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Sequence characterization of the *vir* region of a nopaline type Ti plasmid, pTi-SAKURA

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We isolated a crown gall tumor-inducing nopaline type Ti plasmid from *Agrobacterium tumefaciens* on a Sakura Japanese cherry tree, and designated it as pTi-SAKURA. By primer walking sequencing with long PCR and a newly developed PCR subcloning technique for long insert DNA, we completed DNA sequencing of the most important functional unit, the virulence (*vir*) region of pTi-SAKURA, which is indispensable for T-DNA transfer into the plant's chromosomes. By homology searches with the *vir* genes of other bacterial plasmids, we identified 11 open reading frames (orfs) and 31 genes and 11 vir box, which are 6 bp regulatory sequences. In total, 26 *vir* genes, including the putative *virF* and *virK* and the main *vir* region, were present as the *vir* gene cluster. The presence of vir box, GC content, codon usage and expression analysis in these genes led us to propose a new *vir* region.

INTRODUCTION

The plant tumor-inducing bacterium *Agrobacterium tumefaciens* harbors a large Ti plasmid (see Winans *et al*., 1987; Zambryski, 1988) of which the T-DNA (oncogenes and opine genes) region is transferred into the chromosomes of dicotyledonous plants after infection (Baron *et al*., 1997; Beaupre *et al*., 1997; Chen *et al*., 1991; Das *et al*., 1997; Dang *et al*., 1997; Doty *et al*., 1996; Jayaswal *et al*., 1987; Sundberg *et al*., 1996; Winans *et al*., 1986). The expression of T-DNA genes leads to plant tumor disease. The *vir* genes on Ti plasmids together with a set of genes encoded by the host agrobacterial chromosome are involved in T-DNA transfer (Jones *et al*., 1996). T-DNA transfer has been studied extensively, and the transfer machinery has been used successfully as the most efficient transformation method for plants (Belanger *et al*., 1995). However, Ti-infection in the nature is limited to dicotyledonous plants. Even in dicots, the infection of trees is generally difficult. Though it is surely rare in nature, the recent technology which applies Ti infection, has been established even in monocots.

To understand the molecular basis of infection, genomic data are indispensable. Unfortunately, the nucleotide sequences of Ti plasmids remain largely unknown (Christie, 1997; Hagiya *et al*., 1985). For this reason and because of our interest in the phylogenetic relationship between T-DNA transfer and trans-kingdom conjugation (Inomata *et al*., 1994; Nishikawa *et al*., 1990, 1992, 1998; Sawasaki *et al*., 1996; Yoshida *et al*., 1997), we have isolated a nopaline type Ti plasmid from a wide host range *A. tumefaciens*, which was isolated from a Japanese cherry tree's crown gall, and started to analyze its genome structure. We elucidated the novel structure of the *trb*/*tra*I and *rep* gene region, and subsequently determined the first complete nucleotide sequence of the plasmid (Hattori *et al*., 2000; Suzuki *et al*., 1998; Suzuki *et al*., 2000). In this report, we describe the extensive sequence characterization of the *vir* region using a newly developed sequencing strategy based on PCR, and we propose a new *vir* region.

MATERIALS AND METHODS

Culture of bacteria and phage. *A. tumefaciens* MAFF301001 cells which were isolated from a Japanese cherry's crown gall were cultured in YEM medium at 28°C as described elsewhere (Hattori *et al*., 1997; Suzuki *et al*., 1998). *E. coli* XL1-Blue MRA cells infected with lambda phage were cultured in NZY medium supplemented with $MgSO_4$ at 37°C (Sambrook *et al*., 1989).

Recombinant DNA and sequencing techniques. Ti plasmid was extracted from *A. tumefaciens* by the alkaline-SDS method (Sambrook *et al*., 1989) and subsequently purified as a closed circular form by precipitation with polyethylene glycol 6000 and subsequent CsCl/EtBr isopycnic centrifugation (Ohta, Master thesis in Hiroshima

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Univ. 1995). The cloning vector lambda DASHII DNA was purchased from Stratagene (CA, USA). Restriction enzyme digestion, ligation, *in vitro* packaging of ligated phage DNA, preparation of lambda phage DNA and RNA probes, and filter hybridization were performed essentially as described by Sambrook *et al*., 1989. Primer oligodeoxynucleotides for PCR and sequencing were designed according to Saiki *et al*., 1989. The long PCR method (Hattori *et al*., 1997; Suzuki *et al*., 1997) was applied to prepare template DNAs for sequencing. DNA sequences were determined using an automatic DNA sequencer (Perkin Elmer-ABI, CA USA) and the data were edited using a Seqed Program (Perkin Elmer-ABI). Genes were identified by comparing the sequence data with full sets of the most recent DDBJ/GenBank databases using a FASTA search program.

PCR sequencing. It is easy to obtain DNA sequence data from each end of inserted DNAs of recombinant phages by sequencing with purified phage DNAs as templates and primers. For successive DNA sequencing, we employed the primer walking strategy for the following reasons (Hattori *et al*., 1997): The method is suitable for automatic sequencing and essentially eliminates the need for cloning in the host *E. coli*. As a result, the possibilities of recombination and mutation errors *in vivo* are reduced. Custom-made primers for the forward and reverse directions for sequencing by the primer walking method were designed using the DNA sequences obtained from both end regions of inserts. Extensive sequencing was conducted using the custom-made PCR fragments, T3- and T7- primers and purified recombinant phage DNA templates.

Construction of GUS expression vector. The GUS expression plasmid, pHRP-GUS (see Fig. 4), was constructed from pHRP311 (Parales *et al*., 1993) and pWM5

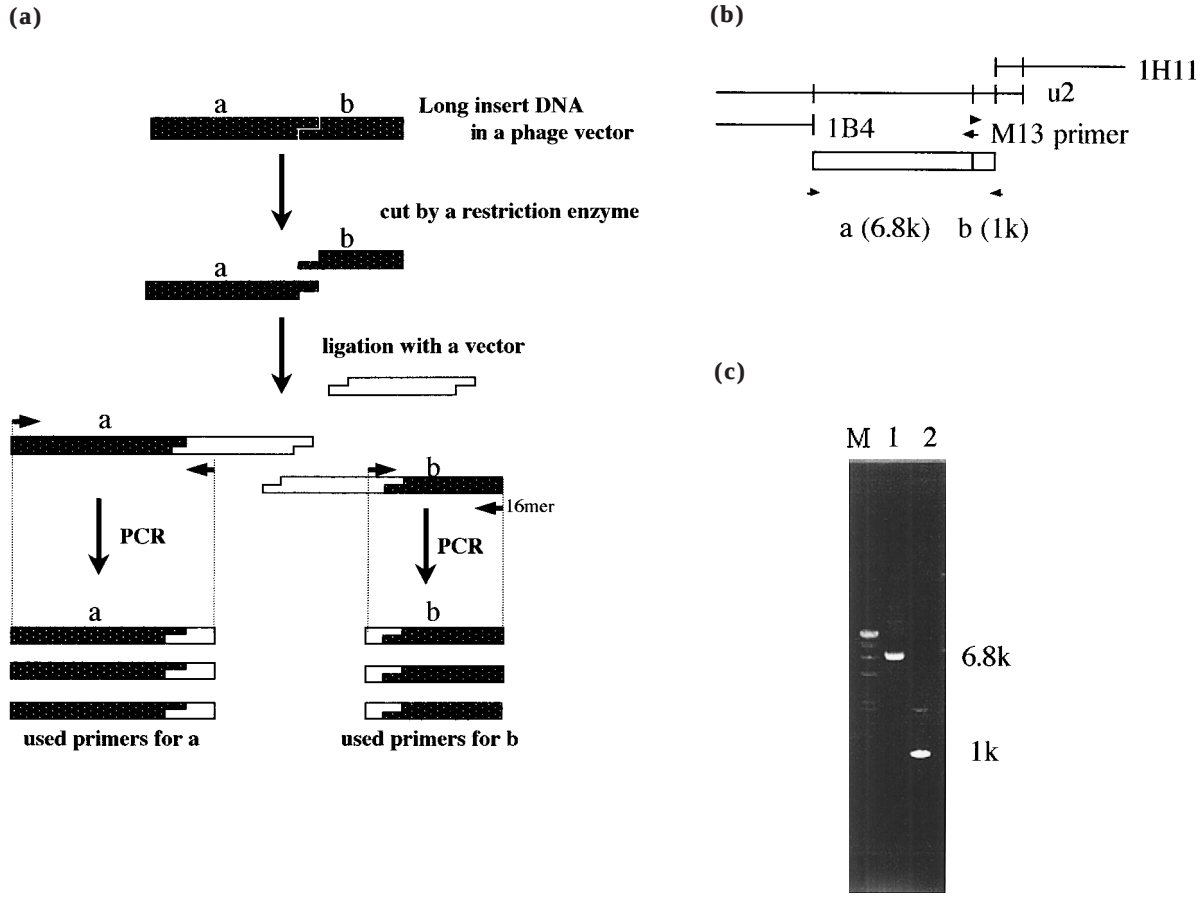

Fig. 1. Our novel PCR subcloning method and its application. (a) Schematic representation of PCR subcloning process. Shaded rectangles indicate long insert DNA in a phage vector, while open rectangles indicate a vector with multiple cloning sites, such as pUC19. Both DNA cut with an appropriate restriction enzyme and resultant fragments were ligated as shown. Horizontal arrows indicate primers. (b) Physical structure of a typical DNA insert to be sequenced. The long insert (a+b) was cut with EcoRI into a (6.8k) and b (1k) fragments. The fragments were inserted into pUC. After ligation, each fragment was amplified by PCR with pUC sequence and fragment sequence primers (see (a)). 1H11, u2 and 1B4 indicate clones of a λ phage-linked library of pTi-SAKURA DNA. (c) Electrophoretic profile of PCR subcloning products. M; size marker, 1; fragment-a product, 2; fragment-b product.

(Metcalf *et al*., 1993). After the removal of the Ω cassette and *lac*Z region by *Hin*dIII digestion, the pHRP311 was self-ligated. The β-glucuronidase cassette from pWM5 was inserted into the *Bam*HI site of the resultant vector. GUS expression was measured using a spectrofluorometer (FP-777, JASCO) according to Jefferson et al.,

Table 1 Genes and orfs in *vir* region and its flanking regions in pTi-SAKURA and their homology with other sequences

Genes or orfs	Nucleotide sequence identity(%)					
pinF1 (*virH1*)	pTiC58	100.0	pTiA6	79.5		
pinF2 (*virH2*)	pTiC58	99.9	pTiA6	81.3		
tpn	pTiC58	100.0				
tiorf132						
virF	pTiC58	100.0				
tiorf134						
virK	pTiC58	100.0				
tiorf136						
tiorf137						
tiorf138						
tzs	pTiC58	100.0				
virA	pTiC58	99.7	pRiA4b	92.0	pTi15955	77.0
virB1	pTiC58	99.5	pTi15955	76.7	pTiA6NC	76.7
virB2	pTiC58	99.7	pTi15955	85.5	pTiA6NC	85.0
virB3	pTiC58	100.0	pTi15955	85.0	pTiA6NC	85.0
virB4	pTiC58	99.6	pTi15955	83.7	pTiA6NC	83.6
virB5	pTiC58	99.8	pTi15955	86.7	pTiA6NC	86.7
virB6	pTiC58	99.7	pTi15955	88.1	pTiA6NC	87.8
virB7	pTiC58	100.0	pTi15955	86.9	pTiA6NC	86.9
virB8	pTiC58	99.3	pTi15955	86.8	pTiA6NC	86.6
virB9	pTiC58	99.9	pTi15955	83.4	pTiA6NC	83.4
virB10	pTiC58	100.0	pTi15955	82.5	pTiA6NC	82.5
virB11	pTiC58	99.3	pTi15955	83.7	pTiA6NC	83.1
virG	pTiC58	100.0	pRiA4b	91.0	pTi15955	81.5
					pTiA6NC	81.4
virC2	pTiC58	100.0	pRiA4b	90.5	pTiA6NC	73.9
virC1	pTiC58	100.0	pRiA4b	93.5	pTiA6NC	81.6
virD1	pTiC58	100.0	pRiA4b	89.2	pTiA6NC	77.3
					pTiA6	77.3
virD2	pTiC58	99.7	pRiA4b	90.2	pTiA6NC	73.7
	pTiA6	73.7				
virD3	pTiC58	99.2	pRiA4b	80.8	pTiA6NC	57.5
virD4	pTiC58	99.4	pRiA4b	91.1	pTiA6NC	83.9
					pTiA6	83.9
tiorf159	pTiC58	99.2	pRiA4b	81.3		
virH	pTiC58	98.2				
tiorf161						
virE1	pTiC58	100.0	pTiA6	70.2		
virE2	pTiC58	99.7	pTiA6	74.7		
tiorf164						
tiorf165						
tiorf166						
tiorf167						
tiorf168						
tiorf169 (*accG*)	pTiC58	72.7	pTi-SAKURA (*tiorf126*)	55.4		
tiorf170 (*accF*)	pTiC58	75.4	pTi-SAKURA (*tiorf125*)	72.0		

Each *tiorf* and its number indicate an open reading frame and its *orf* number in pTi-SAKURA, respectively (Suzuki *et al*., 2000). A blank indicates no other hit in DNA databases. pTiC58 is a nopaline type Ti plasmid. pTi15955, pTiA6 and pTiA6NC are octopine type Ti plasmids and pRiA4b is an Ri plasmid. *Orf*s under the dotted line indicate *orf*s in the flanking region. *tiorf125* and *tiorf126* are *accF* and *accG* in the main *acc* region while *tiorf169* and *tiorf170* are in other region which is close to LB (see Fig. 2).

1987. This assay was repeated more than three times.

RESULTS AND DISCUSSION

PCR subcloning method for efficient primer walking. The primer walking method, which we used in this study, requires repeating a cycle of sequencing and subsequent chemical primer synthesis for the next sequencing cycle based on the resultant data. Without primer data, the next sequencing cycle can not be executed. As a result, this method inevitably requires a long waiting time to complete the sequencing, especially in the case of a long DNA. To overcome this problem, subcloning and shotgun methods have frequently been used to increase the PCR starting points. However, we did not adopt these general methods because they require the laborious use of an *E. coli*-vector system and involve the possibility of insertion and deletion errors. To avoid these problems, we have developed a novel PCR subcloning method.

Table 2 Vir boxes upstream of each *vir* gene operon in pTi-SAKURA

Operon	Sequences in vir boxes
pinF	ATTACAGATGAAAC
virF	ATTCGAGAATCGAAAT
virK	GTTTCAAATGAAGC
virA	GTTTCATTTGAAAC
virB	GCTTCAAATGAAAT
virC	ATTACAATTGAAAT
virD	ATTTCAATTGTAAT
virE	ATTACATATGAAAC
virG	ATTACGTTTGTAGC
virH	GTTTCAGCTGAAAT
tiorf159	GTTTCAGCTGAAAT
Consensus	5' TG(A/T)AA(C/T) 3'

These sequences show the most upstream vir boxes with the inverted vir box. Vir box consensus sequences are underlined.

Fig. 1 (a) schematically shows an outline of the PCR subcloning method. After cleaving a phage vector DNA and/or PCR fragments with an appropriate restriction enzyme, the produced fragments were ligated with a subcloning vector such as pUC19. The DNA fragment containing a target DNA was amplified by PCR using the ligation product as the template and a primer specific for the plasmid or for the Ti DNA. Because the PCR product contained a portion of vector DNA, we could start sequencing within the region of a long Ti DNA fragment by using a universal primer specific for the vector, in this case, pUC19. To avoid a chimeric-amplified fragment, the lengths of the resultant PCR products were analyzed to verify that the sizes matched those calculated based on the source DNA fragments. The PCR products were used for nucleotide sequencing with a universal M13–21 primer, and subsequently new specific primers were designed. The PCR products were used only for the sequencing using the universal primer and never used for further sequencing in order to avoid possible induction of

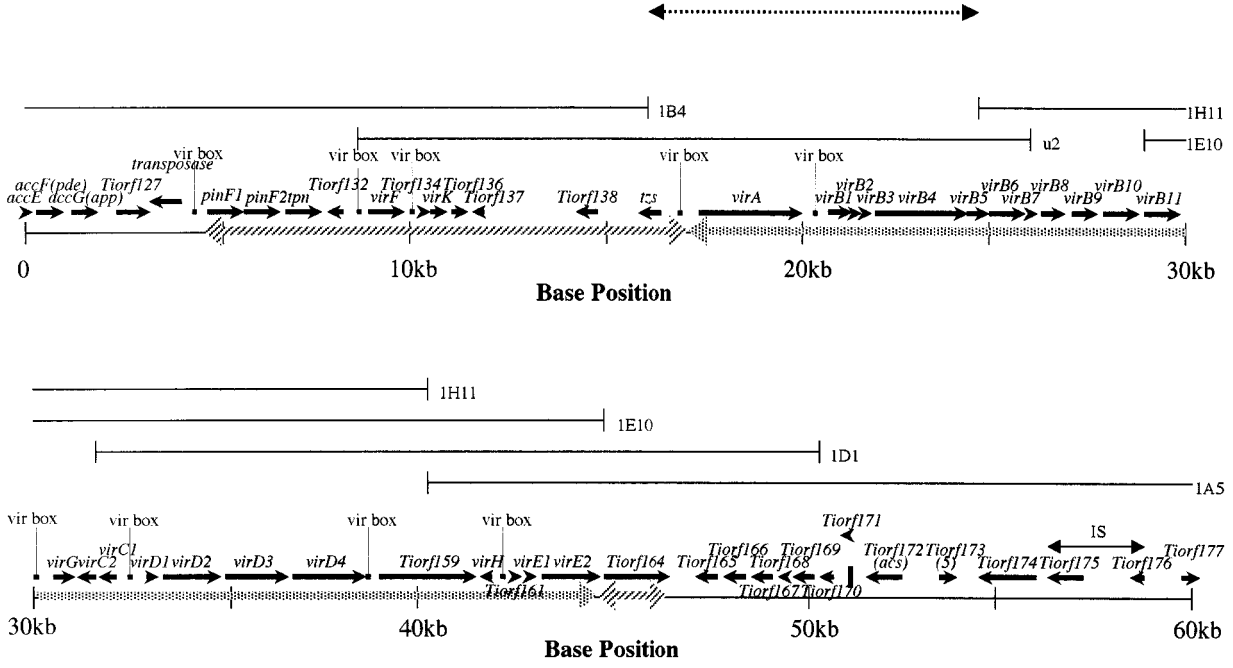

Fig. 2. Gene map of *vir* and *acc* regions, including the T-DNA region. Horizontal arrows indicate genes, of which the names are shown by letter code. Upper thin lines indicate clones of a λ vector library for which sequences were determined. The dotted line indicates the region in which PCR subcloning was carried out. The dotted area on the base size scale indicates the old *vir* region while the dashed area including the dotted area indicates a newly proposed *vir* region in this paper.

rearrangements. Every sequence determination except those made using the M13–21 primer was carried out using either recombinant lambda DNAs or the long PCR products made using the recombinant lambda DNAs. Fig. 1 (b) shows the physical structure of a long DNA (a+b) which we analyzed by PCR subcloning. Fig. 1 (c) shows a typical electrophoretic profile of PCR subcloning products. The profile clearly indicates successful amplification of the and b fragments. This method is essentially applicable to any DNA because multiple cloning sites in major vectors are compatible with many restriction endonucleases.

Sequencing analysis of pTi-SAKURA's *vir* region. By using the primer walking strategy with the above PCR subcloning method, we completely sequenced a 50 kbp region of pTi-SAKURA which contains the important *vir* region and its flanking regions. As shown in Table 1 and Fig. 2, we could finally assign 42 orfs in this region and 11 of these orfs had no homologous orfs in DNA databases. The orfs and their homologies are listed in Table 1, and the map of phage-linked clones used for sequencing is shown in Fig. 2.

Virulence genes (*vir*) are flanked by regions containing agrocinopine catabolism (*acc*) genes on the left and right

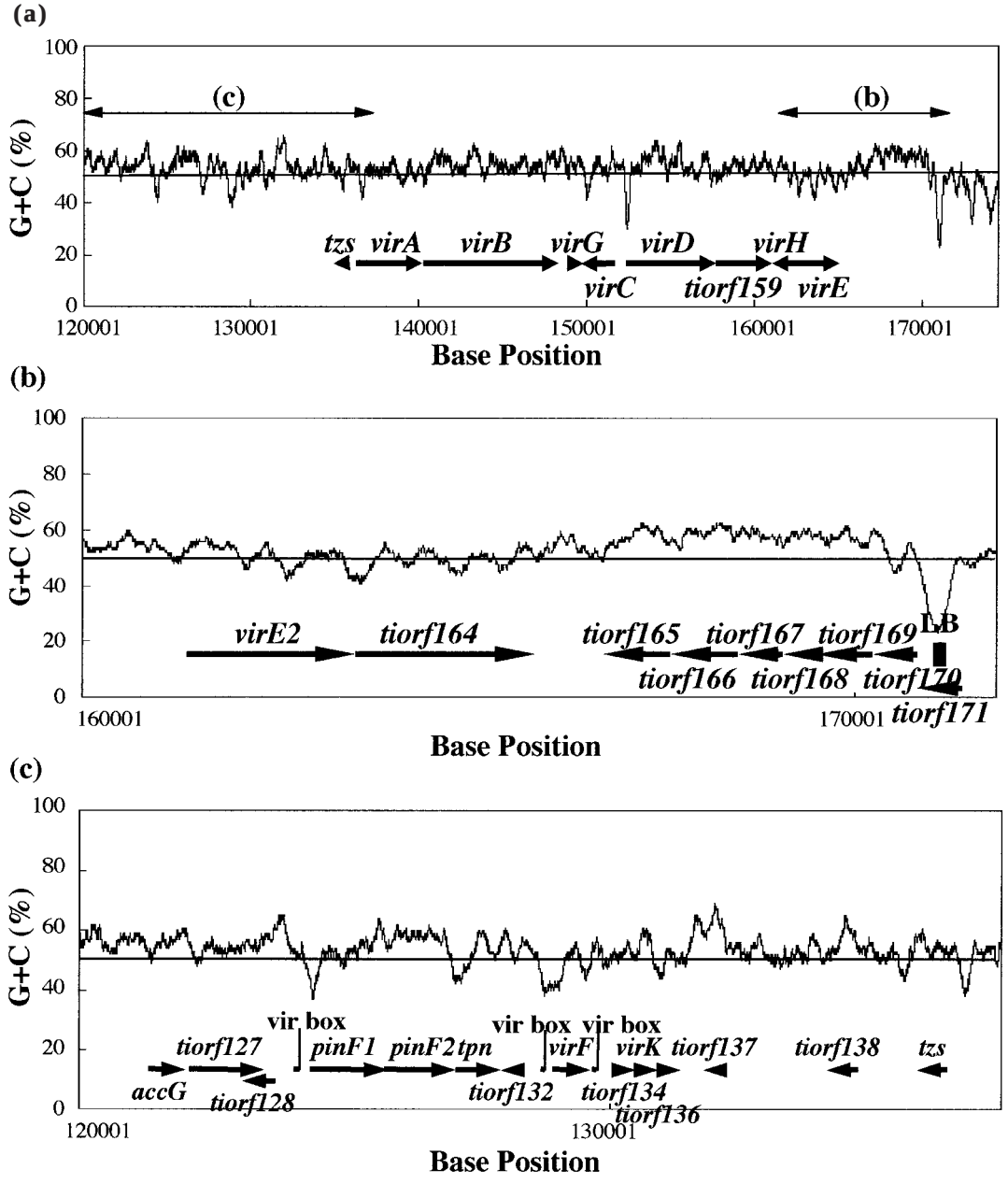

Fig. 3. GC contents of *vir* and *acc* regions, including the T-DNA region. (a) The entire analyzed region, (b) *virE* to LB region corresponding to (b) in (a); (c) *acc* to *tzs* region corresponding to (c) in (a). The ordinate indicates the GC content (%) while the abscissa indicates the base position number in the total 206,479bp of pTi-SAKURA.

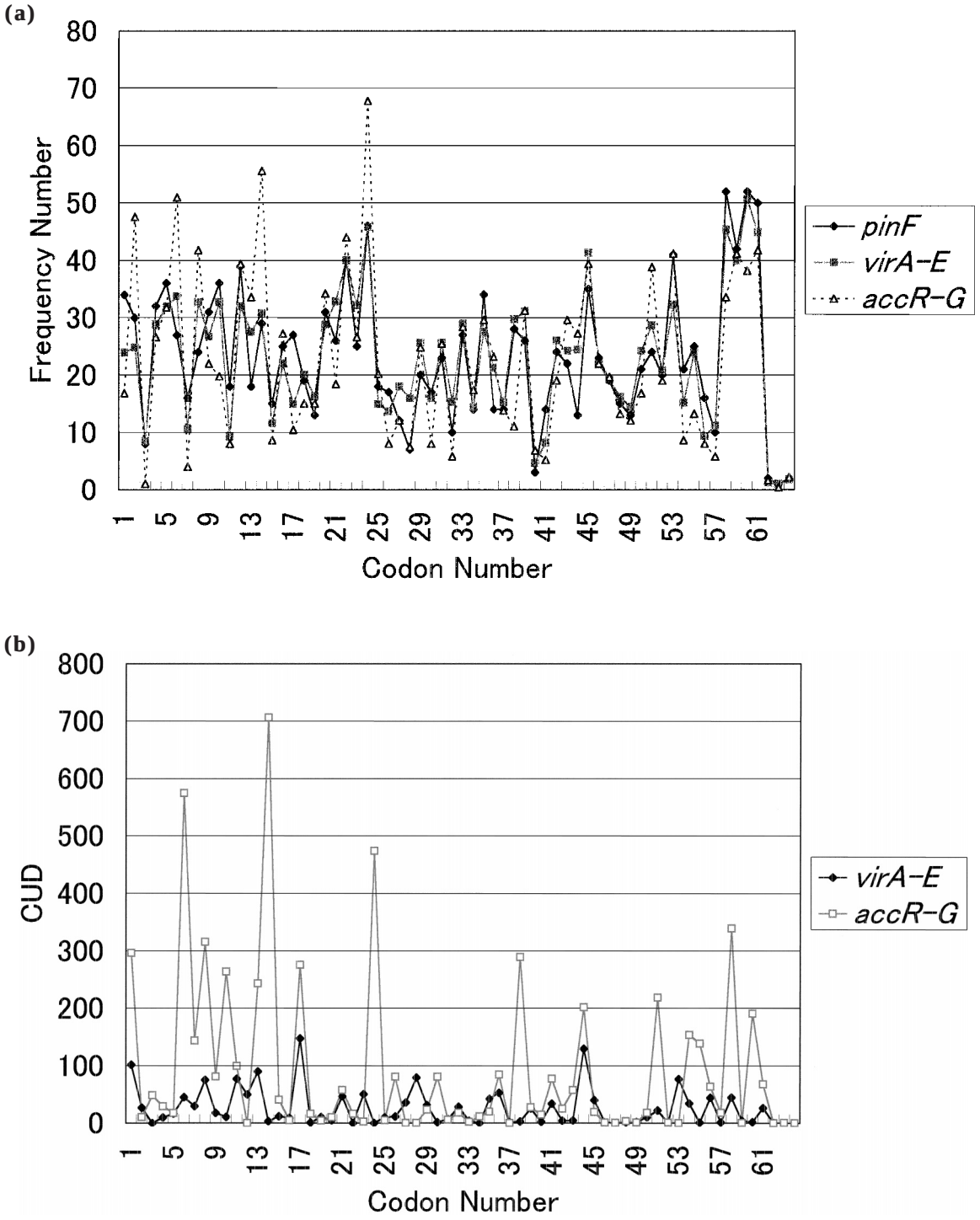

Fig. 4. Similarity of codon usages between *pinF1-tzs* and *virA-E* regions. (a) Frequency number of usage codon in *pinF1-tzs*, *virA-E* and *accR-G*. Each codon of 64 codons is assign to the codon number as follows: 1 ~ 2, F codons; 3 ~ 8, L codons; 9 ~ 11, I codons; 12, M codons; 13 ~ 16, V codons; 17 ~ 20, P codons; 21 ~ 24, A codons; 25, W codons; 26 ~ 31, S codons; 32 ~ 35, T codons; 36 ~ 37, Y codons; 38 ~ 39, Q codons; 40 ~ 41, C codons; 42 ~ 43, N codons; 44 ~ 47, G codons; 48 ~ 49, H codons; 50 ~ 51, K codons; 52 ~ 57, R codons; 58 ~ 59, D codons; 60 ~ 61, G codons; 62 ~ 63, stop codons. A ~ Y indicates one-letter amino acid code. In other words, codon number 1 ~ 25, 26 ~ 47, 48 ~ 57, 58 ~ 61 and 62 ~ 64 assign to nonpolar amino acids, polar amino acids, basic amino acids, acidic amino acids and stops, respectively. (b) Squared codon usage differences (CUD) of *pinF1-tzs* versus *virA-E* and *pinF1-tzs* versus *accR-G*. Each codon number was estimated by total codon number in *pinF1-tzs* × codon usage percent in *virA-E* or *accR-G*. The difference between codon number in *pinF1-tzs* and that in *virA-E* or *accR-G* in each codon was squared and used as CUD of each codon. CUD of each codon was plotted against the codon number which is described in (a). The lower CUD indicates the closer similarity in codon usage.

sides of the T-DNA. Between the right-side boundary of the *acc* region and the left-side boundary of the T-DNA, we found 42 open reading frames (orfs) in total, which included all of the fundamental *vir* genes (Table 1). We identified 4 operons (*virB1~11*, *virC1~2*, *virD1~4*, *virE1~2*) and 2 additional single genes (*virA* and *virG*) based on DNA database homology (Fig. 2 and Table 1). (Aoyama *et al*., 1989; Close *et al*., 1987; Hirayama *et al*., 1988; Kuldau *et al*., 1990; McBride *et al*., 1988; Melchers *et al*., 1986; Powel *et al*., 1987; Thompson *et al*., 1988; Ward *et al*., 1988). *VirA*, *virB*, *virG*, *virD* and *virE* were encoded clockwise while *virC* was encoded counterclockwise, as in other Ti and Ri plasmids (Hirayama *et al*., 1989; Moriguch *et al*., 2001; Winans *et al*., 1986). As shown in Fig. 2, the gene order was as follows: *virA-virB1~11-virG-virC2~1-virD1~4-virE1~2*. All of the main virulence genes had more than 98% homology with the corresponding genes of another nopaline type Ti plasmid, pTiC58 (see Table 1). Upstream of each operon or single gene, there was a vir box regulatory sequence, an element which was first found in pRiA4 (Aoyama *et al*., 1989). Table 2 shows the consensus vir box " TG (T/A) AA (T/C) " determined from the sequence data. The vir box consisted of 1 to 4 six-base-pair motifs in each *vir* gene. These blocks are phased with an interval of 11 base pairs, and the most upstream one in each gene promoter region has an additional block in the inverted orientation (Aoyama *et al*., 1989).

Hitherto, essentially no information was available about the boundary regions. Therefore, we extensively analyzed the right and the left boundaries of the *vir* gene region. In the right side (between *virE2* and LB (Left Border of T-DNA)), we found 8 orfs, including those for the *accF* (*orf169*) and *accG* (*orf170*) like genes. In pTi-SAKURA, there is an *acc* region for agrocinopine catabolism upstream of the *vir* region, as shown in Fig. 2. *AccR* and *accABCDEFG* are all present in this region. However, as shown in Table 1, *tiorf169* and *tiorf170*, which found between *virE2* and LB, showed 72.0% base sequence homology with 76.2% amino acid sequence homology against the *accF* gene and 55.4% base sequence homology with 76.2% amino acid homology against *accG* gene, respectively. Interestingly, the GC content of the right-side boundary area was higher than that of the main virulence gene region, as shown in Fig. 3 (a) and (b). This suggests that the orfs in this region, except for *tiorf164* (see Suzuki *et al*., 2000), are of a different origin. *Tiorf164* had a G+C% value similar to those of the main *vir* genes and was located very close to *virE2* with the same transcriptional direction. These results indicate that *tiorf164* is a distinct member of the *virE* operon. Thus, *tiorf164* occupies the right end of the *vir* region.

In the left side (between *accG* in the *acc* region and *virA*), we found 13 orfs, including those for the *pinF1* (*virH1*), *pinF2* (*virH2*), *virF*, *virK* and *tzs* genes. The GC content of this region was essentially the same as that of the main virulence region, as shown in Fig. 3 (a) and (c). This suggests that the orfs in this area are related to the *vir* genes. Because all of the main *vir* operons have a characteristic six-base-pair regulatory sequence called the vir box, we examined whether or not this sequence is present upstream of the newly found orfs in the left-side boundary. As shown in Fig. 3 (c), we found vir box sequences upstream of the *pin* operon (*virH* operon), *tzs* and *tiorf134* (Suzuki *et al*., 2000) which is present just upstream of *virK*. These vir box sequences have inverted repeat structures (Table2). There was a similar sequence upstream of *virF*, although this sequence was not a perfect inverted repeat sequence. *PinF*, *virK* and *virF* are believed to be involved in the wide host range specificity of *Agrobacterium*. These data indicate that at least *pinF* (*virH*) *1 ~ 2*, *virK*, *tiorf134* and *tiorf136* are regulated by essentially the same mechanism as the main *vir* genes. These genes are the first such genes identified and characterized in nopaline type Ti plasmids. Furthermore, these data suggest that these genes are related to the main essential *vir* region.

The presence of vir box and GC content suggest that *pinF1-tzs* and *tiorf164* (see the dashed arrow lines in Fig. 2) are *vir*-related genes. To confirm this, we compared codon usage in these genes with those in the *vir* region. As shown in Fig. 4 (a), usage of each codon in *pinF1* to *tzs* is closer to that in *virA* to *virE* than that in *accR-accG* which is used as a typical region outside of the *vir* region. However, there is essentially no great difference

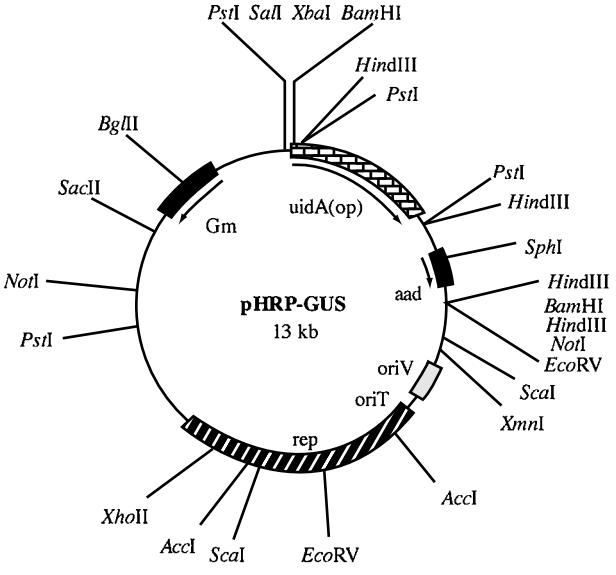

Fig. 5. Constructed GUS expression vector. GUS, Gm, *uidA* (op), *aad*, *oriV*, *oriT* and *rep* indicate β-glucuronidase, gentamycin, GUS coding gene (operon), antibiotic resistance markers for spectinomycin and streptomycin, replication origin, transfer origin and replication gene, respectively. The restriction enzyme cleavage sites are shown outside the circle.

among nonpolar (No. 1–25), polar (No. 26–47), basic (No. 48–57), acidic (No. 58–61) amino acids and stop (No. 62–64) codons. This is more clearly shown in Fig. 4 (b), in which CUD (squared codon usage difference) is defined as (*codon usage in a target gene or area – codon usage in a gene or area to compare*)^2 in each codon. CUD has an inverse relationship to correlation coefficient (Yoshida *et al.*; The statistical details will be published elsewhere). In

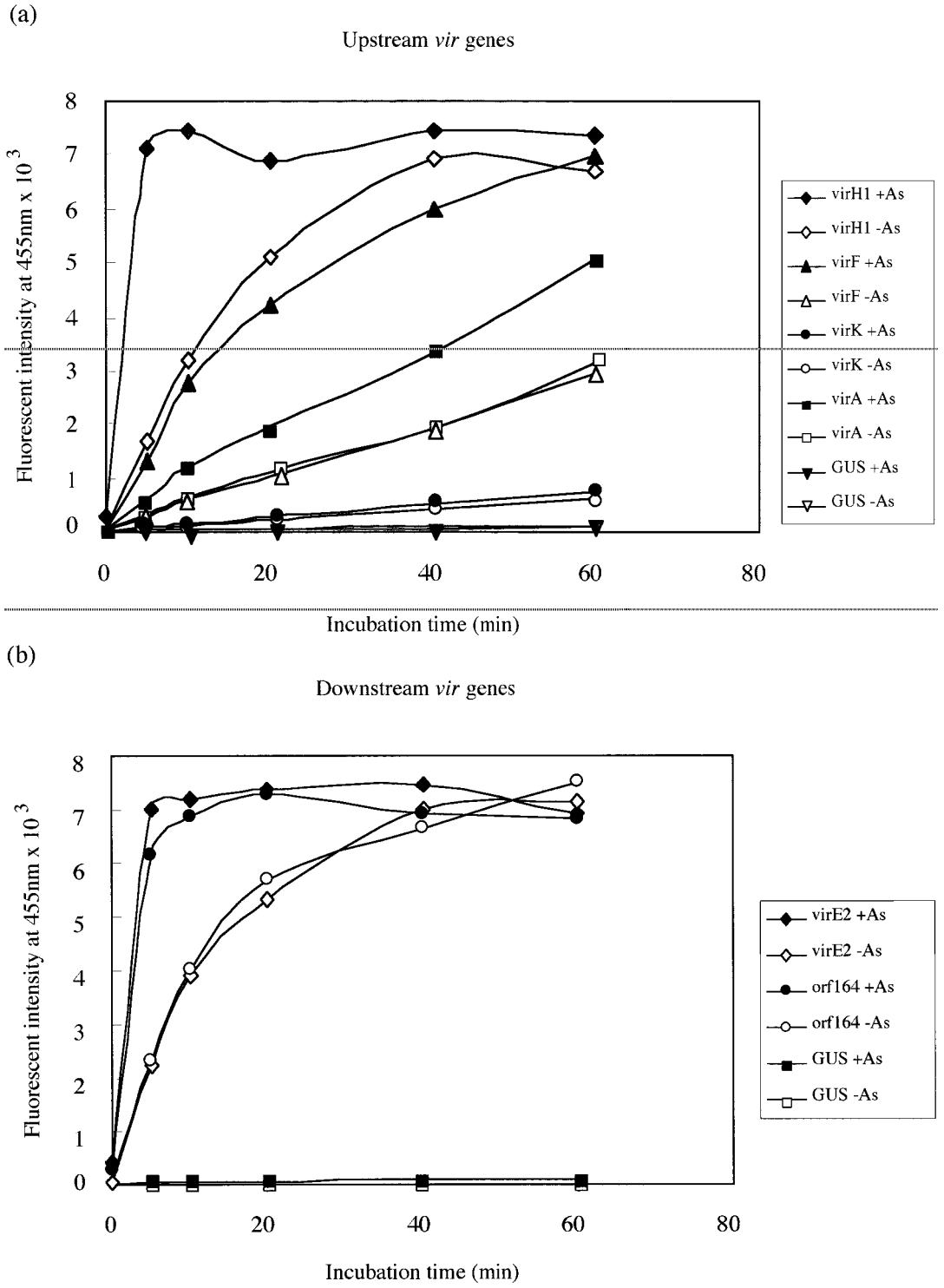

Fig. 6. GUS expression assay. (a) Upstream *vir*-region genes. *VirA* represents a positive control while GUS represents a negative control. (b) Downstream *vir*-region genes. *VirE2* represents a positive control while GUS represents a negative control. The ordinate and abscissa indicate incubation time with (+) or without (–) acetosyringone (As) and the intensity of fluorescence at 455 nm with 350 nm excitation, respectively. This assay was repeated more than three times.

other words, the lower CUD indicates the closer similarity in codon usage. We obtained the essentially same result in *tiorf164* (data not shown). In conclusion, the codon usage of *pinF1-tzs* and *tiorf164* is very similar that of the *vir* region.

Assay of *vir* region expression. Wounded plants release phenolic compounds such as As (acetosyringone). These compounds attract *Agrobacterium* to the wound site and are involved in the induction of *vir* genes (Stachel *et al*., 1985; Spencer *et al*., 1988; Melchers *et al*., 1989). To examine the control of the gene expression of the *vir* region, we constructed a GUS expression plasmid, pHRP-GUS (Fig. 5), by using the vector pHRP311 (Parales *et al*., 1993) and the GUS gene (William *et al*., 1993). The putative promoter region of each newly found *vir* gene was inserted into the *Sal*I, *Xba*I site upstream of the GUS gene in pHRP-GUS. The results of GUS expression analysis are shown in Fig. 6. These assays were done more than three times per one sample. Regarding promoters in the upstream part of the *vir* region, the activity of GUS genes with promoters of *virH1*, *virF* and *virA* was increased in the presence of As (Fig. 6 (a)) while the GUS gene with a *virK* promoter was not expressed regardless of the presence of As. At present, the reason is unknown. Regarding promoters in the downstream part of the *vir* region, the activity of GUS genes with the promoters of *tiorf164* and *virE* was increased in the presence of As (Fig. 6 (b)).

These results clearly indicate that the expression of *virH1*, *virF* and *tiorf164* is regulated by As. Furthermore, this with the above described codon usage and GC content analysis reveal the interesting fact that the *vir* region extends from *pinF1* (*virH1*) to *tiorf164* (see the dashed regions including dotted region on the base size scale in Fig. 2), and not from *virA* to *virE*, as had previously been believed (see the dotted region on the base size scale in Fig. 2).

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