RESEARCH ARTICLE

PLANT SCIENCE

Monodehydroascorbate reductase mediates TNT toxicity in plants

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The explosive 2,4,6-trinitrotoluene (TNT) is a highly toxic and persistent environmental pollutant. Due to the scale of affected areas, one of the most cost-effective and environmentally friendly means of removing explosives pollution could be the use of plants. However, mechanisms of TNT phytotoxicity have been elusive. Here, we reveal that phytotoxicity is caused by reduction of TNT in the mitochondria, forming a nitro radical that reacts with atmospheric oxygen, generating reactive superoxide. The reaction is catalyzed by monodehydroascorbate reductase 6 (MDHAR6), with *Arabidopsis* deficient in MDHAR6 displaying enhanced TNT tolerance. This discovery will contribute toward the remediation of contaminated sites. Moreover, in an environment of increasing herbicide resistance, with a shortage in new herbicide classes, our findings reveal MDHAR6 as a valuable plant-specific target.

R ecalcitrant to degradation, 2,4,6-trinitrotoluene (TNT) is a worldwide pollutant, contaminating manufacturing waste sites, mines, current and former conflict zones, and military land (*1*); the U.S. Department of Defense has an estimated 10 million hectares of operational ranges contaminated with munitions constituents (*2*). Rated a class C carcinogen by the Environmental Protection Agency, TNT has toxic effects on all living organisms: in animals, causing hepatitis, anemia, hyperplasia of bone marrow, and cataracts (*3*), and in soil, severely affecting microbial diversity (*4*) and the establishment of vegetation (*5*). In plants, the majority of TNT remains in the roots (*6*), where growth and development is inhibited, reducing overall biomass (*5*).

Despite these inhibitory effects, TNT can be detoxified by plants to a limited extent (*7–9*), and there is great interest in the development of plant-based explosives remediation (*10*). Seeking to identify plant TNT-detoxifying enzymes, we screened Weigel activation–tagged *Arabidopsis thaliana* plant lines (*11*) for greater root growth in the presence of TNT and isolated a mutant with enhanced TNT tolerance. Through outcrossing with single-nucleotide polymorphism analysis, bulk segregant analysis, and sequencing of candidate genes, this phenotype was mapped to *mdhar6-1* (At1g63940) (fig. S1). The *mdhar6-1* mutant was found to have a thymine deletion 2181 bases from the start codon, introducing a frame shift and an early stop codon and predicted

to truncate over a third of the protein. Two further *mdhar6* mutant lines were sourced (Fig. 1, A and B, and fig. S1), and all three had enhanced shoot and root biomass when grown in TNT-treated soil (Fig. 1, A and B, and fig. S2).

The role of monodehydroascorbate reductase (MDHAR)

The finding that the TNT tolerant line had a frame shift in *MDHAR6*, rather than overexpression of a TNT-detoxifying enzyme, was surprising, because MDHARs are considered to protect plants from oxidative stress by recycling the antioxidant ascorbic acid; MDHARs are flavin adenine dinucleotide–dependent oxidoreductases that reduce monodehydroascorbate (MDA), the free radical oxidation product of ascorbic acid (*12, 13*). Analysis of the *Arabidopsis* genome has revealed five MDHARs; MDHAR1 (At3g52880) and 4 (At3g27820) are targeted to peroxisome matrices and membranes, respectively, whereas MDHAR2 (At5g03630) and 3 (At3g09940) are cytosolic (*14*). MDHAR6 is targeted to mitochondria or plastids, depending on the transcription start site used (fig. S1) (*15*), and is expressed highly in all tissues and developmental stages (*16*).

To confirm whether MDHAR6 deficiency increases TNT tolerance, binary vectors expressing mitochondria or plastid-targeted *MDHAR6* were stably transformed into *mdhar6-1* and the Col7 background line. Plastidial *MDHAR6* partially restored TNT toxicity, and expression of the mitochondrial form completely restored TNT toxicity (Fig. 2 and figs. S3 and S4).
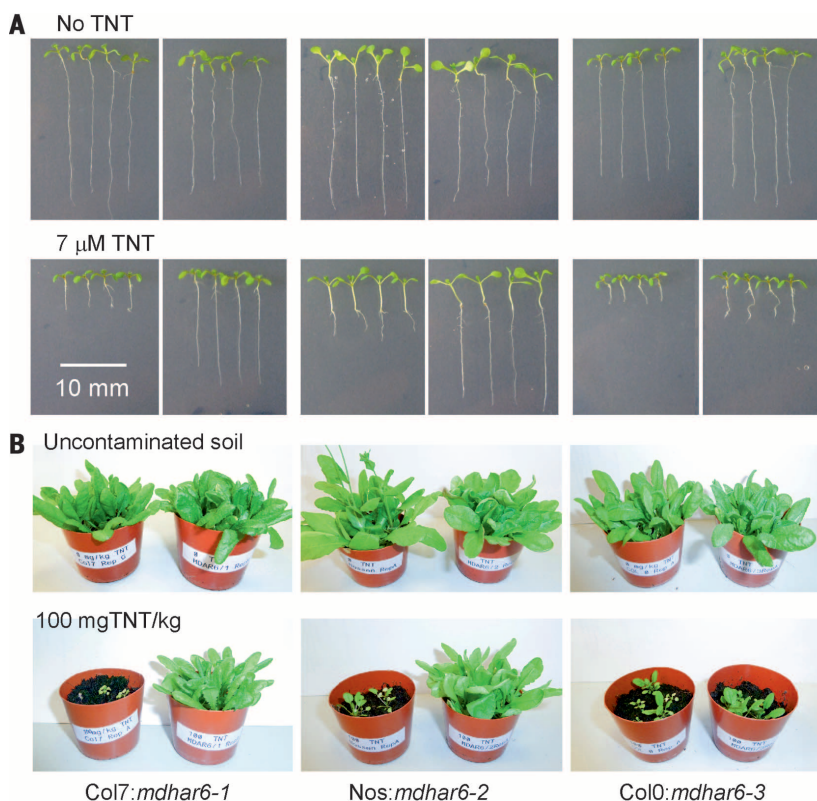

Fig. 1. Plants deficient in *MDHAR6* are more tolerant to TNT. (**A**) Seven-day-old *mdhar6* mutant and WT seedlings germinated on 0 or 7 μM TNT ½ Murashige-Skoog agar. (**B**) Appearance of 6-week-old *mdhar6* plants (right) adjacent to WT backgrounds (left), which were transferred to TNT-treated or untreated soil at 5 days of age.

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To investigate whether the enhanced tolerance was due to reduced TNT uptake, seedlings were transferred to liquid media, which was dosed with TNT, and the media was sampled over 3 days. The mutants removed TNT from the media at equivalent rates to their wild-type (WT) backgrounds, indicating that the TNT tolerance is not due to reduction in TNT uptake (fig. S2).

Uptake of TNT from soil by Col7 and *mdhar6-1* was also tested; TNT binds strongly to humic fractions of soil, and on artillery ranges reaches concentrations over 100 mg TNT/kg soil (*17*). When young *Arabidopsis* seedlings were transferred to 100 mg TNT/kg soil and grown for 5 weeks, 40% more TNT was recoverable from soil treated with Col7 plants compared with *mdhar6-1* (table S1).

Stress tolerance of *mdhar6* mutants is specific to TNT

To investigate whether MDHAR6 deficiency confers resistance to other stresses, Col7 and *mdhar6-1* were germinated on media supplemented with NaCl, sorbitol, methyl viologen (Paraquat), or hydrogen peroxide up to lethal concentrations, and root lengths were measured. No enhanced tolerance in *mdhar6-1* was identified (fig. S5), indicating that the tolerance to TNT is not due to enhanced general defenses.

Considering that MDHAR6 is the only mitochondria and plastid-targeted MDHAR in *Arabidopsis*, it is surprising that plants carrying mutations in MDHAR6 were not more susceptible to environmental stresses. The *mdhar6-1* plants

are indistinguishable from the wild type throughout development and are as robust as the wild type when grown in uncontaminated soil. This raises new questions as to the role of plastidial and mitochondrial MDHAR6 in protecting plants from oxidative stress. Ascorbate deficiency could be complemented by other antioxidant and/or enzyme activity, including dehydroascorbate reductase, which reduces dehydroascorbate, using glutathione as the electron donor (*18*).

Conjugation of reduced glutathione to TNT serves to detoxify the pollutant (*9*). Because ascorbate and glutathione are coupled antioxidants (*18*), we investigated the effect of MDHAR6 deficiency on ascorbate and glutathione levels. There

were no differences between Col7 and *mdhar6-1* seedling leaves; however, *mdhar6-1* roots contained 26% more glutathione than WT roots, with no difference in percentage of oxidation (fig. S6). When whole seedlings were grown in the already more stressful conditions of liquid culture, and treated with TNT or a control treatment, we found no significant differences between Col7 and *mdhar6-1* ascorbate and glutathione levels and percentage of oxidation (fig. S6), suggesting that differences in glutathione levels do not account for the increase in TNT tolerance.

To further investigate whether the increased glutathione level observed in *mdhar6-1* roots (fig. S6) is involved in promoting TNT tolerance, studies
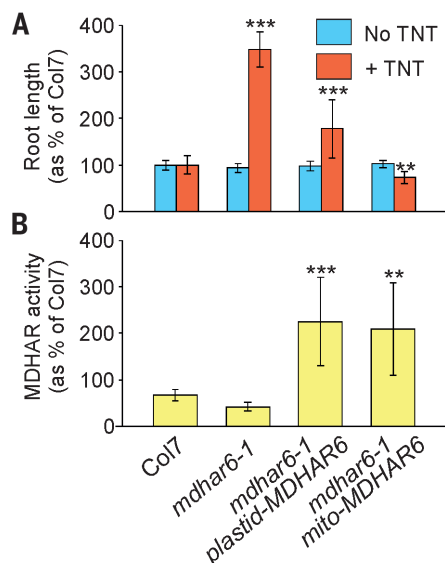

Fig. 2. Complementation of *mdhar6-1* by constitutively expressed plastidial (*plastid-MDHAR6*) and mitochondrial (*mito-MDHAR6*) transcripts. (**A**) Root length of seedlings on solid agar with and without 7 μM TNT (*n* = 6 biological replicates ± SD; **P < 0.01, ***P < 0.01, Student's *t* test compared to Col7). (**B**) Rosette leaf MDHAR activity, with MDA as substrate (*n* = 5 biological replicates ± SD).
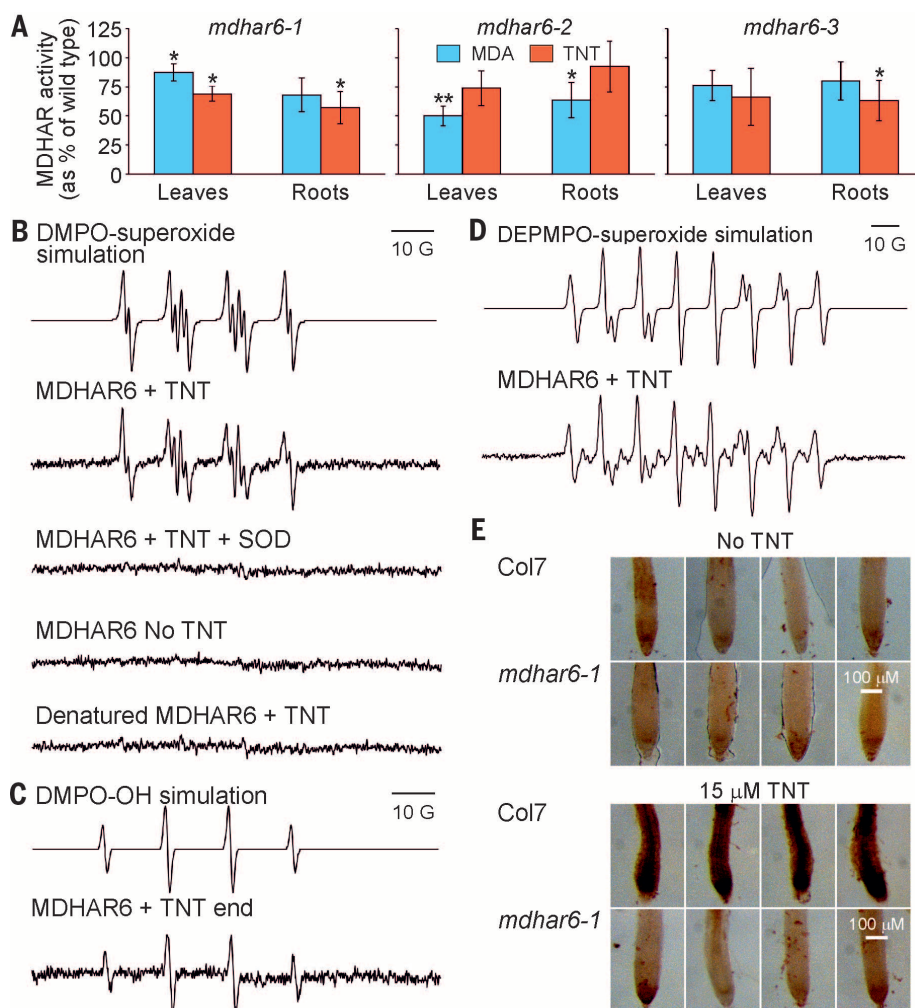

Fig. 3. Purified MDHAR6 reduces TNT, forming a nitro radical that subsequently generates superoxide. (**A**) Crude protein extract activity toward MDA and TNT, as a percentage of activity from WT tissue extract (*n* = 5 ± SD, Student's *t* test between *mdhar6* and corresponding ecotype background; *P < 0.05, **P < 0.01, Student's *t* test compared to wild type). (**B** to **D**) Simulated and experimental EPR spectra of ROS adducts with spin traps. (**B**) Simulated EPR spectrum of the DMPO-superoxide adduct followed by experimental spectra observed when MDHAR6 reacts with TNT in the presence of DMPO, when superoxide dismutase (SOD) is included, when TNT is omitted, and when heat-denatured MDHAR6 is used. (**C**) Simulated spectrum of the DMPO-superoxide degradation product DMPO-OH (*28*), followed by experimental spectrum observed when NADH in the assay is depleted. (**D**) Simulated spectrum of DEPMPO-superoxide adduct, followed by experimental spectrum observed when MDHAR6 reacts with TNT in the presence of DEPMPO. (**E**) Seven-day-old seedlings grown on agar plates in the presence or absence of 15 μM TNT, stained with DAB.

using the glutathione synthesis blocker buthionine sulfoximine (BSO), which inhibits γ-glutamylcysteine synthetase (*19*), were performed. Seedlings were grown for 7 days on agar plates containing increasing levels of BSO in the presence or absence of TNT. In plates containing 250 μM BSO, *mdhar6-1* seedlings remained more tolerant to 7 μM TNT than Col7 (fig. S7), indicating that the tolerance of *mdhar6-1* to TNT is not coupled to a glutathione detoxification system. We also observed that *mdhar6-1* seedlings had enhanced tolerance to BSO compared with Col7 seedlings, in the absence of TNT, indicating that the *mdhar6-1* mutation causes additional subcellular effects.

While phenotyping our mutant lines, we assayed the activity of crude protein extract toward the endogenous substrate MDA and toward TNT. Crude extract MDHAR activity differed between the WT lines (in roots, Col7 659 μmol min^{-1} mg^{-1}, Nossen 505 μmol min^{-1} mg^{-1}, and Col0 353 μmol min^{-1} mg^{-1}) and was reduced in the mutants (Fig. 3A). The decrease in crude extract MDHAR activity from the roots of *mdhar6-1* and *mdhar6-2* (209 and 198 μmol min^{-1} mg^{-1}, respectively) was far greater than the decrease in activity for *mdhar6-3* (70 μmol min^{-1} mg^{-1}), respectively, which corresponds with the degree of enhanced TNT tolerance in the different mutants. The *mdhar6-3* line contains a transferred DNA insert 76 base pairs upstream of the MDHAR6 start ATG and is likely to be a weaker allele, explaining why it is not so tolerant to TNT toxicity. The reduced form of nicotinamide adenine dinucleotide (NADH)–dependent activity in crude extracts toward TNT in the mutants indicated that MDHAR6 could have activity toward TNT.

MDHAR6 generates superoxide from TNT

To investigate MDHAR6 activity toward TNT and whether it could account for TNT phytotoxicity, we expressed *MDHAR6* recombinantly in *Escherichia coli* and purified the protein by affinity chromatography (fig. S8). Oxidation of cofactor NADH in the presence of TNT confirmed that MDHAR6 has activity toward TNT. Kinetics analysis of this activity gave Michaelis constant and maximal velocity values for MDA of 4.1 ± 1 μM and 109 ± 1.7 mmol min^{-1} mg^{-1} and for TNT of 522 ± 57 μM and 0.143 ± 0.0067 mmol min^{-1} mg^{-1}, respectively (fig. S8).

MDHAR6 activity toward TNT did not result in a decrease in TNT concentration when measured by high-performance liquid chromatography (HPLC) (fig. S8). Electron paramagnetic resonance (EPR) spectrometry was therefore used to determine whether the reaction is a one-electron reduction of TNT to produce a nitro radical, which would then autoxidize generating reactive superoxide. This cyclic reaction would explain why no decrease in TNT concentration was observed by HPLC. Reactive oxygen species (ROS) generation has previously been hypothesized as the cause of TNT-induced cataracts when catalyzed by zeta-crystallin (*20*) and neuronal damage when catalyzed by neuronal nitric oxide synthase (*21*).

Analyzing this hypothesis, we used EPR spectrometry with either spin trap 5,5-dimethyl-pyrroline N-oxide (DMPO) or 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO), and spectra correlating with superoxide production were observed (Fig. 3, B to D). The DMPO-superoxide spectrum was not observed in the presence of superoxide dismutase, or when TNT was omitted, or when denatured MDHAR6 was used. These results unambiguously confirm superoxide production during reduction of TNT by MDHAR6. To confirm that this process *in planta* leads to oxidative stress, we used 3,3'-diaminobenzidine (DAB) to visualize H_2O_2 generation. In seedlings grown on agar plates containing 15 μM TNT, the intensity of color (due to DAB oxidation by peroxide) was much higher in WT roots than in *mdhar6-1* roots (Fig. 3E). This finding is indicative of superoxide production by MDHAR6 activity with TNT.

Because *mdhar6* plants show enhanced tolerance to TNT but not to other stresses, we propose that production of superoxide by MDHAR6 with TNT as a substrate is the main mechanism for TNT toxicity in plants. The cyclic nature of the reaction is such that only catalytic amounts of TNT are needed to generate damaging levels of ROS within the mitochondria and plastids (Fig. 4). The reaction also requires NADH, use of which could induce an energy deficit, impeding adenosine triphosphate generation in the mitochondria and subsequent plant growth. The results presented (Fig. 1B and fig. S4) demonstrate that MDHAR6 contributes to the majority of TNT toxicity in *Arabidopsis*. Further toxicity could derive from reduction of TNT by one or more of the remaining four members of the MDHAR family. However, the biochemical environment of the peroxisome, which contains high levels of catalase, would be predicted to ameliorate the production of ROS. While in the cytosol, it is predicted that conjugation to glutathione and glucose would mitigate toxicity by reducing TNT availability (*7, 9*).

Protein sequence alignment (*22*) against the National Center for Biotechnology Information database indicates that MDHAR6 is specific to plants (table S2). MDHAR6 has homologs with high sequence similarity among monocots and dicots, and in *Amborella trichopoda* at the base

Fig. 4. A schematic drawing of the catalytic activity of mitochondrial MDHAR6 toward TNT.
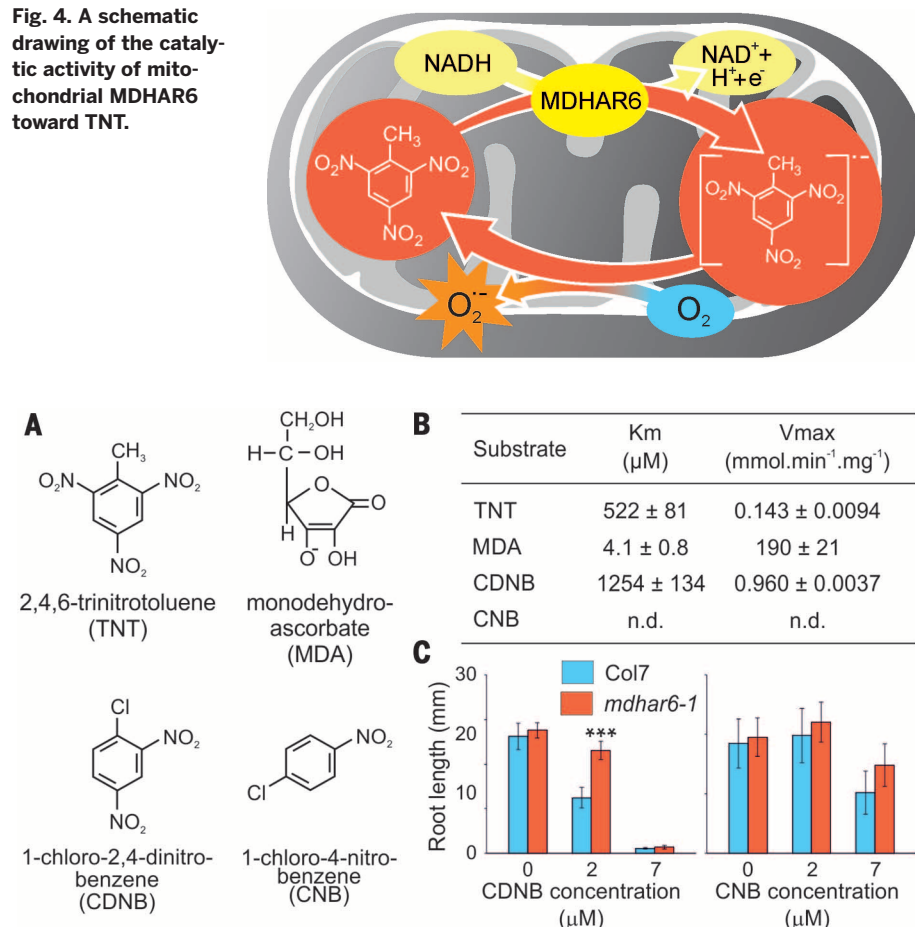

Fig. 5. Enzymatic activity of MDHAR6. (**A**) Structures of substrates tested against purified MDHAR6. (**B**) Kinetics of MDHAR6 activity with substrates, as measured by rates of NADH depletion (n.d., not detected; $n = 3$ technical replicates ± SD). (**C**) Root lengths of 7-day-old Col7 and *mdhar6-1* seedlings germinated on agar containing increasing concentrations of CDNB or CNB ($n = 30$ biological replicates ± SD; Student's *t* test compared to Col7 in same treatment, ***$P < 0.001$).

Substrate	Km (μM)	Vmax (mmol.min^{-1}.mg^{-1})
TNT	522 ± 81	0.143 ± 0.0094
MDA	4.1 ± 0.8	190 ± 21
CDNB	1254 ± 134	0.960 ± 0.0037
CNB	n.d.	n.d.

of the Angiosperm lineage. There are homologs with less similarity in lower plants and algae, and similarity with proteins outside of the Plantae is very low.

Applications

Although herbicide resistance has been increasing steadily since the 1970s (*23*), no new herbicide mode of action has been commercialized since the 1980s (*24*). The toxic effect of the plant-specific MDHAR6 reaction with TNT could be leveraged to develop environmentally acceptable substrates for MDHAR6 as a new class of herbicide. We therefore investigated activity of MDHAR6 toward two additional nitro-group–containing chemicals, 1-chloro-2,4-dinitrobenzene (CDNB) and 1-chloro-4-nitro-benzene (CNB), shown in Fig. 5A. Activity of purified MDHAR6, measured by following NADH oxidation, was detected toward CDNB but not CNB (Fig. 5B and fig. S8F). Measurement using HPLC demonstrated that CDNB was not depleted in assay mixtures (Fig. 5C). As observed for TNT, CDNB inhibited seedling root growth in WT Col7 seedlings on agar plates containing CDNB, with *mdhar6-1* seedlings exhibiting significantly longer roots than the wild type (Fig. 5D). These results demonstrate that MDHAR6 can reduce CDNB, with toxic effect to *Arabidopsis*, further supporting the idea that herbicidal substrates could be developed. Whereas WT seedling root lengths were reduced by ~50% in the presence of 2 μM CDNB, root lengths on 2 μM CNB were unaffected, demonstrating that CNB is significantly less phytotoxic. Alongside this result, we were unable to detect activity toward CNB using recombinant protein (Fig. 5). These results are in agreement with our hypothesis that formation of a nitro radical is the major cause of TNT toxicity in plants.

Although TNT binds to the organic and clay fractions of soil, and is thus not readily mobile in water, a common copollutant in sites contaminated with explosives is royal demolition explosive (hexahydro-1,3,5-trinitro-1,3,5-triazine) (RDX), which is highly mobile in soils and readily contaminates water supplies (*25*). Future work on effective bioremediation of explosives contamination will need both existing RDX-degrading capabilities (*26*, *27*) and resistance to the toxic copollutant TNT. Our findings explain the acute toxicity of TNT to plants and also provide an avenue by which MDHAR6 deficiency can be exploited to increase plant biomass in the presence of TNT, permitting greater rates of remediation for both TNT and RDX. Molecular breeding approaches could be used to identify deletions in *MDHAR6* orthologs, potentially enhancing TNT tolerance in relevant plant species such as switchgrass (*Panicum virgatum*), thus enabling revegetation and remediation of explosives-contaminated sites.

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REPORTS

CHEMISTRY

Transient assembly of active materials fueled by a chemical reaction

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Fuel-driven self-assembly of actin filaments and microtubules is a key component of cellular organization. Continuous energy supply maintains these transient biomolecular assemblies far from thermodynamic equilibrium, unlike typical synthetic systems that spontaneously assemble at thermodynamic equilibrium. Here, we report the transient self-assembly of synthetic molecules into active materials, driven by the consumption of a chemical fuel. In these materials, reaction rates and fuel levels, instead of equilibrium composition, determine properties such as lifetime, stiffness, and self-regeneration capability. Fibers exhibit strongly nonlinear behavior including stochastic collapse and simultaneous growth and shrinkage, reminiscent of microtubule dynamics.

Active self-assembly driven by chemical fuels is at the basis of many processes in living organisms, including cellular transport, cell motility, proliferation, and morphogenesis (*1*). Active self-assembled structures such as actin networks and microtubules (*2*) distinguish themselves from equilibrium self-assembled systems and materials (*3*) by their ability to use the free energy provided by the conversion of the fuel to achieve transient structure formation and to carry out work (*4*, *5*); in addition, their behavior is controlled by the kinetics of fuel consumption instead of by thermodynamic stability. The realization of artificial active materials created through a fuel-driven self-assembly process would further the understanding of kinetically