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Enhanced phytoremediation of multi-metal contaminated soils by interspecific fusion between the protoplasts of endophytic *Mucor* sp. CBRF59 and *Fusarium* sp. CBRF14

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ABSTRACT

The aim of this study was to isolate protoplasts from different metal resistant endophytic fungi and to carry out interspecific fusion of protoplasts to enhance bioremediation of contaminated soils. Three stable fusants (F1, F2, and F3) with resistance to Cd and Zn were constructed by interspecific fusion between inactivated protoplasts from Cd-resistant endophytic *Mucor* sp. CBRF59 and Zn-resistant endophytic *Fusarium* sp. CBRF14. The fusants increased concentrations of water soluble Cd, Pb, and Zn in soils, promoted rape growth, increased metal concentrations in the rape, elevated the extractable metal amount, and increased metal translocation from roots to shoots in soils containing single metals or multi-metals. Especially, the fusants showed much higher extraction efficacy than the parents in soils contaminated by multi-metals. The results indicated that protoplast fusion should be a feasible and efficient fungal method, which could be utilized to construct stable fusants by combining the genetic traits of parents for bioremediation of contaminated soils by multi-metals.

1. Introduction

The threat of heavy metal pollution to public health has led to increased interests in developing methods to remediate the pollution in soils, sediments, and waters. Phytoremediation, using plants to remove pollutants from the environment or to reduce the harm, is considered as a highly promising method for cleaning-up of soils contaminated by metals (Rajkumar et al., 2009). In phytoremediation, the plant-endophyte partnership is an essential field to be further exploited (Weyens et al., 2009). In most contaminated soils, plants and their associated microorganisms have to face mixed contamination. Therefore, it is necessary to introduce multiple detoxifying functions into a single organism (Valls and de Lorenzo, 2002). Engineered endophytic bacteria have been adopted to improve phytoremediation efficiency (Barac et al., 2004; Weyens et al., 2009, 2010). In bacteria, resistance to metals is usually attributable to genes, whose products act as intracellular-to-extracellular transporters, most of which are plasmid-encoded and may be involved in the antibiotic resistance of bacteria (Mehra and Winge, 2004; Deng et al., 2012). Some heavy metal resistant endophytic bacteria isolated from *Brassica napus* also show multiple antibiotic resistances (Sheng et al., 2008). The applications of endophytic bacteria with heavy metal resistance may spread antibiotic resistances among microbes, including human/animal pathogens (Davison, 2005; Ryan et al., 2008).

Fungi, alone or in collaboration with bacteria and plants, display many important features merited by the extensive metabolic capabilities, which can be utilized to design biotechnologies to remediate polluted soils and water. However, the potential use of fungi in bioremediation has not received much attention (Harms et al., 2011). The application of filamentous fungi can be a promising alternative or a valuable complement in situations of bacterial malfunction (Harms et al., 2011). Nevertheless, most fungi are

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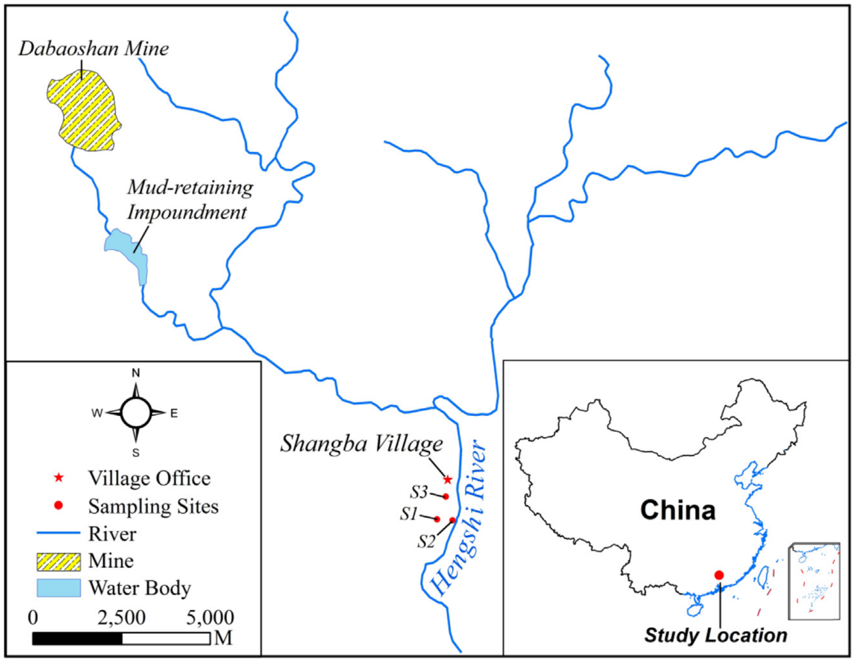

Fig. 1. Sampling locations of soil and rape roots in a heavy metal-contaminated site near Hengshi River (contaminated by mine-washing wastewater from Dabaoshan mine) in Shangba Village, Wengyuan County, Guangdong Province, China.

refractory to transformation, thus genetic or transgenetic approaches can not be undertaken to improve fungal phytoremediation properties (Göhre and Paszkowski, 2006).

Restriction enzyme-mediated integration (REMI) has been used to generate a wide range of insertional mutagenesis of *Trichoderma* and the mutants with elevated Cd resistance can improve the phytoremediation of oilseed rape (Wang et al., 2009). However, the method is limited to construct mutants for single metal contamination. The typical fungal mechanism for regulating intracellular metal ion concentrations is the expression of metallothioneins, which is usually different from the mechanisms of antibiotic resistance. Detoxification of metals by the formation of complexes is used by most eukaryotes (Mejáre and Bülow, 2001; Valls and de Lorenzo, 2002). The introduction and/or overexpression of metal binding proteins have been widely exploited to increase the metal binding capacity, tolerance or accumulation of bacteria and plants (Mejáre and Bülow, 2001). Therefore, engineered fungi constructed with different metal resistance genes may construct mutants with resistance to multi-metals and be explored to remediate multi-metal contaminated soils.

In fungi that lack natural mechanism for recombination of genetic materials, protoplast fusion provides a method to facilitate heterokaryon formation. The method potentially leads to fusion of vegetative nuclei and mitotic recombination even across species and genus barrier, resulting in development of interspecific and intergenetic hybrids (Savitha et al., 2010). Parasexuality can be initiated by hyphal anastomosis and may allow genetic recombination between genomes of fungi from different species and genera (Savitha et al., 2010). Therefore, novel strains with different characteristics can be constructed by protoplast fusion. The method has been widely applied in industrial microbiology but not in bioremediation of the contaminated environment yet (Zhang et al., 2009). In our previous study, the mutant CBRF59T3 constructed by self-fusion of CBRF59 tolerates 4-folds higher Cd concentration than that of parent CBRF59, and can increase the Cd extraction efficacy in Cd-contaminated soils compared to parent CBRF59 and the uninoculated control (Deng et al., 2013). Due to multiple metals in commonly metal-contaminated soils, it is necessary to construct strains with tolerance to different metals for phytoremediation of metal-contaminated soils. Therefore, the objectives of this study were to obtain multi-metal resistant fungi using the interspecific fusion method and to investigate the enhancement of phytoremediation efficiency of multi-metal contaminated soils by the fungi. More specifically, protoplasts from endophytic *Mucor* sp. CBRF59 and *Fusarium* sp. CBRF14 were isolated and fused. The phytoremediation efficiencies of multi-metal contaminated soils by *B. napus* inoculated with the fungi and different fusants were compared.

2. Materials and methods

2.1. Parental strains

Samples of soil and rape roots were collected from a heavy metal-contaminated site near Hengshi River (contaminated by mine-washing wastewater from Dabaoshan mine) in Shangba Village, Wengyuan County, Guangdong Province, China (Fig. 1). Endophytic fungi *Mucor* sp. CBRF59 and *Fusarium* sp. CBRF14 were isolated from the roots of rape growing in the heavy metal-contaminated soil (S1 in Fig. 1) (Deng et al., 2011). The strains were maintained on potato dextrose agar (PDA) and stored at 4 °C. Strain *Mucor* sp. CBRF59 could grow on the PDA medium with 5 mM Cd and 10 mM Pb added, but not survive on the medium with 2 mM Zn. *Fusarium* sp. CBRF14 could grow on the PDA medium with 40 mM Zn and 15 mM Pb added, but not survive on the medium with 0.5 mM Cd.

2.2. Isolation and regeneration of protoplasts

One milliliter of conidial suspension (10^6 spores mL^{-1}) of CBRF59 and CBRF14 was inoculated into 100 mL potato dextrose broth (PDB) and shaken at 150 rpm, 30 °C for mycelium with different ages of 24, 36, 48, and 60 h, respectively. The mycelia with different ages were collected through filter paper and further