CLONING OF BACTERIAL PCB-DEGRADING GENE INTO THE PLANTS

M. Surá[1], M. Macková[1], T. Macek[2], R. Borovka[1], M. Szekeres[3], S. Bancos[3]

[1]Institute of Chemical Technology
Faculty of Food and Biochemical Technology
Department of Biochemistry and Microbiology
Technicka 3, 16628 Prague, Czech Republic
Phone: +420 2 24 35 30 21, Fax: +420 2 24 35 51 67, e-mail:martinasura@hotmail.com
[2]Institute of Organic Chemistry and Biochemistry, CAS
Department of Natural Products
Flemingovo no. 2, 16610 Prague, Czech Republic
[3]Institute of Plant Biology
Biological Research Center of the Hungarian Academy of Sciences
P. O. Box 521 H-6701 Szeged, Hungary

Abstract

The target of this work was cloning of bacterial degrading gene *bphC* to increase biodegradation potencial of polychlorinated biphenyls (PCB) by the plants. For this purpose the gene *bphC* encoding the enzyme 2,3-dihydroxybiphenyl-1,2-dioxygenase from bacteria *Pseudomonas testosteroni B-356* was chosen to be cloned with the detection marker gene GFP (green fluorescent protein). Because of the difficulties with the detection of expression of GFP in plant tissue, also other constructs were prepared. These contain luciferase gene next to the *bphC* gene, beta-glucuronidase gene and/or six histidine motives. Histidine tail facilitated isolation of the enzyme. The presence of *bphC*/GFP DNA and RNA was proved by PCR. Immunochemical analysis confirmed the presence of BphC/GFP fusion in several transformants. The constructs with *bphC*/LUC, *bphC*/GUS and *bphC*/His were transformed into the *Nicotiana tabacum* via agrobacterial infection.

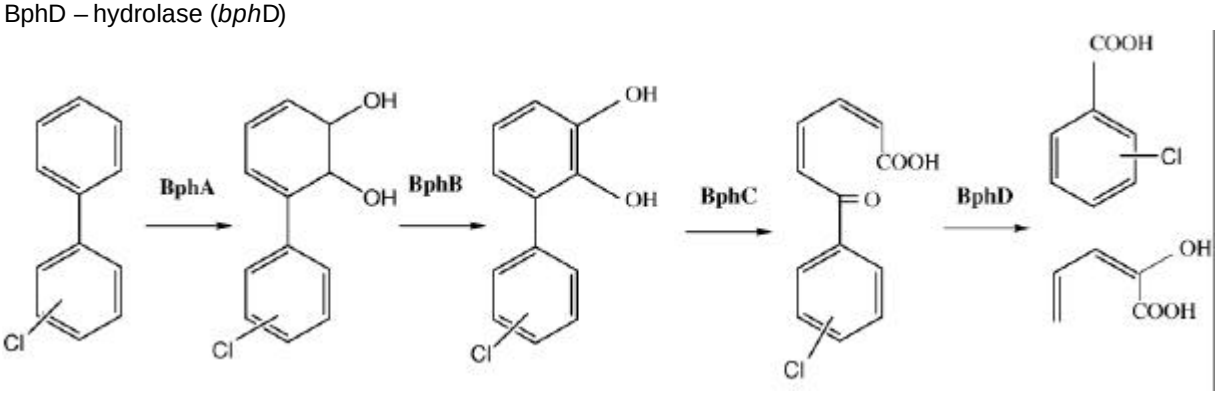
Introduction

Polychlorinated biphenyls (PCBs) are lipophilic substances, which were widely used till the beginning of the eighties. PCBs have very good physical and chemical properties, but these properties negatively affected their persistence in the nature, which can further negatively act on fauna, flora and human health. There are several ways how to decrease the amount of PCBs from the environment. One possibility of the removal of contaminants from soil represents the physico-chemical methods. These techniques are unfortunately highly economically demanding and often can further destroy the environment [1]. Therefore the research has been oriented towards the use of biological remediation methods based on the fact that various organisms can degrade various xenobiotics. One of the biological method is the use of green plants for transfer, accumulation and removal of pollutants from the environment, or at least reduction of their spreading [2, 3] - phytoremediation.

It was shown that plants have limited abilities to mineralise PCBs [4, 5]. Unlike bacteria, plants generally transform PCBs to hydroxychlorobiphenyls without cleavage of the biphenyl ring. This limitation can be overcome by preparation of transgenic plants with known bacterial genes cleaving and destroying biphenyl structure.

The purpose of this investigation was to engineer genetically modified plants bearing the bacterial gene *bphC* coding enzyme 2,3-dihydroxybiphenyl-1,2-dioxygenase. This enzyme is responsible for opening of biphenyl ring and degradation of the molecular structure. It catalyses the conversion of 2,3-dihydroxyclorobiphenyl into 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid (fig. 1).

Figure 1: Bacterial metabolic pathway of PCB
BphA – biphenyl-2,3-dioxygenase (*bph*AEFG)
BphB – biphenyldihydrodioldehydrogenase (*bph*B)
BphC – 2,3-dihydroxybiphenyl-1,2-dioxygenase (*bph*C)
BphD – hydrolase (*bph*D)



Materials and methods

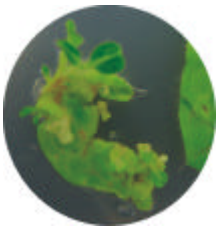
Bacterial and plant strains

In our study following bacterial strains were used - *Comamonas testosteroni* B-356, *Escherichia coli* M15 harboring pREP4 plus hybrid plasmid pQE31 (carrying B356 *bphC*), *Escherichia coli* XL1-Blue, *E. coli* S17-I and *Agrobacterium* GV3101 (pPM90RK).
Aseptic cultures of *Nicotiana tabacum* var. Wisconsin 38 and *Arabidopsis thaliana* var. Wassilewskiya were used for transformation by agrobacterial infection.

Cloning of *bphC* gene into plants

Gene *bphC* (882 bp) was originally isolated from the operon of *Comamonas testosteroni* B-356 [6]. Other genes were for fusion with gene *bphC*. These were gene GFP (gene for green fluorescent protein), GUS (gene for beta-glucuronidase), LUC (gene for luciferase), and also six histidine motives were fused with gene *bphC*. Plasmids, which were used in this study, are following: pBluescript, pQE31, pPCV812i [7], pPCV/LUC+-NOS [7]. Constitutive promoter CaMV 35S was used in each construct. In this study the construct with *bphC*/GFP was already transferred into the plant cells of *Nicotiana tabacum*. Other prepared constructs were first check for their correct primary structure and then transformed to *E. coli* S17-1 later conjugated with *Agrabacterium* GV3101 (pPM90RK). Agrobacteria bearing constructs with the gene *bphC* in cassete with GUS, LUC or His tail were used for transormation of plant cells by agrobacterial infection. Transformed regenerants were selected on media with selective antibiotic (fig. 2). Transgenes were recognized by root formation.

Figure 2: Regenerants of transformed *Nicotiana tabacum*



Confirmation of the presence of the gene *bphC* in plants

Presence of *bphC*/GFP in plants was confirmed after isolation of plant DNA and PCR amplification of the gene using combination of specific primers bphC1/F, bphC2/R, bphC3/F, GFP/F and GFP/R (fig. 3). The following experiments included the isolation of plant RNA, RNA cleaning from DNA and RT-PCR. Also protein studies were done by Western blot analysis with GFP and BphC antibodies.

Presence of *bphC*/LUC in plants *Arabidopsis thaliana* was confirmed on the DNA level by PCR with specific primers bphC1/F, bphC2/R, LUC/F and LUC/R (fig. 4). Also histochemical studies were performed to prove the expression of the protein BphC/LUC.

Figure 3: Fusion of genes *bphC* and GFP. There are shown primers and the size of fragments that they amplifiy.

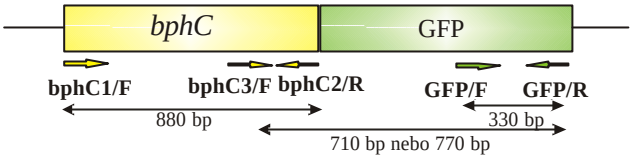
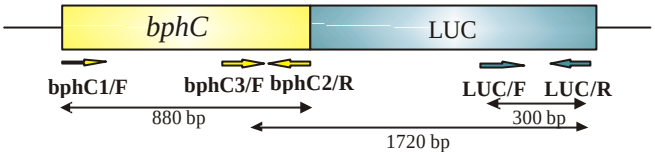


Figure 4: Fusion of genes *bphC* and LUC. The designed primers show the size of amplified fragments.



Results and discussion

Several bacteria can degrade PCB to less toxic chlorobenzoic acid [8]. This degradation pathway of PCB contains four step, from which the third one (catalysed by enzyme BphC), responsible for the cleavage of the biphenyl ring, is crucial. However plant can also transform PCB and the monohydroxylated and dihydrohydroxylated chloroderivatives are occurring as a major product [5]. These experiments show the inability of plants to destroy biphenyl structure. Therefore the aim of this study is to engineer plant harboring gene *bphC* and thus create plant enzyme system that can destroy the biphenyl structure.

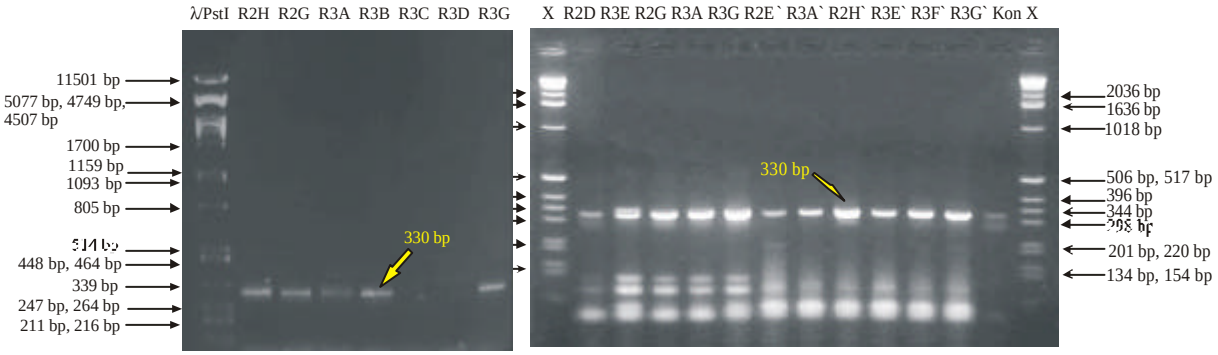
Gene *bphC* (882 bp) encodes the enzyme 2,3-dihydroxybiphenyl-1,2-dioxygenase (see fig. 1) and is originally isolated from *Comamonas testosteroni* B-356.

Four designs to clone gene *bphC* into plants were proposed: To clone it in fusion with the gene for green fluorescent protein (GFP), beta-glucuronidase (GUS), luciferase (LUC) and with histidine tail (His). All these constructs harbor the constitutive promoter CaMV 35S.

The plants of *Nicotiana tabacum* transformed by cassette *bphC*/GFP were already prepared. The detection of presented gene was proved after isolation of plant DNA and PCR amplification of the selected sequences with specific primers (see fig. 3 and fig. 5). The plant RNA was isolated, treated by DNase I and RT-PCR was performed. The presence of appropriate RNA was confirmed (fig. 6).

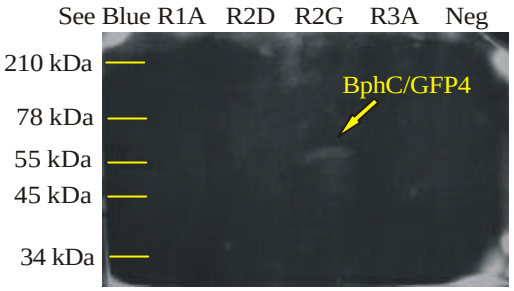
Figure 5:The result of PCR with plant DNA (*bphC*/GFP) with primers GFP/F, GFP/R. Each line is one clone plant.

Figure 6: The result of RT-PCR with plant RNA (*bphC*/GFP) with primers GFP/F, GFP/R. Each line is one clone plant.



The expression of gene *bphC*/GFP was studied on the protein level by Western blot analysis with the BphC antibody and GFP antibody. Experiments were successful only in one case of using GFP antibody (fig. 7). In figure 7 the band about 56 kDa is visible, which size corresponds with the fusion protein BphC/GFP.

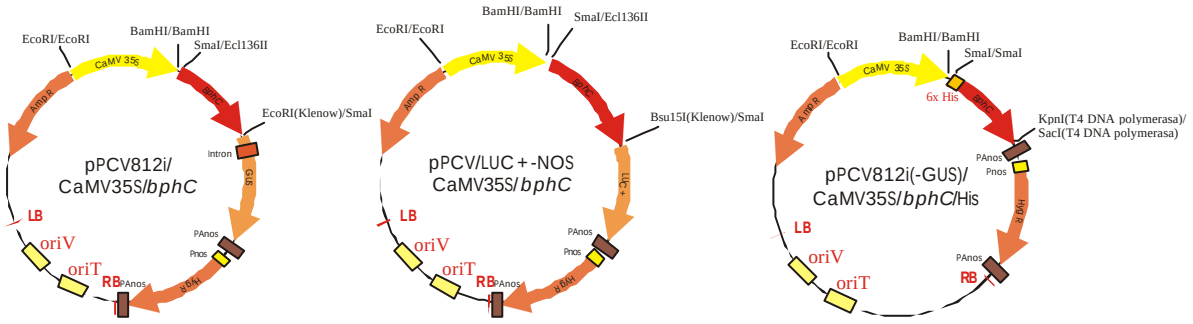
Figure 7: Western blot assays with protein extract. GFP antibody, marker See Blue and negative control (*Nicotiana tabacum* var. Wisconsin 38) were used. Each line is single clone.



Detection of the fusion protein was followed using other techniques. Fluorescent microscopy of protein GFP (green fluorescent protein) did not give satisfactory results, mainly because of the significant autofluorescence of the tobacco tissue.

Because of the difficulties with detection of protein BphC/GFP, additional constructs bearing gene *bphC* were designed. Gene *bphC* was fused with gene for beta-glucuronidase, luciferase and also with histidine tail. These constructs were prepared in plasmids pPCV812i (containing GUS gene) and pPCV/LUC+-NOS (containing LUC gene). The promoter CaMV 35S had to be inserted into these plasmids. The final models are shown in the figure 8.

Figure 8: Prepared constructs containing CaMV35S/*bphC*/GUS, CaMV35S/*bphC*/LUC, CaMV35S/His/*bphC* in pPCV.

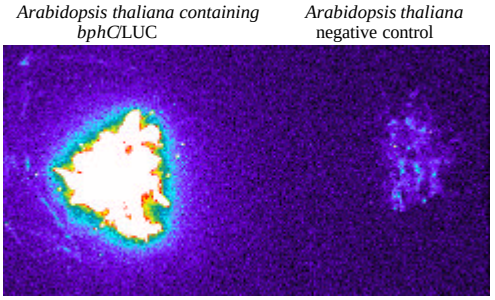


These constructs were cloned in bacteria *E. coli* XL-1Blue, primary structure was proved after isolation of plasmid DNA from bacterial cells. Further experiments were directed to transfer of transgenes to plant cells. First the constructs were cloned to *Escherichia coli* S17-I (helper strain in transfer of the prepared plasmids into the cells of agrobacteria, which is described in the article [7]) and then to bacterial cells of the plant pathogen – *Agrobacteria tumefaciens*. *Agrobacteria* GV3103 (pPM90RK) bearing three different constructs were used for transformation of *Nicotiana tabacum* (with constructs CaMV35S/*bphC*/GUS, CaMV35S/*bphC*/LUC, CaMV35S/His/*bphC*) and *Arabidopsis thaliana* (with construct CaMV35S/*bphC*/LUC) by agrobacterial infection.

In the case of transformation of *Nicotiana tabacum* the regenerants nowadays grow on selective media, where the transgenes are recognized by root formation.

In the case of transformation of *Arabidopsis thaliana* by construct CaMV35S/*bphC*/LUC the transgene plants were already selected. To detect the presence of appropriate gene histochemical detection was followed. Plants were exposed to the effect of luciferine and the occurred luminescence was measured by luminometr. Expression of BphC/LUC was confirmed by detection of the strong luminiscence (fig. 9), so they express the protein BphC/LUC. Plant DNA was isolated and PCR was performed with specific primers (see fig. 4). This experiment demonstrated, that the transgene was inserted into the plant genome.

Figure 9: Luminiscence of *Arabidopsis thaliana* harboring the transgene *bphC*/LUC.



Conclusions

- Different constructs containing gene *bphC* in fusion with gene for green fluorescent protein, luciferase, beta-glucuronidase and histidine tail were prepared.
- The correct primary structure of the fusion genes was proved.
- Fusion genes were transformed into plant cells by agrobacterial infection.
- In transformed plants the presence of DNA (*bphC*/GFP, *bphC*/LUC) and RNA (*bphC*/GFP) was detected.
- Expressed protein BphC/GFP was detected by Western blot analysis.
- Positive expression of BphC/LUC was detected by histochemical assays of luciferase.

Acknowledgements

The work described in above was sponsored by the grants of FRVS No. 1581/G4 and 5FW QLK 3-CT-2001-00101

References

[1] T. Macek, K. Francova, M. Sura and M. Mackova: *Genetically modified plants with improved properties for phytoremediation purposes*. In: Phytoremediation of metals, NATO Science Series, Kluwer Academic Publishers, Dordrecht, in press, (2003).

[2] S. Cunningham, W. Berti and J. Huang: Phytoremediation of contaminated soils, *TIBTECH* **13**, 393-397, (1995).

[3] T. Macek, J. Kas and M. Mackova: Exploitation of plants for the removal of organics in environmental remediation, *Biotechnol. Advances* **18**, 23-34, (2000).

[4] P. Kucerova, M. Mackova, L. Chroma, J. Burkhard, J. Triska, K. Demnerova and T. Macek: Metabolism of polychlorinated biphenyls by *Solanum nigrum* hairy root clone SNC-90 and analysis of transformation products. *Plant and Soil* **225**, 109-115, (2000).

[5] A. Wilken, C. Bock, M. Bokern and H. Harms: Metabolism of different PCB congeners in plant cell cultures. *Environ. Chem. Toxicol*. **14**, 2017-2022, (1995).

[6] P. Hein, J. Powlowski, D. Barriault, Y. Hurtubise, D. Ahmad and M. Sylvestre: Biphenyl-associated meta-cleavage dioxygenases from *Comamonas testosteroni* B-356. *Can. J. Microbiol*. **44**, 42-49, (1998).

[7] C. Koncz, N. Martini, L. Szabados, M. Hrouda, A. Bachmair and J. Schell: *Specialized vectors for gene tagging and expression studies*. In: Plant Molecular Biology Manual **B2**, Kluwer Academic Publishers, Printed in Belgium, 1-22, (1994).

[8] M. Ambramowicz: Aerobic and anaerobic biodegradation of PCBs: a review. *Crit. Rev. Biotechnol*. **10**, 241-245, (1990).