The Role of Oxophytodienoate Reductases in the Detoxification of the Explosive 2,4,6-Trinitrotoluene by Arabidopsis[1][W][OA]

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The explosive 2,4,6-trinitrotoluene (TNT) is a significant environmental pollutant that is both toxic and recalcitrant to degradation. Phytoremediation is being increasingly proposed as a viable alternative to conventional remediation technologies to clean up explosives-contaminated sites. Despite the potential of this technology, relatively little is known about the innate enzymology of TNT detoxification in plants. To further elucidate this, we used microarray analysis to identify Arabidopsis (*Arabidopsis thaliana*) genes up-regulated by exposure to TNT and found that the expression of oxophytodienoate reductases (OPRs) increased in response to TNT. The OPRs share similarity with the Old Yellow Enzyme family, bacterial members of which have been shown to transform explosives. The three predominantly expressed forms, OPR1, OPR2, and OPR3, were recombinantly expressed and affinity purified. Subsequent biochemical characterization revealed that all three OPRs are able to transform TNT to yield nitro-reduced TNT derivatives, with OPR1 additionally producing the aromatic ring-reduced products hydride and dihydride Meisenheimer complexes. Arabidopsis plants overexpressing OPR1 removed TNT more quickly from liquid culture, produced increased levels of transformation products, and maintained higher fresh weight biomasses than wild-type plants. In contrast, *OPR1,2* RNA interference lines removed less TNT, produced fewer transformation products, and had lower biomasses. When grown on solid medium, two of the three *OPR1* lines and all of the *OPR2*-overexpressing lines exhibited significantly enhanced tolerance to TNT. These data suggest that, in concert with other detoxification mechanisms, OPRs play a physiological role in xenobiotic detoxification.

Large amounts of land and water are heavily contaminated by explosives, mainly as a result of the manufacture and military use of munitions. The high financial cost associated with cleaning up these contaminated sites largely precludes the use of many existing remediation technologies, such as soil excavation and incineration or disposal to landfill. There is a great deal of work documenting the global contamination, general toxicity, and microbial metabolism of 2,4,6-trinitrotoluene (TNT) in the environment; however, relatively little is known about the enzymes mediating the detoxification of TNT in plants (for review, see Rylott and Bruce, 2009).

Phytoremediation, the use of plants to remove environmental pollutants, offers a low-cost, sustainable alternative to conventional remediation technologies and is attracting considerable attention as a means to clean up sites contaminated with explosives. While TNT is a potent phytotoxin, plants are able to detoxify low levels of TNT. In an effort to determine how plant tolerance could be further improved, we are investigating the biochemistry and enzymology underlying the innate ability of plants to detoxify TNT. The detoxification of xenobiotics has been loosely categorized into three phases (Sandermann, 1992): activation, conjugation, and compartmentation. The proposed route of TNT detoxification follows these phases. The electron-withdrawing properties of the nitro groups of TNT make the aromatic ring electron deficient. This favors reductive transformation reactions in plants, and the TNT molecule is most commonly activated by the reduction of a nitro group to give hydroxylamino and then amino derivatives (Fig. 1, pathway A). Following the introduction of a functional group, more hydrophilic molecules such as Glc are conjugated to the activated TNT molecule (Gandia-Herrero et al., 2008), facilitating transport and subsequent compartmentation or sequestration.

Data from both our microarray experiments (Gandia-Herrero et al., 2008) and other expression studies (Ekman et al., 2003; Mezzari et al., 2005) have found that members of the small gene family of oxophytodienoate reductases (OPRs) in Arabidopsis (*Arabidopsis thaliana*) are up-regulated following exposure to TNT. The Arabidopsis genome contains three charac-

Figure 1. The transformation of TNT by pentaerythritol tetranitrate reductase. Pathway A shows the transformation of the nitro group to nitroso-dinitrotoluene (NODNT), HADNT, and then ADNT products. Pathway B shows the reduction of the aromatic ring to form hydride and dihydride Meisenheimer complexes, then chemical condensation with HADNT to form diarylamines.

terized OPRs: *OPR1*, *OPR2*, and *OPR3*. In addition, there are three as yet uncharacterized putative OPRs, named here as *OPR4*, *OPR5*, and *OPR6*, with *OPR4* and *OPR5* being identical. The physiological functions of the OPRs remain obscure, with the exception of OPR3, which is involved in jasmonic acid biosynthesis, converting (9*S*,13*S*)-12-oxophytodienoic acid to 3-2(2′ (*Z*)-pentyl)cyclopentane-1-octanoic acid in the peroxisome (Sanders et al., 2000; Stintzi and Browse, 2000; for review, see Wasternack, 2007). *OPR3* is located on chromosome II within the Arabidopsis genome and contains a C-terminal Ser-Arg-Leu type 1 peroxisome-targeting sequence. The remaining OPRs are all located on chromosome I and do not possess any known organelle-targeting sequences. The spatial expression of *OPR1* and *OPR2* across root cells where TNT accumulates (Biesgen and Weiler, 1999; Baerenfaller et al., 2008) favors a role in detoxification.

The OPRs share similarity with the Old Yellow Enzyme family, a group of flavoenzymes that has been repeatedly associated with the transformation of explosives (Binks et al., 1996; Schaller and Weiler, 1997; Snape et al., 1997; Basran et al., 1998; French et al., 1998; Blehert et al., 1999; Pak et al., 2000; Fitzpatrick et al., 2003; Williams et al., 2004). Studies also indicate that Old Yellow Enzyme homologs function as antioxidants, detoxifying the breakdown products of lipid peroxidation and other toxic electrophilic compounds (Kohli and Massey, 1998; Williams and Bruce, 2002;

Fitzpatrick et al., 2003; Trotter et al., 2006). This oxidative stress could result from exposure to xenobiotics including TNT, wounding, or pathogen attack.

Pentaerythritol tetranitrate reductase, an Old Yellow Enzyme homolog isolated from *Enterobacter cloacae* (Binks et al., 1996), possesses two catalytic activities toward TNT (Fig. 1): nitroreduction of TNT to form hydroxylamino-dinitrotoluene (HADNT) and then amino-dinitrotoluene (ADNT), and aromatic ring reduction of TNT to yield hydride and dihydride (2H⁻-TNT) Meisenheimer TNT adducts (French et al., 1998; Williams et al., 2004). The TNT ring-reduced compounds condense via a nonenzymatic reaction with HADNTs to form diarylamines, with the liberation of nitrite (Wittich et al., 2008). Expression of pentaerythritol tetranitrate reductase in tobacco (*Nicotiana tabacum*) confers both resistance to, and the ability to transform, TNT (French et al., 1999). OPR1, OPR2, and OPR3 share 43%, 44%, and 36% identity, respectively, with pentaerythritol tetranitrate reductase, and all possess the conserved active site amino acids crucial for TNT transformation by pentaerythritol tetranitrate reductase and other members of the Old Yellow Enzyme family (Snape et al., 1997; French et al., 1998; Khan et al., 2004), suggesting that they are capable of transforming TNT.

The OPR4/5 protein is predicted to have reduced activity toward TNT, compared with the other OPRs, owing to a C-terminal truncation that removes resi-

dues thought to be important in binding the cofactor NADH, Thus, we investigated OPR1, -2, and -3 as likely candidates for the TNT nitroreduction activity in Arabidopsis.

RESULTS

Expression of OPRs in Arabidopsis in Response to TNT

To identify genes up-regulated in response to TNT treatment, we performed a microarray experiment (Gandia-Herrero et al., 2008). Liquid culture-grown 14-d-old seedlings were treated with 60 μM TNT for 6 h, and then gene expression was analyzed. The microarray data revealed 633 genes that were significantly up-regulated between 2- and 204-fold, with a median value of 3.0. Many were genes involved in the activation and conjugation phases of xenobiotic detoxification, including glycosyltransferases, glutathione reductases, and OPRs. The microarray probes used did not distinguish between *OPR1* and *OPR2*; thus, the 14-fold up-regulation observed represented a pooled value for the expression of both genes. For *OPR4/5*, a 3-fold increase was seen; *OPR3* was not up-regulated, and *OPR6* expression was not detected in the microarray data. Searches of publicly available microarray expression data sets revealed that *OPR6* was rarely up-regulated, and no *OPR6* expression was found in liquid culture samples exposed to TNT using reverse transcription (RT)-PCR (data not shown). To better quantify the expression levels of the OPRs in response to the TNT treatment, quantitative RT-PCR was performed using gene-specific probes. Analysis showed that *OPR1* expression increased by approximately 17-fold, *OPR2* expression by 6-fold, and *OPR4/5* by 3-fold, while no specific increase in *OPR3* expression was seen (Fig. 2). Given that expression levels of *OPR4/5*
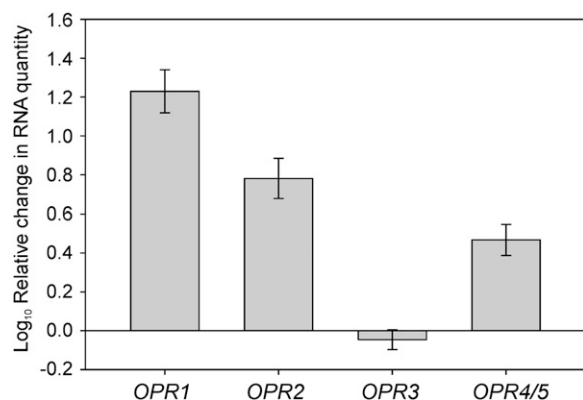
and *OPR6* in response to TNT were low; that expression in the roots, the site of TNT accumulation, was predicted to be low relative to *OPR1*, *OPR2*, and *OPR3*; and that OPR4/5 has a C-terminal truncation predicted to reduce activity, we concluded that OPR4/5 and OPR6 were unlikely to play a significant role in TNT detoxification in planta, and further characterization was focused on OPR1, OPR2, and OPR3.

Transformation of TNT by Recombinant OPRs

To determine whether OPR1, OPR2, and OPR3 were capable of producing reduced TNT products and hydride Meisenheimer complexes, *OPR1*, *-2*, and *-3* were cloned into pET-15b expression vectors and transformed into *Escherichia coli*. Following expression optimization steps, soluble, active recombinant protein was obtained for OPR1, OPR2, and OPR3. The recombinant proteins were purified by affinity chromatography (Fig. 3A), and the identities of these proteins were confirmed using trypsin digestion and matrix-assisted laser-desorption ionization time of flight/time of flight (MALDI-TOF/TOF) tandem mass spectrometry.

The NADH-dependent transformation of TNT by the purified OPRs was followed by HPLC over a period of 3 h. An NADH-dependent alcohol dehydrogenase was employed to keep the levels of NADH constant during the course of the assay. Figure 3 shows that all three OPRs were able to transform TNT to HADNT intermediates. No ADNTs were observed in the time scale monitored, although the decrease in HADNT concentration in the OPR2 and OPR3 assays (Fig. 3, D and E) suggests further transformation. The accumulation of an additional peak, more polar than HADNT, was observed in both OPR2 and -3 assays, which potentially represents dihydroxyl-amino-nitrotoluene (data not shown). Reduction of TNT by OPR1 resulted in the formation of products with retention times of 3.7, 7.3, and 9.2 min and spectra with absorbance maxima at approximately 500 nm (Fig. 3C). These were not seen in reaction mixtures catalyzed by OPR2 and OPR3 and were identified as dihydride Meisenheimer TNT complexes by reference to characterized standards generated by pentaerythritol tetranitrate reductase (Williams et al., 2004). $2H^-$-TNT adducts have been shown to yield four isomers in dynamic equilibrium. These are accounted for as the 1,3- and 3,5-hydride additions to the TNT molecule, with the respective tautomers forming protonated nitroalkanes (Williams et al., 2004). Figure 3C shows that levels of $2H^-$-TNT 3.7 and 9.2 decreased after 40 and 140 min, respectively, but no corresponding increases in any detected compounds were seen. The purified preparation of OPR1 showed the presence of a significant level of a contaminating protein (Fig. 3A). Trypsin digestion and MALDI-TOF/TOF tandem mass spectrometry analysis revealed this to be an *E. coli* 2-deoxy-D-gluconate 3-dehydrogenase. Although deemed unlikely to catalyze the observed reaction
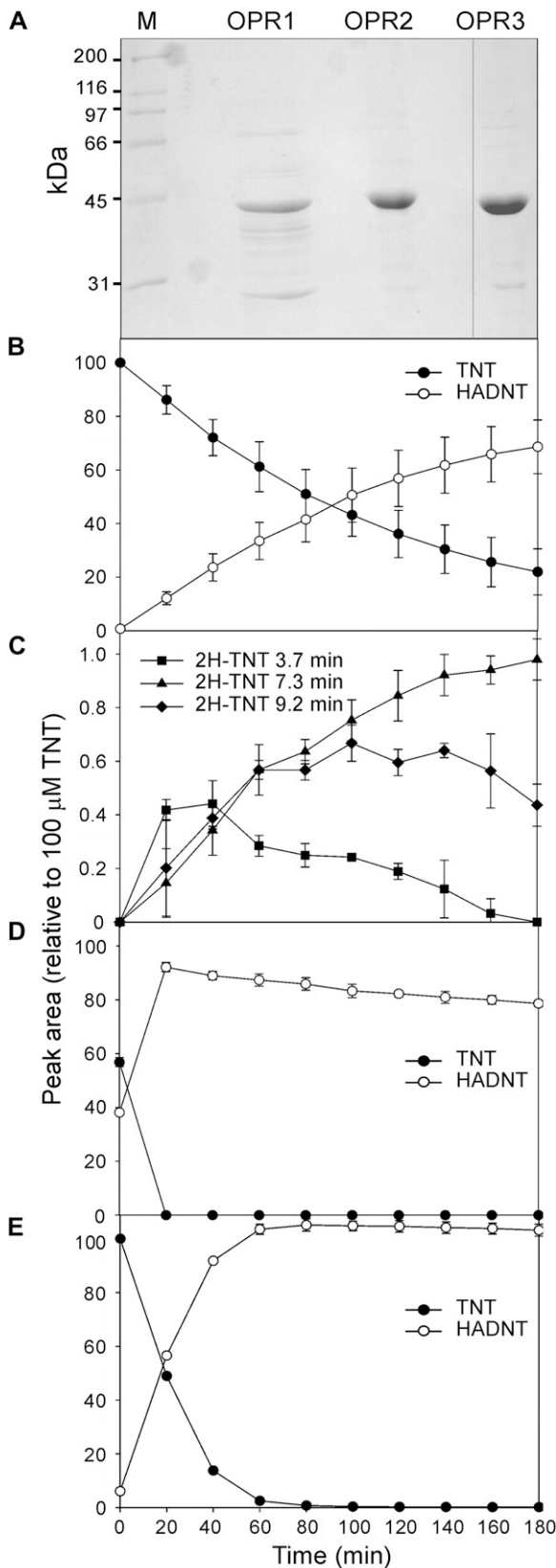

Figure 2. Quantitative RT-PCR of OPR expression in response to TNT treatment. Fourteen-day-old, liquid culture-grown Arabidopsis plants were treated with 60 μM TNT for 6 h, and the level of OPR expression was determined by quantitative RT-PCR. ACT2 mRNA was used to normalize cDNA levels. Results are means of three independent RNA isolations measured in triplicate $\pm$ SE.

Figure 3. Purification and characterization of Arabidopsis OPRs. A, SDS-PAGE analysis of affinity-purified Arabidopsis OPR1, OPR2, and

with TNT, we assayed cell extracts of NovaBlue (DE3) *E. coli*, containing pET-15b with and without *OPR1*. Only cell extract containing OPR1 was able to transform TNT to yield the dihydride-Meisenheimer TNT adducts, confirming that the observed activity with TNT was due to OPR1.

Characterization of Knockout and Overexpression OPR Lines

To dissect the individual contributions of the OPRs toward TNT detoxification in Arabidopsis, T-DNA insertional mutants *opr1* and *opr2* (Alonso et al., 2003) and *opr3* (Stintzi and Browse, 2000) were obtained. Expression analysis on *opr1* and *opr2* demonstrated that *OPR1* transcript was absent from *opr1* and *OPR2* transcript was absent from *opr2* (Supplemental Fig. S1); *opr3* had been characterized previously (Stintzi and Browse, 2000). *OPR1* and *OPR2*, the two OPRs most strongly up-regulated in response to TNT, are closely linked on chromosome I, making the likelihood of generating *opr1opr2* mutants by genetic crossing extremely unlikely. Thus, RNA interference (RNAi) was used to investigate the effect of knockdown expression of both *OPR1* and *OPR2*. The RNAi gene construct included 155 bp of *OPR1* and 451 bp of *OPR2* (construction details are presented in Supplemental Fig. S2). Although the *OPR1* sequence was shorter than the optimum 300- to 600-bp length for efficient RNAi, much of the selected *OPR2* sequence was identical to *OPR1*. The full knockdown *OPR1,2* sequence shared 96% and 92% identity with *OPR1* and *OPR2*, respectively, but only 59% and 73% identity with *OPR3* and *OPR4/5*, respectively. The knockdown *OPR1,2* sequence was under the control of the 35S promoter, resulting in near constitutive expression, thus providing expression in the root tissues. Independently transformed knockdown *OPR1,2* lines were generated with homozygous T3 lines C3, G3, and O1 used for further analysis. There were no phenotypic differences observed in the T3 knockdown *OPR1,2* plants compared with the untransformed wild-type Arabidopsis when grown on plant medium or soil. Quantitative RT-PCR analysis (Fig. 4A) showed that *OPR1* transcript levels were significantly reduced in all three knockdown *OPR1,2* lines, with line O1 showing the greatest reduction in transcript. The levels of *OPR2* were reduced by an equal amount in all three knockdown *OPR1,2* lines. The levels of transcript for *OPR3* and *OPR4/5* were not significantly different from the levels in untransformed wild-type plants, indicating that the knockdown *OPR1,2* transcript was specific to *OPR1* and *OPR2*.

OPR3 from *E. coli*. Lane M contains protein markers. B to E, Time course of TNT disappearance and product formation from purified in vitro assays analyzed by HPLC. B and C, OPR1. D, OPR2. E, OPR3. 2H-TNT, Hydride Meisenheimer complexes and elution times. Results are means of three replicates ± SD.
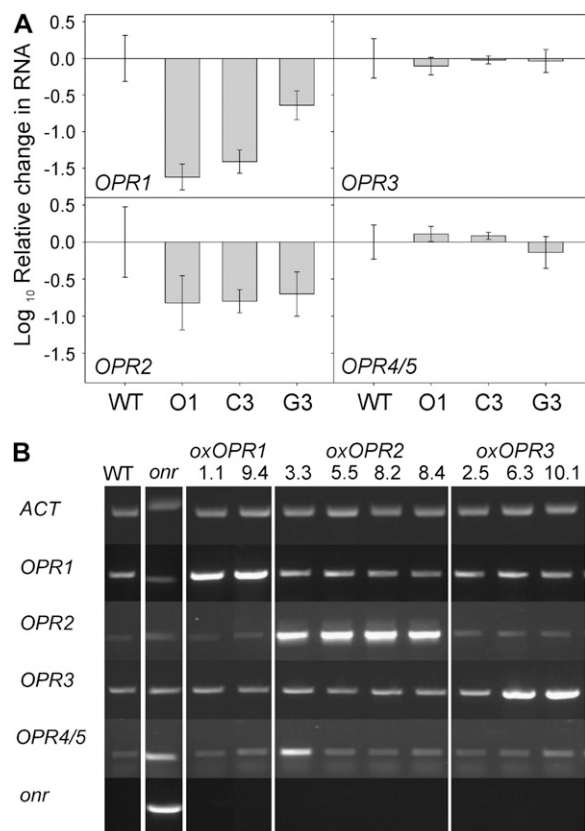
Figure 4. Transcript levels of knockdown and overexpressing OPR lines. A, Quantitative RT-PCR expression analysis on 14-d-old wild-type (WT) plants and knockdown *OPR1,2* lines. Results are means of three independent RNA isolations measured in triplicate ± SE. B, Representative semiquantitative RT-PCR on 14-d-old wild-type plants, overexpressing (*oxOPR*) lines, and pentaerythritol tetranitrate reductase-expressing (*onr*) lines. The experiment was repeated twice with similar results.

The *OPR1*, *OPR2*, and *OPR3* genes were overexpressed using the 35S promoter, with the C-terminal Ser-Arg-Leu type 1 peroxisome-targeting sequence present in *OPR3* conserved in the overexpressing *OPR3* construct. As a control to monitor the detection of ADNT and dihydride Meisenheimer TNT complexes in our experiments, the *onr* gene for the bacterial Old Yellow Enzyme homolog, pentaerythritol tetranitrate reductase, was transformed into Arabidopsis. Transformed lines were generated, and four independent lines were characterized for each gene. As predicted from our earlier studies on tobacco transformed with the same construct, all four *35S-onr* lines exhibited enhanced tolerance to TNT. The expression of line *35S-onr1* is shown in Figure 4B, and this line was used as a control in our liquid culture and solid medium experiments.

To investigate the tolerance of the knockout, knock-down, and overexpressing lines to TNT, these lines were grown on solid medium containing 2 μM TNT. Arabidopsis seedlings expressing *onr* had root lengths

2-fold longer than wild-type plants when grown on 2 μM TNT (Fig. 5A). This enhanced tolerance to TNT agrees with that previously observed in transgenic tobacco (French et al., 1999). Two of the *OPR1*-overexpressing lines (1.1 and 9.4) and all three of the *OPR2*-overexpressing lines had seedling root lengths significantly longer than those of wild-type plants ($P < 0.05$), while root lengths of *OPR3*-overexpressing seedlings were similar to those in the wild type. Decreased root length was observed in the *opr3* mutant seedlings, but root lengths of *opr1*, *opr2*, or knockdown *OPR1,2* lines were not significantly different from those of the wild type. No significant differences were observed, compared with the wild type, in the normal growth and development of the *opr1* or *opr2* knockout or overexpressing lines throughout the life cycle of the plants, with the exception of *opr2*, which exhibited reduced fertility (data not shown), and the previously reported male-sterile phenotype of *opr3* (Stintzi and Browse, 2000). The *opr2* phenotype links with the specific expression of the *OPR2* gene during late pollen development (Biesgen and Weiler, 1999).

TNT Transformation in Liquid Culture

As expected from earlier results (French et al., 1999), the *35S-onr* Arabidopsis plants exhibited enhanced uptake of TNT from liquid culture when compared with the wild type. Of the overexpressing *OPR* lines examined, the *OPR1*-overexpressing lines 1.1 and 9.4 showed enhanced TNT uptake (Fig. 5B), with *OPR2*-overexpressing and *OPR3*-overexpressing lines both behaving like the wild type. After 3 d, ADNT in the medium from *OPR1*-overexpressing lines 1.1 and 9.4 and *35S-onr* lines started to decline, whereas ADNT levels in the medium from the remaining overexpressing *OPR* lines reached a plateau and ADNT levels in medium from wild-type samples continued to rise. No HADNTs were detected in the medium. After 8 h, medium in flasks containing overexpressing *OPR1* 1.1 and 9.4 and *35S-onr* lines turned a translucent red/brown color. Absorbance in this 400- to 500-nm waveband region is characteristic of Meisenheimer adducts, which we observed in recombinant OPR1 assays (Fig. 3C). The spectra of these species shared similarities to characterized Meisenheimer adducts (Williams et al., 2004; data not shown), although the spectra of the wild type, *OPR1* overexpressing, and *35S-onr* were different from each other, suggesting, as in vitro data have shown (Williams et al., 2004), that the profile of Meisenheimer complexes produced is dependent on the Old Yellow Enzyme homolog.

After 7 d of growth in medium containing 200 μM TNT, seedlings of the *35S-onr* line, *OPR1*-overexpressing lines 1.1, and 9.4, and *OPR2*-overexpressing line 8.4 were less chlorotic and had greater biomasses than wild-type seedlings (Fig. 6). Biomass was not significantly altered from the wild type in the control flasks without TNT; however, in the absence of TNT, it was noted that the *OPR*-overexpressing lines showed en-

Figure 5. Root length and liquid culture analysis of Arabidopsis lines with altered levels of OPR transcript. A, Root length of 7-d-old seedlings grown on medium containing 2 μM TNT. Values are expressed as percentage of the root length of seedlings grown on medium without TNT $\pm$ SE. Results are from 40 to 60 seedlings from three independent seed batches. * Significantly different from the wild type ($P <$ 0.05). B, TNT removal and ADNT and Meisenheimer complex formation detected in the medium from 14-d-old, liquid culture-grown Arabidopsis dosed with 200 μM TNT. Results are means of four to five biological replicates $\pm$ SE. kd, Knockdown; NPC, no plant control; *onr*, pentaerythritol tetranitrate reductase-expressing lines; *ox*, overexpressing; Ws, Wassilewskija, background ecotype of *opr3*; WT, Col-0 wild type.
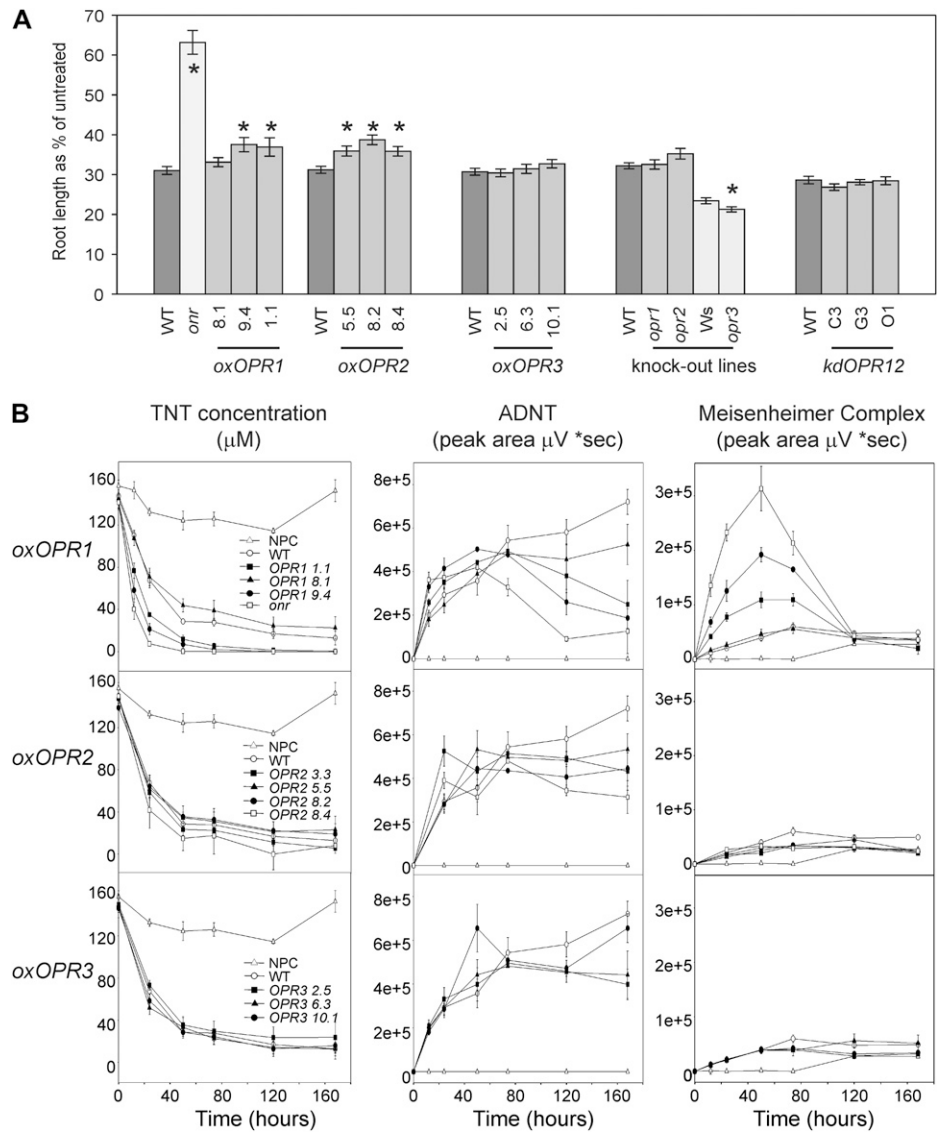
hanced survival under hydroponic conditions (data not shown).

In the flasks containing the knockdown *OPR1,2* lines grown in liquid culture with 300 μM TNT, TNT was taken up more slowly than in the wild type by all three knockdown lines in the first 2 d. Thereafter, no more TNT was removed from the medium by the knockdown *OPR1,2* lines, and significantly less ADNT and Meisenheimer complex compounds were released into the medium than from the wild type (Fig. 7).

DISCUSSION

We have cloned and purified three recombinant Arabidopsis OPRs, OPR1, OPR2, and OPR3, which exhibit significant (43%, 44%, and 36%, respectively) identity with bacterial pentaerythritol tetranitrate reductase, a member of the Old Yellow Enzyme family

that is able to transform TNT. The greatest degree of similarity, both structurally and in regulation, exists between OPR1 and OPR2, and both are up-regulated in the root on exposure to TNT (Biesgen and Weiler, 1999). However, *OPR1* had the greatest increase in expression in response to TNT treatment, suggesting a more dominant role in detoxification. The mechanism of TNT uptake and the intracellular site of accumulation are not known; however, while OPR1 and OPR2 are predicted to be active in the cytosol, OPR3 is targeted to the peroxisome, and this localization is likely to reduce accessibility toward TNT in planta.

The in vitro activity data clearly show that OPR1, OPR2, and OPR3 all have activity toward TNT. In liquid culture, the overexpressing *OPR1* lines 1.1 and 9.4 had increased TNT uptake and produced more TNT transformation products than the wild type, suggesting increased TNT metabolism. Conversely, the knockdown *OPR1,2* lines removed less TNT from
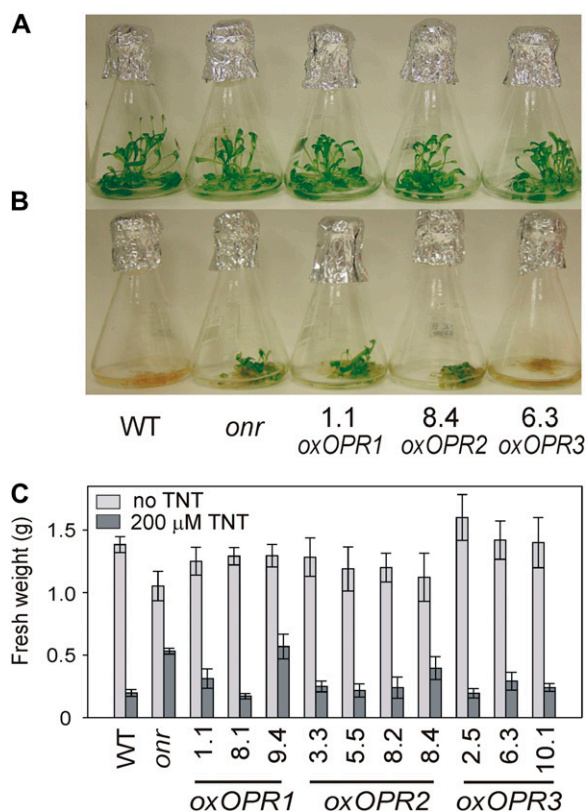
Figure 6. Morphology and biomass of overexpressing OPR (oxOPR) lines treated with TNT. A, Three-week-old, liquid culture-grown Arabidopsis growing without TNT. B, Appearance 7 d after dosing with 200 μM TNT. C, Fresh weight biomass after 2 weeks. Results are means of four to five biological replicates $\pm$ SE. onr, Pentaerythritol tetranitrate reductase-expressing lines; WT, wild type.

liquid culture and produced fewer TNT transformation products. When grown on solid medium, two of the three *OPR1*-overexpressing lines and all of the *OPR2*-overexpressing lines exhibited significantly enhanced tolerance to TNT (as measured by root growth). This agrees with previous data in tobacco expressing *onr*, which showed that enhanced TNT transformation directly increased TNT tolerance (French et al., 1999). These results suggest that OPR1 and OPR2 are involved in TNT detoxification in planta. However, the tolerance to TNT of the *opr1* and *opr2* mutants and knockdown *OPR1,2* seedlings was not significantly less than in wild-type seedlings, suggesting that there is some redundancy between the OPR activities, and other detoxification mechanisms are also likely to be involved (Gandia-Herrero et al., 2008; Rylott and Bruce, 2009). OPR1 is the only OPR in which both nitroreduction and aromatic ring reduction were observed in vitro, and both overexpressing *OPR1* and *35S-onr* plants released measurable levels of Meisenheimer complexes into the medium.

Expression analysis (Fig. 4) showed that in both the *OPR*-overexpressing and knockdown *OPR1,2* transgenic lines, only the targeted *OPR* expression levels

were altered; the expression levels of the remaining *OPR* homologs remained unchanged. While this result shows that *OPR* transcript levels are regulated independently from each other, it is possible that posttranscriptional regulation inhibited the generation of higher levels of active protein in the overexpressing lines. Previous studies using promoter β-glucuronidase fusions with Arabidopsis *OPR1* and *OPR2* showed that although transcript levels transiently increased in response to local and systemic wounding, UV-C illumination, and coldness, no subsequent changes in protein levels were detected (Biesgen and Weiler, 1999). Attempts to determine OPR activity in plant extracts using TNT as substrate were unsuccessful. However, the plant lines with altered levels of *OPR* transcripts did produce significantly different TNT metabolite profiles from those of untransformed
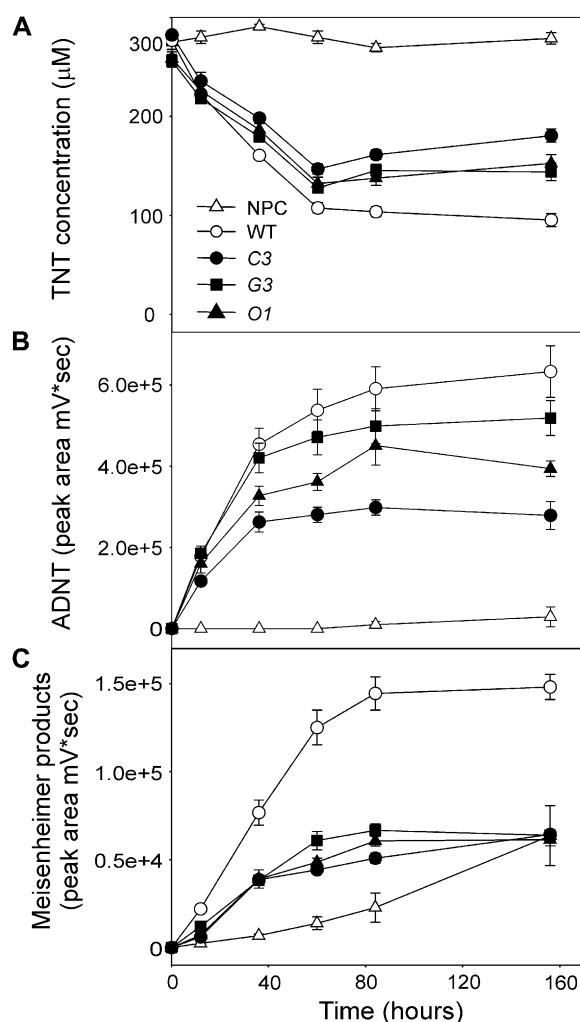
Figure 7. Uptake and product formation of TNT-treated Arabidopsis OPR knockdown lines. A, TNT removal. B, ADNT. C, Meisenheimer complex formation in the medium of 14-d-old, liquid culture-grown Arabidopsis dosed with 300 μM TNT. Results are means of four to five biological replicates $\pm$ SE. NPC, No plant control; WT, wild type.

plants when grown in liquid culture. Furthermore, seedlings of the *OPR1*- and *OPR2*-overexpressing lines had significantly enhanced seedling root growth in the presence of TNT, and these changes were observed in replicate, independently transformed lines. These findings indicate that the alterations seen in transcript levels reflect changes in OPR activity levels.

A wide range of α/β-unsaturated ketones and aldehydes, including vinyl ketones, smaller molecules such as acrolein, and those with more extended $\alpha\beta$-carbonyl chains such as traumatin, are produced in response to stress-related lipid oxygenation as a result of interaction with xenobiotics, pathogen attack, or wounding. Many of these compounds are substrates for Old Yellow Enzyme homologs, and it has been proposed that they could reduce reactive oxygen species in vivo (Vaz et al., 1995). These alternative, physiological substrates could compete with TNT for OPR-mediated catalysis. We have shown here that OPR1, OPR2, and OPR3 all have direct activity toward TNT, and their overexpression confers significantly enhanced tolerance to TNT. However, members of the Old Yellow Enzyme family are better known for their role as antioxidants (Kohli and Massey, 1998; Williams and Bruce, 2002; Fitzpatrick et al., 2003; Trotter et al., 2006), and OPRs may also have activity toward reactive oxygen species and an additional role protecting the plant from the oxidative stress resulting from TNT exposure.

CONCLUSION

In summary, we demonstrate that, in agreement with characterized bacterial Old Yellow Enzyme homologs, OPR1, -2, and -3 display activities toward TNT and OPRs contribute to the detoxification of TNT in the roots of Arabidopsis. A more detailed understanding of the biochemical mechanism of TNT detoxification in plants will allow opportunities to select or breed robust plant phenotypes in a rational way for field applications. This work further supports the view that members of the Old Yellow Enzyme family of flavoproteins play a role in the general detoxification of xenobiotic compounds in plants as well as microorganisms.

MATERIALS AND METHODS

Biochemicals

TNT was kindly provided by the Defence Science and Technology Laboratory at Fort Halstead, Kent, United Kingdom.

Recombinant OPRs

OPR1, *OPR2*, and *OPR3* were cloned from Arabidopsis (*Arabidopsis thaliana* ecotype Columbia-0 [Col-0]) cDNA into pET-15b (Novagen). OPR1 and OPR3 were expressed in NovaBlue (DE3) *Escherichia coli* (Novagen), and OPR2 was expressed in Rosetta-gami B (DE3) *E. coli* (Novagen). Cells were grown to mid-log phase at 37°C and induced with 0.4 mM isopropylthio-β-

galactoside. After 20 h, cells were spun at 12,000g for 10 min at 4°C, washed in buffer A (50 mM potassium phosphate buffer, pH 7), sonicated, and centrifuged at 4°C and 12,000g for 30 min. Soluble extract of OPR1 was applied to a Mimetic Orange 1 affinity chromatography column, and OPR2 and -3 were applied to a Mimetic Blue 1 column (Prometic Biosciences). Columns were washed with buffer A, then protein was eluted using a sodium chloride gradient, concentrated on 10-kD spin filters (Perbio Science), and dialyzed into buffer A. Mimetic Orange 2 matrix was added to the OPR1 fraction to bind and remove contaminants. Protein identities were confirmed following SDS-PAGE and trypsin digest, by MALDI-TOF peptide mass fingerprinting, immediately followed by more detailed protein characterization using MALDI-TOF/TOF tandem mass spectrometry on the same prepared sample.

Transformation of TNT by OPR1, -2, and -3

Assays and HPLC analysis were performed as described previously (Williams et al., 2004). The identities of ADNT and HADNT were confirmed using standards (Accustandard). Hydride Meisenheimer adduct standards were generated using purified pentaerythritol tetranitrate reductase (Williams et al., 2004).

Plant Material and Growth Conditions

Wild-type Arabidopsis ecotypes Col-0 and Wassilewskija were obtained from the Nottingham Arabidopsis Stock Centre at the University of Nottingham. The *opr1* (SALK_145355) and *opr2* (SALK_014855) mutants were obtained from T-DNA express (Alonso et al., 2003). The *opr3* mutant (Stintzi and Browse, 2000), in the Wassilewskija background, was kindly provided by John Browse (Institute of Biological Chemistry, Washington State University). Seeds were imbibed for 4 d at 4°C on agar plates containing 20 mM Suc and half-strength Murashige and Skoog salts (Sigma-Aldrich) with TNT dissolved in dimethylformamide when present (equivalent volumes of dimethylformamide were added to TNT-free flasks). Seedlings were grown in 16 h of light with 125 μmol m^{-2} s^{-1} white light. For liquid culture experiments, eight 1-d-old seedlings were transferred to 100-mL conical flasks containing 20 mL of half-strength Murashige and Skoog medium and 20 mM Suc. Plants were grown under 20 μmol m^{-2} s^{-1} light on a rotary shaker at 130 rpm.

Generation of OPR-Overexpression and Knockdown Constructs and Identification of *opr1* and *opr2*

The *35S* overexpression lines were generated using the pART27 binary cassette system (Gleave, 1992). The *OPRs* were amplified by PCR using overexpressing *OPR* primers and Arabidopsis Col-0 cDNA. Sequences were cloned into pART7 *Kpn*I and *Bam*HI sites for *OPR1*, *Kpn*I and *Xba*I sites for *OPR2*, and *Xho*I and *Cla*I sites for *OPR3*. The resulting *35S-OPR-ocs* cassettes were cloned into pART27 via *Not*I sites. The pART27-onr construct used was as reported previously (French et al., 1999).

The knockdown *OPR1,2* chimera was constructed as follows. *OPR1* and *OPR2* sequences were amplified using kdOPR1 and kdOPR2 primers, and the fragments were digested with *Bfa*I and ligated together. The *OPR1-OPR2* ligated sequence was then reamplified using kdOPR1F and kdOPR2R primers, sequenced, and cloned into pHellsgate 8 (Wesley et al., 2001). Construction details are presented in Supplemental Figure S2.

All constructs was transformed into Arabidopsis Col-0 using *Agrobacterium tumefaciens* strain GV3101. Transformants and homozygous lines were selected using kanamycin resistance.

The T-DNA border regions of *opr1* and *opr2* were amplified for sequencing using T-DNA left border primer LBa1 in combination with OPR1F for *opr1* and OPR2-seq for *opr2*. Insertion sequences are shown in Supplemental Figure S1. Endogenous *OPR1* and *OPR2* were amplified using OPR1F/OPR1R and OPR2F/OPR2R primers. All primer sequences are described in Supplemental Table S1.

RNA Isolation and RT-PCR

The DNase-treated total RNA was isolated from 14-d-old liquid culture-grown plants using the RNeasy kit (Qiagen). The synthesis of cDNA was primed with oligo(dT)$_{12-18}$ using SuperScript II reverse transcriptase (Invitrogen) containing RNasin (Promega) at 42°C for 90 min, before inactivation at 70°C for 15 min.

For quantitative RT-PCR, cDNA was purified using a Promega Wizard SV gel and PCR cleanup system and quantified. The rtOPR primers and probe sets (Supplemental Table S1), specific for each *OPR* gene, were tested for primer efficiency, then TaqMan quantitative PCR was performed using 50 ng of cDNA in an Applied Biosystems 7300 real-time PCR machine with a cycle of 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s, 60°C for 1 min using FAM (5′) and TAMRA (3′) fluorescent dyes. The level of expression was normalized against *ACT2* (GenBank accession no. NM_180280). Data were analyzed using ABI software according to the manufacturer's recommendations.

Semiquantitative RT-PCR was performed on the overexpressing *OPR* lines using serial dilutions of cDNA and sqOPR primers. Reactions were denatured at 94°C for 3 min, cycled 25 times at 94°C for 15 s, 55°C for 30 s, 1 min for 72°C, and then held at 72°C for 10 min. For reactions with *OPR2* primers, the annealing step was 60°C. *ACT2* gene expression using sqACT primers was used as a reference. Primer sequences are shown Supplemental Table S1.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *OPR1* (NM_202428), *OPR2* (NM_106319), *OPR3* (NM_126619), *OPR4* (NM_101664), *OPR5* (NM_179352), and *OPR6* (NM_100810).

Supplemental Data

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

Supplemental Figure S1. Schematic diagram of *OPR1* and *OPR2* loci and transcript analysis for *opr1* and *opr2* mutations.

Supplemental Figure S2. Construction of the RNAi *OPR1,2* vector.

Supplemental Table S1. DNA primer sequences used for cloning OPRs.

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