tcaat TGT AAAt
15955 LB-R	TGGCAGGATATAT accgt TGT AAAt
15955 RB-R	TGGCAGGATATAT gcgg TGT AAAt
T37 RB	TGaCAGGATATAT Tggcg GGT AAAc
T37 LB	TGGCAGGATATAT Tgtgg TGT AAAc
C58 LB	TGGCAGGATATAT Tgtgg TGT AAAc
C58 RB	TGaCAGGATATAT Tggcg GGT AAAc
A4 TL LB	TGGCAGGATATAT Tgggt TGT AAAc
AB2/73 LB	TGGCAGGATATAT Tgggt TGT AAAc
AB2/73 RB	TGGCAGGATATAT cagat TGT AgTg
consensus	tGgCAGGATATAT tvnnd tGT aaac

Fig. 3. Nucleotide sequences of the likely T-region borders of pTiChry5 and their relatedness to border sequences from other Ti and Ri plasmids. The borders of T_L and T_R of pTiChry5 were located and sequenced, and the sequences were compared with those of other T-region borders in the data bases. Nucleotides in bold represent two core regions strongly conserved among known border sequences. Nucleotides shown in uppercase are predominant at any position; those in lower case are the rare substitutions. In the consensus sequence “v” represents an A, G, or C; “d” represents an A, G, or T; and “n” represents any nucleotide.

We located the probable borders of the left T-region by two approaches. First, we used a synthetic oligonucleotide corresponding to the right border sequence of T_R -DNA from pTiChry5 to probe Southern blots of pTiChry5 DNA. Such analyses of *EcoRI*-digested pTiChry5 DNA revealed three signals (data not shown). Two of these correspond to the left (*EcoRI* fragment 5) and right (*EcoRI* fragment 13) borders of T_R sequenced as described above. The third signal, located to *EcoRI* fragment 20, could represent the left border of T_L . This assignment is consistent with the biological data from Kovacs and Pueppke (1994) showing that the T_L -region spans from coordinates 191.6 to 7.6 on the map of pTiChry5. Sequence analysis of *EcoRI* fragment 20 identified an open reading frame with relatedness to the agrocinopine synthase gene, *acs* (data not shown), as well as a sequence, that we propose as T_L LB, with a strong match to known border sequences (Figs. 3 and 4). Furthermore, the sequence of this fragment was nearly identical (>99%) to a sequence located at the left end of the T_L -region of pTiBo542 recently determined in our laboratory (P. Oger, C. Reich, G. J. Olsen, and S. K. Farrand, *in preparation*). However, we were unable to identify any fragments that could code for the right border of the T_L -DNA by Southern analysis. Based on the hypothesis that the T_L -regions of the two Ti plasmids are colinear, we amplified a fragment containing the putative T_L right border of pTiChry5 by polymerase chain reaction (PCR) with primers designed from the right border region of T_L from pTiBo542. Analysis of the single amplicon from this PCR yielded a sequence likely to be the right border of T_L (Fig. 3). Mapping data located this sequence to a region approximately 15 kb to the right of the *acs* gene (Fig. 1). This places the right border of T_L approximately 2 kb to the right of the *6b* gene of pTiChry5 (R. Dinkins, *unpublished sequence*, U88627) (Fig. 4). The sequences of the proposed four T-region borders of pTiChry5 contain the two conserved core regions required by VirD2 for border processing (Fig. 3). However, the likely right borders of the two T-regions are somewhat more diverged from the consensus sequence in the nonconserved regions.

Disarming pTiChry5.

By our analysis, pKYRT1 still contains all of T_R and as much as 9 kb of T_L , including the left border repeat (Fig. 1). Although the presence of a right border greatly increases the frequency, a left border sequence is sufficient to initiate DNA transfer to plants (Ramanathan and Veluthambi 1995; Van der Graaff et al 1996; Kononov et al 1997). Hence, the presence of a single border sequence on a *vir* helper plasmid would result in the transfer of unwanted DNA from the plasmid to the plant (see comment by Smith 1998). In the case of pKYRT1, such transfer is supported by our observation of the integration into the plant genome and expression of the opine synthesis genes carried by T_R (Table 2; Fig. 2). Therefore, we constructed a new disarmed derivative of pTiChry5 that lacks both T-regions and all known border sequences.

The overall strategy, presented in Figure 5, was based upon the isolation of spontaneous deletion derivatives of the Ti plasmid exploiting the sensitivity to sucrose conferred by the *sacB* gene of *Bacillus subtilis* (Hynes et al. 1989). The *sacB-sacR-nptI* cartridge was cloned as a *Bam*HI fragment into the single *Bam*HI site of *EcoRI* fragment 9 of pTiChry5 (Fig. 1). This construction was marker exchanged into the Ti plasmid, resulting in pMx230 (Fig. 5). This plasmid was isolated from its Chry5 background and electroporated into NTL4 (Table 1) for further manipulations. Because the use of sucrose sensitivity as a selection can lead to the loss of the entire plasmid, an antibiotic resistance determinant was introduced into pMx230 to maintain selection for the Ti plasmid. To do this, a gene cassette coding for resistance to erythromycin (Ery) from pR29 (Table 1) was inserted as a *Cla*I fragment into a clone of *EcoRI* fragment 4 from pTiChry5. This fragment corresponds to a region of the Ti plasmid that is required for catabolism of the Amadori opines but is not essential for tumorigenicity (P. Oger, V. Vaudequin-Dransart, Y. Dessaux, and S. K. Farrand, *in preparation*). The cloning resulted in the removal of several small, internal, Ti plasmid *Cla*I fragments (Fig. 5). The mutated *EcoRI* fragment containing the Ery cassette, recloned into pRK415K to form pRKERd4, was marker exchanged into

Fig. 4. Fine structure of the two T-regions of pTiChry5. **A**, Structure of T_R . **B**, Structure of T_L . Locations of open reading frames exhibiting similarity to gene *6b*, to *orf18*, and to agrocinopine synthase (*acs*) from several Ti plasmids are shown by open arrows above the *EcoRI* map. *chs* represents putative chrysopine synthase gene. Black arrowheads indicate locations of the four probable border sequences that define the two T-regions.

pMx230 to give pMx106 (Fig. 5). Correct marker exchange events were confirmed by restriction endonuclease analysis and Southern hybridization (data not shown).

Potential deletion derivatives of pMx106 were obtained by selecting for colonies of NTL4(pMx106) able to grow on nutrient agar (NA) supplemented with 5% sucrose and 150 µg of erythromycin per ml. Approximately 100 sucrose-resistant, kanamycin-sensitive colonies were analyzed by DNA restriction analysis. Twenty-four clones with deletions covering both the T_L- and T_R-regions were further characterized by Southern hybridization. These clones fell into two classes: all members of the first set lack both T-regions as well as part of the *vir* region and are therefore useless as *vir* helper plasmids. Members of the second set retain *vir* but still contain part of T_L and therefore are not completely disarmed. To disarm the plasmid fully, we repeated the selections for sucrose sensitivity starting with pKPSF1, one of the partially disarmed derivatives of pTiChry5 isolated during the first round of screening. This plasmid suffered a deletion that removed the entire T_R-region, but it still retains approximately 3 kb of the left end of T_L (Fig. 1). The *sac* cassette was marker exchanged into *Eco*RI fragment 20 of pKPSF1, which contains the likely left border of T_L, and clones were selected for resistance to sucrose. Of several sucrose-resistant, kanamycin-sensitive clones analyzed, each now lacked this leftmost region of T_L. One clone with a deletion extending from the right end of *Eco*RI fragment 1 through a portion of *Eco*RI fragment 17a (Fig. 1) was chosen as a putative fully disarmed derivative and named pKPSF2. According to our mapping data, the deletion in this plasmid removed a segment extending from several kilobases outside of and to the left of the left border of T_L to several kilobases outside of and to the right of the right border of T_R (Fig. 1). However, the deletion does not extend into the *vir* region. When tested for virulence on tomato plants, strains harboring pTiChry5 produced large, opine-containing tumors, while KYRT1 produced a few, very small, tumorlike growths (Table 2). A strain harboring pKPSF2 was completely avirulent on this host plant (Table 2).

T-DNA transfer by and *vir* helper properties of pKPSF2 and pKYRT1.

We tested the *vir* helper properties of pKPSF2 by constructing two binary strains, NT1(pKPSF2, pBIN19) and Chry5c(pKYRT1, pBINm-gfp5-ER). *A. thaliana* was transformed with each strain and progeny resistant to kanamycin were selected and analyzed for coinheritance of other traits. As judged by the appearance of transformants resistant to kanamycin, both *vir* helper plasmids transferred the binary T-DNA with the same efficiency (Table 3). Furthermore, both transferred the binary T-DNA with an efficiency essentially indistinguishable from that of a standard *vir* helper plasmid, pMP90, present in GV3101 (Table 3). However, while plants transformed with pKYRT1 coinherited opine production at a high frequency, transformants produced by pKPSF2 did not contain detectable levels of these compounds (Table 3).

We also tested for the transfer of unwanted DNA from the *vir* helper plasmid to the plant cells. To do this, we inserted a plant-selectable marker, *nptII*, driven by the 35S promoter of *Cauliflower mosaic virus*, into pKPSF2 and pKYRT1 as a whole-vector, Campbell-type cointegrate (Table 1). In each plasmid, the marker was inserted into *Eco*RI fragment 1 di-

rectly counterclockwise from the left edge of the deletion region to create plasmids pKPSF2-K and pKYRT1-K, respectively (Fig. 1). *A. thaliana* transformed with *Agrobacterium tumefaciens* strains harboring pKYRT1 or pKPSF2, which do not contain the *nptII* gene, did not yield kanamycin-resistant transformants at a detectable frequency (Table 3). Similarly, no transformants were obtained from approximately 27,000 seeds tested following infection with NT1(pKPSF2-K). However, infection with Chry5c(pKYRT1-K) yielded five kanamycin-resistant, transgenic plants from about 10,000 seeds plated (Table 3). Although the frequency is low, these results clearly demonstrate that one or more of the border repeats remaining on pKYRT1 can direct transfer of *vir* helper plasmid DNA to the plant. This transfer occurs in addition to transfer of the T_R-DNA also present on this plasmid. On the other hand, our disarmed plasmid, pKPSF2, does not detectably transfer Ti plasmid DNA to the plant. These results are consistent with our mapping data showing that pKPSF2 lacks both T-regions and their associated border sequences. This plasmid therefore constitutes a safe alternative to pKYRT1 for use as a *vir* helper plasmid.

In conclusion, our analyses indicate that pTiChry5 contains two T-regions. One, T_L, encodes the *onc* functions and also a gene that could code for the production of the agrocinopine-type opines. The second, T_R, confers production of the Amadori-type opines Dfg and chrysopine. A complete T_R, as well as the likely left border of T_L, is retained in pKYRT1, and these borders can and do direct the transfer of pKYRT1 DNA to plant cells when this element is used as a *vir* helper plasmid. On the other hand, pKPSF2, which is deleted for all known T-regions and their borders, retains *vir* helper activity but does not detectably transfer Ti plasmid DNA to infected plants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

Bacteria and plasmids used in this study are described in Table 1. Strains of *Agrobacterium tumefaciens* were grown at

Table 2. Tumorigenicity of *Agrobacterium tumefaciens* harboring pTiChry5 and its deletion derivatives

Plasmid	Characteristics	Tumorigenicity on tomato ^a	Amadori opines in tumors ^b	
			dfg	chy
None		—	—	—
pTiChry5	Wild-type Ti plasmid	++++	++++	++++
pKYRT1	Deletion derivative of pTiChry5	+/- ^c	NT ^d	NT
pKPSF2	Deletion derivative of pTiChry5	—	NA ^e	NA

^a Assessed by inoculating tomato plants with liquid cultures of indicated strain. +++++, Strongly tumorigenic with large, unorganized tumors at all inoculation sites; +/-, very weakly tumorigenic with small growths at a few of the inoculation sites; and —, no tumorous response detected.

^b Assessed by high voltage paper electrophoresis of extracts prepared from normal or tumorous tissues.

^c Very small, tumorous growths distinguishable from wound callus arose at a few wound sites.

^d Not tested. Growth induced by KYRT1 did not yield sufficient tissue for meaningful assays.

^e Not applicable.

Fig. 5. Strategy for construction of pMx106. The *sac-nptI* cassette and erythromycin resistance cassette were introduced into pTiChry5 by two sequential marker exchange events yielding pMx106. This latter plasmid was used to select for deletion derivatives of pTiChry5 culminating in isolation of pKPSF2. Locations of the two T-regions, and the *vir* region on pTiChry5, as well as the sites of the insertions of the two gene cassettes on pMx106, are indicated. Vectors and intermediate clones are not drawn to scale.

28°C on NA (Difco, Detroit, MI, U.S.A.) and in Luria broth (LB) (Sambrook et al. 1989) or in AB minimal medium (Chilton et al. 1974) with mannitol (0.25%) as the carbon source. Strains of *Escherichia coli* were cultured in LB medium at 37°C. All media were solidified with 16 g of Bacto Agar per liter (Difco). Antibiotics were used at the following concentrations in micrograms per milliliter. *E. coli*: ampicillin (Ap), 100; kanamycin (Km), 50; gentamicin (Gm), 30; erythromycin (Em), 100; and tetracycline (Tc), 10; *Agrobacterium tumefaciens*: carbenicillin (Cb), 100; Km, 100; Gm, 100; Em, 150; Tc, 2; and rifampicin (Rif), 100. X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was used at 40 μ g/ml when needed.

DNA isolation, marker-exchange mutagenesis, and recombinant DNA techniques.

Plasmid DNA was isolated from *E. coli* by the alkaline lysis method described by Sambrook et al. (1989). Ti plasmid DNA and recombinant clones were isolated from *Agrobacterium tumefaciens* by an alkaline lysis procedure as described by Hayman and Farrand (1990). Plasmids were introduced into *Agrobacterium tumefaciens* by electroporation (Cangelosi et al. 1991) or by biparental matings with the *E. coli* strain S17-1 (Cook et al. 1997). Marker exchange of mutated fragments was performed and confirmed as described previously (Farrand et al. 1996).

Total genomic DNA of *A. thaliana* was extracted from 10 to 25 mg of freeze-dried plant tissue after pulverization for 5 s at speed setting 4.0 in a FastPrep FP120 shaker (Savant Instruments, Farmingdale, NY, U.S.A.), as described by Wang et al. (1994), omitting the cesium chloride purification step.

Nucleotide sequence analysis.

Complete double-stranded nucleotide sequence determinations were done by the Keck Center (University of Illinois, Urbana, U.S.A.) and by Bio S&T (Lachine, Québec, Canada). Sequences were assembled and analyzed with Sequencher (Gene Codes, Ann Arbor, MI, U.S.A.). Open reading frames were identified with the Map subroutine of the GCG package (Genetics Computer Group, Madison, WI, U.S.A.) and with DNA Strider (Mark 1988). Sequence comparisons were performed with the BLAST protocols accessible on-line from NCBI. Sequences were deposited in the GenBank data base under the accession numbers AF229155 and AF229156 for the right and left borders of T_L, respectively, and AF229157 for the left and right borders of T_R.

PCRs.

A fragment containing the right border of T_L was amplified from purified pTiChry5 DNA by PCR with the primer set

Table 3. DNA transmission by and *vir* helper properties of deletion derivatives of pTiChry5

<i>vir</i> Helper plasmid	Characteristics	Binary plasmid	Fraction of seeds plated growing on selection plates ^a	Fraction of Km-resistant plants tested producing Amadori opines ^b
pMP90	Nopaline-type	pBINm-gfp5-ER	0.0115	NT ^c
pKYRT1	Chrysopine-type	None	<0.00005	NA ^d
		pBINm-gfp5-ER	0.0085	0.45
pKPSF2	Chrysopine-type	None	<0.00005	NA
		pBIN19	0.01	<0.05
pKYRT1-K	Contains a plant-expressible Km resistance gene	None	0.0005	NT
pKPSF2-K	Contains a plant-expressible Km resistance gene	None	<0.00004	NA

^a Assessed by plating seeds of *Arabidopsis thaliana* exposed to infection with *Agrobacterium tumefaciens* harboring indicated plasmids on Murashige-Skoog (MS) medium containing 50 µg of kanamycin per ml. Between 10,000 and 27,000 seeds (approximate) were plated on selective media depending on the experiment.

^b Assessed by high-voltage paper electrophoretic analysis of extracts prepared from kanamycin-resistant T3 plants. Where available, up to 20 plants were screened from each transformation event.

^c Not tested.

^d Not applicable.

RBF (5'-AGAGCGTCGTTGGATTTCG-3') and RBR (5'-CTCCGCACAAGTTACAAAC-3') synthesized by the University of Illinois Keck Center. Conditions for the PCR amplification were as follows. Initial denaturation: 95°C, 3 min. Pre-amplification cycles: 95°C, 30 s; 50°C, 1 min; 72°C, 2 min; 5 cycles; amplification: 95°C, 30 s; 58°C, 1 min; 72°C, 2 min; 30 cycles; final extension: 72°C, 10 min. PCR-generated fragments were purified by agarose gel electrophoresis and sequenced with the amplification primers.

Southern hybridization analysis.

pTiChry5 and its deletion derivatives were digested to completion with *Eco*RI. The fragments were separated by electrophoresis through 0.8% agarose gels, and transferred onto charged nylon membranes (Boehringer-Mannheim, Mannheim, Germany). Probes were prepared from cosmid clones of pTiChry5 (Table 1), including pCH230 (*T_L*-region), pCH3701 (region to the left of *T_L*), and pCH1755 (*T_R*-region), and labeled with the digoxigenin (DIG)-labeling kit from Boehringer-Mannheim as described by the manufacturer. The *T_R* right border oligonucleotide (5'-TGCGAGGATATGACG GTGTCGTG-3'), synthesized by the University of Illinois Keck Center, was end-labeled with the oligonucleotide labeling kit from Boehringer-Mannheim. Southern hybridizations were performed at high stringency in hybridization buffer without formamide at 68°C for cosmid probes and at low stringency at 59°C when the synthetic border probe was used. *Arabidopsis* DNA was digested overnight with *Hind*III. The fragments were separated on 0.8% agarose gels, and transferred onto charged nylon membranes (Amersham-Pharmacia, Piscataway, NJ). The samples were hybridized with a ³²P-labeled probe consisting of a 2.07-kb, *Eco*RI-*Xmn*I fragment of pPOC5E13 that contains an internal portion of *T_R* from pTiChry5. Hybridizations and washes were conducted under conditions of high stringency as described above.

Plant transformation and tumorigenicity testing.

Pathogenicity tests were performed on 3-week-old tomato plants grown in the greenhouse. Stems of plants were inoculated with a needle dipped into an overnight culture of the bacterial strain to be tested. Additionally, the cut apex was inoculated with a loopful of bacteria from the same culture. Production of tumors was scored 5 to 6 weeks after inoculation. Transformation of *A. thaliana* (ecotype Col-0) was accomplished by

dipping flowering plants into suspensions of *Agrobacterium tumefaciens* and selecting transformants from *T₁* seed spread on plates of MS medium supplemented with 50 µg of kanamycin per ml (Clough and Bent 1998).

Analysis for expression of *gfp*.

Expression of *gfp* in transgenic *A. thaliana* plants was assessed by fluorescence microscopy as described by Clough and Bent (1998).

Opine analyses.

Opines in tomato tumors or in transgenic *Arabidopsis* plants were detected by high-voltage paper electrophoresis (HVPE) as described by Dessaux et al. (1992). Nopaline was visualized with the phenanthrene quinone reagent (Dessaux et al. 1992), while the Amadori opines were detected with alkaline silver nitrate chemistry (Dessaux et al. 1992). Mannopine (Sigma Chemical, St. Louis, MO, U.S.A.), which also stains with alkaline silver nitrate, electrophoreses with the same mobility as deoxyfructosyl glutamine (Chilton et al. 1995) and was used as the standard in place of this relatively unstable Amadori opine. Chrysopine was the generous gift of W. S. Chilton (North Carolina State University, Raleigh, U.S.A.).

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