SZENT ISTVÁN UNIVERSITY

ISOLATING AND CHARACTERISING VIRULENCE/PATHOGENECITY GENES OF PHYTOPATHOGENIC *PSEUDOMONAS*ES

Thesis of doctoral dissertation

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PREVIOUS RESEARCHES AND MAIN OBJECTIVES

Despite the rapid expansion of known bacterial genome sequences, slightly is known the function of the majority of genes. The genetic determinants of bacterial virulence are tightly and precisely regulated, mainly at the level of transcription. This allows isolating pathogenicity-associated genes based on their specific *in vivo* (*in planta*) expression. The *in vivo* expression technique (IVET), a promoter probing method, is one of the approaches which allows the positive selection of bacterial mutants for the identification of conditionally expressing genes that are specifically fused with promoterless reporter genes. The final result of this analyses is the identification of bacterial genes that are expressed preferentially *in planta* during the disease process. Several studies report the application of IVET in different animal-bacterial interactions. However, the analysis of virulence mechanisms of plant pathogen bacteria is often limited by the lack of genetic tools (e.g. a suitable IVET promoter probing plasmid) that can be used to identify genes that are preferentially expressed during their interactions with plants. The aim of this study was constructing IVET promoter probing plasmids utilising antibiotic resistance based selection instead of the regularly used auxotroph complementation. For the validation of the plasmids, one of them pIviGK was objected for the identification of genes that encode pathogenicity factors of the soft rot causing bacterium *Pseudomonas viridiflava.*

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacteria used in this study were: *Pseudomonas viridiflava, Escherichia coli* DH5α/λ pir, *E. coli* S17.1 and *E. coli* HB101. Bacterial cultures were maintained and cultivated on Luria Bertani (LB) media. Antibiotics for selection were: 50 μg/ml rifampicin, 10 μg/ml gentamicin and 50 μg/ml kanamycin. Plasmids used in this study were: pRK2013, pBluescriptKS, pGP704, pJQ199 and pAG408.

Recombinant DNA techniques

Plasmid DNA and total genomic DNA isolation, transformation of *E. coli,* agarose gel electrophoresis and all enzymatic treatments were conducted using established methods. DNA fragments were isolated from agarose gels using GELase (EPICENTRE, USA) following the manufacturer's recommendations. For Southern-hybridization we used digoxigenin (DIG) -based probe labeling and detection kit (Boringen Manheim) according to the manufacturer's recommendations.

Conjugative mobilisation

Conjugation was conducted as described by Czelleng et al. (2006).

Construction of pIviGK and pIviGG

A derivative of pGP704, designated pGPMCSII which contains the MCSII synthetic polylinker sequence (Fig. 1) between the *Bgl*II and *Kpn*I sites was created. The gentamicin (*accA1*) and kanamycin resistance genes (*aphA3*) were isolated from pAG408. The ends of both isolated fragments were blunted with T4 polymerase and ligated to *Ssp*I and *Bst*1107I double digested pGPMCSII, resulting in pGPMCSII:gm and pGPMCSII:km.

The *gfp-km* or *gfp-gm* reporter gene pairs were created as follows. Promoterless derivatives of *aphA3* and *accA1* genes (afterwards: *kmS/R* and *gmS/R* respectively) were created by nested PCR. Products of the PCR reactions were digested with *Eco*RI and *Sma*I and ligated to pBluescriptKS resulting in pBKS:gmS/R and pBKS:kmS/R, respectively. These plasmids were digested with *Kpn*I and *Eco*RI and ligated to the atpE-*gfp* gene cassette originated from pAG408, resulting in pBKS:gfp-gmS/R and pBKS:gfp- kmS/R. Reporter gene pairs were isolated by *Kpn*I and *Sma*I double digestions and were ligated to pGPMCSII: km or pGPMCSII: gm resulting in pIviGK and pIviGG plasmids.

Construction of the IVET fusion library

Purified *P. viridiflava* genomic DNA was digested with *Hind*III, fragments of 2,000-3,000 bp were isolated from gel. The 5'-overhanging ends of the isolated fragments were partially backfilled with dATP and dGTP using Klenow DNA polymerase. *Xba*I digested pIviGK that was partially filled with dCTP and dTTP was ligated to *P. viridiflava* genomic DNA fragments. All (approximately 10^4) *Escherichia coli* DH5α/λ pir transformants were used as a donor in a conjugation reaction wherein *P. viridiflava* was the recipient.

Infiltration of green pepper fruits

0.5 cm wide green pepper rings were prepared using two different diameter cork borers. The pepper rings were infiltrated with *P. viridiflava* suspension in vacuum. The infiltration process was completed within 30 seconds.

***In vivo* selection**

An IVET–mutant *P. viridiflava* pool was created from 10^4 colonies and suspensions were used for vacuum infiltration. Two hours post infection

pepper rings were vacuum infiltrated with 50 µg/ml kanamycin solution. After 10-12 hours of incubation the pepper rings were homogenated and bacteria were suspended and plated onto selective LB media containing gentamicin and incubated for 48 hours at 28 °C. Petri dishes were exposed shortly to UV irradiation using a UV transilluminator and colonies lacking green light emission were observed by visual scoring and picked for further characterisation. The selected clones represented candidate genes, which expressed only *in planta*.

Conjugative cloning and sequencing of *ivi*-gene fusions

Plasmids pIviGK with inserts of different *ivi*-genes were mobilised into *E. coli* DH5α/λ pir by retrotransfer. *ivi*-genes were sequenced using the pUC/M13 (-26) sequencing primer.

Infiltration of *Arabidopsis* leaves and assessment of bacterial multiplication *in planta*

The infection of *Arabidopsis thaliana* (ecotype Col-0) leaves using a blunt -end syringe or the dipping inoculation method along with the enumeration of bacteria were carried out using an established method.

Measuring expression dynamics of *ivi*-genes by the fluorescence of GFP

Fluorescence measurements were carried out on a Fluoromax-3 (Jobin Yvon, France) spectrofluorometer. *P. viridiflava* cells conditionally expressing GFP were excited at 395 nm. Fluorescent emission was detected at 512 nm. *P. viridiflava* Ivi-mutants were grown overnight (approx 16 hours) in liquid LB media supplemented with 10 µg/ml gentamicin. Bacteria were centrifuged and pellets resuspended in distilled water, then pepper rings were vacuum infiltrated with the suspension. Pepper rings were

homogenated at different times after infiltration and fluorescence was measured.

Creating PV-dm, an *mviN*$_{pv}$ knockout mutant of *P. viridiflava*

The *mviN*$_{pv}$ containing chromosomal fragment was amplified by PCR from pIviGK::s1/6. The ends of the PCR product were back filled using T4 polymerase and cloned into the unique *Sma*I site of pJQ199. A kanamycin resistance cassette was inserted into the unique *Sma*I site of *mviN*$_{pv}$ and the construction was transformed into the donor *E. coli* S17.1. The mutated *mviN*$_{pv}$ was marker-exchanged as described previously into *P. viridiflava* to construct strain PV-dm. The correct insertion of the *Km*^{R} (*aphA3*) cassette was verified by colony PCR.

Analyses of TIS (translation initiation sequence) via the induction of the fused P$_{BAD}$

The different transposon mutant strains of *E. coli* HB101 containing the D-arabinose inducible P$_{BAD}$ promoter and the analysed translation enhancers containing *gfp* reporter gene fusions were incubated over night in LB media. Bacteria were suspended in LB media containing 0.2% (13.3 μM) D-arabinose. Samples were fluorometrically analysed in every two hours during the first 22 hours and then at the 48th hours of the incubation following promoter induction. The parameters of the used excitation and the detection of emission were the same as it was described above.

RESULTS

Promoter probing plasmid pIviGK

The enhanced features of the newly constructed promoter probing plasmid pIviGK (Fig. 1.) are standing below.

The central part of the MCSII synthetic polylinker sequence contains a multiple cloning site (MCS) which is flanked by the rare cutting *Pac*I restriction endonuclease recognition sequences in both sides and fragments corresponding to the M13/pUC –20 forward and –26 reverse sequencing primers. The *Pac*I recognition sequences make the MCS easily variable. The "universal" M13 sequencing primers can be used for PCR amplification or for the easy sequencing of the inserted DNA fragment.

There is only a single copy from each reporter gene in every bacterial genome, thus efficient translation from the low level of mRNA that is transcribed from the reporter genes is required for an optimal selection. Therefore, the reporter genes of pIviGK were constructed containing translational enhancer sequences in their 5' untranslated region. The 5' non-translating region of the antibiotic resistance encoding reporter gene contains the newly created translation initiation sequence (TIS, Fig. 1.)

Additionally, pIviGK contains three-way translational stop codons in front of both reporter genes (Fig. 1.) This ensures the generation of transcriptional fusions between targeted chromosomal genes and the reporter genes, instead of translational fusions that might produce non-functional fusion proteins.

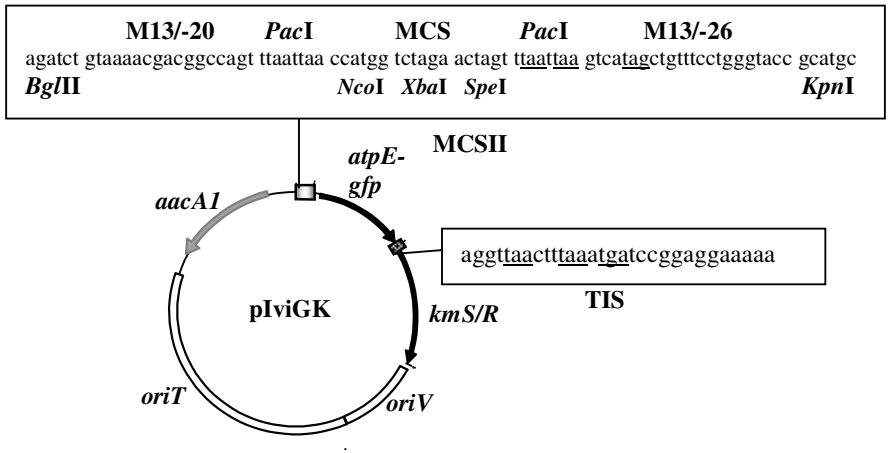

Fig. 1.

Physical map of pIviGK. The MCSII (multiple cloning site) containing *Nco*I, *Xba*I and *Spe*I recognition sites and the flanking region, that contains the *Pac*I rare cutting restriction enzyme recognition site on both sides, and the pUC/M13 sequencing primer recognition sites are shown enlarged in the upper panel. The *Bgl*II and *Kpn*I sites at the ends were used for the insertion of this synthetic DNA fragment into the pGP704 plasmid backbone.
The TIS (translation initiation sequence) is also enlarged on the right panel. The three-way translational stop codons upstream both to the *gfp* and the *kmS/R* (promoterless derivative of *aphA3*) reporter genes are underlined in the sequences. Plasmid pIviGK retained the Pir replication protein-dependent "suicide" replication origo (*oriV*) and the origin of conjugal transfer (*oriT*) from the parent pGP704 plasmid.

Verification of TIS (translation initiation sequence) efficiency in translation

Derivatives of mini-Tn5Km transposon construction were created containing different translational enhancer sequences fused with the promoterless *gfp* gene and the P_{BAD} promoter. The later derived the expression of the *gfp* gene in a D-arabinose dependent manner. The

effect of the new synthetic TIS translational enhancer to translation was determined in monocopy application by the fluorometric measurement of GFP following transposon mutagenesis of *Escherichia coli*.

The new synthetic TIS enhanced 1.5 fold the translation of GFP, compared to the atpE translation enhancer sequence in the first eight hours after induction of P_{BAD} promoter.

Construction of the IVET fusion library and elaborating *in vivo* antibiotic selection

The most useful sized range of the restriction endonuclease digested *Pseudomonas viridiflava* genomic DNA was determined, which containing the most constitutively expressing promoters, that are traceable using pIviGK. Promoter probing library was created in *E. coli* by partial fill-in of restriction endonuclease digested 2-3 kbp length *P. viridiflava* genomic and pIviGK plasmid DNA. Southern-hybridisation verified that the created library was fully comprehensive in the represented genomic fraction.

Using an improved triparental matting the library was introduced into *P. viridiflava*, wherein through a homologues recombination the insert containing pIviGK plasmid integrated into the bacterial genome.

Green pepper rings were infiltrated with *P. viridiflava* pool containing promoter probing library then with kanamycin solution under vacuum. The *in vivo* selection was completed within 10-12 hours post infection.

Isolating bacterial genes that are expressed preferentially during infection

Testing pIviGK and the corresponding antibiotic selection method have enabled the isolation of 132 *in vivo* kanamycin-selected colonies. Ten *ivi*-fusion from the isolated clones were sequenced, from which seven unique open reading frames (ORFs) encoding proteins with known function were identified. The search for protein homology has shown that the proteins encoded by the isolated ORFs may influence pathogenicity of *P. viridiflava* through functions, which contribute infection, colonization and provoking disease symptoms by the pathogen.

One of the isolated ORFs encodes pectate lyase of *P. viridiflava* that attacks pectin, the major constituent of plant cell wall in the soft rot disease of fruits and vegetables. Another identified ORF encodes a protein sharing 100% aa identity with the MviN membrane protein from *Pseudomonas syringae* pv. *tomato* DC3000. Other ORFs that were identified as homologues to *Pseudomonas syringae* pv. *tomato* DC3000 genes may play some marginal role in the pathogenesis of *P. viridiflava*. These include, a gene encoding deoxyguanosine -triphosphate triphosphohydrolase (dGTPase; EC 3.1.5.1), an ABC transporter (ATP-binding/permease protein), and a K^+-dependent Na^+/Ca^{2+} exchange related-protein.

Phenotypic analysis of $MviN_{pv}$

In order to examine the possible function of $MviN_{pv}$, a chromosomal knockout mutant of *P. viridiflava* was created, which is lacking a functioning $mviN_{pv}$ gene.

Soft agar motility assay indicated that the $MviN_{pv}$ protein is required for motility in *P. viridiflava*. Effect of the $mviN_{pv}$ mutation on the virulence of *P. viridiflava* in its compatible host, *Arabidopsis thaliana* was assayed by the growth curve analysis of the knockout mutant. It showed that the loss of motility was responsible for the failure of natural invasion of *Arabidopsis thaliana* leaves by the mutant, while the *in planta* colonisation and disease provoking ability of the mutant remained unaffected just like the provoked symptoms and the time required for symptom development.

Measuring *in planta* expression dynamics of the isolated promoters

The *in planta* activation of the isolated promoters have been examined by measuring the level of the emitted green fluorescent light of GFP reporter protein in Ivi-mutant strains after infection of pepper tissue. Results showed that some of the isolated promoters (e.g. the promoter of $mviN_{pv}$) have a moderate basic activity in complete bacteriological media that can increase under inducing conditions (e.g. *in planta*). However, the majority of the promoters did not show any activity outside of plant.

New scientific results

1. New promoter probing plasmid pIviGK was constructed for the genetic analysis of bacterial promoters, allowing antibiotic resistance-based selection of *in planta* inducible ones.
2. New synthetic translational enhancer (TIS, translation initiation sequence) was created and fused to the reporter gene, encoding antibiotic resistance.
3. A promoter probing gene library was created in the plant pathogen, soft rot causing *Pseudomonas viridiflava* using pIviGK.
4. Appling the new plasmid and the developed antibiotic selection system several *in planta* inducible bacterial genes was selected.
5. These genes included *pel*, the gene encoding pectate lyase, the main virulence factor of the bacteria under study. Another isolated gene is $mviN_{pv}$ encoding a putative novel membrane associated virulence factor of *P. viridiflava*.
6. A knockout mutation in the gene encoding $MviN_{pv}$ protein was shown to influence motility as well as virulence of *P. viridiflava* as far as the mutant was unable to infect host plant when surface inoculated.

DISCUSSION AND FUTURE PERSPECTIVES

In this study a novel IVET promoter probing plasmid, pIviGK and the required improved *in planta* antibiotic selection system was developed. Practically, during the application of the improved method, a special promoter probing gene library was created in *E. coli* using the pIviGK plasmid, containing *P. viridiflava* genomic DNA. In the gene library the genomic DNA fragments fused to a special reporter gene pair harboring on pIviGK plasmid. The prepared gene library was reintroduced into *P. viridifalava*, wherein the cloned genomic fragments along with the fused reporter genes integrated into the genome of the bacteria under study by homologues recombination. Once the host plant, namely green pepper crop was infiltrated with a pool of recombinant merodiploid bacteria, then they was subjected to a special *in planta* antibiotic (kanamycin) selection. The bacteria which survived this selection are kanamycin resistant due to the kanamycin resistance encoding reporter gene that expressed by an active promoter fused with. Thus, the survived bacteria represent constitutively expressing as well as *in planta* inducible promoters. The GFP encoding reporter gene harboring on pIviGK ensures the counter selection of the constitutively expressing promoters through a second *in vitro* selection of the survived bacteria on bacteriological media. It is based on the phenomena, that under *in vitro* circumstances bacterial colonies representing inducible promoters incapable emitting green

fluorescens light, in contrast to the ones, representing the GFP producing constitutively active promoters.

The novel promoter probing plasmid, pIviGK enables the easy sequencing and the determination of expression dynamics of the selected inducible promoters.

The Dissertation describes seven isolated *in planta* inducible genes of *P. viridiflava,* which were isolated using the new pIviGK plasmid construction. One gene of interest was identified as encoding a homologue of MviN putative membrane associated bacterial protein. An $MviN_{pv}$ knockout mutant derivative of *P. viridiflava* was created that was non-motile and, exhibited reduced virulence when was surface inoculated, comparing to the wild type strain, but no differences in virulence were noted when the bacteria were infiltrated into the plant tissue. This indicated first time that *mviN* is a virulence determinant of a plant pathogenic bacterium. The *in planta* expression of $mviN_{pv}$ was monitored using the co-expressed *gfp* reporter gene. It showed that the promoter of $mviN_{pv}$ has a basic activity in LB media that can increase with 20% at the third hours of the infection. In paralel, GFP emission that based on the induction of the pectate lyase promoter, was above the detection baseline at 1.5 hours post infection.

Based on these results the IVET method that utilizes pIviGK would certainly allow identification of additional virulence genes of plant pathogenic bacteria thereby provide deeper insights into the pathogenesis of bacterial diseases of plants.

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