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Enhanced tolerance and remediation of anthracene by transgenic tobacco plants expressing a fungal glutathione transferase gene

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ABSTRACT

Plants can be used for remediation of polyaromatic hydrocarbons, which are known to be a major concern for human health. Metabolism of xenobiotic compounds in plants occurs in three phases and glutathione transferases (GST) mediate phase II of xenobiotic transformation. Plants, although have GSTs, they are not very efficient for degradation of exogenous recalcitrant xenobiotics including polyaromatic hydrocarbons. Hence, heterologous expression of efficient GSTs in plants may improve their remediation and degradation potential of xenobiotics. In the present study, we investigated the potential of transgenic tobacco plants expressing a *Trichoderma virens* GST for tolerance, remediation and degradation of anthracene—a recalcitrant polyaromatic hydrocarbon. Transgenic plants with fungal GST showed enhanced tolerance to anthracene compared to control plants. Remediation of ^{14}C uniformly labeled anthracene from solutions and soil by transgenic tobacco plants was higher compared to wild-type plants. Transgenic plants (T_0 and T_1) degraded anthracene to naphthalene derivatives, while no such degradation was observed in wild-type plants. The present work has shown that *in planta* expression of a fungal GST in tobacco imparted enhanced tolerance as well as higher remediation potential of anthracene compared to wild-type plants.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) with two or more fused benzene rings are major environmental contaminants that occur in oil, coal and tar deposits and produced as byproducts of fuel burning, operations in refineries, fertilizer factories and methanogenesis in jungles [1]. Some of the polyaromatic hydrocarbons have been identified as carcinogenic, mutagenic and teratogenic [2] and high prenatal exposure is associated with lower IQ and childhood asthma. Due to their high degree of toxicity, mutagenicity, carcinogenicity, ubiquitous occurrence and recalcitrance, they cause significant environmental problems [1,2]. They are also known to alter native ecological communities, thus affecting the ecosystem [3]. Many studies have been conducted on biodegradation of individual PAHs and related compounds [2], but the major limitations have been the requirement for long degradation periods and the difficulty in controlling the conditions. Besides, some heavier PAHs (with more than 3 rings), due to their poor water-solubility [1,4] are difficult to be biodegraded. Anthracene is a persistent 3 ring, polyaromatic hydrocarbon present in dyes, wood preservatives, insecticides and coal tar and is known to be mutagenic and carcinogenic to biological tissues [5]. It is one of the 16 PAHs included in the European Union (EU) and the United States Environmental Protection Agency (US EPA) priority pollutant list. Anthracene, due to its structural similarities with the carcinogenic PAHs such as benzo (α) pyrene and benzo (α) anthracene is an important model compound for studies on PAHs degradation [6].

Phytoremediation—the use of plants to remediate pollutants [7,8], due to its aesthetically pleasing, environmentally non-destructive and economically cheaper qualities has gained a lot of attention in the last few years. Phytoremediation can be used for cleanup of polyaromatic hydrocarbons and there are a few studies on decline of PAHs in contaminated soils inhabited by different plants [9,10]. Disappearance of PAHs in those studies was found to be higher in planted soil, compared to unplanted control, indicating that phytoremediation enhanced the removal of these contaminants from the soil [11]. It was assumed that plant roots enhanced biodegradation of PAHs by stimulating the growth of soil microbes [12]. However, the fate of PAHs in plants has not been reported in earlier studies. Plants are known to possess metabolic network for biotransformation of a wide range of xenobiotic compounds [8]. Although plants harbor enzymatic machineries to detoxify the contaminants, which led to the "Green liver concept" proposed by Sandermann [13], they lack the complete metabolic pathways for degradation of xenobiotics, unlike the microorganisms. Plants are known to metabolize xenobiotic pollutants through three sequential steps [13]—phase I involving conversion/activation (oxidation, reduction and hydrolysis) of lipophilic xenobiotics [14], phase II

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resulting in conjugation of xenobiotic metabolite product of phase I to endogenous hydrophilic molecules such as glutathione [15] and phase III, which involves compartmentalization of modified xenobiotics. In phase II, the conjugation with glutathione which results in hydrophilic, less toxic, more polar compounds is mediated by enzymes such as glutathione transferases. Glutathione transferases (GSTs) catalyze the nucleophilic attack of S atom of glutathione on electrophilic groups of a variety of xenobiotic substrates in both prokaryotic and eukaryotic cells [16]. The inherent ability of plants to degrade xenobiotic compounds can be enhanced by introduction of efficient heterologous genes involved in xenobiotic degradation from other sources [8]. Glutathione transferases (E.C. 2.5.1.18), a family of enzymes responsible for detoxification of a broad range of xenobiotics including herbicides by conjugating them with glutathione [16] may be a useful candidate for detoxification of polyaromatic hydrocarbons.

Although there are reports of decline in PAH in planted soil [11], the decline is presumed to be due to rhizosphere biodegradation of PAH by microorganisms. There is no study conclusively reporting *in planta* degradation of PAH either by wild-type plants or by transgenic plants. Several fungi are known to have the property of degradation of PAHs [2]. Cultures of some lignolytic fungi are known to degrade benzo (α) anthracene to naphthalene derivatives and pthalic acid [17]. Using ^{14}C labeled compounds, it was shown that some lignolytic fungi can even degrade PAHs to CO_2 [18]. When biodegradation of fluoroanthene and anthracene from constructed wetlands were studied, *Trichoderma viridae* was one of the species involved in PAH degradation [19]. *Trichoderma virens* is an indigenously occurring economically important fungus, which is used commercially as a biofungicide [20]. In the past, some genes coding for endochitinases from *Trichoderma spp.* have been expressed in plants for improving biotic and abiotic stress tolerance, indicating stable, high level of expression of *Trichoderma* genes in plants [21,22]. Although there are a few reports on phytoremediation of herbicides using transgenic plants overexpressing GSTs [23,24], there is no report on phytoremediation and degradation of polyaromatic hydrocarbons by transgenic plants. We had earlier cloned *Trichoderma virens* GST and expressed it in tobacco plants [25,26]. In the present paper, we report the potential of transgenic *Nicotiana tabacum* plants expressing a glutathione transferase gene from the fungus *Trichoderma virens* to tolerate, remediate and phytodegrade anthracene, a PAH with three fused benzene rings.

2. Materials and methods

Transgenic tobacco plants (T_0 and T_1) expressing *TvGST* gene developed and confirmed [25,26] were used for phytoremediation studies of anthracene. Initially, 14 transgenic plants alongwith controls were assayed for expression of GSTs [25] and six plants showing high levels of GST (6–8 times) as compared to control plants were selected for anthracene uptake studies. T_0 plants were used for hydroponic and soil studies, while T_1 plants were used for studies under *in vitro* conditions.

2.1. ^{14}C Anthracene uptake and degradation studies

2.1.1. Hydroponic studies

Six independent transgenic tobacco lines (T_0) and control tobacco plants (in triplicates) with similar biomass (1.2 ± 0.15 g fw) were grown in hydroponics in Hoagland's medium [27] for 23 days. Anthracene, spiked with ^{14}C anthracene was added to a total of 300 ml medium in each flask to a final concentration of 1 ppm. ^{14}C anthracene (uniformly labeled, specific activity 38.870 MBq/mmol, obtained from IAEA, Vienna) was used for spiking. The activity in the samples was measured using a liquid scintillation counter (Packard, TR2100, US) at 0, 2, 3, 6, 9, 14, 17 and 23 days by taking 0.5 ml of medium from each flask in a liquid scintillation fluid (PPO, 4 g, naphthalene (AR), 60 g, ethylene glycol (AR), 200 ml, methanol (AR), 100 ml and dioxane (AR) to make 1000 ml) for analysis. Background corrections were made for all the samples. Hoagland's medium supplemented with anthracene, but devoid of plant served as a control for estimation of evaporation loss of anthracene. Total ^{14}C activity added to each flask was 10.8 MBq and ^{14}C mass balance of labeled anthracene was determined at the end of the experiment.

2.1.2. Soil studies

^{14}C anthracene (uniformly labeled, specific activity 38.870 MBq/mmole, obtained from IAEA, Vienna) was added to soil to study the uptake and degradation of ^{14}C anthracene by the transgenic plants. Six independent transgenic (T_0) lines and control tobacco plants with similar biomass (in triplicates) were grown in autoclaved red soil (pH 6.5, OC 1.8%, N 1.2%), collected from Trombay experimental field. Anthracene, spiked with ^{14}C anthracene was added to a total of 500 g of soil to a final concentration of 5 ppm and plants were grown for 23 days. Soil containing anthracene without plants and soil containing ^{14}C anthracene with wild-type plants served as controls for the experiment. The activity in the samples was measured using liquid scintillation counter as described above.

2.2. Sample extraction and analysis

After 23 days of experiment, using hydroponics and soil, plants were harvested and extracted using 100 ml of acetone by sonicating for 1 min to release adsorbed anthracene from roots into the solution. An aliquot of 0.5 ml extract was taken for counting by liquid scintillation counter and ^{14}C activity present was estimated. ^{14}C activity left in the hydroponic solution was extracted first with 200 ml acetone and partitioned with 20 ml hexane and 2% sodium sulfate. Hexane extract was concentrated. Aliquots of acetone and hexane extracts were counted separately using liquid scintillation counter. The plant tissues were extracted in 200 ml acetone in soxhlet extraction apparatus for 8 h and concentrated to 30 ml volume and 0.5 ml aliquot of this extract was used for counting. The acetone fraction was further partitioned with 20 ml hexane and 2% sodium sulfate, hexane fraction was separated and concentrated till dryness and reconstituted in 1 ml of acetone and further analyzed using HPLC. Aliquot of hexane extract was subjected to liquid scintillation counting to know the ^{14}C activity present there.

2.3. HPLC analysis

All the extracts were analyzed using HPLC (Waters, U.S.A), model 515 pump and 4.6 mm × 250 mm Symmetry C18 column and detected with a Waters model 2487 dual absorbance detector at 250 nm excitation wavelength and 450 nm absorption wavelength. The mobile phase was 70:30 (v/v) acetonitrile: water mixture at 1 ml min^{-1} flow rate. Anthracene and naphthalene peaks were identified by comparison of their retention time with authentic standards (Sigma). Further comparison of the extracts was done by co-chromatography with the analytical grade standards. The degradation products obtained in the transgenic plant extracts were further analyzed through GC-MS.

2.4. GC–MS analysis

GC–MS analysis was carried out on a Shimadzu GC–MS instrument (Shimadzu Corporation, Kyoto, Japan) equipped with a GC-17A gas chromatograph and provided with a DB-5 (J & W Scientific, CA, USA) capillary column ((5%-Phenyl)-methylpolysiloxane,