Cloning the bacterial *bphC* gene into *Nicotiana tabacum* to improve the efficiency of phytoremediation of polychlorinated biphenyls

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The aim of this work was to construct transgenic plants with increased capabilities to degrade organic pollutants, such as polychlorinated biphenyls. The environmentally important gene of bacterial dioxygenase, the *bphC* gene, was chosen to clone into a plant of *Nicotiana tabacum*. The chosen *bphC* gene encodes 2,3-dihydroxybiphenyl-1,2-dioxygenase, which cleaves the aromatic ring of dihydroxybiphenyl, and we cloned it in fusion with the gene for β-glucuronidase (*GUS*), luciferase (*LUC*) or with a histidine tail. Several genetic constructs were designed and prepared and the possible expression of desired proteins in tobacco plants was studied by transient expression. We used genetic constructs successfully expressing dioxygenase's genes we used for preparation of transgenic tobacco plants by agrobacterial infection. The presence of transgenic DNA, mRNA and protein was determined in parental and the first filial generation of transgenic plants with the *bphC* gene. Properties of prepared transgenic plants will be further studied.

Author’s View

Polychlorinated biphenyls (PCBs) are lipophilic substances that were used worldwide until 1970s and in the former Czechoslovakia PCBs were used until mid 1980s. A PCB consists of two benzene rings substituted by varying amounts of chlorine atoms. PCBs have excellent physical and chemical properties that are well-suited to their use in industrial applications, but some of these properties also make PCBs highly toxic and persistent in the environment, thus having a negative impact on fauna, flora and human health. PCBs accumulate in fat and thus can be transferred through the food chain. One of the investigated negative effects of PCBs is the disruption of endocrine system function.1

There are several ways of reducing the amount of PCBs in the environment. Physical-chemical methods represent one option for the removal of contaminants from soil, but they are costly and often result in further environmental damage.2 Research, therefore, has been oriented towards the use of the environmentally beneficial and less expensive methods of biological remediation, which are based on the ability of various organisms to degrade structurally different xenobiotics.2-3 One such method is phytoremediation, in which plants are used for the transfer, accumulation and removal of pollutants from the environment, or at least to reduce the rate at which they spread.4,5 The process of rhizoremediation, in which plants are used together with rhizospheric microorganisms, is also the subject of growing interest.6-9

In our laboratory we have worked on the topic of biological remediation of soil contaminated by PCB and other pollutants since the early nineties. Our complex approach has yielded a range of published papers concerning the isolation and characterization of microorganism-degrading PCBs, phytoremediation experiments,
cooperation between rhizospheric microorganism and plants in remediation, stimulation of indigenous microorganism utilizing these pollutants and also preparation of transgenic plants with bacterial degradative genes to enhance phytoremediation potential. Our recent paper demonstrates the possibility of cloning of bacterial bphC gene into Nicotiana tabacum plants with the effect of increased degradation of 2,3-dihydroxybiphenyl, a metabolite of PCB.

Various studies have shown that plants have limited abilities to mineralize PCBs, yielding monohydroxylated and dihydroxylated chloro-derivatives without cleaving the biphenyl ring. The higher solubility and bioavailability of these compounds can result in them being more toxic than the original substrate. Conversely, as Figure 1 shows, aerobic bacteria are able to co-metabolically transform PCBs into less toxic chlorobenzoic acids through the biphenyl degradation pathway. The PCB degradation pathway consists of four steps, wherein the third step, the cleavage of the biphenyl ring (catalyzed by the BphC enzyme), is crucial. The various genes that encode this pathway have been cloned and characterized.

Plant metabolism of PCB is the subject of ongoing investigations studied and several metabolites have been found in plants. Among these belong hydroxylated, methoxylated or hydroxymethoxylated metabolites of PCBs. Plants conjugate these metabolites with sugar, glutathion or aminoacids and store them in vacuoles or incorporate them into the cell wall or lignin. We have demonstrated that the mono- and dihydroxybiphenyls formed from the degradation of PCBs by plants can serve as substrates for the bacterial enzyme dihydroxybiphenyl dioxygenase (BphC). The BphC enzyme, which catalyzes the ring cleavage reaction of 2,3-dihydroxybiphenyl into 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid (Fig. 1), is the key enzyme in the aerobic PCB degradation pathway. Thus, the inability of plants to cleave dihydroxybiphenyl can be overcome by the preparation of transgenic plants bearing bacterial genes known to cleave such structures. This approach was recently discussed as a way of enhancing environmental protection. The aim of our study, therefore, was to engineer such a transgenic plant, containing the bacterial bphC gene encoding 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC).

A transgenic plant with a bacterial gene that can be involved in the cleavage of biphenyl was recently prepared. The authors prepared a transgenic Arabidopsis thaliana with bacterial dbfB gene encoding trihydroxybiphenyl dioxygenase from a degradation pathway of dibenzofuran. Other experiments have also aimed to increase phytoremediation of PCBs through the preparation of transgenic tobacco with bacterial genes for biphenyl dioxygenase, the enzyme that catalyzes the first step in PCB degradation.

Our first experiment was to clone bacterial bphC gene from Pandoraea pnomenusa B-356 preparing transgenic N. tabacum containing fusion genes bphC/GFP, the bphC gene in fusion with the gene for green fluorescent protein (GFP), and the LUC gene. The presence of the transgene has been proven by PCR and by the presence of appropriate mRNA by RT-PCR. Nevertheless, the presence of expressed protein was proved only in young plants (seedlings) of the first filial generation, not in older ones (unpublished data). A possible reason for this effect could be gene silencing; therefore we designed other vectors for bphC cloning into a plant genome.

We have designed three plant vectors, where the bphC gene was in fusion with the gene for β-glucuronidase (GUS) gene, with the luciferase (LUC) gene, and with a histidine tail. The GUS and LUC genes

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**Figure 1.** Biphenyl catabolic pathway of Pandoraea pnomenusa B-356. BphAE, biphenyl-2,3-dioxygenase (bphAE); BphF, ferredoxin (bphF); BphG, ferredoxin reductase (bphG); BphB, biphenyl/dihydrodioldehydrogenase (bphB); BphC, 2,3-dihydroxybiphenyl-1,2-dioxygenase (bphC); BphD, hydro-lase (bphD).
were chosen because they can be used as markers for easy detection of transgenic plants. Histochemical detection with β-glucuronidase is a cheaper method than using luciferase; however, it is also a destructive one. The histidine tail was fused with bphC gene to facilitate isolation of the protein expressed in plant tissue.

We first studied the expression of recombinant proteins by transient expression in plants of N. tabacum. Transient expression is a quick method to verify if the particular bacterial protein in question can also be expressed by the plant system and whether or not the protein is degraded just after expression. It can also be used to test activities of various promoters in plants. We proved the possibility of transient expression in plant cells of all three designs—bphC/GUS, bphC/LUC and bphC/His. These results can be compared with results of other authors who also successfully expressed bacterial genes for enzymes from biphenyl degradation pathway BphAE/His, BphF/His and BphG/His in tobacco cells.

During the next step we prepared transgenic plants with the bphC gene in N. tabacum. Tobacco is quite a large plant, and forms a large amount of biomass, making it suitable for phytoremediation. The choice of plant for phytoremediation is tricky, because the chosen plant must have several specific characteristics. The plant should be able to survive in the given pollutant concentration, survive the climate where it will be used, form a large amount of biomass and the final transgenic plant should accumulate the pollutant in high amounts or even degrade the organic pollutant. Tobacco is not endemic to our mild climatic zone (central Europe), however it can survive in our climate or can be used for remediation of contaminated sites in more southern countries. There are also experiments that describe preparing transgenic plants from species such as flax, poplar trees, Arabidopsis thaliana, cannabis, etc. Flax is a technical plant and although it is a plant with low amount of biomass per plant, the amount of biomass per square meter is quite high. At the moment we are trying to prepare transgenic flax with bphC gene, and flax with transgenes aimed to improve heavy metal accumulation and/or increased glutathione content.

Transgenic plants containing bphC gene in three variants (bphC/GUS, bphC/LUC and bphC/His) were prepared via agrobacterial infection. Twelve transgenic lines were chosen for further testing: four of them containing bphC gene in fusion with histidine tail (bphC/His), five lines containing bphC gene in fusion with the gene for luciferase (bphC/LUC) and three lines containing bphC gene in fusion with the gene for β-glucuronidase (bphC/GUS). The presence of transgenic DNA and its appropriate mRNA was proven by PCR or RT-PCR in each of the discussed plant lines. Also, the presence of proteins BphC/GUS, BphC/LUC and BphC/His was verified either by histochemical reaction or with western blot analysis. The presence of BphC/LUC and BphC/GUS has been studied through the whole plant, as there is no preferable place of expression (Figs. 2 and 3).

We see a novel approach of this work in the use of the bacterial bphC gene chosen for cloning into the plant genome. The cloning of this gene is new; therefore, we preferred to clone it in three variants (bphC/GUS, bphC/LUC, bphC/His) to see which one of them will give best results—which would result in the best plant for phytoremediation purposes. Already some pilot experiments have been done to choose the best transgenic line that will give a high amount of biomass, be viable on toxic substrates and have increased phytoremediation abilities. One such experiment evaluated plant viability on high concentrations of 2,3-dihydroxybiphenyl (2,3-DHB). An experiment showing the decrease of 2,3-DHB in media where 2,3-DHB was the substrate for BphC enzyme has also already been performed (data not yet published). The discussed experiment was performed according to ref. 44 where authors tested transgenic plants with TNT as the substrate. An experiment exploiting young plants cultivated with 40 mg/l 2,3-DHB for 10 days was analyzed using HPLC. The decrease of 2,3-DHB was followed in media with different transgenic lines, and confirmed the functionality of the enzyme expressed in the GM-plants, thus proving the concept successful. Several selected transgenic lines showed remarkable decrease of the substrate 2,3-DHB. Also, viability of these lines surpassed that of the controls. Therefore these transgenic plants can be potentially considered as promising for further phytoremediation usage.

We also plan to test the viability of transgenic plants on media containing Delor103 (mix of PCBs) and some selected congeners. We will fully characterize the transgenic plants with genotoxicity test, where the genotoxic effect of PCBs would result in the best plant for phytoremediation purposes. Already some pilot experiments have been done to choose the best transgenic line that will give a high amount of biomass, be viable on toxic substrates and have increased phytoremediation abilities. One such experiment evaluated plant viability on high concentrations of 2,3-dihydroxybiphenyl (2,3-DHB). An experiment showing the decrease of 2,3-DHB in media where 2,3-DHB was the substrate for BphC enzyme has also already been performed (data not yet published). The discussed experiment was performed according to ref. 44 where authors tested transgenic plants with TNT as the substrate. An experiment exploiting young plants cultivated with 40 mg/l 2,3-DHB for 10 days was analyzed using HPLC. The decrease of 2,3-DHB was followed in media with different transgenic lines, and confirmed the functionality of the enzyme expressed in the GM-plants, thus proving the concept successful. Several selected transgenic lines showed remarkable decrease of the substrate 2,3-DHB. Also, viability of these lines surpassed that of the controls. Therefore these transgenic plants can be potentially considered as promising for further phytoremediation usage.

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in transgenic lines. Future aspects of the work include cloning of bacterial enzyme of wider substrate specificity into plants, e.g., toluene dioxygenase or improving the active site of BphC enzyme by site-specific mutagenesis. Although phytoremediation technology has been extensively reviewed in the literature, very few reviews have focused specifically on PCBs or include information on transgenic organisms, PCB and plant-associated bacteria. Tobacco as the target plant might be replaced soon by some energy or industrial crop (e.g., flax) to ensure some financial gain for the whole process of remediation or species better suited for some particular contaminated environment. As recently summarized, transgenic plants and associated bacteria constitute a new generation of genetically modified organisms for efficient and environment-friendly treatment of soil and water contaminated with polychlorinated biphenyls (PCBs).

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