Supplementary Materials for

Monodehydroascorbate reductase mediates TNT toxicity in plants

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Materials and Methods

The TNT was kindly provided by the Defence Science and Technology Laboratory (DSTL) (Fort Halstead, Kent, UK).

Plant material

Wild-type *Arabidopsis thaliana* (Arabidopsis) ecotypes Columbia-0 (Col0), Columbia-7 (Col7) and Nossen (Nos) were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (University of Nottingham, UK). The *mdhar6-1* mutant, in Col7 background, was isolated from a T-DNA activation-tagged population (*11*) obtained from NASC. The mutation in *mdhar6-1* was mapped to a thymine deletion 2,181 bp from the *MDHAR6.2* start ATG, in exon 11 of the genomic sequence of *MDHAR6*. The *mdhar6-2* mutant, in Nos background, was obtained from the RIKEN Arabidopsis transposon-tagged mutant (RATM) lines (*29*), RIKEN BioResource Center (Japan). Sequencing of the region flanking the transposon using primers 5'-TTCCCTGCGTCTGTGGTGAG-3'and 5'-CCGTCCCGCAAGTTAAATATG-3' confirmed the transposon to be inserted 538 bp from the *MDHAR6.2* ATG, between exons 3 and 4. The *mdhar6-3* mutant, in Col0 background, was obtained from the GABI-Kat T-DNA mutagenized lines (line ID 258H07) (*30*). Sequencing of the region flanking the border region of the T-DNA using primers 5'-CTTTCTCCACCACCTCCAAC-3' and 5'-ATATTGACCATCATACTCATTGC-3' confirmed a T-DNA insert 76 bp upstream of the *MDHAR6* start ATG.

Liquid culture and soil studies

Uptake of TNT from liquid culture and soil, and extraction of TNT from soil, were performed as described by Gunning et al. (*9*).

Complementation of mdhar6-1

Primers 5'-GGTACCATGTCTGCAGTTCGTAG-3' and 5'-GGATCCCTAACTCTGTAGAGCG-3' were used to amplify the mitochondrial targeted form of *MDHAR6* (At1g63940.2) and primers 5'-GGTACCATGGCGTTAGCATCAACC-3' and 5'-GGATCCCTAACTCTGTAGAGCG-3' were used to amplify the plastidial targeted form (At1g63940.1) from cDNA. Sequences were cloned into pART7, and then the binary vector pART27 (*31*). These constructs were, separately, stably transformed into Arabidopsis Col7 and *mdhar6-1* through Agrobacterium-mediated floral dipping (*32*). Intron-spanning primers 5'CTTCCAGAAGCCTCGTCACT3' and 5'-AGCCGGTCTCTCATAAGGTG-3'were used to confirm the presence of the cDNA-derived MDHAR6 transgene integrated into the genomic DNA shown in Fig.S4.

Root length analysis

Roots lengths of photographed seedlings grown vertically on ½ MS agar (supplemented with test treatment), were measured using ImageJ (*33*).

Treatment with TNT in liquid culture for ascorbate and glutathione analysis

Two-week old seedlings in ½ MS, 20 mM sucrose, 1 x Gamborg's vitamin solution were treated with 60 µM TNT or control treatment (0.06 % v/v DMF) for 6 h, as described by Gandia-Herrero et al. (*7*).

Metabolite measurements

Following TNT or control treatment of plants grown in liquid growth media as described by Gandia-Herrero et al. (*7*), ascorbate was measured as described in Queval

and Noctor (*34*). Root and leaf ascorbate levels were measured using the protocol described by Kamfenkel et al. (*35*). Glutathione was measured as described in Queval and Noctor (*34*).

Enzyme activities

The MDHAR activity measurements on crude plant extracts was carried out as described by Colville and Smirnoff (*36*). To measure activity of extracts towards TNT, rate of decrease in absorbance at A_{340} was measured before and after addition of 1 mM TNT (10 % v/v final DMSO concentration).

The kinetic studies with MDA using purified MDHAR6 contained 50 mM Tris 1 mM EDTA (pH 7.6), 100 µM NADH, 509 ng/ml MDHAR6 and 1.12 U ascorbate oxidase (Sigma A0157) with increasing concentrations of sodium ascorbate. Concentration of MDA was estimated by measuring A_{360} after addition of ascorbate oxidase, in assays without NADH or MDHAR6, and assuming an absorbance coefficient for MDA of 3.3 mM^{-1} cm^{-1} as described by Hossain *et al.* (*37*).

The reaction mixtures for kinetic assays for TNT and CDNB contained 10.3 µg/ml and 128 µg/ml MDHAR6 respectively, in 50 mM Tris 1 mM EDTA (pH 7.6), 100 µM NADH and 15% DMSO. Michaelis-Menten parameters were calculated using Sigma Plot v12.0.The reactions for HPLC analysis contained 52 µg/ml MDHAR6, 200 µM NADH and 200 µM TNT or CDNB in 50 mM Tris 1 mM EDTA (pH 7.6), 15 % DMSO. HPLC analysis was carried out as outlined in Gunning et al. (*9*).

Expression and purification of MDHAR6

Codon-optimized *MDHAR6*, lacking organelle targeting sequences, was cloned into pET52b, introducing an N-terminal STREP-tag and C-terminal HIS-tag. The vector pET52b-MDHAR6 was transformed into *Escherichia coli* strain Arctic Express (Agilent Technologies) following the manufacturer's instructions. A transformed colony was used to inoculate 50 ml Luria Broth (LB) starter culture (grown at 37 °C, 250 rpm, 15 h), 10 ml of which used to inoculate 500 ml LB (50 µg/ml carbenicillin) within a 2 L conical flask, incubated at 37 °C 250 rpm until the OD_{600} approximated 0.6. At this point 0.6 mM isopropyl β-D-1-thiogalactopyranoside was added to induce *MDHAR6* expression, and the culture was incubated at 15 °C with 180 rpm for 24 h. Following centrifugation (4,000 rpm, 5 min, 4 °C), pellets were re-suspended in 30 ml binding buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 0.1% Tween20; 70 µl 0.1 M phenylmethanesulfonylfluoride in isopropanol) then sonicated at amplification 70 for 4 min (3 sec on, 7 sec off), centrifuged (15,000 rpm, 15 min, 4 °C) and the supernatant syringed through a 22 µm-filter. Strep-tagged MDHAR6 was then purified on a 5 ml StrepTrap column (GE HealthCare) that had been equilibrated with 7 ml water then 25 ml binding buffer. The column was then washed with a further 15 ml binding buffer, before the addition of elution buffer (binding buffer with 2.5 mM desthiobiotin) over a gradient of 0-100 % in 20 min. Purified MDHAR6 was dialyzed in 50 mM KH_2PO_4, pH 7 at 6 °C for 2 h, then with fresh buffer for a further 18 h.

EPR spectrometry

Spectra were recorded on a Bruker EMX Micro spectrometer at X band (9.86 GHz), with modulation amplitude 1 G, microwave power 5 mW, scan time 80 s and time constant 80 ms. The activity assays contained 1.5 mg/ml MDHAR6 in 50 mM KH_2PO_4 (pH 7), 80 mM DMPO or DEPMPO, 300 µM NADH and 500 µM TNT in DMF (end DMF concentration 1 % v/v). For assays containing SOD (Sigma S8409), 2,500 U/ml

SOD in 100 mM KH_2PO_4 pH 7.5 was added to the assay to an end concentration of 50 U/ml, before addition of TNT and NADH. For a control experiment, MDHAR6 was denatured by boiling for 5 min. EPR spectra were simulated using WinSim freeware available from the National Institute of Environmental Health Sciences (http://www.niehs.nih.gov/research/resources/software/tox-pharm/tools/). The line widths and hyperfine constants used in the simulations were optimized to fit the experimental spectra. Simulation parameters for DMPO-superoxide adduct: a_N = 14.09 G, $a_{\beta\text{-}H}$ = 11.33 G, $a_{\gamma\text{-}H}$ = 1.23 G, DMPO-OH a_N = 14.97 G, $a_{\beta\text{-}H}$ = 14.68 G, DEPMPO-superoxide adduct, isomer 1 (42%): a_N = 13.03 G, $a_{\beta\text{-}H}$ = 11.85 G, $a_{\gamma\text{-}H}$ = 0.68 G, a_P = 50.76 G, and DEPMPO-superoxide adduct, isomer 2 (58%): a_N = 13.15 G, $a_{\beta\text{-}H}$ = 10.29 G, $a_{\gamma\text{-}H}$ = 0.61 G, a_P 49.63 G.

DAB staining

Seedlings were vacuum infiltrated in 1 mg/ml 3,3'diaminobenzidine (DAB) in 50 mM Tris-acetate (pH 5) for 30 min. The vacuum was then released, and seedlings incubated for a further 2.5 h, in the dark and at room temperature. Images were taken using a Nikon SMZ800 dissection microscope with AxioVision Rel. 4.5 software.

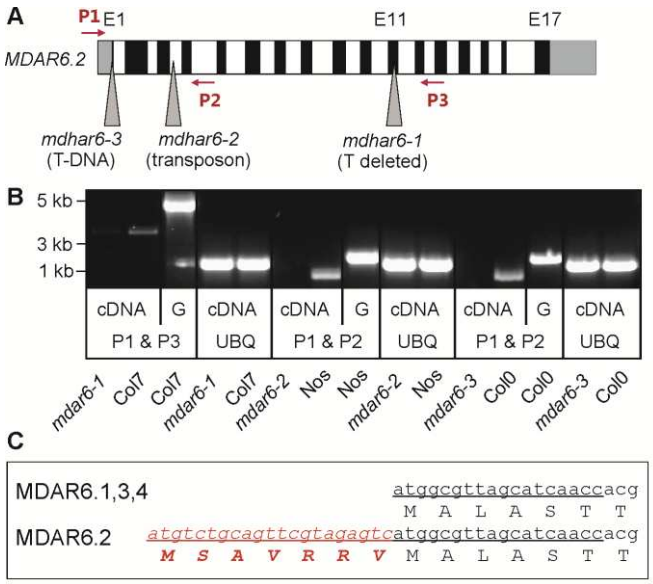

Fig. S1. Representation of the three *mdhar6* mutant alleles studied

(**A**) Scale representation of *MDHAR6.2* (the splice variant for mitochondria-targeting of *MDHAR6*), with mutations present in the mutant lines indicated. Black boxes; exons. White boxes; introns. Grey boxes; untranslated regions. Primers used in (**B**) indicated. (**B**) RT-PCR analysis of cDNA and genomic (G) DNA using primers indicated in (**A**) and ubiquitin control primers. (**C**) N-terminal amino acid sequences for plastid- (MDHAR6.1, MDHAR6.3, MDHAR6.4) and mitochondria- (MDHAR6.2) targeted variants of MDHAR6.

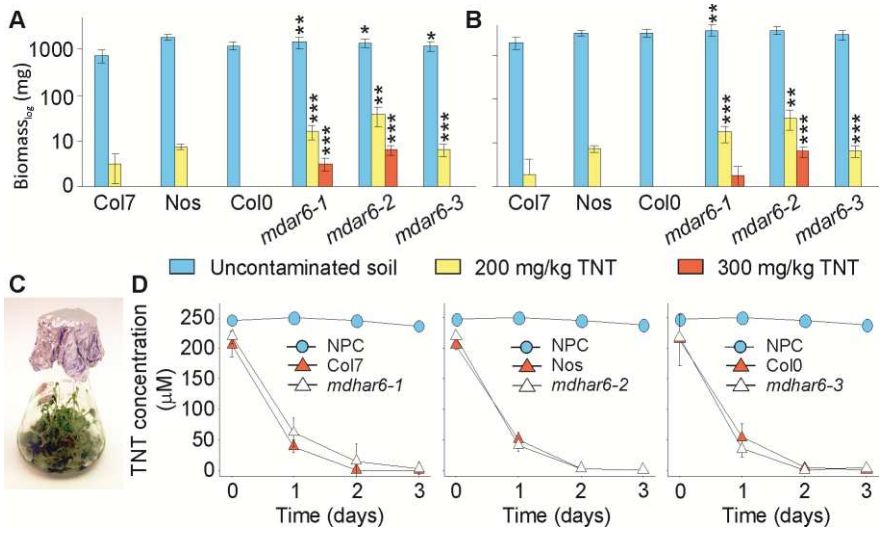

Fig. S2. The *mdhar6* mutants have increased biomass in TNT-contaminated soil, but take-up TNT from liquid media at the same rate as their wild-type backgrounds

Fresh weight of (**A**) shoot and (**B**) roots from six-week old plants which were transferred to TNT-treated or control-treated soil at 5 d of age (n = 8 biological replicates ± s.d.). (**C**) Appearance and (**D**) TNT removal rates of three-week old plants grown in 20 ml ½ MS, dosed with 250 µM TNT (n = 8 biological replicates ± s.d. , t- test between *mdhar6* and corresponding ecotype background * P<0.05, ** P<0.01, *** P<0.001). NPC; no plant control.

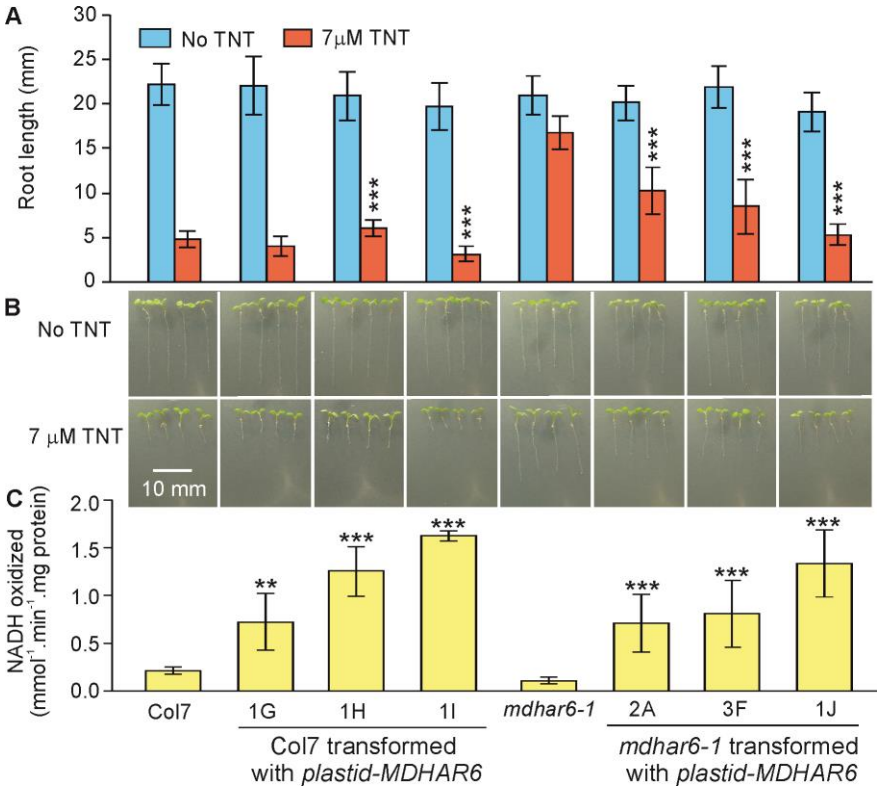

Fig. S3. Overexpression of plastidial *MDHAR6* partially complements TNT toxicity in *mdhar6-1*

Wild-type Col7 and *mdhar6-1* were transformed with 35S:*MDHAR6.1* (the splice variant for plastid-targeted). (**A**) Root lengths and (**B**) appearance of seven-day old seedlings germinated on ½ MS agar containing 0 μM or 7 μM TNT (n = 30 biological replicates ± s.d.). (**C**) MDHAR activity of rosette leaf crude protein extract from four-week old plants (n = 5 biological replicates ± s.d., t- tests are between complemented and parental lines ** P<0.01, *** P<0.001).

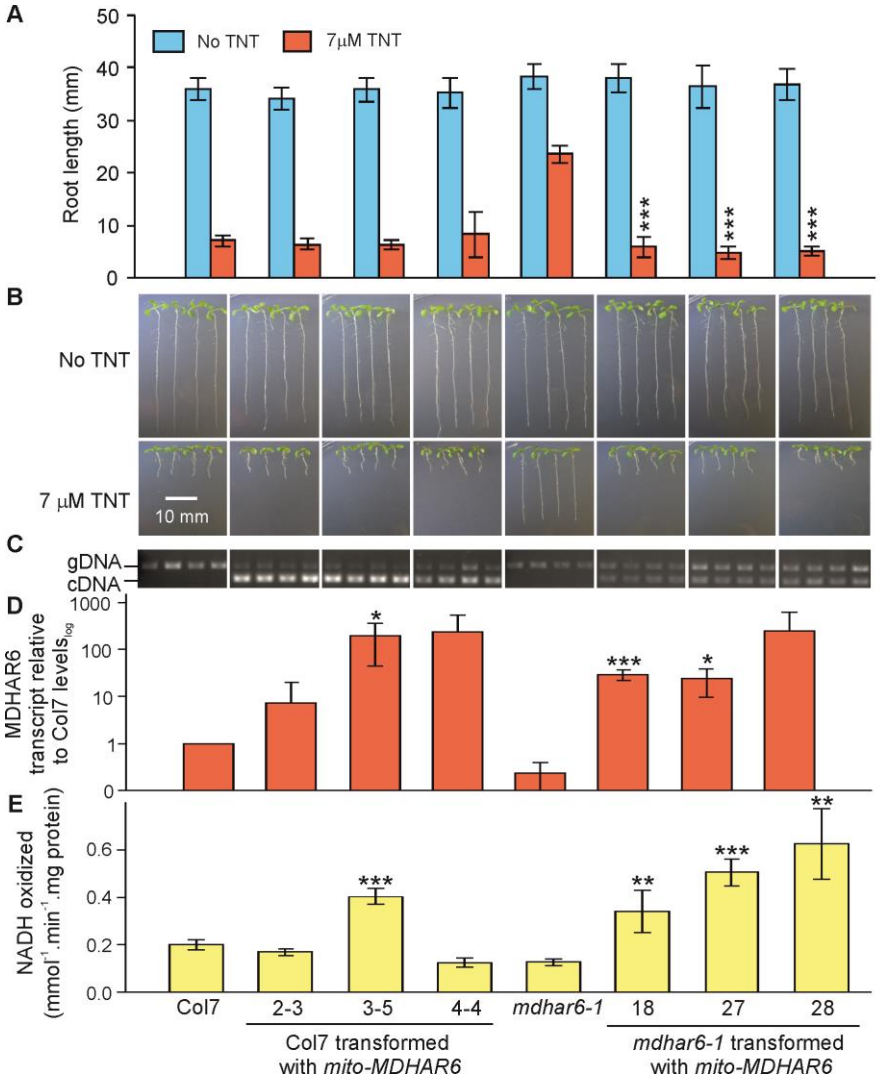

Fig. S4. Overexpression of mitochondrial *MDHAR6* fully complements TNT toxicity in *mdhar6-1*

Wild-type Col7 and *mdhar6-1* were transformed with 35S:*MDHAR6.2* (splice variant for mitochondria targeted MDHAR6). (**A**) Root lengths and (**B**) appearance of seven-day old seedlings germinated on ½ MS agar containing 0 µM or 7 µM TNT (n = 30 biological replicates ± s.d.). (**C**) PCR analysis on rosette leaves showing presence of genomic DNA (gDNA) in Col7 and *mdhar6-1* alongside *MDHAR6.2* cDNA in 35S:*MDHAR6.2* over-expression lines. (**D**) *MDHAR6* transcript and (**E**) MDHAR activity in rosette leaf crude protein extract from four-week old plants (n = 5 biological replicates ± s.d., t- test between complemented and parental lines * P<0.05, ** P<0.01, *** P<0.001).

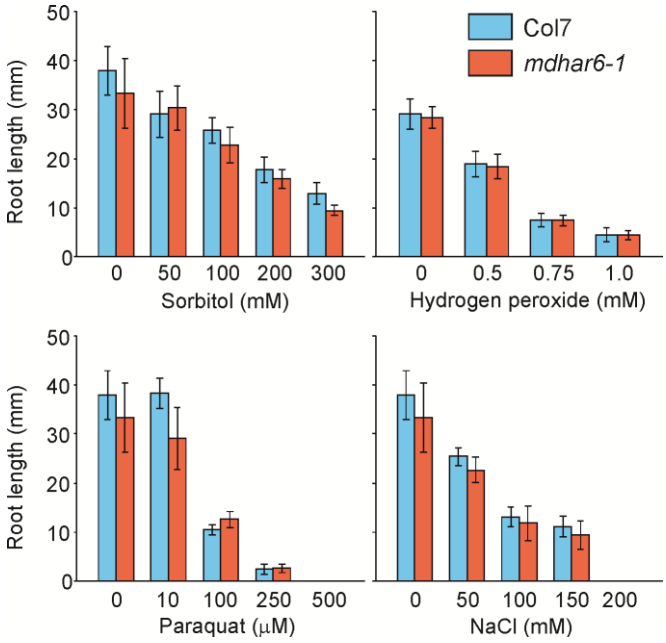

Fig. S5. The *mdhar6* mutants are no more tolerant than wild-type to stresses other than TNT treatment

Root lengths of seven-day old Col7 and *mdhar6-1* seedlings (n = 30 biological replicates ± s.d.).

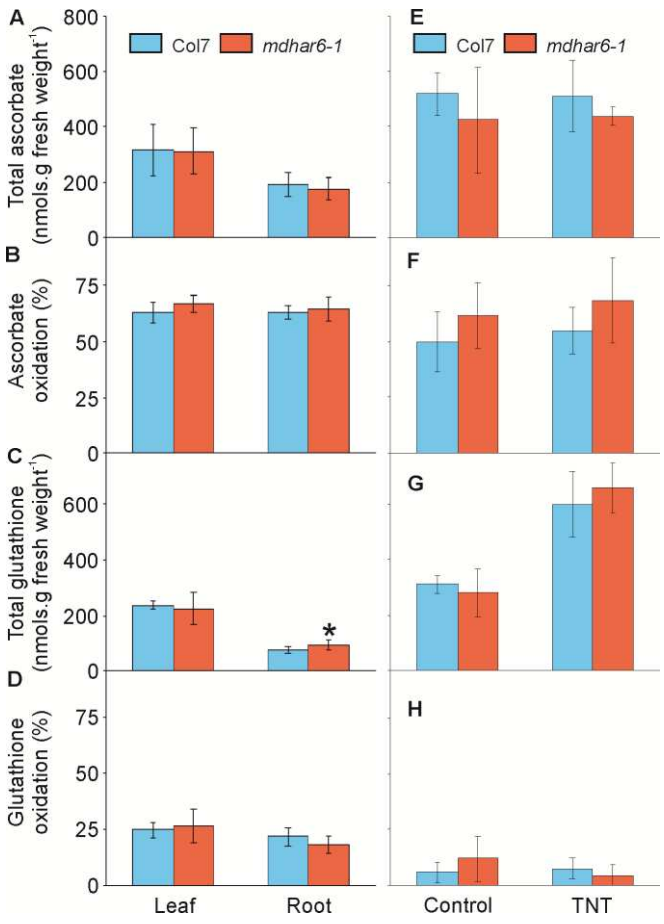

Fig. S6. Roots of *mdhar6-1* have significantly more glutathione than wild-type Col7 when grown on ½ MS agar, but not when grown in liquid culture and treated with TNT

Ascorbate and glutathione content of Col7 and *mdhar6-1*. (**A - D**) Leaf and root tissue from 2-week old seedlings germinated horizontally on ½ MS 20 mM sucrose agar (n = eight biological replicates ± s.d.). (**F - H**) Fourteen-day old seedlings. Ten one-day old seedlings were transferred from ½ MS agar to 100 ml ½ MS 20 mM sucrose 1 x Gamborg's vitamin solution, and at 14 days of age treated with 60 µM TNT or a control (0.06 % v/v DMF) treatment for 6 h (n = 6 biological replicates ± s.d., t- test between Col7 and *mdhar6-1* * $P<0.05$).

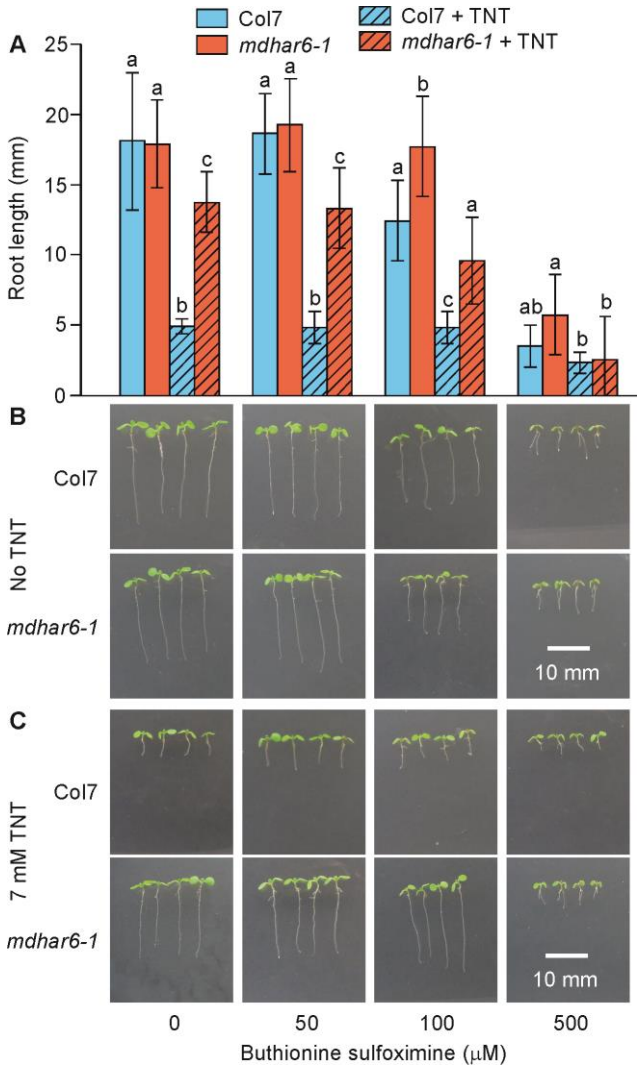

Fig. S7. Glutathione synthesis inhibitor buthionine sulfoximine does not affect TNT tolerance in *mdhar6-1*

(**A**) Root lengths and appearance of seven-day old seedlings germinated on ½ MS agar (**B**) without or (**C**) with 7 μM TNT, and with a range of buthionine sulfoximine concentrations (n = 25 biological replicates ± s.d letters above bars indicate significant difference within BSO treatments (ANOVA, with *post hoc* Tukey HSD $p < 0.05$).

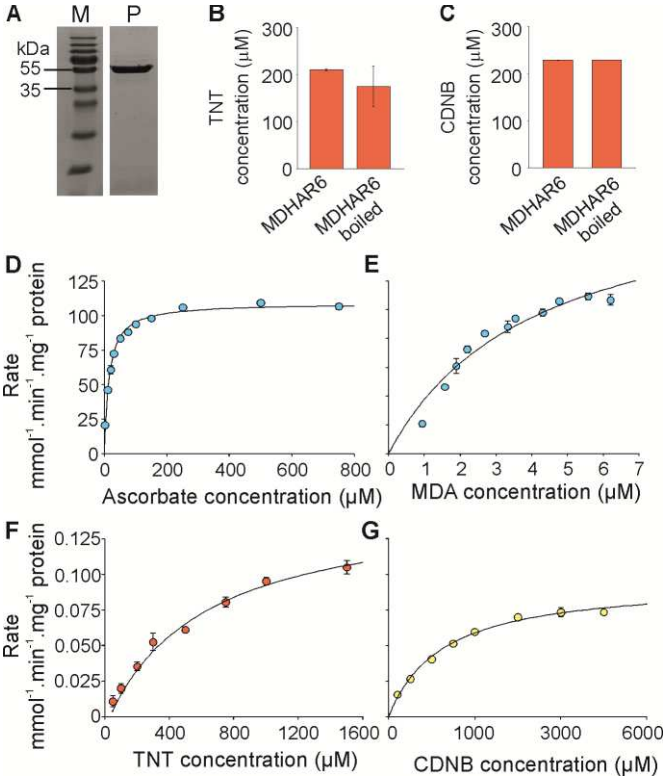

Fig. S8. Purification and Michaelis-Menten kinetics data for MDHAR6

(**A**) Coomassie stain of SDS-PAGE gel, P; purified MDHAR6 (52 kDa), M; molecular markers. (**B**) TNT and (**C**) CDNB concentration at end of reaction with purified MDHAR6, when NADH is depleted, as determined by HPLC (n = 5 technical replicates ± s.d.). Michaelis-Menten plots showing purified MDHAR6 activity, and containing 50 mM Tris 1 mM EDTA (pH 7.6), 100 µM NADH, 509 ng/ml MDHAR6 and 1.12 U ascorbate oxidase, with increasing concentrations of (**D**) ascorbate. (**E**) Concentration of MDA. (**F**) TNT and (**G**) CDNB (n = three technical replicates ± s.d.).

Table S1.
Extractable TNT from 100 mg TNT/kg soil (n = 8 ±.) after 5 weeks treatment with no plants, Col7 or *mdhar6-1* seedlings.

Sample	Extractable TNT (nmol/g dry soil)
No plants	31 ± 2.77
Col7	21 ± 1.64
mdhar6-1	15 ± 0.92

Table S2.

Protein sequence similarity analysis
Basic local alignment search of protein sequences in the National Center for Biotechnology Information database against MDHAR6 (The Arabidopsis Information Resource AASequence 1009107687).

Species	Relevance	Accession and Annotation of Closest Hit	Total Score	% Coverage	% Identity	E value
Amborella trichopoda	Placed at base of Angiosperm lineage	XP 011628912.1 predicted MDHAR	734	99	72	0.0
Physcomitrella patens	Lower plant (moss)	XP 001776830.1 predicted protein	374	84	48	1e-124
Ostreococcus tauri	Alga	XP 003079182.1 MDHAR	361	83	46	3e-119
Picea sitchensis	Gymnosperm	ABK24288.1 unknown	360	81	46	1e-119
Ginkgo biloba	Gymnosperm	AGG40646.1 isoflavone reductase-like protein	29.6	13	31	0.067
Synechococcus sp. PCC 7335	Cyanobacteria, of interest when considering genetic origin of *MDHAR6*	WP 006457515.1 NAD(FAD)-dependent dehydrogenase	177	89	28	2e-48
Populus trichocarpa	Remediation relevant genus	XP 002299509.2 hypothetical protein	795	100	78	0.0
Homo sapiens	Important when considering herbicide-target applications	BAH14413.1 unnamed protein product	137	72	27	4e-34