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Genetic Transformation Of The Phytopathogenic Bacterium *Xanthomonas Euvesicatoria* With Synthetic Plasmids

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Introduction

Recombinant DNA technologies are one of the leading trends in molecular biology. The developments in question are increasingly being used in various fields, including microbiology. Microorganisms, and bacteria in particular, are the causative agents of a number of pathogenic diseases in humans, animals and plants. DNA technologies have developed a harmless way to visualize microorganisms, based on the incorporation into their genome (either directly in their DNA or through additional plasmid DNA molecules) of fluorescent compounds of completely natural origin. This enables us to trace a number of processes that are otherwise invisible to us. Thus, for example, in the transformation of the phytopathogenic bacterium *Xanthomonas euvesicatoria* with a synthetic plasmid leading to the expression of green fluorescent protein (GFP), we could easily follow the movement and spread of the bacterium in question in infected plants under UV light and, accordingly, observe results in its treatment (effective or not).

Aim of the study

The aim of the present study was to obtain a transformed bacterium that, based on constant expression of green fluorescence, could be easily tracked in artificially infected pepper plants with a view to the in-depth study of the relationship between the host plant and the phytopathogenic bacterium (Fig. 1).

Fig. 1

Methods

The transformations were carried out via electroporation and molecular methods (PCR and RFLP) were used for plasmid and species identification. Three synthetic plasmids were selected to be transferred into *X. euvesicatoria* cells: pBbB13k (GFP, NeoR), pKRR5 (mRFP, GmR) and pWVR5 (mRFP, GmR)(Table 1). The electroporated cultures of *X. euvesicatoria* were grown on LB agar with appropriate antibiotic (AB) (K - kanamycin or G - gentamicin) depending on the plasmid.

Fig. 4: Agarose gel electrophoresis for visualization of products from long range PCR

Table 2: Viability of transformed bacteria after four passages on LB agar with gentamicin

number	growth				number	growth				number	growth			
	*I**	*II*	*III*	*IV*		*I*	*II*	*III*	*IV*		*I*	*II*	*III*	*IV*
t1					t11					t21				
t2					t12					t22				
t3					t13					t23				
t4					t14					t24				
t5					t15					t25				
t6					t16					t26				
t7					t17					t27				
t8					t18					t28				
t9					t19					t29				
t10					t20					t30				

I* - succession of reseeding; green - good growth; yellow - weak growth, but visible; blue - lack of growth

Fig. 5: Agarose gel electrophoresis of restriction enzyme reaction of ABC and BDC constructs with *Bgl* I endonuclease

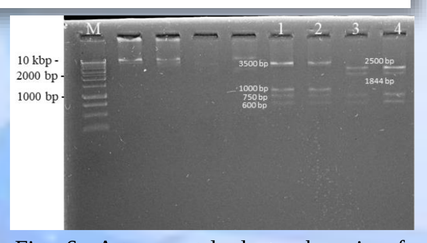

Fig. 6: Agarose gel electrophoresis of restriction enzyme reaction of AB+C and BD+C with endonuclease *Bgl*I

Table 1: Characteristics of plasmids

Plasmid	***Lenght (bp)***	***Origin of replication***	***Fluorescent protein***	***Marker gene***
pBbB13k	3321	pBBR	GFPuv (green)	NeoR/KanR (kanamycin)
pKRR5	8289	RK2 and pUC	mRFP (red)	GmR (gentamicin)
pWVR5	6840	pSa	mRFP (red)	GmR (gentamicin)

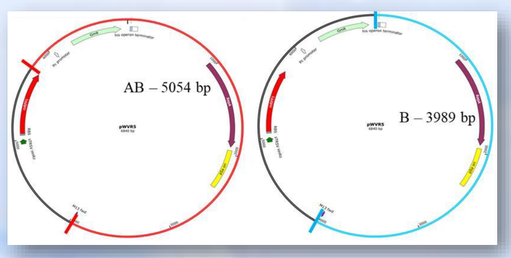

Fig. 2: Position of the amplified fragments from pWVR5 plasmid

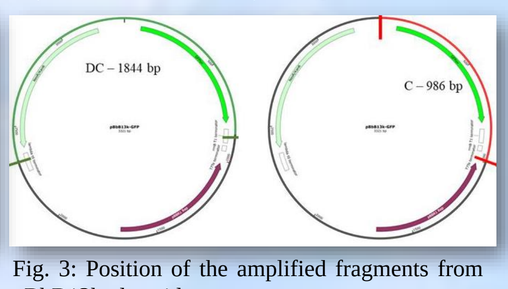

Fig. 3: Position of the amplified fragments from pBbB13k plasmid

Results

After cultivation bacterial growth was observed only in the culture transformed with plasmid pWVR5. A total of 20 colonies were isolated and their belonging to *X. euvesicatoria* was proven by species-specific PCR. However, the expression of AB resistance gene turned out to be rather unstable, since after several re-inoculation of the LB + 20µg/mL G, the transformants gradually lost their viability. After the second pasage, 73% retained their ability to grow on medium with G, after the third - 20%. No growth was observed after the fourth reseeding (Table 2). However, the marker gene in this plasmid (mRFP) is not constitutive and depends on the presence of rhamnose in the media. That's why the experiments continued with the construction of new synthetic plasmid, containing parts from pBbB13k (GFP) and pWVR5 (GmR and pSaOri). The needed fragments (C, containing GFP and AB - GmR and pSaOri)(Fig. 2 and 3) were amplified via long-range PCR and subsequently ligated into new plasmid (Fig. 4). The positive result for plasmid construction was confirmed by RFLP analysis using endonuclease *Bgl*I (Fig. 5 and 6). The new synthetic plasmid was used to transform *X. euvesicatoria* cells by electroporation (Fig. 7). After reported growth of the transformed bacterium on LB agar + G, we isolated 23 clones. Species belonging to *X. euvesicatoria* was confirmed by PCR (Fig. 8). However, we observed a trend towards a gradual loss of the ability of the transformed bacteria to grow on the LB agar +G after each subsequent re-inoculation. Finally, only two clones retained relative viability (Fig. 9, 10 and 11).

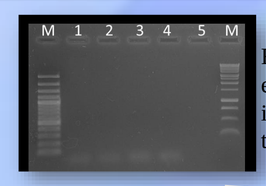

Fig. 9: Agarose gel electrophoresis of plasmid isolated from ABC 16 transformant

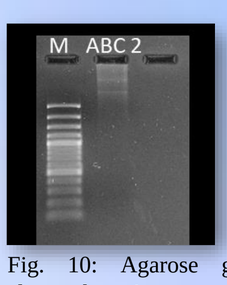

Fig. 10: Agarose gel electrophoresis of plasmid isolated from ABC 2 transformant

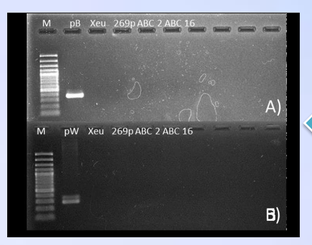

Fig. 11: Agarose gel electrophoresis with primers GFP_F1/R1 (A) and primers Gen_F1/R1 (B)

2 clones → 9 clones → 23 clones

Fig. 8: PCR amplification of proving species affiliation of *X. euvesicatoria*

colonies of *X. euvesicatoria* 269p (wild strain) on LB agar + 20 µg/mL gentamicin (A)

colonies of *X. euvesicatoria*269p after electroporation (B)

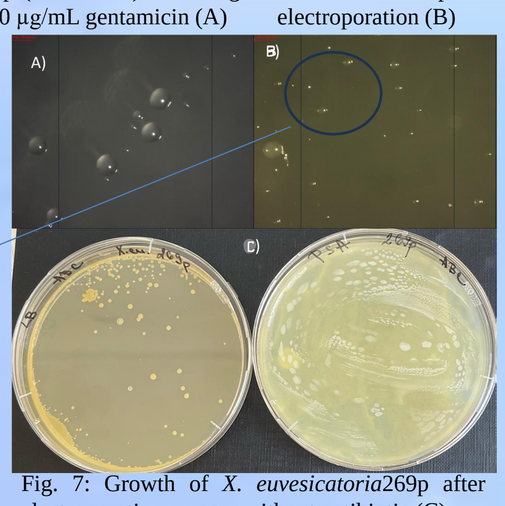

Fig. 7: Growth of *X. euvesicatoria*269p after electroporation on agar without antibiotic (C)

Conclusion

At the end of this thesis, there are many questions remain: Can *X. euvesicatoria* transform as easily as other bacterial species; is a correct construct formed after the assembly reaction; how long the transformed bacteria retain the plasmids in the cell after transformation; and from what depends how long this time will be.

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