Plant Transformation Pathways of Energetic Materials (RDX, TNT, DNTs)

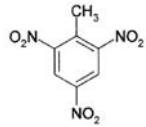
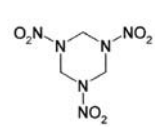
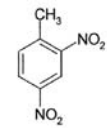
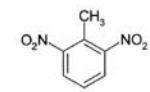
Jong Moon Yoon, David J. Oliver and Jacqueline V. Shanks
Iowa State University
Ames, IA

2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) are commonly found in surface soil and groundwater at ammunition-production and military-training sites. Approximately 2,000 US Department of Defense facilities are contaminated with explosives both in soil and in groundwater (Medina *et al.*, 2003). Dinitrotoluenes (DNTs), such as 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT), contaminate the sites as by-products of TNT. TNT and DNTs are classified as nitroaromatic explosives having aromatic ring structures, whereas RDX is a nitramine explosive possessing N-nitro groups (Hannink *et al.*, 2002). The explosives are transported to soil and groundwater after open detonation, seepage and/or improper disposal at military and munition-production sites. TNT and DNTs have higher octanol-water partition coefficient (K_{ow}) values than does RDX, suggesting that they are strongly bound to soil organic matter, whereas RDX is mobile as a result of poor sorption. The explosives are not volatile due to their low vapor pressures. Physical and chemical properties of the explosives are shown in Table 1.

Phytoremediation is a promising technology using plants to clean up contaminated soil and groundwater in situ *because of low cost of maintenance and operation, and public acceptance.*

Several studies have reported abiotic methods of treatment of explosives, such as incineration, carbon adsorption, alkaline hydrolysis, and catalytic and advanced oxidation (Garg *et al.*, 1991; Rodgers and Bunce, 2001). Harmful by-products, requiring further treatment, and transport of contaminated soils or groundwater have drawn attention to bioremediation. Phytoremediation is a promising technology using plants to clean up contaminated soil and groundwater *in situ* because of low cost of maintenance and operation, and public acceptance (Schnoor *et al.*, 1995). The fact that plants are able

Table 1. Chemical and physical properties of TNT, RDX, and DNTs.

	TNT	RDX	2,4-DNT	2,6-DNT
Molecular weight	227.15	222.26	182.14	182.14
Molecular formula	$C_7H_5N_3O_6$	$C_3H_6N_6O_6$	$C_7H_6N_2O_4$	$C_7H_6N_2O_4$
Log K_{ow}	1.6–1.84	0.81–0.87	1.98	1.9–2.10
Solubility in water (mg/L)	100	42	270–273	910
Vapor pressure (mm Hg)	1.99×10^{-4}	$1.0–4.0\times10^{-9}$	1.47×10^{-4}	5.67×10^{-4}
Henry's constant (atm-m^3/mole)	4.57×10^{-7}	1.2×10^{-5}	1.3×10^{-7}	9.26×10^{-8}
Molecular structure				

Data from Yinon and Zitrin (1993), Talmage *et al.* (1999) and HSDB (2000).

to accumulate metals to high concentrations in their tissues is well known (Salt *et al.*, 1998). Phytoremediation research has been conducted on organic pollutants ranging from pesticides, *e.g.* atrazine (Burken and Schnoor, 1997), to industrial pollutants such as trichloroethylene and polycyclic aromatic hydrocarbons (PAH) (Newman *et al.*, 1997; Paquin *et al.*, 2002; Vervaeke *et al.*, 2003). The transformation products of xenobiotics by plants are less toxic than parent compounds. In addition, root exudates are reported to enhance microbial activity for degradation of xenobiotics (Miya and Firestone, 2001; Anderson *et al.*, 1993). The cost for soil remediation is $10 to $100 per cubic meter whereas vegetative cleanup of contaminated soils costs only $0.02 to $1 per cubic meter (Cunningham *et al.* 1995).

This short review summarizes the toxicity of energetic materials (TNT, RDX, and DNTs) and pathways of transformation by plants.

Toxicity of the Explosives

TNT and its degradation products have been reported to be mutagenic and toxic to several organisms. Survival of the midge (*Chironomus tentans)* decreased significantly after exposure to 200 mg/kg of TNT, 1,3,5,-trinitrobenzene (TNB), and 2,4-diamino-6-nitrotoluene (2,4-DANT); and the amphipod (*Hyalella azteca*) was more susceptible to TNT, TNB, and 2,4-DANT than the midge (Steevens *et al.*, 2002). Gogal *et al.* (2002) reported that northern bobwhite quail showed decreases in total red blood-cell counts and plasma protein as dietary TNT intake increased, and they determined a low observed-adverse-effect level of 178 mg of TNT per kg of weight per day. Survival and growth

of two freshwater invertebrates were not affected after a 10-day exposure to 1,000 mg of RDX per kg of sediment (Steevens *et al.*, 2002). The growth and survival of benthic invertebrates, *Neanthes arenaceodentata* and *Leptocheirus plumulosus*, were not affected by exposure up to 1,000 µg RDX per kg dry weight of sediment (Lotufo *et al.*, 2001). Inhibition to growth and reproduction of adult earthworms can occur at less than 95 mg of RDX per kg of artificial soil (Robidoux *et al.*, 2000, 2001), but acute toxicity was not observed up to 756 mg per kg dry soil for RDX.

Gong *et al.* (2002) investigated the influence of RDX on indigenous microbial activities. They measured soil dehydrogenase activity, potential nitrification activity, heterotrophic nitrogen fixation activity, substrate-induced respiration, and basal respiration for 12 weeks. Significant reductions (up to 30% of control) in these parameters were observed in RDX-spiked soil. In the case of a luminescent marine bacterium (*Vibrio fischeri*), the EC_{50} value of RDX (116 mg/L) was above the solubility in water (42 mg/L for RDX) after incubation periods of 90 min (Drzyzga *et al.*, 1995).

Neither 2,4-DNT nor 2,6-DNT were mutagenic with the Ames assay, whereas their hydroxylamine isomers proved to be mutagenic (Padda *et al.*, 2003). Using the uptake response of H4IIE rat hepatoma cell cultures to neutral red, the NR_{50} values were 45 mg/L for 2,4-DNT, 50 mg/L for 2,6-DNT, and 7 mg/L for TNT, suggesting dinitrotoluenes are less cytotoxic than TNT (Mitchell and Burrows, 1995).

TNT was toxic to hybrid poplars at a concentration of 5 mg/L in hydroponic solution (Thompson *et al.*, 1998) and at 50 mg/kg soil there were adverse effects on germination and seedling growth of cress and turnip (Gong *et al.*, 1999). Alfalfa did not grow at 0.55 m*M* (100 mg/kg) 2,4-DNT in soil (Dutta *et al.*, 2003), and lettuce was more sensitive than wheat, mustard, and lentil, indicating that phytotoxic effects of nitroaromatic explosives depend on plant species (Picka and Friedl, 2004). The highest non-observed adverse effect concentrations (NOAEC) for the growth of lettuce were 20 mg/kg for TNT, 2 mg/kg for 2,4-DNT and 10 mg/kg for 2,6-DNT. Hydroponic toxicity of RDX to maize and wheat was estimated to be 21 mg/L RDX, while soybean and sorghum did not show a toxic effect up to 21 mg/L for 30-day exposures (Chen, 1993). RDX was not toxic to hybrid poplars up to 21 mg/L (Thompson, 1997).

Mechanisms of Degradation of Xenobiotics by Plants

Prior to the introduction of xenobiotics to plant cells, they must be taken up through the roots. Several studies reviewed predictive relationships between the uptake rate of a compound and its physical-chemical properties (Briggs *et al.*, 1982; Burken and Schnoor, 1998). Root uptake and translocation of the compounds are related to the logarithm of the octanol-water partition coefficient, log K_{ow}. Root concentration factor (RCF), defined as the the concentration sorbed to the roots divided by the concentration in the aqueous phase, is generally proportional to the log K_{ow} value. The relationship is proposed as follows:

$$\log (RCF–3.0) = 0.65 \log K_{ow} – 1.57 \text{ by Briggs } \textit{et al.} \text{ (1982)}$$
$$\log (RCF–0.82) = 0.77 \log K_{ow} – 1.52 \text{ by Burken and Schnoor (1998)}$$

The transpiration stream concentration factor (TSCF) is calculated as the concentration in the transpiration stream divided by the aqueous concentration. The values of TSCF for various chemicals show Gaussian distribution curves over the range of log K_{ow} values, indicating that hydrophilic compounds (log $K_{ow} < 1.8$) are not able to pass through lipid membranes of roots, whereas hydrophobic compounds (log $K_{ow} > 3.8$) tend to bind strongly to root tissues and are not then translocated to shoots (Dietz and Schnoor, 2001). The relationship between TSCFs and log K_{ow} is proposed as follows:

$$TSCF = 0.784 \exp\{-(\log K_{ow} - 1.78)^2/2.44\} \text{ by Briggs } \textit{et al.} \text{ (1982)}$$
$$TSCF = 0.756 \exp\{-(\log K_{ow} - 2.50)^2/2.58\} \text{ by Burken and Schnoor (1998)}$$

Enzymatic transformation of xenobiotics by plants follows the green-liver model and involves three steps. First, the foreign compounds taken up by plants are transformed by enzymes such as cytochrome P450, carboxylesterases, and peroxidase (Sandermann, 1994). Secondly, the transformed xenobiotics are conjugated with D-glucose, glutathione, or amino acids (Komoba *et al.*, 1995) by enzymes such as glutathione *S*-transferases, glucosyltransferases and malonyltransferases, resulting in either soluble or insoluble products. The third step is storage and compartmentation; the soluble compounds are stored in vacuoles or as cell-wall materials by further processing, and the insoluble compounds are generally assumed to be stored in the cell wall (Schroder and Collins, 2002).

Uptake of the Energetic Materials by Plants

Nitroaromatic explosives showed different uptake and fate in plant systems than nitramine explosives. According to Thompson *et al.* (1998), 95% of the TNT was removed from solution in less than 24 h by hybrid poplar, whereas 71% of the RDX was removed from hydroponic solution in 7 days (Thompson *et al.*, 1999). The uptake of both RDX and TNT from soil was slower than in the hydroponic systems because of decreased bioavailability in soil. Bush beans took up less than 16% of RDX in soil after 60 days; in contrast 60% was removed from solution after 7 days (Harvey *et al.*, 1991).

Over 60% of radioactivity of ^{14}C-RDX taken up by hybrid poplars was found in the leaves after 2 days. In contrast, 78% of radioactivity of ^{14}C-TNT taken up by the poplars remained in the roots after the same exposure time (Thompson *et al.*, 1998), suggesting that RDX is translocated more readily. In addition, an overall low recovery of RDX with no significant mineralization by plants suggested that the final transformation products are volatile compounds (Just and Schnoor, 2000). Recently, poplar nodule cultures were reported to mineralize RDX under sterile conditions (Van Aken *et al.*, 2004).

Regarding DNTs, knowledge of uptake by plants and transformation products is limited compared to information on TNT and RDX. Best *et al.* (2001) applied wetland systems to remove explosives from groundwater at ammunition plants, resulting in average removals of 58% and 61% for 2,4-DNT and 2,6-DNT, respectively, in a 115-day operation at the Volunteer Army Ammunition Plant, Chattanooga, TN. Todd and Lange (1996) observed that 67% of 2,4-DNT from soil was removed in a phytoremediation system using parrot feather (*Myriophyllum brasiliense*). They found 4-amino-2-nitrotoluene (4A2NT) in the

plant tissues after 90 h of treatment prior to 2-amino-4-nitrotoluene (2A4NT) which was detected after 190 h of exposure. However, other transformation products of the DNTs, as well as their fate in plants, are unknown.

Transformation Pathways

2,4,6-trinitrotoluene (TNT)

Subramanian and Shanks (2003) proposed the TNT transformation pathway by plants based on experiments with periwinkle (*Catharanthus roseus*) and parrot feather, as shown in Figure 1.

Two monoamino compounds [2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene(4ADNT)] have been found as the primary reduction products by plants (Palazzo and Leggett 1986; Thompson *et al*. 1998; Bhadra *et al*., 1999a). Diaminotoluenes (2,4-diamino-6-nitrotoluene and 4,6-diamino-2-nitrotoluene) and azoxy compounds were observed under strong reducing conditions and by the condensation of hydroxylamines, respectively (Pavlostathis *et al*., 1998; Sens *et al*., 1998; Thompson *et al*., 1998).

As for oxidative transformation of TNT in plant systems, Bhadra *et al*. (1999b) isolated six oxidized metabolites such as 2-amino-4,6-dinitorbenzoic acid, 2,4-dinitro-6-hydroxy-benzyl alcohol, 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde, 2,4-dinitro-6-hydroxytoluene, and two binuclear metabolites from azoxytetranitro toluenes. In addition, they showed that oxidation of TNT by the plant could occur before the reductive transformation. This was based on results where monoamino compounds were added to plants and the oxidized metabolites of TNT were not produced. To date, oxidized metabolites have only been found in parrot feather; they were not detected in *Catharanthus* or *Arabidopsis* (Subramanian, 2004).

2-hydroxylamino-4,6-dinitrotoluene (2HADNT) and 4- hydroxylamino-2,6-dinitrotoluene (4HADNT) were observed following reduction of nitro groups of TNT in non-axenic and aquatic plant systems (Pavlostathis *et al*., 1998; Wang *et al*., 2003). Measurement of hydroxylamines was difficult due to their instability. Wang and Hughes (1998) developed an efficient assay for hydroxylamines by derivatization with acetic anhydride. Recently, these hydroxylamines were shown to be present in axenic hairy roots of *Catharanthus* and axenic *Arabidopsis* seedlings (Subramanian, 2004; Subramanian *et al*., 2005). The hydroxylamines are considered the first transformation products resulting in other metabolites of TNT by reduction, oxidation, conjugation, and polymerization (Subramanian and Shanks, 2003; Wang *et al*., 2003).

The transformed products of TNT are further conjugated and sequestered in plant cells. Over 80% of the TNT label was associated with plant biomass, suggesting that the labeled carbon from TNT was sequestered in the plant tissues (Harvey *et al*., 1991). Thompson *et al*. (1998) showed that 75% of the radioactivity of ^{14}C-TNT was present in unextractable and bound residues in the poplar roots. Bhadra *et al*. (1999a) characterized the four conjugates of TNT metabolites with a 6-carbon moiety by *Catharanthus roseus* and *Myriophyllum aquaticum*. They found that two of them have molecular structures similar

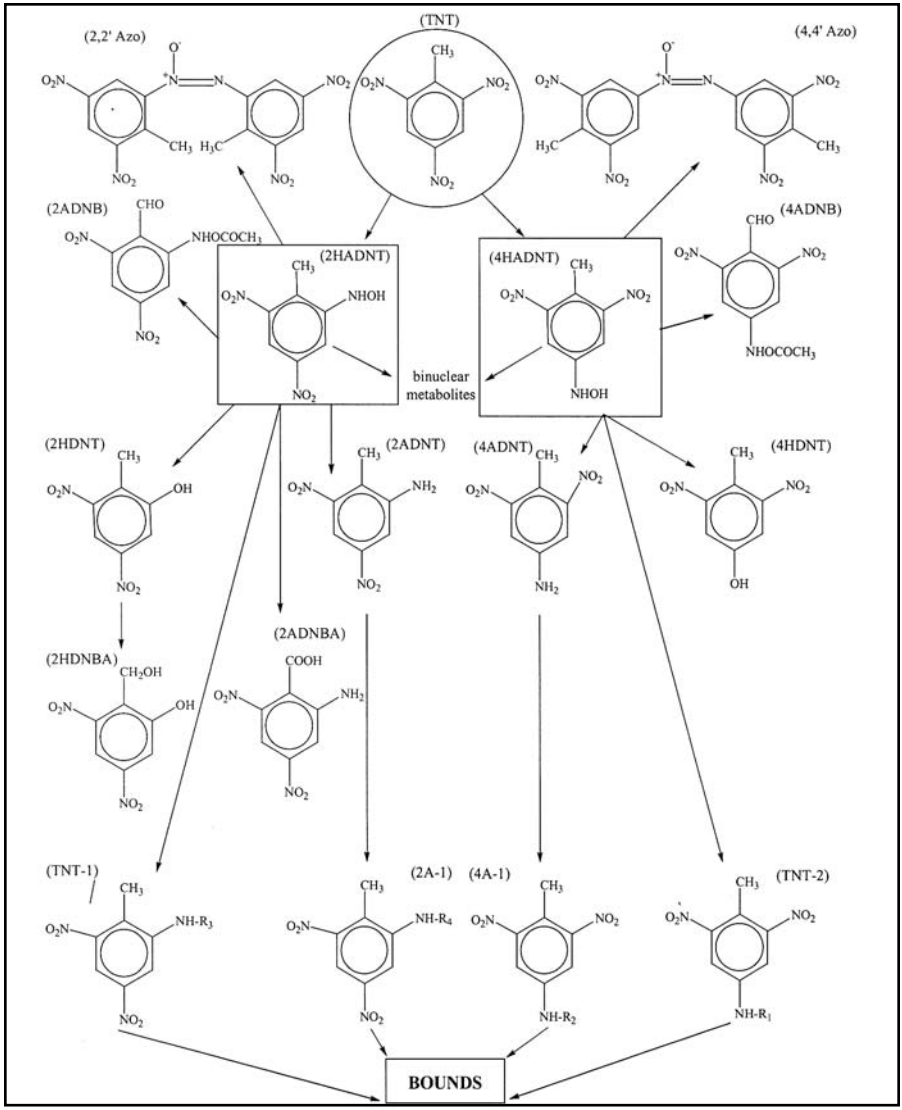


Figure 1. TNT transformation pathway by Subramanian and Shanks (2003). Abbreviations—TNT: 2,4,6-trinitrotoluene; 2ADNT: 2-amino-4,6-dinitrotoluene; 4ADNT: 4-amino-2,6-dinitrotoluene; 2HADNT:2-hydroxylamino-4,6-dinitrotoluene; 4HADNT: 4-hydroxylamino-2,6-dinitrotoluene; 4,4'Azo: 2,2',6,6'-tetranitro-4,4'-azoxytoluene; 2,2'Azo: 4,4',6,6'-tetranitro-2,2'-azoxytoluene; 2HDNT: 2-hydroxy-4,6-dinitrotoluene; 4HDNT: 4-hydroxy-2,6-dinitrotoluene; 2ADNB: 2-*N*-actamido-4,6-dinitrobenzaldehyde; 2ADNB: 4-*N*-actamido-2,6-dinitrobenzaldehyde; 2HDNBA: 2-hydroxy-4,6-dinitrobenzyl alcohol; and 2ADNBA: 2-amino-4,6-dinitrobenzoic acid. TNT-1, TNT-2, 2A-1 and 4A-1 represent conjugates with six carbon sugars (R1, R2, R3, and R4).

to that of 2ADNT (labeled TNT-1 and 2A-1) and the others were similar to 4ADNT (TNT-2 and 4A-2), indicating that the monoamines were precursors to the conjugates. Recent studies have elucidated these TNT conjugates. The conjugates of TNT metabolites by tobacco cell cultures are formed by conjugation of glucose on the hydroxylamine group of either 2HADNT or 4HADNT, and various diglycoside conjugates with gentiobioside or sophoroside forms were identified, including monoglycosides (Vila *et al.*, 2005). In precursor-feeding studies, Subramanian (2004) and Subramanian *et al.* (2005) found evidence for conjugation of monoamines and hydroxylamines with plant sugars.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)

Studies on the transformation of RDX by plants are rare, whereas several microbial transformation pathways have been proposed and some established. After being taken up and translocated to leaf tissues, direct photolysis of RDX in the leaves is a feasible fate under natural sunlight. Just and Schnoor (2004) proposed the photodegradation pathway of RDX by reed canary grass, as shown in Figure 2. They identified ring-cleavage products, such as nitrous oxide (N_2O) and 4-nitro-2,4-diazabutanal in leaves under simulated sunlight, including nitrite (NO_2^-) and formaldehyde (CH_2O) in solution. Van Aken *et al.* (2004) proposed three processes for the pathway of degradation of RDX by using poplar tissue cultures and crude extracts from leaves, as shown in Figure 2. First, reduction products such as hexahydro-1-nitroso-1,3-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) were produced by intact plant cells regardless of light. In the second step, the reduced metabolites were further transformed to formaldehyde and methanol, both in crude extracts and in intact cultures under light. In the final step, light-independent mineralization of one-carbon metabolites by intact plant cultures, but not crude extracts, occurred. Some transformed products may be re-incorporated into plant cells. Formaldehyde may be conjugated by plant enzymes to form compounds like *S*-formyl-glutathione (Just and Schnoor, 2004). Small quantities of CO_2 produced by degradation of RDX by plants may be re-assimilated by photosynthesis (Van Aken *et al.*, 2004).

Dinitrotoluenes (DNTs)

In contrast with bacterial systems, little information is available on the transformation of DNTs by plants. We are aware of only one study: monoamino isomers, 2A4NT and 4A2NT, were reductive transformation products in plants (Todd and Lange, 1996). The bacterial reduction of dinitrotoluenes can take place under aerobic and anaerobic conditions, resulting in the production of monoamines isomers (Hughes *et al.*, 1999). Hydroxylaminotoluenes and dihydroxylaminotoluenes were produced anaerobically in cell cultures of *Clostridium acetobutylicum* (Hughes *et al.*, 1999). Further transformed products, aminohydroxylaminotoluenes and diaminotoluenes, were observed in the cell extracts. *Hydrogenophaga palleronii* and *Burkholderia cepacia* produced oxidative intermediates and mineralized DNTs to CO_2 by mono- or dioxygenases (Nishino *et al.*, 1999). The bacteria converted 2,6-DNT into 3-methyl-4-nitrocatechol with release of nitrite, and then 2-hydroxy-5-nitro-6-oxohepta-2,4,-dienoic acid and 2-hydroxy-5-nitropenta-2,4,-dienoic acid (Nishino *et al.*, 2000).

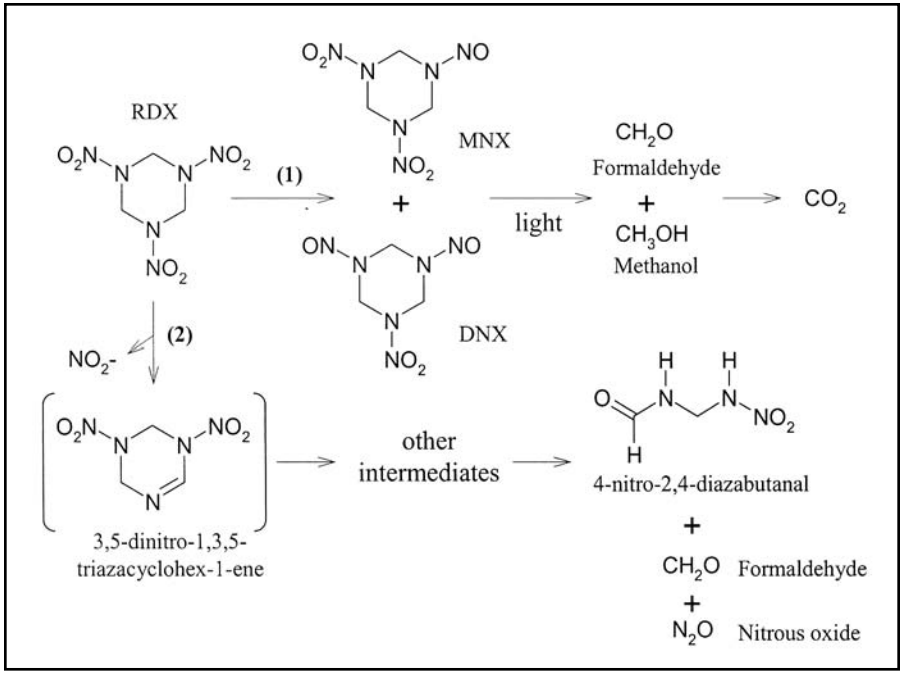


Figure 2. RDX degradation pathways proposed by Van Aken *et al.* (2004) and Just and Schnoor (2004). The bracketed compound was not observed. Abbreviations—RDX: hexahydro-1,3,5-trinitro-1,3,5-triazine; MNX: hexahydro-1-nitroso-1,3-dinitro-1,3,5-triazine; and DNX: hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine.

Transgenic tobacco plants expressing nitroreductases of Enterobacter cloacae *showed enhanced ability to tolerate and remove TNT at high concentration.*

Transgenic Plants and Gene Expression

In the past 6 years there has been significant activity in using genetic approaches to enhance transformation and to reduce phytotoxicity of energetic materials. Genetically modified plants expressing bacterial genes have been developed for phytoremediation. Transgenic tobacco plants expressing nitroreductases of *Enterobacter cloacae* showed enhanced ability to tolerate and remove TNT at high concentration (0.25 m*M*), which is toxic to wild-type tobacco (Hannink *et al.*, 2001). Another transgenic tobacco line expressing pentadrythritol tetranitrate reductase from the bacterium showed better germination and growth in the presence of TNT (0.05 m*M*) than did wild-type plants (Rosser *et al.*, 2001; French

et al., 1999). In addition, these researchers showed enhanced RDX removal in tobacco engineered with an XplA cytochrome P450 from *Rhodococcus rhodochrous* (unpublished results). Clearly, genetic modification with microbial redox enzymes has the potential to enable faster transformation of TNT and RDX and reduced phytotoxicity.

Transcriptomic studies are providing clues to endogenous plant genes involved in transformation. Specific genes such as those for glutathione-*S*-transferases and cytochrome P450 in *Arabidopsis* were proposed by Ekman *et al*. (2003) to be involved in transformation of explosives. They used serial analysis of gene expression (SAGE) to compare 14-day-old *Arabidopsis*, exposed to 15 mg/L of TNT after 24 h, to untreated plants. A glutathione-*S*-transferase was found to be induced up to 27-fold. Among the highly induced genes were those encoding cytochrome P450 (CYP81D11-A-TYPE), an ABC transporter that is known to expend ATP energy to transport hydrophobic molecules into or out of the cytoplasm, and a 12-oxophytodienoate reductase having high homology to nitroreductases of *Enterobacter* sp. (Ekman *et al*., 2003). However, as noted previously, oxidative compounds were not found in *Arabidopsis* (Subramanian, 2004), thus the role of P450s in transformation pathways in *Arabidopsis* is unclear. In microarray experiments, *Arabidopsis* gene expression was monitored after long-term exposure (10 days) to various concentrations of TNT (Mentewab *et al*., 2005). In response to TNT amendment, fifty-two genes were upregulated and forty-seven were downregulated, many of which have cell-defense and detoxification functions. Glutathione-S-transferases and cytochrome P450s were not found to be significantly upregulated in this study. Most of the genes differentially expressed were observed at the higher concentration of TNT amendment (10 μM) and genes expressed at 1 and 10 μM rarely overlapped. They confirmed the gene expressions of pathogenesis-related protein-1 precursor, DNA-binding protein, and ABC transporter-like protein by real-time PCR analysis.

The transcriptome studies provide clues to genes that may be involved in TNT transformation. Upregulation of some of the genes may be the result of a generalized stress response without synthesis of enzymes involved in the TNT phytotransformation pathway or in a reduced phytotoxicity response. Reverse-genetics approaches using the genes identified should enable further clarification of the transcriptome results. In a forward-genetics approach, ten activation-tagged *Arabidopsis* mutant lines showing significantly better germination rates than the wild type on the TNT-amended medium were isolated from 300,000 mutant seeds (Moon *et al*., 2004).

Selection of high-performing native plants, engineering plants with enhanced transformation capabilities, identifying the fate of transformation products in plants, and designing the external variables to operate a more effective phytoremediation process are all dependent on a knowledge base of the genetic structure, enzymatic structure, and biochemical reaction pathways. The genetic approaches discussed here will enable the design of effective strategies for remediation of energetic materials in the future.

Conclusions and Future Directions

Plants can remove contaminants from soil and groundwater, and transform them into less harmful compounds. Based on information on transformation pathways and gene

Research on the post-harvest fate of explosives is required.

expression, further studies on metabolic engineering and genetic modifications may make plants tolerant to higher concentrations of xenobiotics by inducing faster rates of uptake and using less toxic metabolic pathways. In addition, the explosives taken up by plants can be released by action of water—*e.g.* rain and river—and thus may be returned to the environment as hazardous contaminants. Research on further treatments and the post-harvest fate of explosives is required. Information about phytoremediation of dinitrotoluenes is lacking compared to that for TNT and RDX; thus, it also merits further investigation.

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Jacqueline Shanks is a professor of chemical engineering at Iowa State University (ISU) and an adjunct professor of bioengineering at Rice University. She received her BS from ISU in 1983 and her PhD from the California Institute of Technology in 1989. She joined Rice in 1988 and ISU 1999.

Dr. Shanks' research interests include engineering of secondary metabolites in plants, nuclear magnetic resonance spectroscopy techniques for metabolic flux analysis, phytoremediation of explosives and related nitroaromatics, and production of valuable products from biorenewable resources.

She received the NSF Young Investigator Award in 1992 and ISU's Professional Progress in Engineering Award in 1994. She was elected as fellow to the American Institute of Medical and Biological Engineers in 2000, and served as a member of the NRC Committee on Biobased Industrial Products. She is a member of the ACS's Biochemical Technology (BIOT) and Environmental Chemistry (ENVI) Divisions. She received the Van Lanen Award for service in the BIOT division in 2004. Shanks has served as co-editor of the Biochemical Engineering section of *Current Opinion in Biotechnology* and as co-editor for a 2002 issue of *Metabolic Engineering* devoted to plant metabolic engineering. She is a member of the Editorial Advisory Board for *Biotechnology Progress*.