

The Key Role of Chlorocatechol 1,2-Dioxygenase in Phytoremoval and Degradation of Catechol by Transgenic Arabidopsis^{1[W]}

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Transgenic exploitation of bacterial degradative genes in plants has been considered a favorable strategy for degrading organic pollutants in the environment. The aromatic ring characteristic of these pollutants is mainly responsible for their recalcitrance to degradation. In this study, a *Plesiomonas*-derived chlorocatechol 1,2-dioxygenase (TfdC) gene (*tfdC*), capable of cleaving the aromatic ring, was introduced into *Arabidopsis* (*Arabidopsis thaliana*). Morphology and growth of transgenic plants are indistinguishable from those of wild-type plants. In contrast, they show significantly enhanced tolerances to catechol. Transgenic plants also exhibit strikingly higher capabilities of removing catechol from their media and high efficiencies of converting catechol to *cis,cis*-muconic acid. As far-less-than-calculated amounts of *cis,cis*-muconic acid were accumulated within the transgenic plants, existence of endogenous TfdD- and TfdE-like activities was postulated and, subsequently, putative orthologs of bacterial *tfdD* and *tfdE* were detected in *Arabidopsis*. However, no TfdC activity and no putative orthologs of either *tfdC* or *tfdF* were identified. This work indicates that the TfdC activity, conferred by *tfdC* in transgenic *Arabidopsis*, is a key requirement for phytoremoval and degradation of catechol, and also suggests that microbial degradative genes may be transgenically exploited in plants for bioremediation of aromatic pollutants in the environment.

Decades of modern agricultural practices and industrializations have resulted in the extensive accumulation of organic pollutants in soil and water. Aromatic compounds, such as 2,4-dichlorophenoxyacetic acid, 3-chlorobenzoate, and chlorocatechol, are among the most hazardous due to their (or their derivatives') potential carcinogenesis and recalcitrance to degradation (Neilson et al., 1991; Capasso et al., 1995). On the other hand, the increasing accumulation of these compounds in the environment has prompted native bacteria (*Pseudomonas*, *Alcaligenes*, *Ralstonia*) to develop capabilities of using them as carbon and/or energy sources. Intensive studies have led to the identification and elucidation of a modified *ortho*-degradation pathway (Fig. 1; Perkins et al., 1990; Laemmler et al., 2000). Generally, the aromatic compounds are first converted to their respective chlorocatechols. These chlorocatechols are then sequentially converted to the 3-oxoadipate through the actions of four enzymes encoded by *tfdC*, *tfdD*, *tfdE*, and *tfdF*, respectively. The 3-oxoadipate is further funneled to the 3-oxoadipate pathway, leading to the production of succinate and acetyl-CoA,

which can be incorporated into the fundamental metabolic and/or catabolic pathways (Perkins et al., 1990; Reineke, 1998). Cleavage of the aromatic ring in the *ortho*-position, between the hydroxyl groups (Harayama and Reikik, 1989), to form *cis,cis*-muconic acids is a rate-limiting step for the degradation of chlorocatechols and reduction of their toxic activity. Chlorocatechol 1,2-dioxygenase (TfdC; EC 1.13.11), encoded by the *tfdC* gene, catalyzes the reaction. It has been shown that the chlorocatechol oxidation reaction is the key step in the 3-chlorobenzoate degradation pathway of bacteria (Perez-Pantoja et al., 2003).

The *tfdC* was first cloned from plasmid pJP4 of JMP134, which can degrade and utilize chloroaromatics (Perkins et al., 1990). In our previous study, a similar *tfdC* gene was identified and cloned from *Plesiomonas*, which was isolated from the activated sludge collected from a wastewater-recycling factory and can use chlorobenzene as the sole source of carbon and energy. After being cloned into expression vector and expressed in *Escherichia coli*, the expected 33-kD protein was observed on the SDS-PAGE and a significant TfdC activity was detected from its cell extracts (Luo and Li, 1998; Ma et al., 2000).

Bioremediation is considered to be a cost-effective, environmentally friendly way for the cleanup of contaminated soils and surface water. However, persistence of the aromatic pollutants in the environment suggests the less effectiveness of natural-microorganism-promoted biodegradations *in situ*, probably because their efficiency is largely dependent on the suitability of environmental conditions in maintaining the growth of microorganisms. Phytoremediation has therefore

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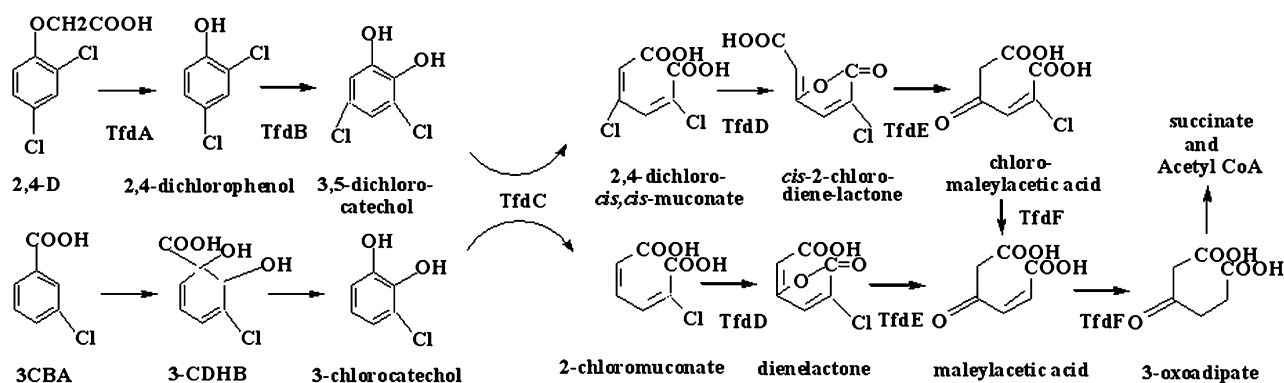


Figure 1. Proposed degradation pathways of chlorinated aromatics. Enzymes catalyzing the reactions are as follows: TfdA, 2,4-dichlorophenoxyacetic acid α -ketoglutarate dioxygenase; TfdB, chlorophenol hydroxylase; TfdC, chlorocatechol 1,2-dioxygenase; TfdD, chloromuconate cycloisomerase; TfdE, dienelactone hydrolase; TfdF, maleylacetate reductase.

received increasing attention in recent years. Plants are used instead for remedial purposes not only because they generally have extensive root systems, larger biomass, and consequently higher decontamination efficiency, but also because they are highly differentiated and protective organisms capable of tolerating adverse environmental factors effectively. It is also important that they can improve and/or provide conditions favorable for microbial degradation. Besides, as a renewable energy resource and a local climate regulator, plants have potential economic and environmental benefits (Kramer, 2005; Newman and Reynolds, 2005; Peuke and Rennenberg, 2005; Pilon-Smits, 2005).

Using genetic engineering to increase/create the remedial capacity of plants has been increasingly explored as a sustainable technology (Rugh et al., 1996; French et al., 1999; Hannink et al., 2001; Dhankher et al., 2002; Wang et al., 2004). A preliminary work exploring the potential of a chlorocatechol dioxygenase gene (*cbnA*) in phytoremediation was reported in rice (*Oryza sativa*), and its conversion of catechol to muconic acid was observed in transgenic calli and leaf tissues. However, the remediation potential was not demonstrated at the plant level (Shimizu et al., 2002).

To fully explore the potential of chlorocatechol dioxygenase gene in phytoremediation of soil contaminated with aromatic compounds, we modified the *tfdC* gene from *Plesiomonas* spp. and transferred it into *Arabidopsis* (*Arabidopsis thaliana*). Our results show that transgenic plants expressing the modified *tfdC* gene obtained a striking ability of tolerating, removing, and biodegrading catechol, implying that the *tfdC* gene has the potential of being explored for phytoremediation of sites contaminated with aromatic compounds.

RESULTS

Construction of Transgenic Arabidopsis

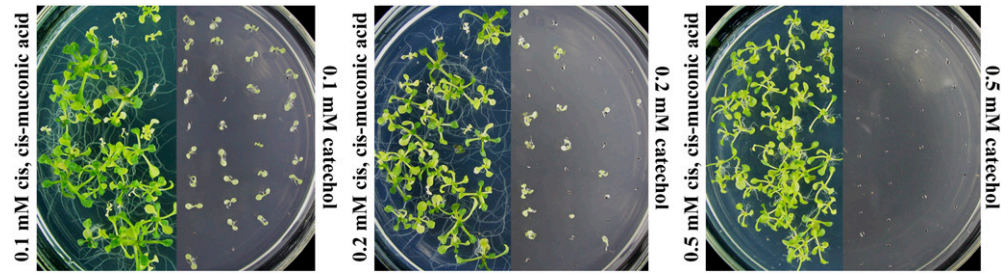
To efficiently express the *tfdC* gene from *Plesiomonas* in the plant, a plant consensus start sequence AAATGA was introduced using PCR method. The

modified gene was subsequently inserted into the binary vector pPZPY122 (Hajdukiewicz et al., 1994) under the control of cauliflower mosaic virus 35S promoter, and introduced into *Arabidopsis* via *Agrobacterium* (LBA4404)-mediated transformation (Clough and Bent, 1998). Empty vector transgenic (VT) plants were also constructed to be used as a control. Twenty-seven *tfdC* transgenic (CT) plants (T_1) were initially identified from 29 putative transgenic plants regenerated on half-strength Murashige and Skoog (MS) agar plates containing gentamicin by PCR. All the transgenic plants were phenotypically indistinguishable from wild-type plants when they were grown either on half-strength MS agar plates or in soil in the growth room, suggesting that the insertion of the *tfdC* gene in these plants caused no visible morphological effects.

Relative Toxicities of Catechol and *cis,cis*-Muconic Acid to *Arabidopsis*

Relative toxicities of catechol and *cis,cis*-muconic acid to the wild type as well as to transgenic *Arabidopsis* were determined on agar-solidified, half-strength MS plates. Seeds of the wild type were germinated on half-strength MS agar plates containing either 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM catechol, or 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM *cis,cis*-muconic acid. On plates containing even 0.1 mM catechol, some wild-type seeds were either not able to germinate or died shortly after germination, whereas on plates containing 0.5 mM *cis,cis*-muconic acid, they could still develop into seedlings (Fig. 2). This significantly differential toxic effect implies that the tolerance to catechol can be used as a valid indicator of the function of active *tfdC* gene in transgenic *Arabidopsis*. However, at the higher concentrations of *cis,cis*-muconic acid (above 0.4 mM), a visible toxicity to root development was also observed (Fig. 2, right). At 0.1 and 0.2 mM catechol, T_2 transgenic lines, although segregating, generally showed enhanced tolerances compared to wild-type plants (data not shown). Four transgenic

Figure 2. Relative toxicities of catechol and cis,cis-muconic acid to Arabidopsis. Morphologies of wild-type plants on half-strength MS agar plates containing 0.1 (left), 0.2 (middle), and 0.5 mM (right) catechol or cis,cis-muconic acid.



lines, CT1-2, CT1-5, CT1-16, and CT1-19, were chosen for further analyses.

Stable Expressions of the *tfdC* Gene and TfdC Activities in Transgenic Plants

Expressions of the *tfdC* gene were determined in the T₃ plants of homozygous transgenic lines using reverse transcription (RT)-PCR. An RT-PCR product of approximately 800 bp, corresponding to the *tfdC* coding sequence, was detected in all four CT lines analyzed, whereas no signals were detected in VT plants (Fig. 3A). With *ACT11* as a reference, relatively higher expression levels were detected in CT1-5 and CT1-19 compared to those in CT1-2 and CT1-16.

To determine the tissue-specific expression pattern, RT-PCR analysis was performed with total RNAs extracted from roots, rosette leaves, cauline leaves, and stems of CT1-2 plants. Transcripts of *tfdC* were detected in all the tissues examined, with the highest being observed in the stems and the lowest in the roots (Fig. 3B).

TfdC activities in the CT and VT lines were assayed with crude enzyme extracts from their seedlings. The TfdC activity was expressed as the amount of cis,cis-muconic acid produced per milligram of protein. All the four CT lines showed significantly higher TfdC activities during the period of incubation. In contrast, after incubating even for 30 min, no detectable amount of cis,cis-muconic acid was found in the treatment with the enzyme extract prepared from the VT plants (Fig. 3C), indicating that there is no significant intrinsic TfdC activity in Arabidopsis. Nevertheless, significantly differential TfdC activities were observed in different CT lines, which can be well correlated to the transcript levels of the *tfdC* in these lines.

Enhanced Tolerances of Homozygous CT Plants to Catechol on Plates

Forty seeds from each of VT and CT lines were sown on half-strength MS agar plates containing 0.0, 0.1, and 0.2 mM catechol, respectively, and grown vertically for 2 weeks. On the control medium without catechol, both CT and VT plants appeared equally healthy and produced similar biomasses (about 4 mg/plant). As the concentration of catechol in the medium increased, however, the CT plants generally exhibited significantly higher tolerances, showing greener and broader

leaves as well as higher growth rates (Fig. 4A). On the other hand, growth of the VT plants was retarded even at 0.1 mM, with their average fresh weight being about 60% of those of the CT plants (Fig. 4B). At 0.2 mM, however, VT plants were severely inhibited, grew too small and abnormal so that the weights of individual plants could not be determined accurately. Not surprisingly, at higher concentrations of catechol, growth and development of the CT plants were also inhibited (Fig. 4A). These observations indicated that the inhibitions on the growth and development of VT as well as CT plants were solely due to the presence of catechol.

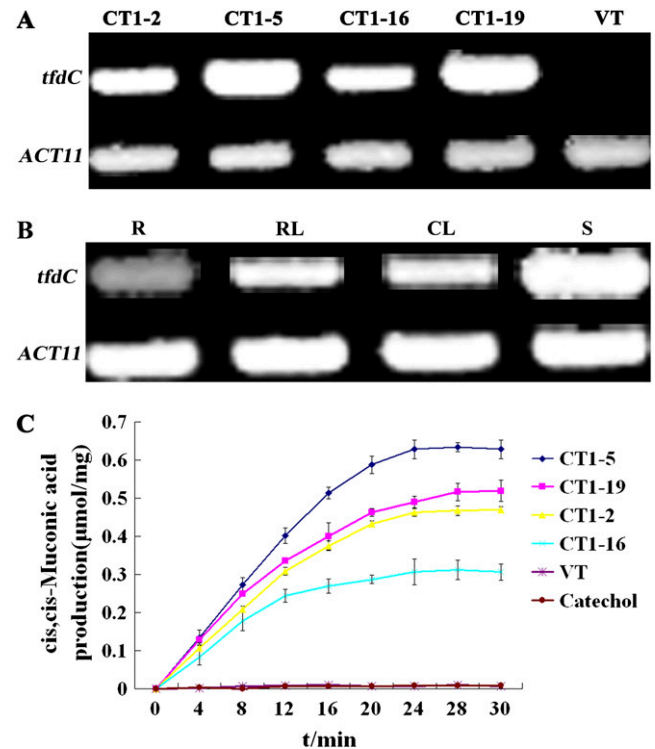


Figure 3. Expression levels of *tfdC* and activities of TfdC in transgenic Arabidopsis. A, RT-PCR-amplified *tfdC* fragments from the plants of VT and CT lines (CT1-2, CT1-5, CT1-16, CT1-19) with *ACT11* as a reference. B, RT-PCR-amplified *tfdC* fragments from roots (R), rosette leaves (RL), cauline leaves (CL), and stems (S) of the transgenic line CT1-2. C, Activities of TfdC in the VT and CT plants. Catechol, Added substrate catechol in the reaction mixtures but no crude enzyme. The data represent mean \pm SD ($n = 3$).

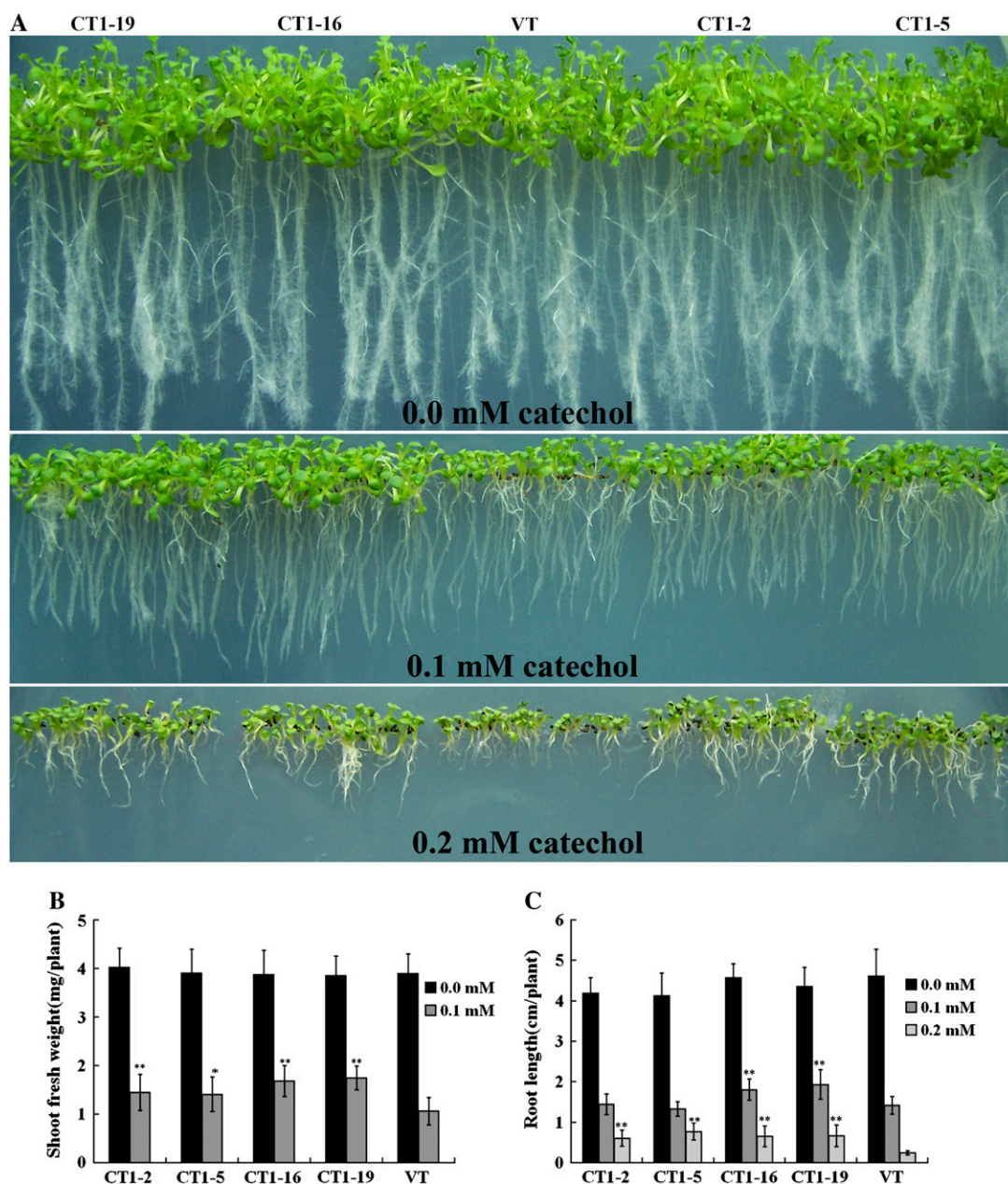


Figure 4. Enhanced catechol tolerances of CT plants on plates. A, VT and CT T_3 plants germinated and grown vertically for 2 weeks on half-strength MS agar plates containing 0.0, 0.1, or 0.2 mM catechol. B, Fresh weight of 2-week-old VT and CT plants grown on half-strength MS agar plates containing 0.0 and 0.1 mM catechol. C, Root length of 2-week-old VT and CT plants grown on half-strength MS agar plates containing 0.0, 0.1, and 0.2 mM catechol. The data represent mean \pm SD ($n = 10$). Statistical analysis of differences in CT lines with respect to VT was performed using two-tailed *t* test. Significant difference is denoted with one asterisk ($P < 0.05$) or two ($P < 0.01$).

Efficient root and shoot formation is a crucial characteristic of transgenic plants designed for potential application in phytoremediation. Both VT and CT plants developed similar root systems with extensive secondary root branches and root hairs on the control medium without catechol, with the longest reaching about 4.5 cm. On the medium containing 0.1 mM catechol for 2 weeks, transgenic plants displayed minimal sign of phytotoxic effects, producing extensive

root branches and showing a normal shoot development, especially CT1-16 and CT1-19, while VT seedlings exhibited a stunted development, producing few root hairs. At 0.2 mM catechol, although the shoot development of CT plants was affected, they could still develop gradually to later stages and produced secondary roots and visible root hairs. The average root length of CT plants was 0.6 cm. At the same concentrations, however, shoot development of the VT plants was halted at

the two-leaf stage, and neither secondary roots nor visible root hairs were observed. The average root length of VT plants was about 40% of those of CT plants (Fig. 4, A and C). In addition, the CT plants examined also showed an enhanced tolerance to catechol in soil in the growth room (see Supplemental Fig. S1).

Higher Catechol Removal Efficiencies of CT Plants

Fifty seeds from each of the VT and CT lines were germinated and grown in flasks containing half-strength MS liquid medium with 0.0, 0.1, and 0.2 mM catechol, respectively, on a shaker for 2 weeks. In the medium without catechol, both CT and VT plants grew healthy and produced similar fresh weights of about 2.8 g per flask. At 0.1 mM, however, the growth of VT plants was significantly inhibited, with the average fresh weight being about 0.4 g per flask, about 30% of those of CT1-16 and CT1-19 and about 50% of those of CT1-2 and CT1-5. At 0.2 mM, only some of the VT plantlets could survive the incubation, producing a final fresh weight of 0.055 g. In contrast, the seeds of the CT lines germinated well and the seedlings showed a relatively normal morphology and growth rate, producing an average final fresh weight of approximately 0.2 g (Fig. 5, A and B).

Concentrations of catechol and its catabolite, *cis,cis*-muconic acid, in the medium were then determined by HPLC. Catechol and *cis,cis*-muconic acid were identified by referring to their respective retention time (Fig. 6A, top). After 2-week incubation, a sharp contrast on catechol concentration was detected in the media of VT (Fig. 6A, middle) and CT (Fig. 6A, bottom) plants. According to the amount of catechol detected in the medium and that initially added, the degraded proportions were calculated. CT plants generally showed 3 to 5 times higher degradation rates to those of VT plants (Fig. 6B). No detectable amount of *cis,cis*-muconic acid was found in both media. A further HPLC analysis revealed that no detectable amounts of catechol were accumulated in the extracts of either CT or VT plants. However, *cis,cis*-muconic acid was found to accumulate significantly in the seedlings of CT plants (CT1-2, 0.057 ± 0.007 nmol g⁻¹ fresh weight; CT1-5, 0.060 ± 0.006 nmol g⁻¹ fresh weight; CT1-16, 0.030 ± 0.001 nmol g⁻¹ fresh weight; CT1-19, 0.047 ± 0.001 nmol g⁻¹ fresh weight; Fig. 7).

Far-Less-Than-Calculated Amounts of *cis,cis*-Muconic Acid Retained in the CT Plants

Approximately 5 nmol catechol was initially added into the medium of CT plants, but only trace levels were detected either in the medium or within the CT plants at the end of incubation. Theoretically, equivalent amounts of *cis,cis*-muconic acid should have been produced and retained in the CT plants (no *cis,cis*-muconic acid was detected in the media previously). Unexpectedly, only 0.01 nmol *cis,cis*-muconic acid (about 0.2% of the amount of catechol disappeared)

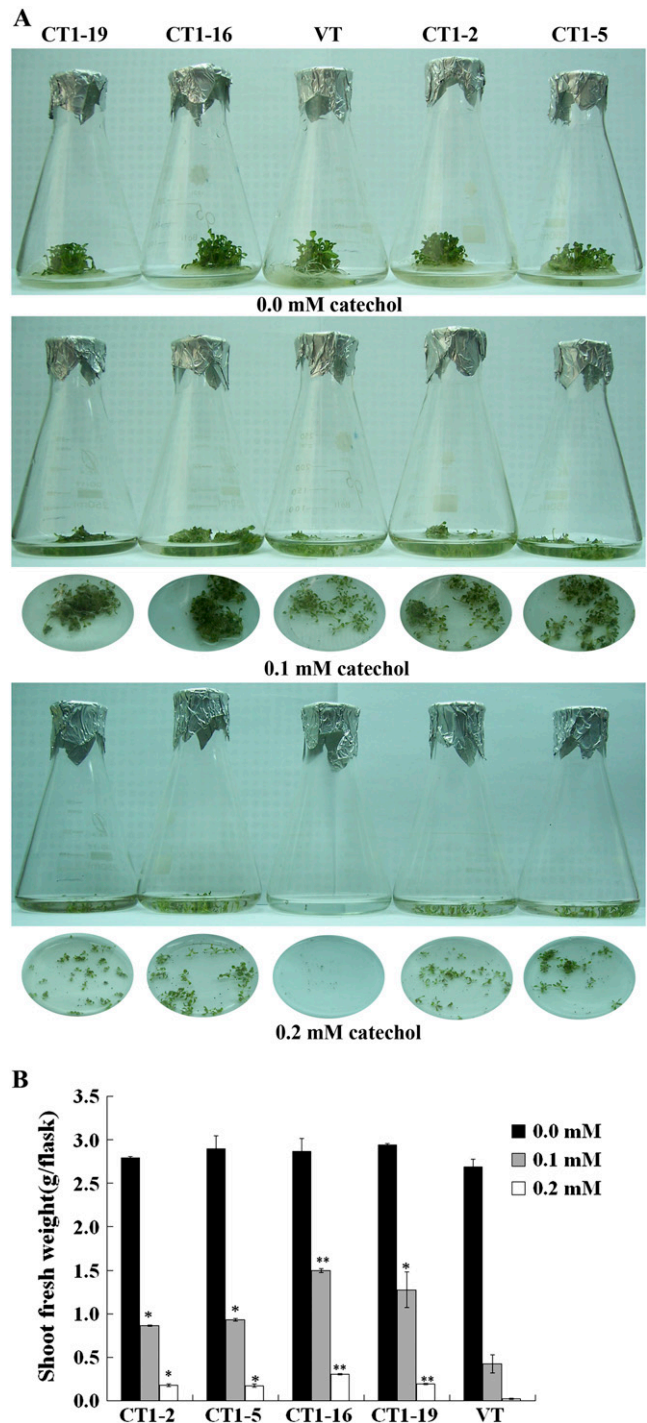


Figure 5. Morphologies of VT and CT lines in catechol-containing liquid medium. A, Fifty seeds of VT and CT plants in flasks were allowed to grow for 2 weeks in half-strength MS liquid medium containing 0.0, 0.1, and 0.2 mM catechol. B, Fresh weights of seedlings in the individual flasks in the above treatment. The data represent mean \pm SD ($n = 3$). Statistical analysis of differences in CT lines with respect to VT was performed using two-tailed *t* test. Significant difference is denoted with one asterisk ($P < 0.05$) or two ($P < 0.01$).

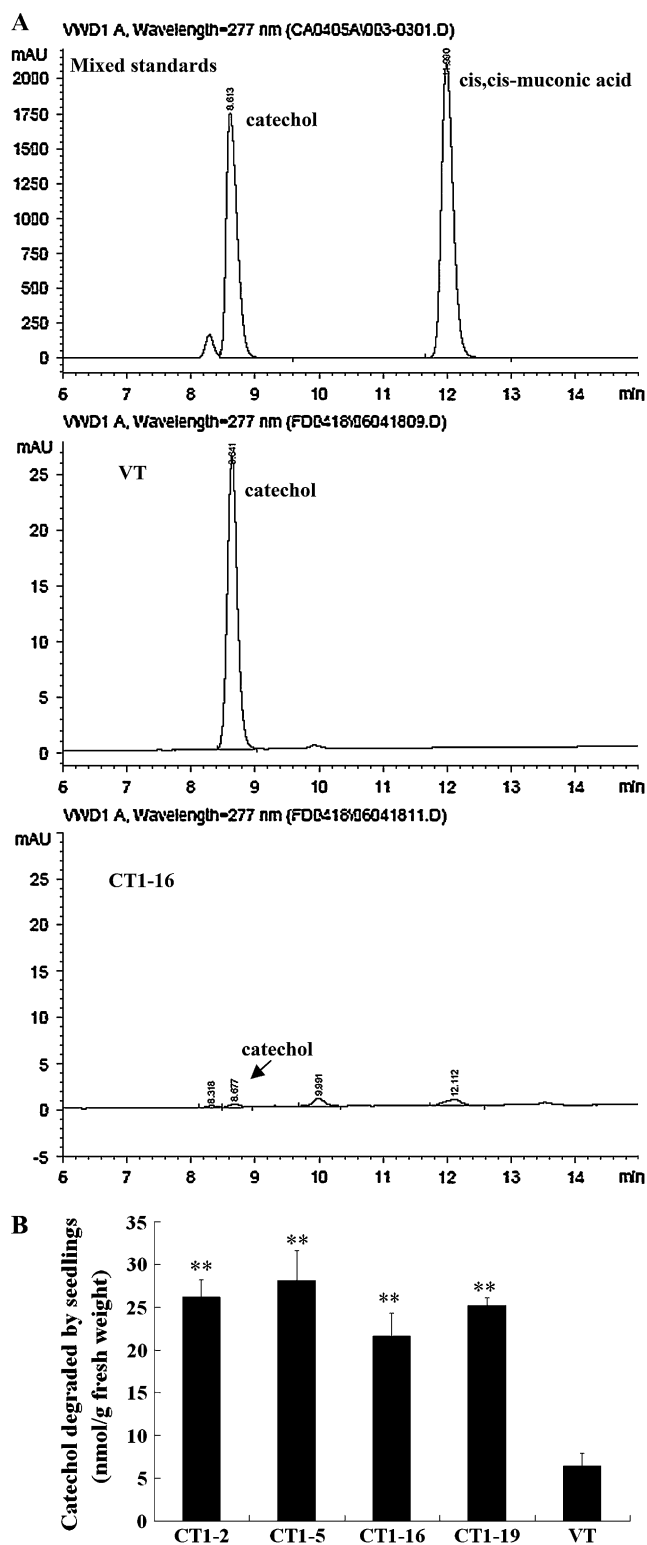


Figure 6. HPLC analysis of removal of catechol in media. A, HPLC analyses of catechol and cis,cis-muonic acid in the media; after seedlings were grown in half-strength MS liquid media containing 0.2 mM catechol for 2 weeks, concentrations of catechol and cis,cis-muonic acid in VT (middle) and CT media (bottom) were determined by HPLC; mixed standards (top). B, Catechol degradation rates after 2-week incubation. Catechol amounts degraded were calculated by

was detected within the plants (Table I). To explain the discrepancy, we assumed the existence of endogenous TfdD-, TfdE-, and/or TfdF-like activities. A search of the Arabidopsis protein database against the amino acid sequences of bacterial TfdC, TfdD, TfdE, and TfdF, by WU-BLAST 2.0, revealed the existence of expected putative orthologs of *tfdD* and *tfdE*. An experiment was also carried out to measure the activities of TfdD and TfdE in the cell extracts of Arabidopsis using spectrophotometric methods. Our preliminary results indicated that the cell extracts did have the activities of both TfdD and TfdE. In the proceeding cause of enzymatic reaction, dienelactone, the catalyzed product of cis,cis-muonic acid by TfdD, was first accumulated and then declined, which was the characteristic reflection of the sequential functioning of TfdD and TfdE in the extracts (see Supplemental Fig. S2). However, according to the *P*-value criterion ($P < 0.01$), no putative orthologs of *tfdC* and *tfdF* have been identified in the database (Table II).

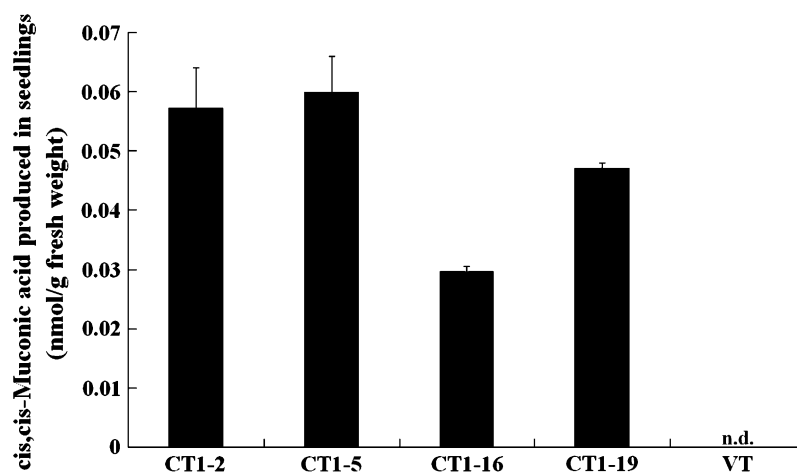
DISCUSSION

Catechol and its catabolites have cellular toxicities to bacteria and animals, as stated by a fact sheet of U.S. Environmental Protection Agency (<http://www.epa.gov/ttn/atw/hlthef/pyrocate.html>). However, to our best knowledge, there have been no data showing their toxicities to plants so far. In this study, a significant toxic effect of catechol to Arabidopsis was demonstrated. It not only severely inhibited the germination and growth of the wild-type Arabidopsis, but also significantly stunted the development of both the roots and shoots of the wild-type plants germinated otherwise in the absence of catechol. The toxicity of catechol is considered a consequence of its chemical properties and chemical reactions with biomolecules. Many reactions can occur between catechol and biomolecules (DNA and proteins) or membranes, causing them to break, to be inactivated, and/or to be destroyed (Schweigert et al., 2001).

Fortunately, a significantly lower toxicity of cis,cis-muonic acid to Arabidopsis was identified and, consequently, a screening approach was used in analyzing transgenic plants in this study (Fig. 2). In the case of either an equal or even higher toxicity identified, an otherwise complicated, labor-intensive approach would have to be adopted to analyze the functional effect of the transgenic *tfdC* gene. This work implies that single microbial degradative genes can be transgenically exploited to analyze the phytodegradation of aromatic pollutants straightforward, provided that the immediate products are less toxic.

deducting the amount of the catechol left in medium from the amount of catechol added initially into medium, and degradation rates were expressed as nmol g^{-1} fresh weight. The data represent mean \pm SD ($n = 3$). Statistical analysis of differences in CT lines with respect to VT was performed using two-tailed *t* test. Significant difference is denoted with two asterisks ($P < 0.01$).

Figure 7. HPLC analysis of plant extracts. After seedlings were grown in half-strength MS liquid media containing 0.2 mM catechol for 2 weeks, extractions were performed. Concentrations of *cis,cis*-muconic acid were quantitatively determined by HPLC. The data represent mean \pm SD ($n = 3$); n.d., not detected.



Expression of the *tfdC* gene was detected in all the tissues examined, including the root (Fig. 3B). However, higher expression levels were generally detected in tissues above the ground, with the highest found in the stem and the lowest in the root, which shares a general tendency with Shimizu's observation (Shimizu et al., 2002). Importantly, CT plants produced in this study can tolerate significantly higher concentrations of catechol than VT plants, producing higher biomass and more normal root in the presence of catechol (Figs. 4 and 5). Even more importantly, they can remove catechol from their surrounding media (Fig. 6) and degrade it much more efficiently than VT plants (Fig. 7). In addition, it was found that seedlings germinated first in the absence of catechol and then transferred to catechol-containing plates grew much better than seedlings germinated and grown on plates containing catechol all the time (Fig. 2; Supplemental Fig. S3). A similar phenomenon also was observed by Hannink (Hannink et al., 2001). According to Hannink et al.'s understanding, either the inhibitory effect of catechol to wild-type seedlings is more significant at the early stage of plant development, or increased biomass or possibly elevated enzyme activities in older seedlings allows more effective detoxification of catechol. However, we also believe that both of the factors may contribute to the differential tolerances observed.

Biodegradations of organic pollutants can occur either in vivo (Hannink et al., 2001) or in vitro (Wang et al., 2004). To microorganisms, the complete in vivo degradation of organic pollutants may mean assimilation of the carbon and capture of the energy they contain, as in the case of *Plesiomonas*, from which the *tfdC* gene was isolated. In this study, based on the observation that almost all the added catechol was removed but no *cis,cis*-muconic acid was detected in the media of CT seedlings (Fig. 6), we also consider that the ring cleavage, catalyzed by the exogenous TfdC, mainly took place in vivo. Furthermore, no catechol was found to accumulate in vivo, but significant amounts of *cis,cis*-muconic acid were detected in the CT seedlings (Fig. 7). These results together indi-

cate that catechol was absorbed into the CT plant and degraded efficiently. A similar situation also was observed in the case of 2,4,6-trinitrotoluene phytodegradation (Hannink et al., 2001). No matter whether the immediate catabolites are toxic or not, it is a favorable characteristic to trap catabolites within the plants for phytoremediation purpose.

Stoichiometrically, a reduction in the nmol of catechol should equal an increase in the nmol of *cis,cis*-muconic acid in the liquid culture system. Far less amounts of *cis,cis*-muconic acid retained in the CT plants (Table I) prompt us to postulate that *cis,cis*-muconic acid was either gradually degraded further by endogenous TfdD-, TfdE-, and/or TfdF-like activities (Fig. 1), or sequestered within the plant in a form that may not be easily extractable. Considering the fact that *cis,cis*-muconic acid has been detected in the extracts of CT plants, we assume that the former was likely the case. The assumption is supported by the identification of putative orthologs of bacterial *tfdD* and *tfdE* in the *Arabidopsis* genome (Table II) and by the demonstration of the existence of TfdD- and TfdE-like activities in the cell extracts of *Arabidopsis* plants.

Table I. Amounts of catechol degraded and *cis,cis*-muconic acid detected within VT and CT plants

Amounts of catechol degraded were calculated by deducting the amount of the catechol left in medium at the end of incubation from the amount of catechol added initially into medium, and the amount of *cis,cis*-muconic acid accumulated in plants was calculated according to HPLC data. The data represent mean \pm SD ($n = 3$).

Lines	Catechol Degraded (A)	<i>cis,cis</i> -Muconic Acid Detected in Plants (B)	A/B
	nmol	nmol	%
CT1-2	4.698 \pm 0.011	0.0102 \pm 0.0004	0.22
CT1-5	4.891 \pm 0.005	0.0104 \pm 0.0002	0.21
CT1-16	4.937 \pm 0.001	0.0091 \pm 0.0000	0.18
CT1-19	4.909 \pm 0.002	0.0092 \pm 0.0002	0.19
VT	0.358 \pm 0.130	n.d. ^a	

^aNot detected.

Table II. Identification of putative orthologs of bacterial *tfdC*, *tfdD*, *tfdE*, and *tfdF* in *Arabidopsis* by BLAST

Arabidopsis protein database was searched against the amino acid sequences of the bacterial TfdC, TfdD, TfdE, and TfdF, which are located sequentially on the *ortho*-pathway, by WU-BLAST 2.0. Candidates were picked up according to the *P*-value criterion ($P < 0.01$).

Enzyme	AGI Code	Description	<i>P</i> Value
TfdC	NF ^a		
TfdD	AT3G18270.1	Mandelate racemase/muconate lactonizing enzyme family protein	1.40E-11
	AT1G68900.1	Mandelate racemase/muconate lactonizing enzyme	0.0019
TfdE	AT2G32520.1	Dienelactone hydrolase family protein	1.00E-08
	AT3G23570.1	Dienelactone hydrolase family protein	0.0011
TfdF	NF		

^aNot found.

In a broader sense, it is also plausible for the existence of these orthologs in *Arabidopsis* since plants have versatile detoxification systems to counter the phytotoxicity of the wide variety of natural and synthetic chemicals (Coleman et al., 1997). The finding that no obvious putative orthologs of *tfdC* and *tfdF* exist in the *Arabidopsis* genome supports our observation of no endogenous TfdC activity. These results together demonstrate that the TfdC activity conferred by *tfdC* in transgenic *Arabidopsis* is the key requirement for phytodegradation of catechol.

In conclusion, this study clearly shows that the bacterial *tfdC* gene can be manipulated to cleave the aromatic ring of a simple organic pollutant, catechol, in the model plant *Arabidopsis*, and that the catechol can be efficiently removed and degraded from the media of transgenic plants. An immediate future work would be to engineer its upstream genes, *tfdA* and *tfdB*, on the *ortho*-pathway (Fig. 1), as well as *tfdC*, ideally combined with pollutant (chlorobenzene, for example)-responsive cis-elements on the bacterial plasmid, into *Arabidopsis* to test the feasibility of phytodegrading more complex aromatic pollutants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All the wild-type and transgenic *Arabidopsis* (*Arabidopsis thaliana*) lines used in this study are in the Columbia-0 background. When grown in plates, seeds were surface sterilized, placed in the dark at 4°C for 2 d, and then sown on plates containing half-strength MS salts with 2% (w/v) Suc. When grown in soil pots, seeds were sown in square pots (10-cm sides) with soil (peat soil:vermiculite:perlite [v/v/v] = 3:9:0.5; purchased from Shanghai Institute of Landscape Science) presoaked with plant nutrient medium. All the plants were grown in a growth room at 22°C ± 2°C and with approximately 100 μmol s⁻¹ m⁻² light intensity under standard long-day conditions (16 h light/8 h dark).

Construction of Binary Vectors

The *tfdC* gene was isolated from the pL1 plasmid of *Plesiomonas* using PCR method with a forward primer (5'-ACGGAGGCAAAGTGAACAAAAGAG-3') and a backward primer (5'-ACTGCTTCAATCGCGTCAATC-TTC-3'), and modified using the forward 5'-ACGGAGGCAAATGAACA-AAAGAG-3' (base changes underlined) and the same backward. The gene was modified to contain the plant consensus start sequence. PCR products

were purified with glass beads and then ligated into pGEM-T Easy vector (Promega). The *tfdC* fragment was cleaved with *Bam*HI and *Sac*I, subcloned into *Bam*HI-*Sac*I sites of a plant transformation vector containing cauliflower mosaic virus 35S promoter pPZPY122, and designated pPZPY122/*tfdC*. Constructs were verified by sequencing. All DNA manipulations were performed according to standard protocols (Sambrook et al., 1989).

Transformation of *Arabidopsis*

Construct pPZPY122/*tfdC* was introduced into the *Agrobacterium tumefaciens* LBA4404 strain by electroporation. *Arabidopsis* (Columbia-0) was transformed using floral-dipping method. T₁ seeds were screened for gentamicin resistance on half-strength MS agar plates containing 90 mg L⁻¹ gentamicin. Genomic DNAs were isolated from transgenic and wild-type plants according to protocol (ftp://ftp.arabidopsis.org/home/tair/Protocols/compleat_guide). PCR was used to screen for putative T₁ transgenic plants, with the conditions described previously. T₂ homozygous transgenic lines were selected based on the segregation analysis of gentamicin resistances of their self-fertilized T₃ seedlings. The homozygosity of T₂ transgenic lines was checked again by growing T₃ seedlings on media containing 0.1 mM catechol. T₃ homozygous seeds were used for all subsequent analyses.

Total RNA Extraction and RT-PCR

Total RNAs were extracted from both transgenic and wild-type seedlings using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed with 2 μg of total RNA using a first-strand cDNA synthesis kit (Shenergy Biocolor), and the products were quantitatively equalized for RT-PCR using *ACT11* cDNA as a reference. The RT-PCR reactions were performed with the following primers: *tfdC-1* (5'-ACGGAGGCAAAGTGAACAAAAGAG-3'), *tfdC-2* (5'-ACTGCTTCAATCGCGTCAATC-TTC-3'), *ACT11-1* (5'-GATTTGGCATCACACTTCTACAATG-3'), and *ACT11-2* (5'-GTTCCACCCTGAGCACAATG-3'). Amplified fragments were separated on a 1.2% agarose gel, cloned in pGEM-T (Promega), and then verified by sequencing.

Extraction of TfdC from Seedlings

Fresh leaves were homogenized in a Waring blender with precooled 0.05 M phosphate buffer, pH 7.0, and the homogenate was filtered through six layers of gauze. The filtrate was then centrifuged at 13,000g for 30 min at 4°C. Crude enzyme was quantified by Coomassie Brilliant Blue G250 (Spector, 1978) and used for activity assay.

Measurement of cis,cis-Muconic Acid with Spectrophotometer

Reaction mixtures contained 3 μmol EDTA, 0.3 μmol catechol, 26.1 μmol sodium phosphate, pH 7.0, and cell extracts (0.3 mg of total protein) in a final volume of 3 mL. Reactions proceeded at 25°C for 30 min. The increase in A₂₆₀ was used as a measure of the accumulation of cis,cis-muconic acid. When 1 μmol catechol changes to cis,cis-muconic acid, A₂₆₀ increases 5.6. TfdC

activity was expressed as amount of *cis,cis*-muconic acid produced per milligram of protein (Spain and Nishino, 1987).

Tests of Plant Tolerances

For test on plates, VT and CT plant seeds were sown on half-strength MS agar plates containing either catechol or *cis,cis*-muconic acid, which were dissolved in water and filtrated (0.22- μ m filter; Millipore) before added to plates. About 40 seeds of each line were placed on plates under aseptic conditions. After grown vertically for 2 weeks, the longest root length and fresh weight were measured for each treatment of approximately 10 seedlings.

For test in soil, VT and CT plant seeds were germinated in soil soaked with plant nutrient medium containing 0, 1, 2, 3, 4, and 5 mM catechol, respectively. After 2 weeks, the plantlets were sprayed with 0, 1, 2, 3, 4, and 5 mM catechol solutions every 3 d for 3 weeks before photos were taken.

Measurements of Catechol and *cis,cis*-Muconic Acid by HPLC

VT and CT plant seeds (50 per flask) were surface sterilized, germinated in 25 mL of half-strength MS liquid medium containing catechol, and grown for 3 weeks with rotary shaking at 120 rpm, 23°C. Samples were taken from the media and seedlings. The medium samples were directly analyzed with Agilent 1100 HPLC using an Agilent Eclipse XDS-C18 column. Methanol-water containing 1% (v/v) acetic acid was used as the eluant at a flow rate of 0.8 mL/min. The column effluent was monitored simultaneously at 277 nm by a diode array detector. A 10- μ L aliquot of each sample was injected onto the column. The retention times of known standards were used to identify catechol and *cis,cis*-muconic acid. Catechol was purchased from Sinopharm Chemical Reagent. *cis,cis*-Muconic acid was purchased from Sigma-Aldrich (Fluka).

Extraction of Catechol and Its Metabolites from Plants

The seedling samples were homogenized in a Waring blender with 200 μ L of precooled methanol and then sonicated for 10 min. After centrifugation at 13,000g for 30 min at 4°C, the supernatant was filtered (0.45- μ m filter; Millipore). Ten milliliters of each sample was then analyzed by HPLC using the conditions described above to determine the concentration of catechol and *cis,cis*-muconic acid in the seedlings. The peak areas were converted to molar amounts according to the standard curves of the standards and normalized to the wet weight of samples.

Measurement of TfdD- and TfdE-Like Enzymatic Activities

Preparation of the cell extracts of Arabidopsis plants and assay of TfdD- and TfdE-like enzymatic activities were carried out as reported (Schmidt and Knackmuss, 1980; Kuhm et al., 1990; Schlomann et al., 1990).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NC_005912 for *tfdC*.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Enhanced tolerances of CT plants in soil.

Supplemental Figure S2. Assay of TfdD- and TfdE-like activities.

Supplemental Figure S3. Enhanced tolerances of CT plants in plates.

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