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Transgenic poplar trees expressing yeast cadmium factor 1 exhibit the characteristics necessary for the phytoremediation of mine tailing soil

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HIGHLIGHTS

- We introduced a heavy metal resistance gene, *ScYCF1*, into poplar.
- The transgenic poplar was tested in soil taken from a closed mine site.
- The transgenic poplar exhibited enhanced growth and reduced toxicity symptoms.
- The transgenic poplar accumulated more heavy metal(loid)s than the wild type in mine soil.

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ABSTRACT

Genetic engineering of plants for phytoremediation is thought to be possible based on results using model plants expressing genes involved in heavy metal resistance, which improve the plant's tolerance of heavy metals and accumulation capacity. The next step of progress in this technology requires the genetic engineering of plants that produce large amounts of biomass and the testing of these transgenic plants in contaminated soils. Thus, we transformed a sterile line of poplar *Populus alba* X *P. tremula var. glandulosa* with a heavy metal resistance gene, *ScYCF1* (yeast cadmium factor 1), which encodes a transporter that sequesters toxic metal(loid)s into the vacuoles of budding yeast, and tested these transgenic plants in soil taken from a closed mine site contaminated with multiple toxic metal(loid)s under greenhouse and field conditions. The *YCF1*-expressing transgenic poplar plants exhibited enhanced growth, reduced toxicity symptoms, and increased Cd content in the aerial tissue compared to the non-transgenic plants. Furthermore, the plants accumulated increased amounts of Cd, Zn, and Pb in the root, because they could establish an extensive root system in mine tailing soil. These results suggest that the generation of *YCF1*-expressing transgenic poplar represents the first step towards producing plants for phytoremediation. The *YCF1*-expressing poplar may be useful for phytostabilization and phytoattenuation, especially in highly contaminated regions, where wild-type plants cannot survive.

1. Introduction

When toxic heavy metal(loid)s, including Cd, As, Pb, Hg, and Zn, accumulate in the ecosystem, they can cause yield losses in plants, as well as diseases and heavy metal poisoning in organisms at all levels of the food chain. Heavy metal(loid)s are particularly hazardous to human beings, which are located at the top of the food chain. Once absorbed by the body, heavy metal(loid)s have a long half-life (25 yr for Cd), which results in chronic poisoning. Thus, the removal of contaminating heavy metal(loid)s from the environment is important for the health of entire ecosystems as well as for human societies. However, remediation of an environment polluted by heavy metal(loid)s is a difficult task, because these substances are highly toxic, immobile in soil, and non-biodegradable.

Phytoremediation, the use of plants for remediation, is environmentally friendly and cheaper than other methods of remediation (Pilon-Smits, 2005), and has a positive impact on the physical, chemical, and biological aspects of the environment (Salt et al., 1998). Phytoremediation can be subdivided into phytoextraction and phytostabilization. Phytoextraction aims to rapidly extract metals from contaminated soil and accumulate metals in aerial parts, whereas phytostabilization aims to immobilize the

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contaminants using plants that can hold the pollutants in their extensive root systems, and thereby reduces soil erosion and the dispersal of pollutants in wind-blown dust or floods. In practice, short-term phytoextraction is rarely possible, due to the low capacity of plants to absorb and tolerate heavy metal(loid)s. Instead, plants are used to stabilize the environment and gradually extract toxic metal(loid)s in the long term. Such practical considerations led to the recent proposal of a new phytoremediation concept termed phytoattenuation (Meers et al., 2010). This approach combines phytoextraction over an extended period of time with the economic use of the products of the remediating plants. For instance, the products can be used as a source of renewable energy, which could generate additional income, and thus serve as motivation for this approach.

Cd, which accumulates in organisms and is extremely toxic even at low concentrations, is known to be one of the most dangerous pollutants (Alloway, 1995). Toxic levels of Cd are often found in industrial and mining areas, and in agricultural land where Cd-containing phosphate fertilizers have been used. Long-term exposure to Cd can cause itai-itai disease, which is characterized by the softening of bones and kidney failure (Nogawa, 1981). *Arabidopsis halleri* and *Thlaspi caerulescens* have been tested for their capacity to remove Cd (Liang et al., 2009). Whereas these phytoaccumulators can efficiently absorb toxic heavy metals, they are not suited for phytoremediation, because these herbaceous plants grow slowly and produce only a small biomass and shallow root system compared to tree species. Thus, the genetic engineering of tree plants for improved tolerance and accumulation is being suggested for the phytoremediation of Cd-polluted soil (Kärenlampi et al., 2000). Many properties of poplar trees make them excellent candidates for genetic engineering for phytoremediation. First, they produce a high biomass in their aerial parts, at a comparable rate to herbaceous plants, which allows them to store large amounts of heavy metal(loid)s. Second, they have an extensive root system, which can hold and stabilize large amounts of soil particles, and constitutes a large surface area to solubilize and absorb toxic metal(loid)s bound to soil particles. Third, poplars are not a source of food for farm animals, and thus the risk of heavy metal(loid)s entering the food chain is low. Fourth, the fact that poplar plants take a long time to flower as opposed to reproducing every year, as do herbaceous plants, makes it relatively easy to control undesirable genetic spreading. Finally, since poplar trees are readily propagated by cuttings, it is possible to amplify one clone with preferred characteristics without seed propagation, which is a useful characteristic for genetic engineering.

Therefore, poplar plants have been genetically engineered to exhibit improved tolerance to Cd. However, the target genes of previous studies are limited to facilitating the biosynthesis of chelators such as gamma-glutamylcysteine (γ-EC) and glutathione (Arisi et al., 2000; Koprivova et al., 2002). Recently, genetically engineering plants to sequester chelated heavy metal(loid)s in the vacuole has been proposed as a promising phytoremediation strategy (Tong et al., 2004), because vacuolar storage is an essential step in heavy metal(loid)s detoxification and accumulation, and vacuoles constitute the majority of the cell volume. Numerous vacuolar transporters of heavy metal(loid)s have been described, including the *P*-type heavy metal ATPase3 (Morel et al., 2009; Ueno et al., 2011), heavy metal proton antiporter calcium exchanger2 (Schaaf et al., 2002), and ATP-binding cassette (ABC) transporters ABCC1 and ABCC2 (Song et al., 2010; Park et al., 2012). A vacuolar transporter of yeast has successfully been used to improve heavy metal sequestration into plant vacuoles. *Saccharomyces cerevisiae* Cadmium factor1 (*ScYCF1*), a yeast transporter for Cd detoxification first reported by Li et al. (1997), pumps glutathione-conjugated Cd (GS–Cd) into the vacuole (Li et al., 1997). *Arabidopsis* plants transformed with this yeast ABC transporter exhibited increased GS–Cd uptake activity, resulting in plants with an improved Cd tolerance and uptake activity (Song et al., 2003). Similarly, *Brassica juncea* (Indian mustard) plants expressing *YCF1* were more tolerant to Cd and Pb, and accumulated more heavy metals than control plants (Bhuiyan et al., 2010).

Although these earlier studies established the concept that genetically engineered plants could be used to improve the efficiency of phytoremediation, it is not clear whether the results of these studies will be replicated in the field, since these studies often used only model plants, and the experiments were performed on soils artificially contaminated with one or two metal ions in laboratory or greenhouse conditions. The next step toward actually using this technology for the remediation of contaminated sites requires the genetic engineering of plants with a large biomass, the testing of their performance in contaminated soil, and finally a field-scale test (Fig. SM-1 in Supplementary Material (SM)). To start this next stage of development, we engineered poplar trees to express *YCF1*, and monitored the growth of these transgenic plants under both greenhouse and field conditions using soil from a closed mine site in South Korea that is heavily contaminated with multiple heavy metal(loid)s.

2. Materials and methods

2.1. Plant materials and transformation of poplar with ScYCF1

Transformation of poplar plants was conducted using *Agrobacterium*-mediated transfer of genes according to Choi et al. (2007). A non-flowering mutant poplar clone, 'Bonghwa' (BH) (*Populus alba* X *P. tremula var. glandulosa*, BH1), was used as the transformation background. Briefly, the internodes of young aseptically grown poplar plants were excised and co-incubated on callus induction medium (CIM) [4.4 g L^{-1} Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), 1.0 mg L^{-1} 2,4-D, 0.1 mg L^{-1} α-naphthaleneacetic acid (NAA), 0.01 mg L^{-1} 6-benzylaminopurine solution (BAP), 3% sucrose, pH 5.8] with *Agrobacterium* harboring *YCF1* and the hygromycin-resistance gene, *HPT*, in the recombinant plasmid vector EnPCAMBIA1302 (Song et al., 2003). Light was blocked during the first 2 d of co-incubation with *Agrobacterium* to increase the efficiency of transformation. When calli developed at the cut ends of the internodes, they were transferred to shoot induction medium [2.46 g L^{-1} Woody Plant Medium, 1 mg L^{-1} zeatin, 0.1 mg L^{-1} BAP, 0.01 mg L^{-1} NAA, 3% sucrose, pH 5.5], and then to root induction medium [2.2 g L^{-1} MS, 0.2 mg L^{-1} Indole-3-butyric acid, 3% sucrose, pH 5.8] after a few leaves developed. Hygromycin (4 mg L^{-1}) was included in both the shoot and root induction media to allow growth of only the seedlings that express *YCF1*. The transgenic and control plants in the test tubes were maintained by cutting and transferring to new medium every 3 months.

2.2. Expression analysis

YCF1 expression in the independent transformants was examined by RT-PCR. Total RNA was extracted from 3-month-old poplar plants using Trizol buffer and then reverse transcribed into cDNA using the Promega cDNA Synthesis Kit (Promega, WI, USA). The primers described in Table SM-1 were used for amplification of *YCF1* and the small subunit of Rubisco (*SSU*) (Rishi et al., 2004). Expression of *SSU* was used as a control to ensure that similar amounts of RNA were used in each preparation.

2.3. Intactness and copy number analysis of the transgene cassette in the transgenic poplars

Genomic DNA from poplar plants was isolated from 3 to 5 g of fresh leaf tissue, in accordance with the method described by