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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,

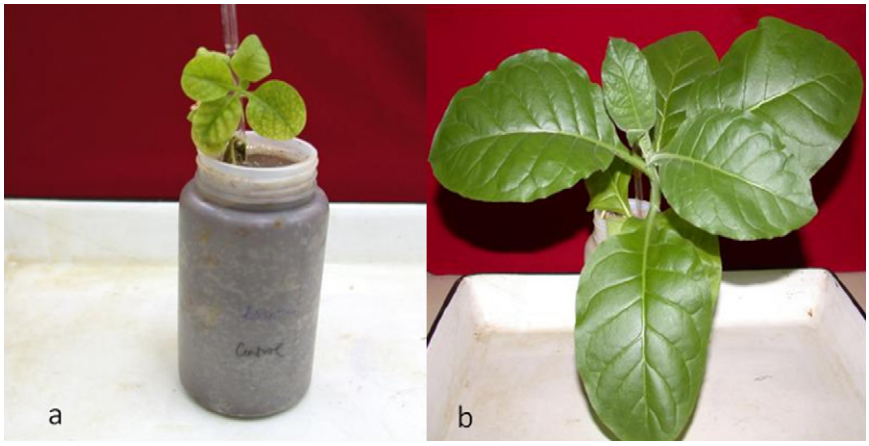

Fig. 1. Wild type (a) and transgenic T_0 plants (b) after 23 days of addition of anthracene (5 mM) in soil.

length, 30 m, id., 0.25 mm and film thickness, 0.25 μm). The operating conditions were: column temperature programmed from 60 to 200 °C at the rate of 4 °C/min, held at initial temperature and at 200 °C for 5 min and further to 280 °C at the rate of 10 °C/min, held at final temperature for 20 min, injector and interface temperatures, 210 and 230 °C, respectively, carrier gas helium (flow rate, 0.9 ml/min), ionization voltage, 70 eV, electron multiplier voltage, electron multiplier voltage 1500 V. Analyses were carried out in the splitless mode. The major peaks in each fraction were tentatively identified by comparing its mass fragmentation pattern with that of standard spectra available in the spectral library (Wiley/NIST Libraries) of the instrument as well as from the literature data [28].

2.5. Growth of T_1 seedlings on anthracene under in vitro conditions

Tobacco T_1 seedlings were germinated aseptically on MS medium [29] supplemented with 25 mg l^{-1} hygromycin. Ten seedlings each (0.5–1 cm) selected were transferred to MS medium supplemented with anthracene at 2 and 4 mM and grown under *in vitro* conditions in glass bottles. The growth of transgenic T_1 plants alongwith control plants grown on MS medium supplemented with 2 and 4 mM anthracene were recorded at the end of 30 days. Transgenic plants (T_1) alongwith control grown on anthracene supplemented medium were collected, samples extracted and used for HPLC analysis as described above.

2.6. Statistical analysis

All experiments were conducted as randomized block design with three replicates and repeated at least twice. For analysis, all the data were subjected to Duncan's test and one way ANOVA using the software IRRISTAT.

3. Results and discussion

Trichoderma virens TvGST gene was cloned, introduced into the binary plant expression vector pCAMBIA 1301, transgenic tobacco plants developed through *Agrobacterium*-mediated co-culture method and confirmed as described earlier [25,26]. Confirmed T_0 and T_1 plants were used to study the uptake and degradation of anthracene.

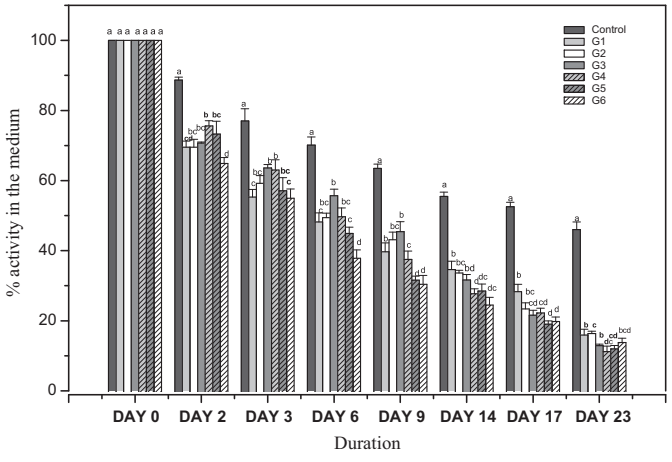

Fig. 2. Anthracene uptake by transgenic and control plants. Hydroponically grown plants were exposed to ^{14}C anthracene (specific activity 38.870 MBq/mmole) for 23 days and activity left in the solution was extracted and counted using liquid scintillation counter. All the values are means of three replicates ± S.E. Different letters indicate significantly different values at $p \leq 0.05$. Uptake of anthracene in transgenic plants was 88.6% as compared to 57% in control plants. All the values are means of three replicates ± S.E. Different letters indicate significantly different values at $p \leq 0.05$ at a particular duration (day) by Duncan's test.

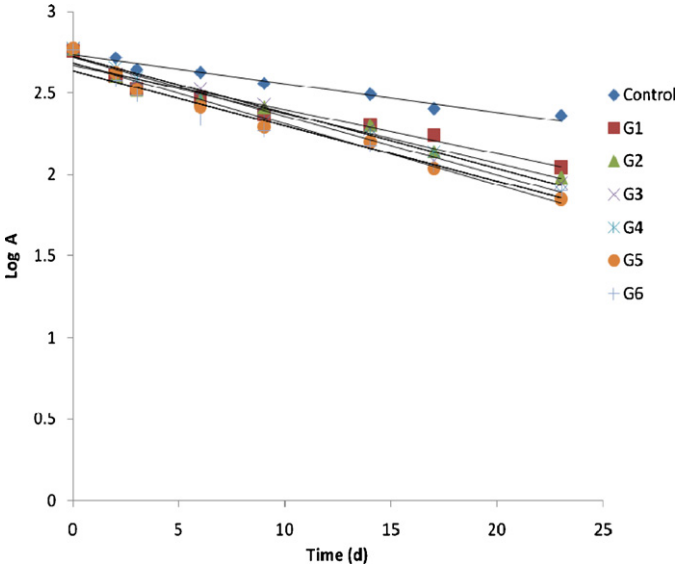

Fig. 3. First order kinetics of anthracene removal by control and transgenic plants.

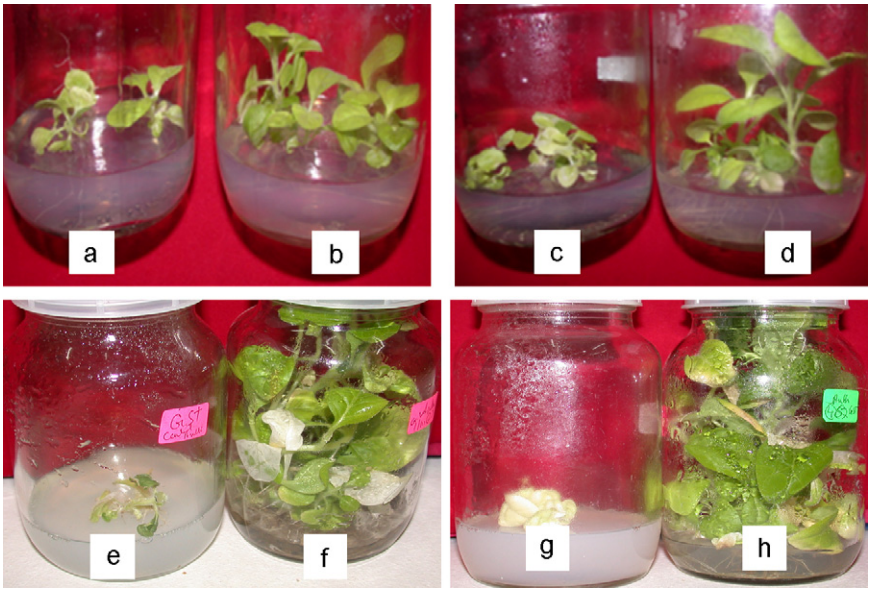

Fig. 4. Wild type tobacco after 30 days of addition of 2 mM anthracene (a), T_1 transgenic plants after 30 days of addition of 2 mM anthracene (b) wild type tobacco after 30 days of addition of 4 mM anthracene (c) and T_1 transgenic plants after 30 days of addition of 4 mM anthracene (d). Wild type tobacco after 90 days of addition of 2 mM anthracene (e), T_1 transgenic plants after 90 days of addition of 2 mM anthracene (f) wild type tobacco after 90 days of addition of 4 mM anthracene (g) and T_1 transgenic plants after 90 days of addition of 4 mM anthracene (h).

3.1. Tolerance, uptake and degradation of anthracene in transgenic plants

When six independent transgenic (T_0) and wild-type plants were grown in hydroponics supplemented with ^{14}C anthracene, transgenic plants were seen to tolerate anthracene and remained healthy, while control plants showed toxicity symptoms and remained stunted. The dry weight of transgenic plants exposed to anthracene (1 mM) at the end of 23 days was higher (0.183 ± 0.02 g) compared to control plants (0.03 ± 0.005 g). In soil spiked with anthracene (5 mM), transgenic plants showed better growth compared to control at the end of 23 days (Fig. 1) The dry weight of transgenic plants exposed to anthracene (5 mM) in soil at the end of 23 days was higher (0.29 ± 0.014 g) as compared to control plants (0.019 ± 0.007 g). The transgenic plants could take up 1.5 times more anthracene as compared to control plants when grown in hydroponics (Fig. 2). The kinetics of anthracene removal by both control and transgenic plants fitted the first-order kinetics (Fig. 3). All transgenic plants showed significant improvement of anthracene removal in comparison to the control. Rate of anthracene removal (k) increased by two fold in case of transgenic line of G3-G5 (Table 1). The result indicates the role of glutathione transferase gene introduced into the transgenic plants for anthracene removal. ^{14}C anthracene mass balance analysis showed a mass balance of 90–95% in hydroponics (Table 2). The difference in uptake of anthracene between different transgenic lines may be due to different expression levels of GST gene. In soil, uptake of anthracene by transgenic plants was 1.3 times higher as compared to wild type plants. The results of the present study that transgenic plants with a *TvGST* gene showed enhanced uptake of anthracene is comparable to earlier reports using other genes, where aspen plants expressing a bacterial nitroreductase gene showed enhanced uptake of TNT over wild type plants [30]. Studies with T_1 transgenic seedlings grown *in vitro* on MS medium supplemented with anthracene at 2 and 4 mM also showed that transgenic plants were more tolerant to anthracene compared to control plants (Fig. 4a–d). The dry weight of transgenic plants was found to be 0.22 ± 0.04 g at 2 mM anthracene and 0.079 ± 0.005 g at 4 mM anthrcene as compared to 0.030 ± 0.006 g at 2 mM anthracene and 0.021 ± 0.007 g at 4 mM anthracene. The T_1 transgenic plants showed increased height and they also had more number of leaves compared to wild-type plants when exposed to anthracene at the end of 30 days (Fig. 4a–d) and 90 days (Fig. 4e–h) of *in vitro* culture.

HPLC analysis of T_0 plants grown in hydroponics, soil and T_1 plants in *in vitro* showed that anthracene taken up was degraded in transgenic plants, but not in wild-plants (Fig. 5a and b). HPLC analysis of extracts from wild-type plants revealed three major

Table 1
First-order kinetics rate constant for ^{14}C anthracene removal from medium.

Plants	First order rate constant (k), d^{-1}	r^2
Control	−0.039	0.964
G1	−0.062	0.949
G2	−0.069	0.961
G3	−0.078	0.98
G4	−0.08	0.981
G5	−0.085	0.972
G6	−0.076	0.94

Table 2
^{14}C anthracene mass balance. Total activity added to each flask was180,000 dpm and ^{14}C anthracene mass balance was studied at the end of the experiment. ^{14}C anthracene mass balance analysis showed a mass balance of 90–95%.

Components	C	G1	G2	G3	G4	G5	G6
Activity left in solution at the end of the experiment	45.9	15.9	16.4	13	11.3	12	13.8
Evaporation loss	28.8	28.8	28.8	28.8	28.8	28.8	28.8
Physical adsorption	3.94	1.8	1.05	1.73	2.3	3.6	2.2
Extractable activity (soxhlet)	18.1	49.2	47.3	48.8	49.13	44.87	49.5
Total recovered activity (%)	96.7	95.7	93.5	93.2	91.53	89.2	94.3

All the values are in % activity.

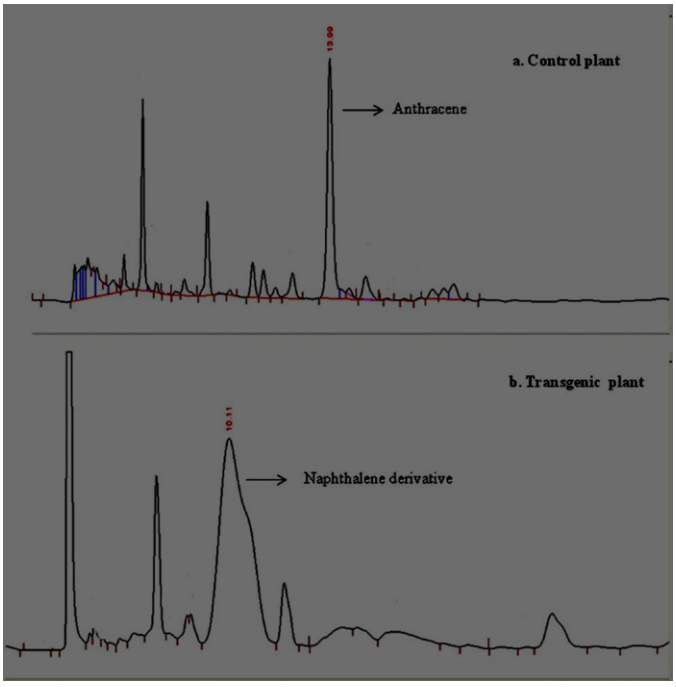

Fig. 5. HPLC analysis of control and transgenic plant grown in soil spiked with 5 ppm of anthracene. (a) HPLC analysis of control plant revealed a peak at 13.99 retention time corresponding to anthracene peak (b) HPLC analysis of transgenic plant showed a peak at 10.11 retention time which corresponds to naphthalene derivative.

peaks. Out of the different peaks, the peak at 13.99 min retention time was identified as anthracene. This was also confirmed by co-chromatography with anthracene. HPLC analysis of transgenic plant extract showed the presence of three major peaks. Transgenic plant extract did not show any peak at 13.99 min corresponding to anthracene while a major peak was observed at 10.11, which was comparable to napthalene. GC–MS analysis and subsequent identification with the GC–MS PAH library showed that non transgenic plant revealed one major peak (peak no 1) at 31.2 min retention times identified as anthracene (Fig. 6a) whereas transgenic plant sample revealed peaks (1 and 2) at 32.9 min and 41.3 min retention time which corresponded to naphthalene derivatives tetrahydro hexamethyl naphthalene and diethyl, tetrahydro tetramethyl naphthalene respectively (Fig. 6b–d). Similar results were seen for hydroponically grown T_0 plants and *in vitro* grown T_1 plants. The present observation showed that transgenic plant with *TvGST* gene could take up more anthracene and could degrade anthracene to naphthalene derivatives. The transgenic plants were not only tolerant to anthracene, but also could remediate and degrade it to naphthalene derivatives.

The cytosolic glutathione-s-transferase comprises a pivotal enzyme system for protecting the cell from electrophilic compounds and is known to play a major role in detoxification of xenobiotics [17]. Polycyclic aromatic hydrocarbons are known to induce GSTs in human liver [31]. Heterologous expression of GSTs in plants is expected to mediate degradation of xenobiotics [8]. Glutathione transferases are well known for enzymatic detoxification of xenobiotic compounds in mammals and fungi. Some of the fungal GSTs are related to alpha and mu classes of GSTs found in rat [32,33], suggesting a relationship between fungal and mammalian GSTs. Development of transgenic plants where critical steps in the catabolic pathway for degradation of recalcitrant, xenobiotics are incorporated could further enhance their potential for xenobiotic degradation [34,35]. Hence, cloning and introduction of a fungal GST gene into plants is expected to improve the detoxification potential of externally added xenobiotics.

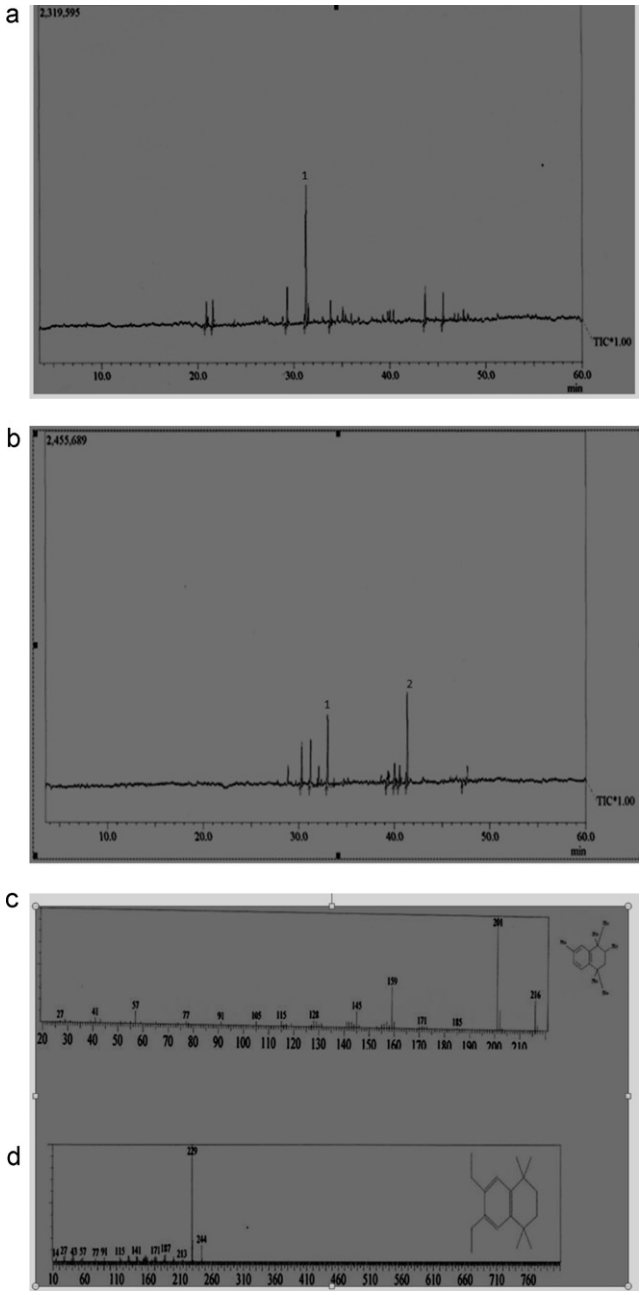

Fig. 6. Total ion chromatography of non transgenic and transgenic plants grown in anthracene spiked soil. (a) Chromatogram of non transgenic plant revealed one major peak (peak no 1) at 31.2 min retention time identified as anthracene. (b) Chromatogram of transgenic plant sample revealed peaks (1 and 2) at 32.9 min and 41.3 min retention time which corresponded to naphthalene derivatives (tetrahydro hexamethyl naphthalene and diethyl, tetrahydro tetramethyl naphthalene respectively). (c) Mass spectrum of peak at 32.9 min retention time in transgenic plant sample which corresponds to naphthalene derivative (tetrahydro hexamethyl naphthalene), identified using GC–MS PAH library. (d) Mass spectrum of peak at 41.3 min retention time in transgenic plant sample which corresponds to naphthalene derivative (tetrahydro tetramethyl naphthalene), identified using GC–MS PAH library.

In the present study, transgenic tobacco plants expressing a GST gene from the biocontrol fungus *Trichoderma virens* were developed and the plants showed enhanced uptake of anthracene compared to control plants. The transgenic plants also showed better growth and enhanced tolerance compared to wild-type plants in the presence of anthracene. HPLC analysis and further GC–MS analysis (present study) with confirmation from PAH library showed that anthracene taken up was degraded to naphthalene derivatives by

transgenic tobacco plants expressing a fungal GST. The transgenic plants could degrade anthracene – a three fused benzene ring compound to naphthalene derivatives – two ring compounds, while in control plants, anthracene remained unchanged. Degradation of anthracene to naphthalene derivatives were observed not only in T_0 plants grown in hydroponics, but also in *in vitro* grown T_1 tobacco plants. This confirmed that transgenic plant could phytodegrade anthracene to naphthalene derivatives. In the present study, GST gene of *Trichoderma virens* when transferred to tobacco could achieve this step of degrading a 3-fused benzene ring compound to 2-ring compounds. Although there are a few reports on decrease in PAH content in soil inhabited by plants [36–39], the degradation of the compounds tested in those studies was attributed to the action by microbes present in the rhizosphere. Using *in vitro* grown plants, we have shown that transgenic plants with GST gene could degrade anthracene to naphthalene derivatives. In our knowledge, this is the first report on an *in planta* degradation of anthracene to naphthalene derivatives. The high levels of expression of a fungal GST in transgenic plants help in enhancing the tolerance to anthracene. Earlier reports on overexpression of endogenous GST in plants have enhanced their tolerance and detoxification potential of various herbicides [23,40,41]. Plants do not have metabolic pathways for complete degradation of anthracene and other PAHs, while bacteria and fungi have evolved efficient machinery for anthracene biodegradation [42]. Microorganisms have an arsenal of enzymes for degradation of xenobiotic pollutants and transfer of genes involved in xenobiotic degradation into plants will further enhance the potential of plants for degradation of recalcitrant xenobiotics. However, for further degradation of naphthalene derivatives, perhaps other genes involved in naphthalene degradation need to be co-transferred to the plant along with GST gene.

4. Conclusions

The present findings successfully demonstrate a novel approach for developing tolerance, uptake and partial degradation of a recalcitrant environmental pollutant such as anthracene using transgenic approach. The present studies using T_0 and T_1 transgenic plants expressing a fungal GST gene have shown that transgenic tobacco plants expressing a *Trichoderma virens* GST were more tolerant to anthracene. Uptake of anthracene by transgenic plants was higher and they degraded anthracene – a three benzene ring compound to naphthalene derivatives – a two ring compound, while in wild-type plants, anthracene was not degraded. The present studies can have potential applications for remediation of other poly aromatic hydrocarbons and possibly other related xenobiotics.

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