A T-DNA from the *Agrobacterium tumefaciens* Limited-Host-Range Strain AB2/73 Contains a Single Oncogene

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Agrobacterium tumefaciens strain AB2/73 isolated from *Lippia canescens* has been described as a limited-host-range strain. Its tumor-inducing (Ti) plasmid has been found to lack DNA homology to known T-DNAs (L. Unger, S. F. Ziegler, G. A. Huffman, V. C. Knauf, R. Peet, L. W. Moore, M. P. Gordon, and E. W. Nester. J. Bacteriol. 164:723-730, 1985). We have isolated a T-DNA from AB2/73 by using a heterologous border sequence as a probe. The AB2/73 T-DNA sequence (3,504 bp) is flanked by canonical border sequences, has no detectable DNA homology with other T-DNAs, and contains only two genes: *lsn* (*Lippia* strain nopaline synthaselike gene) and *lso* (*Lippia* strain oncogene). The *lso* gene induces nondifferentiating tumors on a limited number of hosts when transferred by a Ti plasmid from a wide-host-range strain. Part of the predicted Lso protein is weakly homologous to other *Agrobacterium* oncoproteins encoded by *rolB*, *rolB^{TR}*, *orf13*, gene *e*, gene 5, and gene 3'. A 28-kb fragment corresponding to the *virA* to *virE* region was cloned by using a heterologous *vir* fragment as probe. The AB2/73 *vir* region is homologous to most of the C58 virulence region; however, the *virA* gene is most related to the *virA* gene of the *Agrobacterium vitis* limited-host-range strain Ag162.

Virulent *Agrobacterium* strains induce plant growth by transfer of one or several oncogenes into plant cells. The oncogenes are carried by one to three fragments of the tumor-inducing (Ti) or root-inducing (Ri) plasmid called T-DNA. T-DNA oncogenes may induce roots (as in the case of the *iaa* auxin biosynthesis genes or the *rol* genes), shoots (the cytokinin synthesis gene *ipt*), or tumors (the *6b* gene) (reviewed in Gaudin et al. 1994).

T-DNA transfer is under the control of chromosomal and Ti plasmid-located virulence (*vir*) genes. Wounded plant cells secrete phenolic compounds that are detected by the *virA* gene product, which then activates the transcriptional regulator

protein VirG. The latter induces various *vir* genes, leading to the synthesis of a single-stranded copy of the T-DNA (T-complex) and its transport to the plant cell nucleus where the T-DNA is integrated. The T-DNA is limited by 25-bp border sequences that are recognized by the VirD2 endonuclease. In some cases, T-DNA synthesis is enhanced by the so-called "overdrive sequence" located outside the T-DNA close to the right border. This sequence is recognized by the VirC1 protein (reviewed in Zupan and Zambryski 1995, 1997; Sheng and Citovsky 1996; Winans et al. 1996).

In 1979, an *Agrobacterium tumefaciens* strain with unusual host range properties was reported: AB2/73 (Anderson and Moore 1979). AB2/73 belongs to biotype 2, does not use octopine, nopaline, agropine, or mannopine, and induces tumors on *Lippia canescens* (from which it was isolated), the cucurbit family, and *Nicotiana glauca*, but not on other commonly used test plants like Kalanchoe, tobacco, or tomato. Thus, it has a limited host range. Molecular and genetic analysis showed that AB2/73 carries a 500-kb Ti plasmid (called pAtAB2/73d) with homology to the *vir* region from pTiA6. However, AB2/73 was found to lack homology to the T-DNA of A6 (*A. tumefaciens*) or to the TL-DNA of A4 (*A. rhizogenes*), suggesting that AB2/73 contained an as yet unknown type of oncogene or oncogene combination (Unger et al. 1985). Here we report the identification of an AB2/73 T-DNA sequence flanked by classical T-DNA borders. This T-DNA contains a nopaline synthaselike gene and a single oncogene. A protein homology search showed that the AB2/73 oncoprotein belongs to a highly diverged family that includes the products of the following T-DNA genes: gene 3' (Barker et al. 1983), gene *e* (Broer et al. 1995), gene 5 (Körber et al. 1991), *rolB* (Schmülling et al. 1988), *rolB^{TR}* (Lemcke and Schmülling, *in press*), and *orf13* (Hansen et al. 1993), and possibly those of gene *6b* (Tinland et al. 1989), *6a* (Messens et al. 1985), and *orf14* (Hansen et al. 1993).

RESULTS

Isolation of part of the AB2/73 virulence region.

AB2/73 DNA hybridizes to probes derived from the A6 *vir* region (Unger et al. 1985), suggesting that the general mechanism of T-DNA transfer is similar to that of other Ti plasmids. To isolate the AB2/73 *vir* region, we first constructed *Bam*HI, *Pst*I, and *Xma*I banks of total AB2/73 DNA in pUC18. A *vir*

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Nucleotide and/or amino acid sequence data are to be found at GenBank as accession nos. AF029715 to AF029725 and U96413.

probe derived from pTiAch5 allowed the cloning of a 12-kb partial *Pst*I fragment from the AB2/73 *vir* region that was then used to clone adjacent fragments (see Materials and Methods); the final region has a length of 28 kb and contains most of the classical *vir* genes: *virA* to *virE*. The cloned region was mapped with restriction enzymes and partially sequenced (GenBank accession nos. AF029715 to AF029725, representing 15 kb; Fig. 1). It aligns for most of its length with the C58 virulence region (Rogowsky et al. 1990); DNA homology values in the *virB* to *virD* region range from 59 to 82%. To the left, a *virA* gene sequence is found with 74% homology to the *virA* gene of the limited-host-range strain pTiAg162 (Leroux et al. 1987) and 59% homology to the *virA* gene of C58. Like the C58 *vir* region, the AB2/73 *vir* region lacks IS66v1 and IS66v2 found on pTiA6 (Machida et al. 1984). To the right, the cloned region ends in the middle of *virE2*. Homology values in the *virE* region vary. The similarities between most of the AB2/73 and C58 *vir* regions suggested to us that an AB2/73 T-DNA could be flanked by classical border sequences. We therefore searched a genomic AB2/73 bank for clones with homology to a heterologous T-DNA border.

Isolation and sequence of an AB2/73 T-DNA.

In preliminary experiments, T-DNAs from several Ti plasmid types (pTiAch5, pTiTm4, pTiS4; Otten et al. 1992) were hybridized to Southern blots with restriction enzyme-digested AB2/73 DNA. None hybridized (results not shown), confirming the results of Unger et al. (1985). Subsequently, a 23-bp oligonucleotide sequence containing the left border of the Tm4 TA region (Fig. 2A) was hybridized to a *Bam*HI clone bank of AB2/73 and yielded a weakly positive clone (pPM3804) with a 10-kb insert. pPM3804 (10,681 bp) was sequenced (GenBank accession no. U96413) and showed a left and a right T-DNA border sequence (Figs. 2 and 3) at positions 4024 to 4048 and 7503 to 7527 flanking two open reading frames (ORFs) that may code for typical T-DNA functions (see below). To the right of the right border, an overdrive-like sequence was found (Fig. 2C). The characteristics of the 4024 to 7527 sequence indicate

that this is a small (3.5 kb) but functional T-DNA; moreover, like other T-DNAs it is surrounded by sequences with homology to bacterial insertion sequences (see below).

Search for other T-DNAs.

Several Ti plasmids carry more than one T-DNA. To detect the presence of possible additional AB2/73 T-DNAs, two oligonucleotides representing the left and right AB2/73 T-DNA borders (see Materials and Methods) were hybridized to a Southern blot with total AB2/73 DNA digested with *Eco*RI, *Pst*I, *Sal*I, and *Sac*I. Only fragments corresponding to the already identified T-DNA borders were detected, even after overexposure of the autoradiographs (results not shown).

The Lso protein is homologous to a family of T-DNA oncoproteins.

A BLAST search (Altschul et al. 1990) with the AB2/73 T-DNA sequence did not yield related DNA sequences but a BLASTP search (Gish and States 1993) showed that the ORF in the left portion of this sequence (4428 to 5156) may code for a protein of 242 amino acids that resembles (Figs. 4 and 5) various oncoproteins encoded by other T-DNA genes: *rolB*, *rolB^{TR}*, and *orf13* (from various Ri plasmids), gene 3' (TR region of pTiAch5, T-DNA of pTiAB4 and pTi82139), gene 5 (TL region of pTiAch5), and gene *e* (pTiC58); see Table 1. Homology values are weak (25 to 35% identity, 30 to 72% similarity) and concern only part of the proteins. When the products of the two 3' genes and *rolB/rolB^{TR}* are excluded, none of the proteins of this group are strongly related (similarity scores from 0.35 to 0.50). Since the protein encoded by gene 5 has been well characterized (Körber et al. 1991), we have also included the comparison of this protein with the others (Fig. 4, italics). As will be shown below, the ORF from AB2/73 can induce tumors without help from other oncogenes. It was therefore called *Lippia* strain oncogene or *lso*.

The ORF in the right portion of the AB2/73 T-DNA (5504 to 6652) may code for a protein of 383 amino acids with homology (47% identity) to the C58 and AB4 nopaline syn-

Fig. 1. Map of the AB2/73 virulence region. Top: Organization of the pTiC58 virulence region (Rogowsky et al. 1990). *vir* loci are indicated by A–E. SEQ: sequenced parts of AB2/73 *vir* region. Black: regions homologous to pTiC58 *vir* region (Rogowsky et al. 1990); white: no homology to other DNA sequences. *virE1/virE2* and *virC1/virC2* are named according to A6 *vir* sequences X04784 and M14480. pPM3801, pPM3803, and pPM3816: pUC18 derivatives carrying parts of AB2/73 *vir* region. P1, P2, and P3: probes used for cloning; P1 is from pTiAch5; P2 and P3 are from pPM3801. Dashed lines indicate regions with protein homology only.

thases and was called *Lippia* strain nopaline synthaselike gene or *lsn* (alignment not shown). The activity of this gene remains to be tested. Thus, the cloned T-DNA is a minimal structure that potentially encodes the two essential T-DNA functions: tumor induction and opine synthesis. The biological activity of the *lso* gene was tested by transfer into a disarmed vector and infection of different plant species.

The *lso* gene is a limited-host-range oncogene.

The *lso* gene was cloned on a 4-kb *Eco*RI fragment (U96413 coordinates 1695 to 5762) into the T-DNA of the

A.
CGGCAGGATATATTGAATTGTAAAT AB3/Tm4, TA
 AB4
 CG474
C.....C ACH5/15955, TL
T.G.....A S4, T1
 T.....TGA.....C A4, TL
 T.....TGG.....C C58
 T.....CA..G.....G S4, T3
 T.....C..GG.....A ACH5/15955, TR
 T.....G.....TCA.....A S4, T2
 T.....C.TTA.....TC AB3/Tm4, TB
 T.....GTG.....C AB2/73
 -GGCAGGAT-TAT-----TGTA-- invariable

B.
 TGACAGGATATATACCGTTGTAATT AB3/Tm4, TA
 ..G..... CG474
 ..G..... ACH5/15955, TL
 ..G.....G..... ACH5/15955, TR
GGT.A...C.CG S4, T2
GTTC...C..G A4, TL
CTT..G..C.CG A4, TR
TGCGG...AC C58/AB4
T...CGTTA...C..G S4, T1
 ..G.....T...TG.TAA...C..C S4, T3
 ..G.....G...CAGA...G.G AB2/73
 TG-CAGGAT-TAT-----GT---- invariable

A. and B.
 -G-CAGGAT-TAT-----GT---- invariable

C.
 RB-14nt-TAAATCGCTGTGTATGTTTGTGTTG AB3/Tm4
 RB-14nt-.....A.....A..... CG474
 RB-14nt-...G..... ACH5/15955,
 RB-13nt-....TT....A.T..... ACH5/15955,
 RB-15nt-CCCC.AT...C...AC..... AB2/73
 RB-8nt -.TTG.GTGGAG.....A A4, TR
 T-----T---T-TGTTT-

Fig. 2. Comparison between T-DNA borders from AB2/73 and other *Agrobacterium* strains. **A**, left borders; **B**, right borders; **C**, overdrives. Underlined: sequence used as a probe to isolate pPM3801. Most sequences are from Van Haaren et al. (1988), others from our own work (L. Otten, unpublished). The AB3/Tm4 left and right border are taken as arbitrary standards.

binary vector pBin19 (Bevan 1984). This *Eco*RI fragment includes the left border of the AB2/73 T-DNA. Two orientations were obtained: the one in which the left borders of the pBin19 T-DNA and the AB2/73 T-DNA were in the same orientation (pPM3805) was retained. pPM3805 was transferred into the *Agrobacterium* helper strain LBA4404, which carries the virulence region of the wide-host-range Ti plasmid pTiAch5 (Hoekema et al. 1983). Different plant species were infected with LBA4404(pPM3805). AB2/73 and LBA4404(pBin19) were used as positive and negative controls, respectively. In a first series of experiments, stems of intact plants were infected under greenhouse conditions. The results (summarized in Table 2 and shown for *N. rustica* and *K. tubiflora* in Figure 6) show that the *lso* gene induces outgrowths on a limited number of hosts. Interestingly, whereas AB2/73 induces small outgrowths on *N. glauca* and *N. rustica*, the *lso* gene does not.

In a second series of experiments, AB2/73, LBA4404 (pPM3805), and LBA4404(pBin19) were used to infect leaf fragments of *N. rustica* and *N. glauca* in vitro on medium without hormones. Under these conditions, AB2/73 induces necrosis and no cell growth is observed (results not shown). The *lso* gene induces calli on *N. rustica* leaf fragments contrary to the LBA4404(pBin19) control, which only leads to some root induction due to wounding (Fig. 7); no growth is observed on *N. glauca* fragments (not shown). The properties of the *lso* gene and those of the other members of the *rolB* family are listed in Table 1.

Surroundings of the T-DNA.

The immediate surroundings of the AB2/73 T-DNA (4 kb to the left and 3 kb to the right) were searched for other pTi-like sequences. Starting at the left of the T-DNA, the 1206 to 2307 region has homology to the transcriptional repressor *DegA* from *Bacillus subtilis* (Bussey and Switzer 1993). The sequence from 3233 to 3866 may code for a truncated IS3-like transposase (about 80 amino acids shorter; Timmerman and Tu 1985). We could not detect insertion element (IS)-characteristic inverted repeats around this sequence.

To the right of the T-DNA (4024 to 7527), the 7737 to 8517 region corresponds to a degenerated transposase gene (Mariani et al. 1993) with a frameshift at position 8192. This region is bracketed by an inverted repeat (7736 to 7758 and 9110 to 9132) that delimits an IS-like element and is followed

Fig. 3. Overall organization of the AB2/73 T-region. Protein similarity values are indicated above the putative coding sequences. *degA*, *tpIS3*, *lso*, *lsn*, *tp*, *orf4*, and *orf5*: sequences homologous to *degA* from *Bacillus subtilis*, a truncated transposase gene of IS3, *lso* gene, *lsn* gene, a degenerated transposase gene from *Mycobacterium tuberculosis*, *orf4* and *orf5* from IS866 from *Agrobacterium vitis*, respectively. LB and RB: left and right T-DNA border.

by a 1.4-kb region (9209 to end of sequence) with 81% homology to part of the 2.7-kb IS866 sequence of *A. vitis* (Bonnard et al. 1989a) and 89% homology to an IS866-like element of *Rhizobium meliloti* (U66830.Gb_Ba; S. Au, J. Bergeron, and P. Dion, unpublished). Thus, as in the case of other T-DNAs, the AB2/73 T-DNA is surrounded by ISs or remnants of ISs. The various features of the 10-kb sequence are summarized in Figure 3.

DISCUSSION

We have isolated a T-DNA from strain AB2/73 that is surrounded by classical T-DNA borders and an overdrive sequence. This T-DNA carries a nopaline synthaselike gene (*lsn*) and a gene that encodes a protein with weak homology to various T-DNA oncoproteins (*iso*). The enzymatic properties of the Lsn protein and the structure of the opine(s) in AB2/73-induced tumors remain to be tested. In view of the homology between Lsn and the classical nopaline synthase gene it may be interesting to isolate and characterize the corresponding opine degradation genes of AB2/73, especially since Unger et al. (1985) reported that AB2/73 does not utilize nopaline, octopine, mannopine, or agropine.

The *iso* gene induces tumors on *K. tubiflora* and *K. daigremontiana* stems but not on *N. glauca*, *N. tabacum*, *N. rustica*, or *L. esculentum* stems. On *N. rustica* leaf fragments the *iso* gene induces calli in the absence of hormones that continue to grow for several weeks in the absence of bacteria; the *iso* gene may therefore be considered to be a T-DNA oncogene. The limited host range of the *iso* gene could be one of the factors that limit the AB2/73 host range.

Fig. 4. Alignment of the Lso protein with other oncoproteins from the RolB family. The alignment was constructed with the Pileup program (UW-GCG software). Underlined: sequence fragments with similarity to the Lso protein as found by the BLASTP program. Italics: sequence fragments with similarity to the gene 5-encoded protein as found by the BLASTP program. Abbreviations: A4rlb, Rlbtr, Av3pr, At3pr, Iso, octg5, genee, A4r13: *rolB* from the TL-DNA of *Agrobacterium rhizogenes* strain A4 (X03433), *rolB^{Tr}* from the TR-DNA of A4 (X15952), 3' gene from *A. vitis* strain AB4 (X77327), 3' gene from the TR-DNA of *A. tumefaciens* strain Ach5 (X00493), *iso* from AB2/73 (this work), gene 5 from the TL-DNA of Ach5 (modified from X00493), gene *e* from *A. tumefaciens* strain C58 (modified from Z29717), *orf13* from the TL-DNA of A4 (K03313).

Fig. 5. Schematic representation of the protein similarities of various oncoproteins. Thickness of the lines indicates degree of sequence similarity. Proteins encoded by gene 6a (X00493) and *orf14* (K03313) were compared with the protein encoded by gene *e*. Scale indicates numbers of amino acids.

Since AB2/73 induces small tumors on *N. glauca* and *N. rustica* stems, whereas the *iso* gene does not, the *iso* gene may not be the only AB2/73 host range determinant. It is possible that AB2/73 contains an additional T-DNA with further oncogenes. Their activity may be detected by mutating the *iso*

	1				50
A4rlb	.MDPKLLFLF	RFQPVDLTPA	WSOINLEFEG	RFAFAIYSRD	YSKPLLLHFOK
Rlbtr	.MGSRLILRE	YFPPRNLCBS	QDATLLREL	QDAFDSYNOS	FRGEIMRFOK
Av3pr	...MEYNRE	MFRPRYVKFV	SDSLGDFRL	RESRDAYVGF	INSTLASAOK
At3pr	...MEYNRE	HFQPTYVNF	SASOVNSIL	AGSKEAYLTY	LNSTLASAOK
Octg5	...MYDQGP	IFNIDSSNL	QDRRELKVL	IHTENAYRSS	AQSLIASOR
Lso	...MEENRE	VEIPSNLCV	ERPEOYAI	ETALNVYRKE	VVEDLMKROK
Genee	...MMVPPRP	SSAPLSLEST	NDRMEMHFL	TSACILYHYI	IRDNLIIYQE
A4r13	MARYCSGGSG	RLHVDDFRNI	KEPTRLKAQL	INVVEYTKAA	QTETLKYITS
A4rlb	51				100
Rlbtr	RWALAVLDLK	ENSPPYILK	QLAELLKNKV	CYHPMPFVSQ	PDLARENDQH
Av3pr	AFABRVLNMV	EARYPVNQLK	INAILTRKV	CYHSPAH.DP	LDLALEROQY
At3pr	VLEESILKAK	VCAA...DGR	LSHFSEAPCL	QGPSFDTL..	.YSVLPPTD
Octg5	SWAKSIKAG	LMIS...DEN	LECFSEVPCL	HGPDFDSL..	.DVVLPPDYDL
Lso	SWINFINTD	VPIDPAKDEV	VKCSRKVACC	PDPTDIPF..	.DI...LNVSL
Genee	EWATVPLTA	DEYWRVAGE	FFTKPLLDG.	.PAKGRGE..	.REVOLDRAL
A4r13	IWTKY.WRGR	LAESALKNEI	SALYLRLPFP	RPPTLQSTAE	QEVICKDAAP
	SATERVAHVE	AAEVNNAEME	L.....		.HPAGLKYPL
A4rlb	101				150
Rlbtr	VFVYLSREKM	QKVLKEQSIT	FGM...EAVL	ATTIOPYRSE	LALOEMLRVH
Av3pr	LFYVVSCEKI	QELLFNRRFL	VG...EAAV	ATSIOPYRSE	LSRGEMARVH
At3pr	MYVYSSDQI	HRFVECGEFL	CTH...ASRFV	ACTESPYTGG	VTSQTLWKWY
Octg5	LVFYGTSHV	QQFVECGNPL	CTH...ASRFV	ACTESPYTGG	VTAQTLRWY
Lso	LVVYCSQEM	RRYAQORFYD	GVS...DGGAV	ISTVPPYAEI	ITKOTMRLWQ
Genee	LVVYSGPRDEI	ADYVKTRTLH	RPR...RODVT	ACTMPPFERGE	ICKOETLRHL
A4r13	LVVYCSREAM	LSHVQSKDLG	QFG...RANLL	ACTLAPYRTG	VTEMVRCLE
	SVFVTSLAVA	TACKENKHL	CEEHLGDLI	SCVPPPYQTN	VSLAALREHL
A4rlb	151				200
Rlbtr	NL...AWPH	SRTTEPDLEC	FIATASSLF	IHLLELKVNT	VYGREVACTF
Av3pr	NL...AWRR	ROTKESEDMC	FVATESSTLF	VSWYEARAVD	IYGEVLCPPF
At3pr	NQISPGSRL	...SLKDVFA	FEAFPLNLSF	.FKTNYSLLE	IHGMRHOKFL
Octg5	NKISPCSRL	...SLNDYGA	FEAFPLNLSF	.LSTVHTVVE	IHGTVHVKFL
Lso	NKLVONTSK	...ETHDLDA	VTALLPNASL	.QNPNSHMK	IGNSFLSFL
Genee	NELSEVSYN	...TDADVEY	VYALLNCTE	.AEGPSLEL	SSGEYCGREH
A4r13	KRLRDGNRVE	AGTGEEDLSH	FVAFLPTTAE	.RRGPRFYL	...GGENVQFE
	NSISGGGYO	...ROADMDY	FVAITPDNDF	DYQSCBIDTR	SCGKGLCKIY
A4rlb	201				250
Rlbtr	FLRRGTENRP	YD.VVACGTT	QFTKNALGIS	RPAASSPEPD	LTL...RLSGP
Av3pr	LTRQATGDRD	YD.VVAYGFT	KEAENATSRR	LPAASSFLD	LKLGPVVSAT
At3pr	API.PGSRFR	CE.VVAFGEA	F.....PPS	PKLVDPKIV	SVER.....L
Octg5	APA.AGSWER	GE.VVAFGAA	F.....PPR	PELRPTASV	SVAK.....R
Lso	APS.RVDPIC	VE.IVAVGKA	LFQDRRPKE	PKVRWAMALS	SLNK.....R
Genee	A...DGVRAK	YD.IIAFGRS	HERNSSPNND	TLFDPISLPE	AIQOEVSRTK
A4r13	LRE.GGLELP	CH.IIAFGRA	LESSTLDGGS	SWDWKSVGES	SRLNHVRASV
	SRELGGQPLA	YDAILATGKV	LLLE*.....		
A4rlb	251				300
Rlbtr	DQEGEGVMK	PAAVNLKKEA			
Av3pr	DDEADGEPT	LGPGETRTSA	*		
At3pr	SKWSYSGCFR	C.....			
Octg5	SVWAYFNCFQ	C.....			
Lso	LV.....				
Genee	GFKFVSCFY	GN*.....			
A4r13	ELSRNVPTAE	TTDNWAEAL	GTDVAVYVPR	PNLTPTQDHP	CKPLLSGNRV
Genee	301				326
	PNERHRPWKR	FFKKLRVSVK	HFYFNR		

gene. Unfortunately, we were unable to transfer plasmids from *Escherichia coli* to AB2/73 by conjugation with commonly used mobilization strains in order to mutate the *lso* gene, neither did we succeed in transferring the 500-kb pAtAB2/73d Ti plasmid to other *Agrobacterium* backgrounds. Further experiments are required to obtain *lso* mutants. However, in Southern experiments with labeled border sequences and restriction enzyme-digested AB2/73 DNA we could not detect more than two T-DNA borders. This strongly argues against the existence of additional T-DNAs. Bacterial hormone synthesis genes or virulence genes can also determine host range. In the case of pTiC58, the *trans*-zeatin (*tzs*) gene strongly influences tumor induction (Bonnard et al. 1989a), and the *virF* gene of pTiAch5 is an important host range determinant (Hooykaas et al. 1984). AB2/73 may contain such genes that would enhance tumor induction by the *lso* gene.

Although most of the *vir* region of AB2/73 is similar to the classical *vir* regions, Unger et al. (1985) reported lack of homology to the *virA* and *virE* region from A6. Our sequence data confirm this. They show that AB2/73 carries *virA* and *virE* genes with low homology to the A6 and C58 genes; the *virA* region resembles the *virA* gene of the *A. vitis* limited-host-range strain Ag162 (the *virE* region of this strain has not yet been sequenced). The limited host range of Ag162 has been attributed to its T-DNA oncogene content (Buchholz and Thomashow 1984; Yanofsky et al. 1985; Huss et al. 1990) and its *virA* and *virC* loci (Yanofsky et al. 1985). It remains to be tested whether the AB2/73 *virA* gene plays a role in host range similar to that of the Ag162 *virA* gene (Yanofsky et al. 1985).

Three IS-related sequences were found close to the AB2/73 T-DNA. One of these, an IS3-like sequence, is not surrounded

by inverted repeats. This sequence may be part of an incomplete or mutated IS element. Various IS elements have been found close to or within other T-DNAs and IS866 is part of this group (Bonnard et al. 1989b; Otten and Canaday 1992; Otten et al. 1992). The reason for such frequent associations between T-DNA and IS sequences remains unknown.

Protein homology searches and alignments show that the *lso* gene is a member of a larger family composed of genes

Table 1. Functional properties of oncogenes belonging to the *rolB* family

Oncogene	Properties	Reference
<i>rolB</i>	Cell-autonomous root induction	Schmülling et al. 1988
<i>rolB^{TR}</i>	Stunted growth, shoots at base ^a	Lemcke and Schmülling, <i>in press</i>
<i>orf13</i>	Shoot induction by diffusible factor ^a	Hansen et al. 1993
Gene <i>e</i>	No phenotype by itself, increases tumor ^a	Broer et al. 1995; L. Otten, <i>unpublished</i>
Gene 5	Conversion of tryptophan to indole-lactate, slight reduction in growth rate ^a	Körber et al. 1991
Gene 3'	Tumor induction on limited number of hosts	L. Otten, <i>unpublished</i>
<i>lso</i>	Tumor induction on limited number of hosts	This paper

^a Placed under control of a strong promoter to enhance phenotypic effects.

Table 2. Infections of *Nicotiana* spp., *Kalanchoe* spp., and *Lycopersicon esculentum* with AB2/73, the *lso* gene cloned in pBin19 LBA4404(pPM3805), and the negative control LBA4404(pBin19)^a

Infection source	<i>N. tabacum</i>	<i>N. tabacum</i> LF	<i>N. rustica</i>	<i>N. rustica</i> LF	<i>N. glauca</i>	<i>K. daigremontiana</i>	<i>K. tubiflora</i>	<i>L. esculentum</i>
AB2/73	—	+/-	N	+	N	+/-	+	—
LBA4404(pPM3805)	—	—	+	—	—	+/-	+	—
LBA4404(pBin19)	—	—	—	—	—	—	—	—

^a +: tumors; +/-: very small tumors; —: no growth. N: necrosis. Results were scored 6 weeks after infection. LF: leaf fragments, the other infections were done on stems of whole plants.

Fig. 6. Tumor induction by AB2/73 and the *lso* oncogene on stems. Top: *Nicotiana rustica*; bottom: *Kalanchoe tubiflora* 1, 2, and 3: infection with LBA4404(pBin19), AB2/73, and LBA4404(pPM3805) respectively, pPM3805: *lso* gene cloned in pBin19. Scale: 1 cm.

with different plant growth induction properties. Broer et al. (1995) noted homology between the protein products of the *A. tumefaciens* C58 gene *e* and gene 5, and the *A. rhizogenes* A4 gene *rolB*. Gene *e* is essential for tumorigenesis on tomato in spite of the fact that C58 carries *iaa* and *ipt* genes (Broer et al. 1995). The *Lso* protein is related to this group but also to the products of gene 3' and *orf13*. A BLASTP search for each of these proteins revealed further weak homology between the protein products of gene *e* and genes *6a* and *orf14*. The *6a* product in its turn is weakly homologous to the *6b* product (not shown). At least six and possibly nine T-DNA genes are therefore distant relatives of each other with a long evolutionary history. They may or may not code for similar basic functions. Our results partially confirm and extend an earlier T-DNA gene classification by Levesque et al. (1988) who grouped several genes of pTi15955 (gene 3' of the TR-DNA, genes 5, 7, *6a*, *6b*, and the 5' part of *iaaM* of the TL-DNA), and pRiA4 (the 5' part of *iaaM*, *rolB*, *rolC*, *orf13*, and *orf14*) on the basis of weak protein homologies; they proposed to call this group the "plast" family. We could not confirm the inclusion of gene 7, the 5' part of *iaaM*, or *rolC*, and propose therefore to call the more restricted 3'-5'-*e-rolB-orf13-lso* group the *rolB* family after one of its most-studied members, *rolB*.

Possibly, one of the early T-DNAs acquired the ancestor of this *rolB* family, which subsequently diverged as part of a further evolving T-DNA. In this case the T-DNA is an extremely

old genetic element since the divergence between the present-day *rolB* family members is considerable. Alternatively, the *rolB* ancestor gene could have diverged as a bacterial gene well before the first T-DNA appeared. The various members were then recruited independently by different T-DNAs. In the latter case, T-DNA borders and virulence genes would be less diverse than the *rolB*-like genes. Sequence data for borders and virulence genes including those presented in this paper seem to support the latter hypothesis but the mosaic nature of the Ti plasmids (Otten et al. 1992) complicates the analysis.

The biological effects and the mode of action of the *rolB*-like genes are only partially understood. Gene 5 codes for the conversion of tryptophan into indole-3-lactate (Körber et al. 1991). The gene itself does not induce growth on *N. tabacum*, *N. rustica*, *K. daigremontiana*, or *K. tubiflora* even when placed under strong promoter control (L. Otten, unpublished results). Young seedlings of transgenic tobacco plants with gene 5 under control of the strong cauliflower mosaic virus 35S promoter are slightly reduced in growth rate (Körber et al. 1991). Gene 3' from the Ach5 TR-DNA or the AB4 T-DNA induces small tumors on *K. tubiflora* but has no effect on other hosts (L. Otten, unpublished). The oncogenic activity of gene *e* has not yet been studied but a C58 gene *e* mutant was reported to be avirulent on tomato stems (Broer et al. 1995). The *rolB* gene induces roots in a cell-autonomous manner and necrotic lesions when placed under control of the 35S promoter (Schmülling et al. 1988). This gene has been reported to encode a β -glucosidase able to hydrolyze indoxyl- β -glucosides (Estruch et al. 1991) but its proposed role in increasing the internal levels of indole-3-acetic acid (IAA) has been put into question (Nilsson et al. 1993). Tobacco plants containing the *rolB^{TR}* gene under control of the 35S promoter are stunted, have shoots at the stem base, and bent and wrinkled leaves with epinastic growth (Lemcke and Schmülling, *in press*). Tobacco plants with *orf13* under 35S promoter control are small, with variable internode lengths and poorly developed roots. These plants also exhibit reduced apical dominance and carry shoots at the base. Their leaves are small, irregularly shaped, and dark green. They produce a diffusible factor that induces the *orf13* phenotype on normal tobacco plants in grafting experiments, contrary to *rolB* plants. *orf13* under control of its own promoter is symptomless, as is *orf14* (Hansen et al. 1993). Gene *6b* induces tumors (Hooykaas et al. 1988; Tindland et al. 1989). Gene *6a* has been reported to encode an opine transporter (Messens et al. 1985).

Like the *6b* and 3' genes, the *lso* gene induces nondifferentiating calli without help from other T-DNA oncogenes. Part of the functional differences between the *rolB*-like oncogenes may be due to promoter differences or to differences in experimental conditions in the various studies. It would therefore be interesting to insert these genes into the same expression cassette and compare their effects under standard conditions. Such comparisons may also provide a hint as to the fundamental mechanism by which the *lso* gene induces cell division.

MATERIALS AND METHODS

Cloning of AB2/73 *vir* region and T-DNA.

Total AB2/73 DNA was partially digested with *Pst*I and *Bam*HI, and completely by *Xma*I. Fragments were sized on

Fig. 7. Tumor induction on *Nicotiana rustica* leaf fragments by the *lso* gene in vitro on medium without hormones. Top: Control infection with LBA4404(pBin19); empty vector strain. Bottom: Infection with LBA4404(pPM3805) carrying the *lso* gene. Control infection leads to some root induction due to wounding; infection with the *lso* gene leads to callus induction.

sucrose gradients and used to prepare genomic banks in pUC18. The *Bam*HI 1.8-kb fragment of *vir* clone pGV204 from pTiAch5 (related to pTiA6; De Vos et al. 1981) carrying part of the *virD* locus (Fig. 1, P1) was used to isolate a 12-kb partial *Pst*I fragment from the AB2/73 *vir* region (pPM3801). A 1.9-kb *Eco*RI fragment from pPM3801 (fragment P2) and a 2.2-kb *Pst*I fragment from pPM3801 (P3) were used to isolate adjacent regions: a 15-kb partial *Bam*HI fragment (pPM3803) and a 12-kb *Xma*I fragment (pPM3816).

The GCAGGATATATTGAATTGTAAAT oligonucleotide (the left border of the pTiTm4 TA-DNA, accession number M91189) was used to isolate the AB2/73 T-DNA (pPM3804) from the genomic *Bam*HI bank.

Test for additional T-DNA borders.

The presence of additional T-DNA borders in AB2/73 was tested with TGGCAGGATATTGGTGTGTAAAC and TACAATCTGATACATCCTGCCA (the left and right T-DNA border of AB2/73, the latter one as complementary sequence) as probes in Southern experiments with total AB2/73 DNA at standard stringency conditions (50% formamide and 45°C). Under these conditions the pTiTm4-derived oligonucleotide (see above) hybridized to a number of heterologous T-DNAs. For general molecular biology techniques see Sambrook et al. (1989).

Sequencing.

pUC18 clones were sequenced on both strands with an Applied Biosystems (Foster City, CA) 373 Sequencer according to the instructions of the supplier, with primers derived from sequences on both sides of the pUC18 polylinker or from earlier established sequences. Sequences were analyzed on an alpha workstation with the GCG sequence analysis software package version 8.0 (Devereux et al. 1987). DNA homology searches were carried out with the BLAST (Altschul et al. 1990) and BLASTP (Gish and States 1993) programs. The *Ro*LB-like proteins were aligned with the Pileup program (UW-GCG software). Homology to the *Lso* protein as found by the BLASTP program was underlined, homology to the gene 5 protein was italicized.

Plant infection.

Stems of *Lycopersicon esculentum*, *Kalanchoe daigremontiana*, *K. tubiflora*, *Nicotiana tabacum*, *N. glauca*, and *N. rustica*, and leaf fragments of *N. rustica* and *N. glauca* were infected by inoculation with overnight *Agrobacterium* cultures as described (Huss et al. 1990). Leaf fragments were dipped in a fivefold-diluted *Agrobacterium* suspension in 10 mM MgSO₄ and placed on MS medium (Murashige and Skoog 1962) without hormones. Two days later the fragments were washed with liquid MS medium without hormones containing 500 mg of carbenicillin and 500 mg of claforan (MSCC) per l to eliminate the bacteria, and placed on solid MSCC at 24°C (16 h light, 8 h dark). Results were scored 6 weeks later.

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