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Short Communication

Improved degradation of organophosphate dichlorvos by *Trichoderma atroviride* transformants generated by restriction enzyme-mediated integration (REMI)

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ABSTRACT

A simple technique, REMI (restriction enzyme-mediated integration), was used to construct transformants of *Trichoderma atroviride* with improved capability of degrading organophosphate pesticide dichlorvos. Linearized DNA of plasmid pV2 bearing the hygromycin B phosphotransferase (*hph*) gene was inserted into chromosomes of wild strain T23 and transformation was confirmed by PCR and Southern blot analysis, respectively. Of 247 transformants, 76% showed improved dichlorvos degradation ability as compared to the parent strain T23 based on the least significant difference (LSD) test at $p = 0.01$. Among them, 8 transformants exhibited 30% higher in degradation rate than the parent isolate. The highest dichlorvos degradation rate of the transformants was up to 96%. This study provided an effective approach for improving organophosphate pesticide-degrading capability of *T. atroviride*.

1. Introduction

Organophosphate pesticide is heavily used and causes a major threat to ecosystems and public health. In China, with the banned use of some highly toxic organophosphate pesticides, such as parathion and methyl parathion, dichlorvos (*O*,*O*-dimethyl-2,2-dichlorovinyl phosphate; $C_4H_7Cl_2O_4P$), a relative low toxic organophosphate pesticide has been applied for plant protection against varied pests. Due to its water solubility, the pesticide residue in agricultural practices is capable of infiltrating through soil into surface water and eventually causing damage to aquatic organisms (Zhang et al., 1999). Therefore, it is very important to have an effective way to reduce the residual levels of dichlorvos in the environment.

There have been reports on microbial degradation of dichlorvos (Wang et al., 2006). Fu (2005) isolated a *Trichoderma* strain for dichlorvos degradation from dichlorvos-contaminated soil. *Trichoderma* spp. have also been used to remove heavy metals (Guillermina et al., 2002) and degrade cyanide pollutants (Ezzi and Lynch, 2005). However, *Trichoderma* strains reported above are mostly screened from natural soil through laborious work and those strains display low activity when used for degrading organophosphate pesticide residues in soil due to some adverse soil factors. Therefore, searching for genetically modified *Trichoderma* strains with improved abilities will satisfy the growing needs to remediate organophosphate pesticide contaminated soil.

Restriction enzyme-mediated integration (REMI) is a commonly used method to construct engineered mutants. We have previously reported that degradation of cyanides was improved by *Trichoderma* transformants generated by REMI (Zhou et al., 2007b).

The purpose of this study was to construct *Trichoderma* transformants with REMI method and subsequently to screen strains with higher dichlorvos degrading ability.

2. Methods

2.1. Construction of REMI transformants

Trichoderma atroviride strain T23 was used for REMI manipulation in this study. Plasmid pV2 (5080 bp in length) was kindly provided by Prof. Youliang Peng (China Agricultural University, Beijing, China). REMI transformants were constructed according to Huang et al. (2005) and Zhou et al. (2007a).

2.2. PCR analysis

To confirm the integration of the plasmid into the host chromosome, PCR analysis was performed. The parent strain T23 and REMI transformants were grown in 100 mL liquid potato dextrose medium (PD) for 48 h at 28 °C and *Trichoderma* genomic DNA was routinely extracted by CTAB protocol. Two pairs of PCR primers were

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used to amplify the fragments corresponding to *hph* and *amp* genes (Zhou et al., 2007b). The reaction conditions and amplification profiles were described by Zhou et al. (2007b). Ultrapure H_2O and DNA from parent T23 were served as negative controls and pV2 was used as positive control.

2.3. Southern blot analysis

To further confirm the plasmid integration into *Trichoderma* T23 chromosome, particularly the exact copy numbers of the heterologous gene in each transformant, 10 randomly selected transformants were analyzed through Southern blot, in which there was 5 transformants with a 510 bp *hph* gene fragment as the probe and other 5 with an 840 bp *amp* gene fragment as the probe. Fungal genomic DNA was digested overnight at 37 °C with SacI which does not cut within the probe region. The pV2 linearized with HindIII served as a positive control. The digested DNA was run on a 1.0% agarose gel for 2.5 h in 1 × TAE buffer. After electrophoresis, the gel was depurinated (0.3 M HCl) for 12 min, denaturated (1.5 M NaCl and 0.5 M NaOH) for 55 min and neutralizated (1.5 M NaCl and 0.5 M tris base, pH7.5) for 30 min. Then the digested DNA products were blotted onto Hybond N+ nylon membrane. DNA probe labeling (510 bp *hph* or 840 bp *amp* fragment amplified by PCR, respectively) hybridization and signal detection were performed using the Gene Images CDP-star detection system according to the manufacturer's protocols (AmershamGene Images CDP-Star Detection Kit, UK).

2.4. Assessment of tolerance to dichlorvos

A 5-mm mycelial plug of each transformant was transferred to a potato dextrose agar (PDA) plate with 600 μg mL^{-1} dichlorvos. A single plug of parent strain T23 on a PDA plate with the same concentration of dichlorvos was served as control. After incubation at 28 °C for 4 days, average diameters of *T. atroviride* colonies were measured in triplicates. The transformants with larger diameter than parent strain T23 were selected for further screening. The experiment was done twice.

2.5. Screening transformants with higher dichlorvos degrading ability

A fluorimetric method was used to confirm dichlorvos degradation level after the transformants tolerance test. The parent strain T23 and its selected transformants with improved tolerance to dichlorvos were incubated at 28 °C at 180 rpm for 3 days in flasks containing 100 ml PD. Then, mycelia were collected by filtration through Whatman No. 1 filter paper, and 5 g of mycelia were transferred to a flask containing 300 μg mL^{-1} dichlorvos in liquid Burk medium (g/L: $MgSO_4 \cdot 7H_2O$, 0.2, KH_2PO_4, 0.8, K_2HPO_4, 0.2, $CaSO_4 \cdot 2H_2O$, 0.1, $(NH_4)_2SO_4$, 1.0, $MnSO_4$, 0.01, $FeSO_4 \cdot 7H_2O$, 0.005, $Na_2MoO_4 \cdot 2H_2O$, 0.0033, glucose, 0.5, pH 7.0). Uninoculated flasks with the same concentration of dichlorvos were maintained as control. The flasks were incubated at 28 °C on a rotary shaker (180 rpm). After 60 h of incubation, the concentration of residual dichlorvos was detected according to a modified resorcinol-fluorimetric method (Yu and Tan, 1995). Constant volume of 10 ml mixture, including 4 ml 10% ethanol, 0.96 ml 0.5% NaOH, 0.24 ml fresh resorcinol and 4.8 ml sample were reacted for 3 min in boiling bath and cooled to room temperature for detection. The excitation wavelength and emission wavelength for fluorimetric detection were set at 491.6 nm and 521.1 nm, respectively. The degradation rate (%) of dichlorvos was calculated by formula $(C1 - C2)/C1^{*}100$ ($C1$ and $C2$ represent original and final concentration of dichlorvos, respectively). All treatments were in triplicate and the experiment was repeated 3 times.

3. Results

3.1. REMI transformants of T. atroviride strain T23 and stability test

By consecutive subculture test, a total of 247 transformants invariably displayed the resistance to hygromycin B as compared with the parent strain, which indicated that insertional mutagenesis of *Trichoderma* was stable.

3.2. PCR analysis

All 247 transformants generated from *T. atroviride* T23 were analyzed by PCR. Only representative results from randomly selected transformants were showed in Fig. 1. For *hph* primer, an anticipated 510 bp specific band was detected by the agarose gel electrophoresis in all transformants with pV2. This band was not found in parent strain T23 (Fig. 1A). For *amp* primer, a specific 840 bp band was amplified in all the transformants and the positive control (Fig. 1B). These results preliminarily confirmed that the fragment of linearized pV2 plasmid was successfully inserted into chromosomal DNA of T23.

3.3. Southern blot

The results showed that hybridization bands were present in all selected transformants and positive control, while there was no hybridization signal detected in the negative controls (Fig. 2).

3.4. Assessment of tolerance to dichlorvos

All transformants derived from *T. atroviride* T23 were measured for their tolerance to dichlorvos based on their colony diameters on the PDA with a given concentration of dichlorvos. A total of 207 transformants was found with better growth than the parent T23 in the medium with 600 μg mL^{-1} dichlorvos.

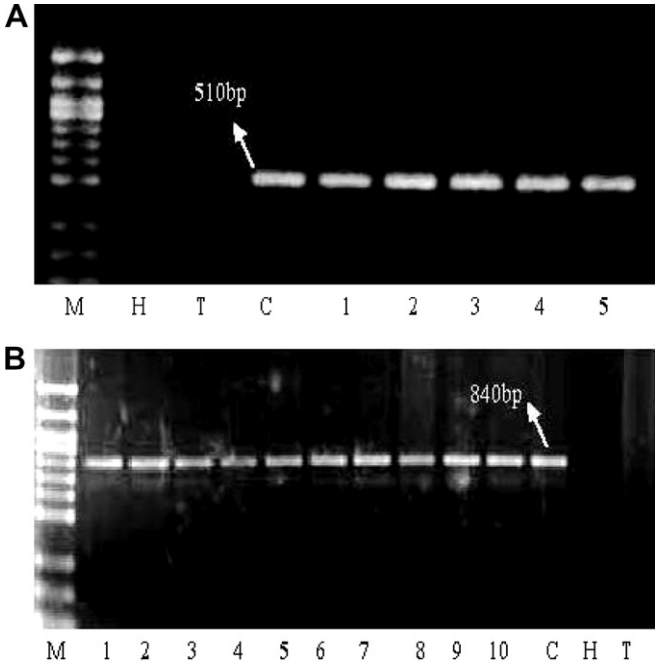

Fig. 1. PCR analysis of randomly selected transformants of *T. atriviride* T23 for the presence of *hph* gene (A), and *amp* gene (B). M, DNA Marker. T, wild type T23 strain. H, H_2O. C, plasmid pV2. 1–10, selected T23 transformants.

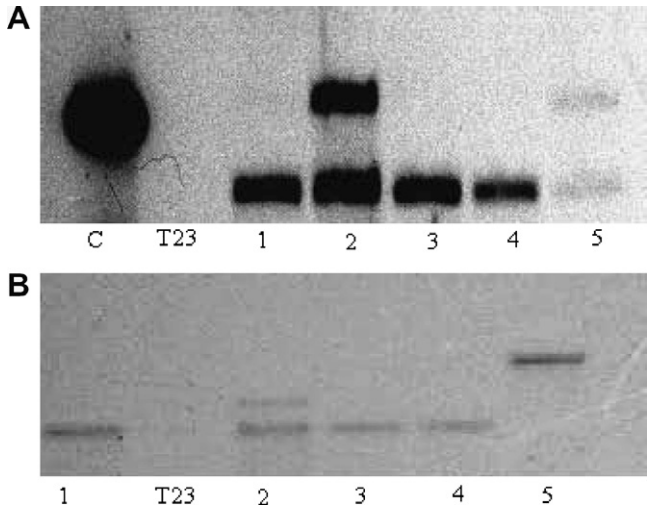

Fig. 2. Southern blot analysis of randomly selected T23 transformants for the presence of the *amp* gene (A), and *hph* gene (B). C, plasmid pV2. T23, wild type T23 strain. 1–5, different transformants.

3.5. Assay of dichlorvos degradation ability

Dichlorvos-degrading ability of the transformants was measured by colorimetric method. Calibration curves were made for each individual detection with $R^2 = 0.99$. All 207 transformants showed increased tolerance to dichlorvos as above were screened for its ability to degrade dichlorvos. The dichlorvos degradation rates of the transformants ranged from 81% to 96% as compared to 72% of the wild strain. Of these transformants, 169 transformants (82%) exhibited an increased ability at different extents in degradation of dichlorvos and showed significant difference as compared with the parent strain T23 based on the least significant difference (LSD) test at $p = 0.01$. Among them, 8 transformants were improved over 30% in the degradation ability and 132 transformants were improved 20–30% in the degradation rate, which accounted for 64% of the transformants with significant improved dichlorvos degradation rate over T23. One transformant showed the highest dichlorvos degradation rate as 96%.

4. Discussion

Bioremediation of environmental pollutants has been extensively studied (Rahman et al., 2003; Labana et al., 2005; Ha et al., 2007; Malaviya and Rathore, 2007). Many works have been performed on molecular improvement of microorganisms for bioremediation (Kang et al., 2002; Liu et al., 2006; Yu et al., 2006). Organophosphate pesticide-degradation activities have been found in numerous microorganisms, such as *Pseudomonas*, *Azospirillum*, *Flavobacterium*, and *Agrobacterium* (Horne et al., 2002; Siddavattam et al., 2003; Foster et al., 2004). However, only a few works on organophosphate pesticide-degradation involved *Trichoderma* spp. (Fu, 2005).

In our study, the *T. atroviride* strain T23 has been used as a biocontrol agent against plant diseases for many years, thus those transformants derived T23 should simultaneously keep biocontrol effectiveness. Results showed that most transformants were still able to provide good inhibition against the growth of various plant pathogens *in vitro*, such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotinia sclerotium*, and *Phytophthora* spp. as compared with the parent strains (data not shown). Our results first demonstrate that transformants generated by REMI could satisfy the requirements for soil remediation by removal of dichlorvos as well as reduction of pathogen population in soil.

Trichoderma strains in biocontrol or bioremediation are usually screened from the natural microbial community (O'Neill et al., 1996; Harman et al., 2004). It requires laborious work and target genes are difficult to be further cloned. REMI technique has proven to be an effective way to find mutants with single copy flanking gene surrounding insertional sites, which then facilitates cloning and localizing genes corresponding to functional mutation by plasmid rescue or inverse PCR. REMI has been widely applied to the mutagenesis and gene tagging (Akamatsu et al., 1997; Leem et al., 2003; Rogers et al., 2004; Takano et al., 2006; Zhou et al., 2007b; Han et al., 2007; Wang et al., 2007). From our results, the majority of transformants (70%) showed low copy numbers of the heterologous gene, which thereby offers a great many of potential strains for future cloning diversified functional genes as well as for further understanding the mechanisms of the biodegradation of organophosphate pesticide in *Trichoderma* spp. Due to random insertion of foreign DNA, *Trichoderma* transformants showed diverse characteristics e.g. lower or enhanced dichlorvos degradation ability.

Additionally, our laboratory has developed a cell immobilization technique to degrade ferrocyanide (Zhou et al., 2007a). We suppose that cell immobilization might be more powerful to further improve the effectiveness of REMI transformants for pollutant removal from water not just from soil.

5. Conclusions

Our work proves that REMI is a very useful tool to construct insertional mutants with wider range of genetic variance, and provide us more opportunities to screen ideal strains with higher degradation of organophosphate pesticide residues in ecosystem. Through this approach, we successfully obtained some *Trichoderma* transformants with significantly improved dichlorvos degradation activity as compared with the parent isolate.

Although this study just targeted degradation of organophosphate pesticide dichlorvos with *Trichoderma* transformants, our results also make great sense for screening other ideal bioremediation microbes.

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References

Akamatsu, H., Itoh, Y., Kodama, M., Otani, H., Kohmoto, K., 1997. AAL-toxin deficient mutants of *Alternaria alternata* tomato pathotype by restriction enzyme-mediated integration. Phytopathology 87, 967–972.

Ezzi, M.I., Lynch, J.M., 2005. Biodegradation of cyanide by *Trichoderma* spp. and *Fusarium* spp.. Enzyme Microb. Technol. 36, 849–854.

Foster, L.J.R., Kwan, B.H., Vancov, T., 2004. Microbial degradation of the organophosphate pesticide, Ethion. FEMS Microbiol. Lett. 240, 49–53.

Fu, W.X., 2005. Research on the growth condition of organophosphate pesticides degrading strain – *Trichoderma* sp. FM10. Biomagnetism 5, 29–31.

Guillermina, M., Romero, M.C., Cazau, M.C., Bucsinszky, A.M., 2002. Cadmium removal capacities of filamentous soil fungi isolated from industrially polluted sediments, in La Plata (Argentina). World J. Microbiol. Biotechnol. 18, 817–820.

Ha, J.Y., Engler, C.R., Wild, J.R., 2007. The gene biodegradation of chloroferon and diethylthiophosphate by consortia enriched from waste cattle dip solution. Bioresour. Technol. 98, 1916–1923.

Han, Y.K., Kim, M.D., Lee, S.H., Yun, S.H., Lee, Y.W., 2007. A novel F-box protein involved in sexual development and pathogenesis in Gibberella zeae. Mol. Microbiol. 63, 768–779.

Harman, G.E., Lorito, M., Lynch, J.M., 2004. Uses of *Trichoderma* spp. to alleviate or remediate soil and water pollution. Adv. Appl. Microbiol. 56, 313–330.

Horne, I., Sutherland, T.D., Harcourt, R.L., Russell, R.J., Oakeshott, J.G., 2002. Identification of an *opd* (organophosphate degradation) gene in an *Agrobacterium* isolate. Appl. Environ. Microbiol. 68, 3371–3376.

Huang, Y.Q., Zhou, X.Y., Chen, J., 2005. Insertion mutagenesis of *Trichoderma atroviride* by restriction enzyme-mediated DNA integration. J. Shanghai Jiaotong University (Science) E-10, 161–166.

Kang, D.G., Kim, J.Y.H., Cha, H.J., 2002. Enhanced detoxification of organophosphates using recombinant *Escherichia coli* with co-expression of organophosphorus hydrolase and bacterial hemoglobin. Biotechnol. Lett. 24, 879–883.

Labana, S., Singh, O.V., Basu, A., Pandey, G., Jain, R.K., 2005. A microcosm study on bioremediation of *p*-nitrophenol-contaminated soil using *Arthrobacter protophormiae* RKJ100. Appl. Microbiol. Biotechnol. 68, 417–424.

Leem, Y.E., Ross, I.K., Choi, H.T., 2003. Tagging and localization of a phospholipase D gene in *Coprinellus congregatus* by restriction enzyme-mediated integration and pulsed-field gel electrophoresis. FEMS Microbiol. Lett. 225, 285–289.

Liu, Z., Hong, Q., Xu, J.H., Jun, W., Li, S.P., 2006. Construction of a genetically engineered microorganism for degrading organophosphate and carbamate pesticides. Int. Biodeterior. Biodegrad. 58, 65–69.

Malaviya, P., Rathore, V.S., 2007. Bioremediation of pulp and paper mill effluent by a novel fungal consortium isolated from polluted soil. Bioresour. Technol. 98, 3647–3651.

O'Neill, T.M., Elad, Y., Shtienberg, D., Cohen, A., 1996. Control of grapevine grey mould with *Trichoderma harzianum* T39. Biocontrol Sci. Technol. 6, 139–146.

Rahman, K.S.M., Rahman, T.J., Kourkoutas, Y., Petsas, I., Marchant, R., Banat, I.M., 2003. Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. Bioresour. Technol. 90, 159–168.

Rogers, C.W., Challen, M.P., Green, J.R., Whipps, J.M., 2004. Use of REMI and *Agrobacterium*-mediated transformation to identify pathogenicity mutants of the biocontrol fungus, *Coniothyrium minitans*. FEMS Microbiol. Lett. 241, 207–214.

Siddavattam, D., Khajamohiddin, S., Manavathi, B., Pakala, S.B., Merrick, M., 2003. Transposon-like organization of plasmid-borne organophosphate degradation (*opd*) gene cluster found in *Flavobacterium* sp.. Appl. Environ. Microbiol. 69, 2533–2539.

Takano, Y., Takayanagi, N., Hori, H., Ikeuchi, Y., Suzuki, T., Kimura, A., Okuno, T., 2006. A gene involved in modifying transfer RNA is required for fungal pathogenicity and stress tolerance of *Colletotrichum lagenarium*. Mol. Microbiol. 60, 81–92.

Wang, J., Zhang, G., Li, J., Liu, X., Cheng, H.R., 2006. Research of screening and decomposing performance of a bacterium producing dichlorvos-decomposing enzyme. J. Environ. Health 23, 310–312.

Wang, Y.C., Guo, B.H., Miao, Z.Q., Tang, K.X., 2007. Transformation of taxol-producing endophytic fungi by restriction enzyme-mediated integration (REMI). FEMS Microbiol. Lett. 273, 1–7.

Yu, B., Ma, C.Q., Zhou, W.J., Zhu, S.S., Wang, Y., Qu, J.Y., Li, F.L., Xu, P., 2006. Simultaneous biodetoxification of S, N, and O pollutants by engineering of a carbazole-degrading gene cassette in a recombinant biocatalyst. Appl. Environ. Microbiol. 72, 7373–7376.

Yu, Y.B., Tan, P.G., 1995. Detection of dichlorvos in the air by resorcinol-fluorimetric method. Chin. J. Anal. Chem. 23, 1470.

Zhang, L.P., Hong, H.S., Chen, Z.T., Chen, W.Q., Calamari, D., 1999. An initial environmental risk assessment of pesticide application in Xiamen seas. J. Xiamen University (Nature Science) 38, 96–102.

Zhou, X.Y., Liu, L.X., Chen, Y.P., Xu, S.F., Chen, J., 2007a. Efficient biodegradation of cyanide and ferrocyanide by Na-alginate beads immobilized with fungal cells of *Trichoderma* koningii. Can. J. Microbiol. 53, 1033–1037.

Zhou, X.Y., Xu, S.F., Liu, L.X., Chen, J., 2007b. Degradation of cyanide by *Trichoderma* mutants constructed by restriction enzyme mediated integration (REMI). Bioresour. Technol. 98, 2958–2962.