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Cloning and characterization of a *Nicotiana tabacum* methylputrescine oxidase transcript

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Abstract

The oxidative deamination of *N*-methylputrescine is an essential step in both pyridine and tropane alkaloid biosynthesis. Reverse genetic approaches have not resulted in the cloning of a methylputrescine oxidase gene (*MPO*). However, we have used a homology-based approach to clone a full-length tobacco *MPO1* cDNA. The *MPO1* gene is part of a small multigene family comprised of approximately six members. *MPO1*-like transcript levels increased in roots that were either deprived of auxin or treated with methyl jasmonic acid. Similar to other known nicotine biosynthetic genes in domesticated tobacco, *MPO1*-like mRNA levels were lower in roots with the mutant *a* and *b* alleles. The MPO1 protein was expressed in bacteria as a recombinant Thioredoxin–His^6–MPO1 fusion protein. The recombinant MPO1 protein utilized *N*-methylputrescine more efficiently than other diamines. Therefore, the kinetic properties of the MPO1 enzyme may play an important role in determining the pyridine alkaloid profiles observed in tobacco roots.

Keywords: Amine oxidase; Burley 21; Cadaverine; Methylputrescine oxidase; *N*-methylputrescine; Nicotine; Nicotiana tabacum; Tobacco; Solanaceae; Putrescine methyltransferase; Pyridine alkaloid; Quinolinate phosphoribosyl transferase; Tropane alkaloid

1. Introduction

Many Solanaceous plants synthesize biologically active alkaloids that increase plant fitness in the natural environment. Pyridine and tropane alkaloids are two important classes of plant natural products whose biological activities are used by humans for medicinal purposes (Schmeller and Wink, 1998; Wink, 1998). Pyridine alkaloids and tropane alkaloids share two early biosynthetic steps that produce a common biosynthetic intermediate: the *N*-methylpyrrolinium salt (**4**) (Fig. 1). The first committed step in pyridine and tropane alkaloid biosynthesis is the *N*-methylation of the symmetrical diamine putrescine (**1**) by putrescine methyltransferase (PMT), resulting in *N*-methylputrescine (**2**) (Mizusaki et al., 1972). PMT genes have been cloned from a variety of Solanaceous plant species (Hashimoto et al., 1998; Hibi et al., 1994; Riechers and Timko, 1999; Stenzel et al., 2006; Suzuki et al., 1999).

The second step in the biosynthesis of the *N*-methylpyrrolidine ring is the oxidative deamination of *N*-methylputrescine (**2**), producing *N*-methylaminobutanal (**3**). *In vitro*, *N*-methylaminobutanal (**3**) spontaneously cyclizes to form the *N*-methylpyrrolinium salt (**4**) and it is generally assumed this is also the case *in vivo* (Mizusaki et al., 1972). The oxidative deamination of the primary amine is catalyzed by a copper-containing diamine oxidase activity (DAO) (Davies et al., 1989; Hashimoto et al., 1990; Mizusaki et al., 1972). This DAO activity has been detected in *Atropa belladonna* (Mizusaki et al., 1973), *Datura stramonium* (Mizusaki et al., 1973), *Hyoscyamus niger* (Hashimoto et al., 1990), and *Nicotiana tabacum* (Mizusaki et al.,

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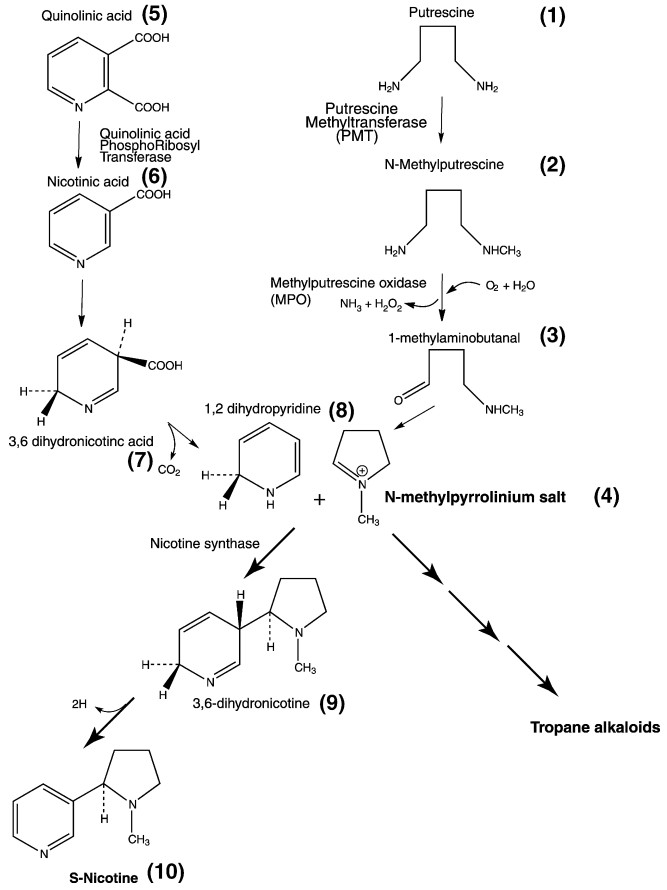

Fig. 1. Biosynthesis of the pyridine alkaloid nicotine (**10**) with *N*-methylpyrrolinium salt (**4**) as an intermediate to both nicotine (**10**) and tropane alkaloid biosynthesis.

1972, 1973; Saunders and Bush, 1979; Walton and McLauchlan, 1990) roots and preferentially oxidizes *N*-methylputrescine (**2**) relative to putrescine (**1**) (1,4-diamino butane) or cadaverine (1,5-diaminopentane) (Hashimoto et al., 1990; Haslam and Young, 1992; Walton and McLauchlan, 1990). Because of the preference for *N*-methylputrescine (**2**) as a substrate, this methylputrescine oxidase activity is frequently called MPO. MPO belongs to class of amine oxidases that require copper as a cofactor (EC 1.4.3.6). The copper ion is needed to oxidize a conserved tyrosine residue into a topaquinone, which serves as an essential functional group at the catalytic site of this class of enzymes (Matsuzaki et al., 1994). In addition, MPO activity isolated from *N. tabacum* has a requirement for 2-mercaptoethanol, suggesting the enzyme must be in a reduced state to be active (Davies et al., 1989; Haslam and Young, 1992; Mizusaki et al., 1972).

Pyridine and tropane alkaloid biosynthesis occurs in the roots. The pyridine alkaloid nicotine (**10**) is synthesized in excised tobacco roots (Dawson, 1942b). Consistent with root specific nicotine (**10**) biosynthesis, tomato scions grafted on tobacco roots accumulate nicotine (**10**) in the tomato leaves and fruit, indicating tobacco roots are responsible for nicotine (**10**) biosynthesis (Dawson, 1942a). Also consistent with roots as the site of nicotine (**10**) biosynthesis, MPO enzyme levels are highest in the roots of plants that produce either pyridine or tropane alkaloids (Boswell et al., 1999; Hashimoto et al., 1990; Mizusaki et al., 1972; Wagner et al., 1986).

MPO enzyme levels in *N. tabacum* increase during conditions that stimulate nicotine (**10**) biosynthesis. Removal of the tobacco shoot apical meristem modestly increases MPO enzyme levels in the roots and subsequently increased nicotine (**10**) accumulation levels in the leaves (Mizusaki et al., 1973). In domesticated tobacco, the *A* and *B* loci regulate the levels of several nicotine (**10**) biosynthetic enzymes including MPO. For example, tobacco roots with the mutant *aabb* genotype show decreased MPO, PMT, and quinolinate phosphoribosyl transferase (QPT) enzyme levels (Saunders and Bush, 1979). The decreased PMT and QPT enzyme levels correlate with decreased mRNA levels for these enzymes (Cane et al., 2005; Hibi et al., 1994; Kidd et al., 2006; Reed and Jelesko, 2004), indicating that the *A* and *B* loci are regulatory genes affecting all known nicotine (**10**) biosynthetic genes.

Several attempts to purify plant MPO to homogeneity have not been successful and have lead to conflicting results. Using native gel filtration chromatography, the *N. tabacum* and *H. niger* MPOs show an apparent molecular weight of 130–135 kDa (Hashimoto et al., 1990; Heim and Jelesko, 2004). Copper amine oxidases are homodimers. Using a suicide inhibitor that reacts with quinone prosthetic groups, the tobacco MPO subunit molecular weight was estimated at ~70 kDa by SDS–PAGE (Davies et al., 1989). In contrast, other reports of MPO purification suggest a 54 kDa subunit size (Haslam and Young, 1992; McLauchlan et al., 1993). A common observation in these attempts to purify MPO to homogeneity is a propensity for MPO to become inactive during extensive purification steps, particularly after reversed phase chromatography. Nevertheless, using an unusual purification schema that emphasized fractions containing MPO activity with the least protein complexity rather than fractions with the highest total MPO activity, McLauchlan et al. (1993) reported purifying a 53 kDa protein with purported MPO activity. This 53 kDa protein was used to make a polyclonal rabbit immune serum that immunodepleted MPO activity from tobacco cell-free root extracts. However, subsequent characterization of this 53 kDa antiserum indicated that it binds to tobacco *S*-adenosylhomocysteine hydrolase (SAHH), suggesting that SAHH and MPO may associate in a multienzyme complex *in vivo* (Heim and Jelesko, 2004). The association between SAHH and MPO (either direct or indirect) may be part of a hypothesized multienzyme nicotine metabolic channel.

Here we report a homology-based cloning strategy that resulted in the successful cloning of a cDNA encoding a *bona fide* tobacco MPO activity (MPO1). The *MPO1* mRNA expression patterns and MPO enzyme activity were consistent with previous reports of MPO in *N. tabacum*.

2. Results and discussion

2.1. Cloning and transcript accumulation levels of N. tabacum MPO-like cDNAs

We utilized a degenerate oligonucleotide strategy to amplify a gene fragment encoding a putative *N. tabacum* MPO enzyme. Five predicted plant proteins with similarity to copper amine oxidases were aligned and used to identify conserved amino acid sequences. Degenerate oligonucleotide primers were designed corresponding to these conserved peptide sequences. These oligonucleotide primers were used in conjunction with a phagemid tobacco root cDNA library in PCR reactions to amplify a tobacco *MPO*-like cDNA fragment. This resulted in the amplification of a 986 bp PCR fragment that was subcloned to produce plasmid pWGH10. BLASTX analysis of this subcloned PCR fragment showed highest similarity (*E*-value $= 1 \times 10^{-173}$) to an Arabidopsis gene (At2G42490) encoding a copper-containing amine oxidase belonging to the same enzyme class as tobacco MPO (EC 1.4.3.6).

To determine if the corresponding transcript was subject to genetic regulation by the *A* and *B* loci, axenic *N. tabacum* cv. Burley 21 roots (B21) with wild type *AABB* genotype and *N. tabacum* cv. Low-Alkaloid Burley 21 roots (LA21) with the double mutant *aabb* genotype were grown either in media resulting in low expression of nicotine (**10**) biosynthetic genes (i.e. supplemented with indole butyric acid (IBA)) or in media that increases expression of nicotine (**10**) biosynthetic genes, i.e. media without IBA (Hibi et al., 1994; Reed and Jelesko, 2004). Total RNA was isolated from these primary root cultures and subjected to RNA blot analysis. Fig. 2A shows that the *MPO-like* transcript levels in the mutant LA21 roots (i.e. *aabb* genotype) were reduced relative to those in wild type B21 roots (i.e. *AABB* genotype). In contrast, a probe corresponding to the housekeeping gene *β-ATPase* showed similar hybridization levels to RNA from the wild type and the mutant *aabb* genotypes. Thus, the MPO-like transcript levels were regulated by the *A* and *B* loci. Because MPO enzyme levels are known to be regulated by the *A* and *B* loci, the cDNA fragment in pWGH10 was a good candidate for an *MPO* gene. Therefore, the pWGH10 insert was used as a hybridization probe to isolate a full length cDNA (i.e. plasmid pWGH15, Genbank DQ873385) from the same cDNA library used to amplify the PCR fragment in pWGH10.

All known nicotine (**10**) biosynthetic genes increase their mRNA accumulation levels in response to either reduced auxin levels or methyl jasmonic acid treatment (Cane et al., 2005; Kidd et al., 2006; Shoji et al., 2000). To evaluate the mRNA accumulation patterns of the

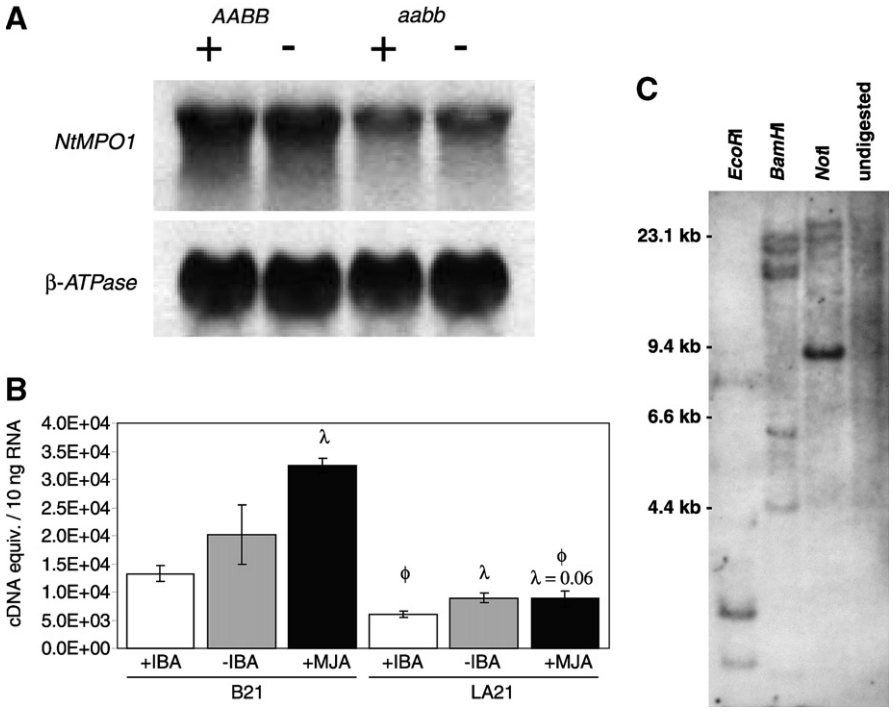

Fig. 2. Steady state MPO transcript levels and gene copy number. (A) RNA blot analysis using a pWGH10 specific probe. Wild type *AABB* roots, B21; mutant *aabb* roots, LA21. Media containing IBA (+IBA) does not induce high levels of nicotine biosynthetic gene expression, whereas media lacking IBA (−IBA) increases mRNA accumulation of several known nicotine biosynthetic genes (Hibi et al., 1994; Kidd et al., 2006; Reed and Jelesko, 2004). (B) Quantitative Real Time PCR of *MPO1*-like transcript accumulation levels. The number of cDNA equivalents per reaction were estimated from a standard curve using pWGH15 as template. GLM ANOVA pair wise comparisons ($n = 3$): λ, treatment-specific effect with *P*-value $\leqslant 0.05$; ϕ, genotype-specific effect with *P*-value $\leqslant 0.05$; "$\lambda = x$" (where $x = P$-value) indicates near-significant treatment-specific effect. (C) DNA blot analysis of *MPO1*-like gene copy number. B21 genomic DNA digested with indicated enzymes and hybridized with a *MPO1* Digoxygenin-dUTP-labeled probe.

MPO-like multigene family, oligonucleotide primers were designed to a region of pWGH15 that is highly conserved with pWGH10. These oligonucleotide primers were used in QRT-PCR analyses of RNA isolated from B21 and LA21 root cultures that were treated with either media lacking IBA or media supplemented with 100 μM methyl jasmonic acid (MJA). Fig. 2B shows that *MPO*-like transcript levels significantly increased during MJA treatment in B21 roots and were significantly reduced in the mutant LA21 roots during both control and MJA treatments. The *MPO*-like transcripts also showed a modest, though not statistically significant, increase during IBA deprivation in B21 roots. Removal of IBA from the media of tobacco root cultures simulates the reduced auxin biosynthesis caused by the topping of tobacco plants. Previously, it was shown that the topping of wild type B21 tobacco plants resulted in a modest 23% increase in MPO enzyme levels (Saunders and Bush, 1979). In the same study, MPO enzyme levels were approximately 50% lower in the mutant LA21 *aabb* genotype, relative to wild type B21. Thus, the *MPO*-like mRNA levels in the B21 and LA21 primary root cultures with and without IBA, mirrored the MPO enzyme levels in the roots of intact and topped plants (respectively) previously reported by Saunders and Bush (1979). Similarly, both the increased *MPO*-like mRNA levels during MJA treatment and decreased mRNA levels in the *aabb* mutant genotype parallel the differential mRNA accumulation levels of several known nicotine (**10**) biosynthetic genes (e.g. *PMT*, *QPT*, *ODC*, and *ADC*) (Cane et al., 2005; Hibi et al., 1994; Kidd et al., 2006; Reed and Jelesko, 2004; Riechers and Timko, 1999; Shoji et al., 2000). Therefore, the *MPO*-like mRNA levels showed differential expression patterns similar to those reported for other nicotine (**10**) biosynthetic genes.

2.2. MPO1 is part of a small multigene family

The nucleic acid sequence of pWGH10 was 98.6% similar to the corresponding region in pWGH15, suggesting there may be a MPO multigene family (Fig. 3). To test this assertion, DNA blot analysis of B21 genomic DNA digested with three restriction enzymes was performed using a hybridization probe derived from pWGH15. Fig. 2C shows that Burley 21 wild type genomic DNA digested with *Bam*HI yielded five to six hybridizing fragments between 23.1 and 4.4 Kb with approximate equal hybridization intensity. In contrast, *Eco*RI digests yielded hybridizing fragments smaller than the 2.8 Kb MPO-like cDNA in pWGH15, suggesting internal *Eco*RI restriction sites that make copy number predictions difficult to determine. *Not*I digests yielded two large fragments of equal hybridization intensity, as well as a 9.1 Kb fragment that showed more intense hybridization. The *Not*I hybridization pattern suggests there are more than three MPO-like genes. Taken together the *Not*I and *Bam*HI results suggest five to six MPO-like sequences in the *N. tabacum* genome.

2.3. The predicted MPO1 protein shares essential structural motifs with other copper amine oxidases

Plasmid pWGH15 was an apparent full length cDNA that encoded a predicted copper amine oxidase. The assigned ATG start codon at position 143 was preceded by stop codons in all reading frames, suggesting pWGH15 was a full-length cDNA. The largest open reading frame encoded a 790 amino acid polypeptide with a predicted molecular weight of 88 kDa. BLASTX analysis of the cDNA insert in pWGH15 identified many homologous proteins belonging to a class of predicted copper amine oxidase proteins (data not shown). Based upon the predicted class of enzyme and mRNA expression patterns, this gene was tentatively named *MPO1*. The predicted MPO1 protein sequence was aligned with four copper amine oxidases for which X-ray crystal structures are available (i.e. *Arthrobacter globoformis*, *Escherichia coli*, *Hansenula polymorpha*, and *Pisum sativum*) and two Arabidopsis orthologs (At2G42490 and At4G14940). The highly conserved Asp-Tyr^{509}-Glu/X motif, containing the tyrosine residue that is post-translationally oxidized by a bound copper ion into a topaquinone (Matsuzaki et al., 1994; Tanizawa et al., 1994) was conserved in the predicted tobacco MPO1 protein (Fig. 4). Similarly, three histidines that are responsible for coordinating a copper ion near the reactive tyrosine^{509}/topquinone^{509} were also well conserved in the predicted MPO1 protein. The WoLFPSORT program predicted that MPO1 may be localized to the peroxisome because it has a carboxyl terminal – AKL tripeptide, which is similar to the – SKL consensus motif that directs proteins to the peroxisome. The Arabidopsis At2G42490 protein was also predicted to localize to the peroxisome, whereas the At4G14940 protein lacks this motif and was not predicted to localize to the peroxisome.

2.4. Expression and enzyme kinetics of a recombinant TRX–His^6–MPO1 fusion protein

To provide direct evidence that the protein encoded by pWGH15 has MPO activity, a recombinant MPO1 protein was expressed in *E. coli* as a fusion protein with thioredoxin. Specifically, the *MPO1* cDNA was subcloned into pET32a+ creating plasmid pJGJ389, which encoded a 106 kDa TRX–His^6–MPO1 fusion protein. Crude cell-free extracts of *E. coli* Rosetta cells harboring pJGJ389 did not show a demonstrable 106 kDa recombinant protein, indicating the recombinant TRX–His^6–MPO1 fusion protein was not expressed at high levels. However, after purification through metal binding chromatography (Fig. 5A) a 106 kDa recombinant TRX–His^6–MPO1 protein was observed. Moreover, the TRX–His^6–MPO1 enriched extracts showed putrescine (**1**)-specific amine oxidase activity (Fig. 5B). In contrast, extracts from Rosetta cells harboring the pET32a+ plasmid did not show any putrescine (**1**)-specific amine oxidase activity. Therefore, the TRX–His^6–MPO1 recombinant fusion protein had

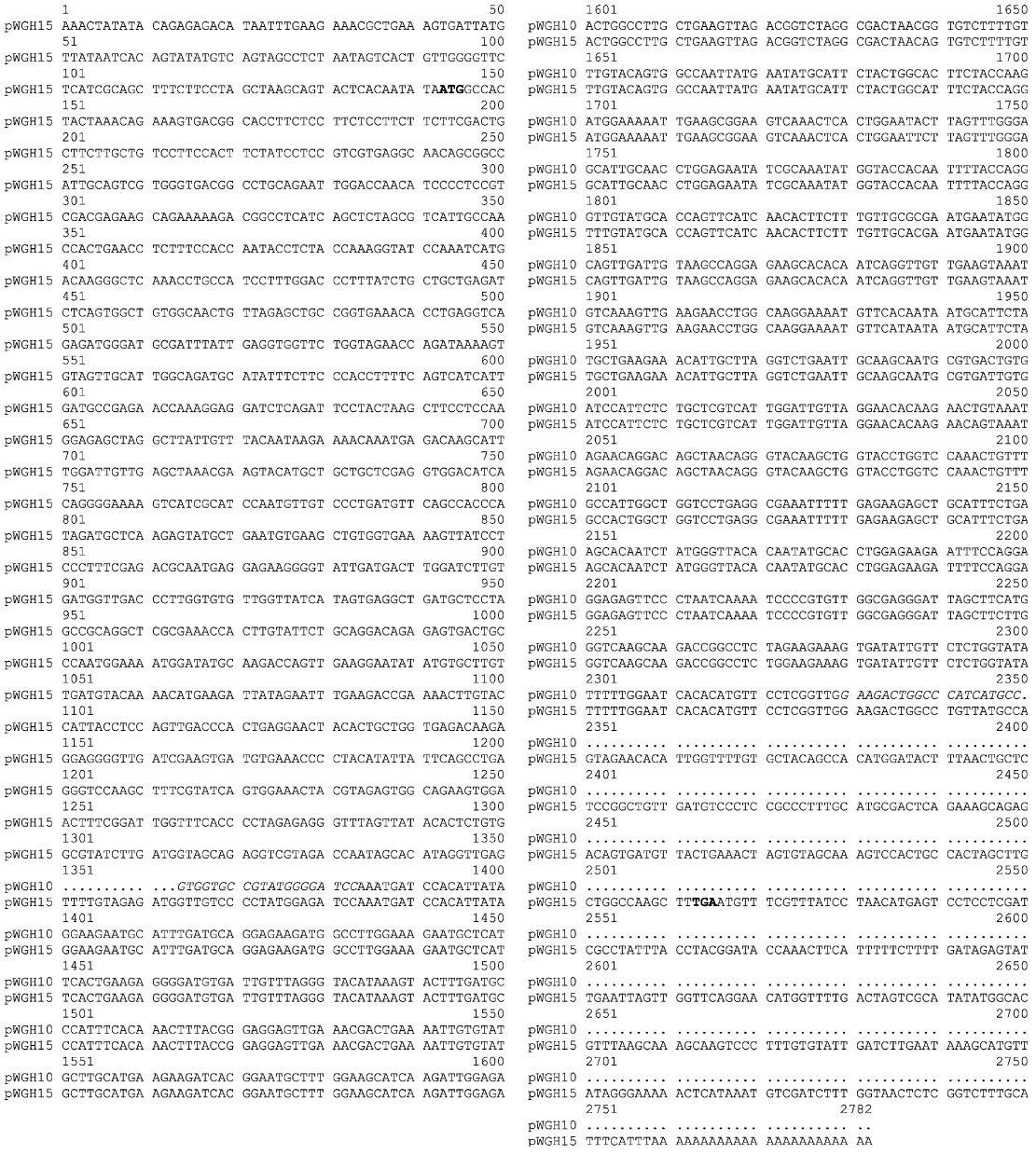

Fig. 3. Multiple sequence alignment of pWGH10 and pWGH15 DNA sequences. Bold ATG indicates predicted methionine start codon and bold TGA indicates predicted stop codon.

DAO activity, whereas the residual proteins from the *E. coli* host did not exhibit substrate-specific DAO activity.

To determine if the TRX–His^6–MPO1 recombinant fusion protein was a generalized DAO or a specialized MPO, the V_{max} and K_m kinetic parameters were determined using several diamines. Table 1 indicates that the TRX–His^6–MPO1 protein bound *N*-methylputrescine (**2**) with the lowest K_m value and displayed the fastest V_{max} of the four substrates tested. In all but one case, the V_{max} and K_m values for putrescine (**1**), cadaverine, and 1,3-diamino propane were statistically different from those observed for *N*-methylputrescine (**2**) (Table 1). We used the ratio of V_{max}/K_m as an estimate of how readily the MPO1 fusion protein utilized a particular substrate. Table 1 illustrates that *N*-methylputrescine (**2**) was utilized 10-fold more efficiently than putrescine (**1**) and 51-fold better than cadaverine. Therefore, based upon the *in vitro* kinetic parameters of the recombinant TRX–His^6–MPO1 protein, it was concluded that pWGH15 encoded a *bona fide* tobacco MPO enzyme (i.e. MPO1).

Previous reports about the *N. tabacum* MPO activity have produced quite variable estimates of MPO enzyme kinetic properties. Using a radiolabel-assay, the K_m of MPO in partially purified root extracts for *N*-methylputrescine (**2**) was 0.08 and 0.45 mM (Mizusaki et al., 1972; Walton and McLauchlan, 1990). Using an HPLC assay for MPO activity in unfractionated tobacco root cell-free extracts, the K_m of MPO for *N*-methylputrescine was estimated at 1.9 mM (Feth et al., 1985). Thus, there is not a good consensus for the K_m of MPO for *N*-methylputrescine.

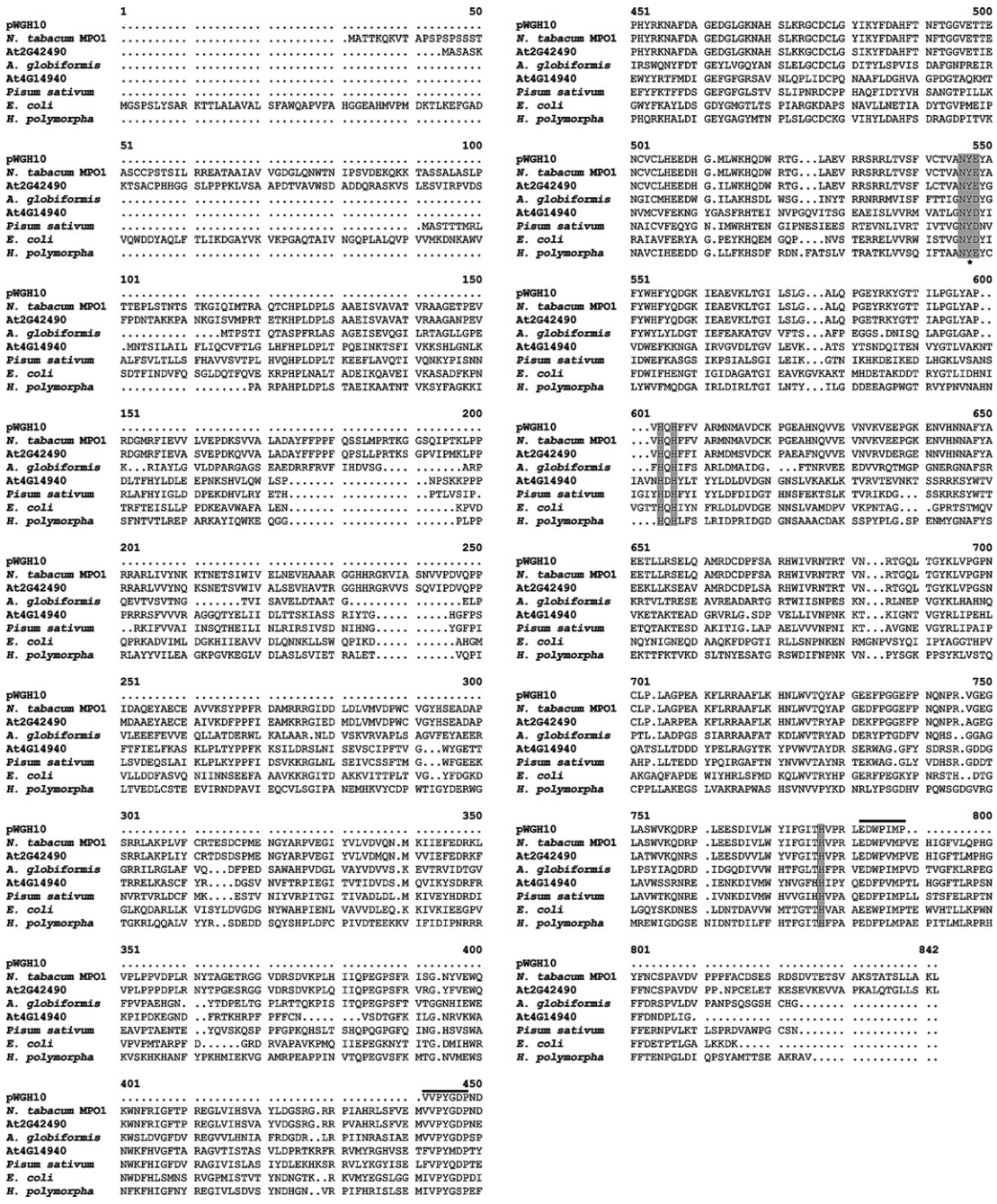

Fig. 4. Protein sequence alignments of copper amine oxidase proteins. Horizontal line indicates region amplified by degenerate oligonucleotides used to identify 986 bp PCR fragment. Shaded amino acids are conserved amino acids influencing the catalytic site. Asterisk indicates tyrosine that is converted to a topaquinone.

The MPO assay utilized in this report was a spectrophotometerically-based MPO assay previously reported to correlate well with the radiolabel-based MPO assay used during the assay of highly enriched *H. niger* DAO/MPO enzyme preparations (Hashimoto et al., 1990). Using the spectrophotometerically-based MPO assay, our estimate for the K_m of TRX–His^6–MPO recombinant protein for *N*-methylputrescine (**2**) (0.19 mM) is intermediate to previously estimated K_m values (i.e. 0.08 and 0.45 mM) from partially purified tobacco root extracts (Mizusaki et al., 1972; Walton and McLauchlan, 1990). Likewise, our results from the recombinant TRX–His^6–MPO1 fusion protein (i.e. 0.19 mM) were also in reasonable agreement with the K_m (i.e. 0.33 mM) estimated for *H. niger* MPO for *N*-methylputrescine (**2**) that is involved in tropane alkaloid biosynthesis (Hashimoto et al., 1990). The kinetic properties of the recombinant TRX–His^6–MPO1 fusion protein were likely not significantly altered by the amino-terminal TRX–His^6-fusion tag, because the observed MPO1 kinetic properties were within the range of previous reports for endogenous MPO. Moreover, the TRX domain completely suppressed the requirement for 2-mercaptoethanol during protein

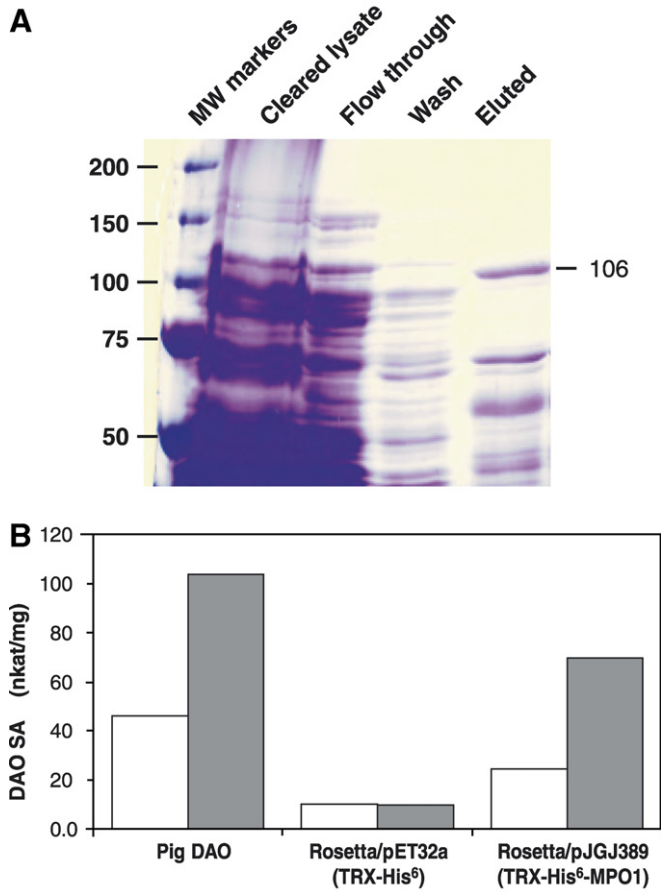

Fig. 5. Expression of recombinant TRX–His^6–MPO1 protein in *E. coli*. (A) Partial purification of recombinant TRX–His^6–MPO1 over Ni-NTA column. Fractions are as indicated and separated on SDS–PAGE stained with Coomassie Brilliant Blue. (B) Amine oxidase assays of porcine DAO and two *E. coli* extracts isolated over Ni-NTA column (as labeled). White bars indicate amine oxidase specific activity without substrate. Gray bars indicate amine oxidase specific activity using 1 mM putrescine as substrate.

purification, suggesting the TRX fusion protein helped maintain MPO's requirement for reduced sulfhydryl groups.

The observed kinetic properties of the TRX–His^6–MPO1 fusion protein provide interesting insights about the pyridine alkaloid composition in *N. tabacum*. Most Nicotiana species predominately accumulate nicotine (**10**). However, they also accumulate minor pyridine alkaloids such as nornicotine and anabasine (Saitoh et al., 1985). It is formally possible that the oxidative deamination of putrescine (**1**) by a generalized DAO would produce an unmethylated-pyrrolinium salt, resulting in the direct biosynthesis of nornicotine in roots. Similarly, the oxidative deamination of cadaverine would give rise to the piperdine ring in anabasine. However, a specialized MPO, preferring *N*-methylputrescine (**2**) as substrate, would produce an *N*-methylated pyrrolinium salt, resulting in mostly nicotine (**10**) biosynthesis. *In vitro*, the recombinant TRX–His^6–MPO1 enzyme preferentially utilized *N*-methylputrescine (**2**) $\gg$ putrescine (**1**) $>$ cadaverine. This order of MPO1 substrate preference (see Table 1) mirrors previously reported accumulation levels of predicted pyridine alkaloid products: nicotine (**10**) (82%) $\gg$ nornicotine (6.0%) $>$ anabasine (2.0%) in *N. tabacum* roots (Saitoh et al., 1985). Thus, the kinetic properties of MPO1 may play an important role in determining the alkaloid composition in tobacco roots.

3. Experimental

3.1. Isolation of a full length MPO1 cDNA and gene copy number estimate

Five predicted copper-containing amine oxidases (i.e. *Arabidopsis thaliana*, AF034579; *Canavalia lineate*, AF172681; *Brassica juncea*, AF449459; *Glycine max*, AF089851; and *Zea mays*, AY103626) were subjected to ClustalW alignment using DS Gene version 1.0 software (Accerlys Inc., San Diego, CA). Based upon this sequence alignment two degenerate oligonucleotides were designed and synthesized: oWGH27 (5′-GTIGTICCITAYGGIGAYCC-3′) and oWGH29 (5′-GGCATIAYIGGCCARTCYTC-3′), where Y is C or T; R is A or G; W is A or T; and I is inosine to reduce degeneracy (Integrated DNA Technologies, Coralville, IA). Eight identical PCR reactions were prepared consisting of 2 μl of a Burley 21 root cDNA library (Heim and Jelesko, 2004) and 1 μM of each oligonucleotide primers (oWGH27 and oWGH29) in a final reaction volume of 25 μl (Ausubel et al., 2006). These eight PCR reactions were subjected to the following thermocycling parameters: 3 min at 94 °C for one cycle, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing across a temperature gradient from 50 to 57 °C for 1 min, and extension at 72 °C for 1.5 min on a RoboCycler Gradient 40 (Stratagene, La Jolla, CA). The PCR products were then extended for 10 min at 72 °C. A 10 μl

Table 1
Enzyme kinetics of recombinant TRX–His^6–MPO1 protein

Substrate	V_{max} (nkat)	K_m (mM)	V_{max}/K_m (min^{-1})	Rel. sub. efficiency (%)
N-methylputrescine	28.8 ± 2.2	0.19 ± 0.02	87.2×10^{-4}	100
Putrescine	11.1 ± 2.2 ($P = 0.0003$)	0.76 ± 0.16 ($P = 0.0156$)	8.7×10^{-4}	10
Cadaverine	5.2 ± 0.8 ($P < 0.0001$)	1.79 ± 0.16 ($P < 0.0001$)	1.7×10^{-4}	2
1,3-diamino propane	11.3 ± 0.8 ($P = 0.0003$)	0.35 ± 0.03 ($P = 0.7021$)	19.3×10^{-4}	22

Substrate specific amine oxidase rates were used to generate Lineweaver–Burk plots and then estimate the TRX–His^6–MPO1 kinetic properties. Each substrate was assayed with three independent recombinant TRX–His^6–MPO1 preparations. Averages ± SE are indicated. GLM ANOVA *P*-values of pair wise comparisons to results with *N*-methylputrescine are bounded by parentheses.

aliquot of each PCR sample was separated on a 0.8% (w/v) TAE agarose gel containing 0.5 μg/ml ethidium bromide (Ausubel et al., 2006), and imaged on a Bio-Rad Gel Doc 2K System (BioRad, Hercules, CA). PCR products of the expected size were excised from a gel slice using the QIAEX® II Gel Extraction Kit (Qiagen, Valencia, CA) and subcloned into pCR2.1 using the TOPO TA Cloning kit and TOP10 competent cells (Invitrogen, Carlsbad, CA), resulting in plasmid pWGH10. The insert in pWGH10 was fully sequenced on both strands using the Big Dye Terminator (version 3.0) Ready Reaction kit (Applied Biosystems, Foster City, CA) in conjunction with the oligonucleotide primers: M13 Forward; M13 Reverse; oWGH31, (5′-TTCACAAACTTTACGGGAGGAG-3′); oWGH32, (5′-TCGAGCGAGTATCAAAGAAAT-3′); oWGH33, (5′-CGTGACTGTGATCCATTCTCTGCT-3′); and oWGH34, (5′-TGTAACCCATAGATTGTGCTT CAG-3′). The cycle sequencing reactions were analyzed at the Core Laboratory Facility at the Virginia Bioinformatics Institute (Virginia Polytechnic Institute and State University, Blacksburg, VA) using an ABI 3100 (Applied Biosystems) capillary sequencer. The resulting DNA trace files were assembled into contigs and edited using the SeqMan Windows 32 version 5.07 in the Lasergene software package (DNASTAR, Madison, WI). The genomic DNA blot analysis was performed with B21 genomic DNA and hybridized using high stringency conditions to a dUTP-digoxygenin-labeled PCR fragment amplified using oJGJ156 (5′-TCCATGGCCACTACTAAACAGAAAG-3′) and oJGJ179 (5′-TAACAGGCCAGTCTTCCAACC-GAG-3′) using pWGH15 as template DNA.

Plasmid pWGH10 DNA and oligonucleotide primers oWGH27 and oWGH29 were used to generate a PCR amplified Digoxygenin-dUTP-labeled DNA fragment that was used as a hybridization probe for screening a B21 root cDNA phagmid library using the same methods as previously described in (Heim and Jelesko, 2004). This resulted in the isolation of pWGH15 containing an approximate 2.8 Kb cDNA insert. Plasmid pWGH15 was randomly mutagenized with the GeneJumper transposon (Invitrogen) to introduce novel oligonucleotide priming sites that facilitated complete DNA sequencing of the insert. The nucleotide sequences of pWGH10 and pWGH15 were aligned using CLUSTALW in DS GENE version 1.5 (Accelrys Inc). The predicted protein sequences encoded by these two plasmids were also aligned with four amine oxidase proteins for which X-ray crystal structures have been solved (Kumar et al., 1996; Li et al., 1998; Parsons et al., 1995; Wilce et al., 1997). BLASTX searches on non-redundant Genbank databases were also performed using DS GENE.

3.2. Primary root cultures and mRNA expression analysis

B21 and LA21 primary root cultures were grown and RNA extracted as previously described (Heim and Jelesko, 2004). The same pWGH10 digoxygenin-dUTP labeled probe that was used for screening the cDNA library was also used to monitor the steady state mRNA levels of *MPO1*-like genes. Hybridization with a *β-ATPase* digoxygenin-dUTP labeled PCR fragment (Reed and Jelesko, 2004) was used to examine the steady state mRNA levels of a housekeeping gene that does not change during these conditions (Heim et al., 2006; Reed and Jelesko, 2004; Riechers and Timko, 1999; Xu et al., 2004). Quantitative Real Time PCR was performed on B21 and LA21 root RNA using oligonucleotide primers oJGJ178 (5′-TCAAAA TCCCCGTGTTGGCGAG-3′) and oJGJ179 (5′-TAACA GGCCAGTCTTCCAACCGAG-3′) using pWGH15 to generate a standard curve, as previously described (Kidd et al., 2006).

3.3. Assay of recombinant TRX–His^6–MPO1 in bacteria

In order to facilitate the cloning of the *MPO1* gene into a recombinant expression vector, *BamH*I and *Nco*I sites were introduced upstream of the predicted ATG start codon, using oligonucleotide primers oJGJ166 (5′-GGAT-CCCCATGGCCACTACTAAACAGAAAG-3′) and oJGJ157 (5′-TGGTAGAGGTATTGGTGGAAAG-3′) to amplify a 241 bp PCR fragment using pWGH15 as template DNA. This modified fragment was cloned into pCR2.1 (Invitrogen) to yield pJGJ367. A 165 bp *BamH*I-*Sal*I fragment was cut from pJGJ367 and ligated into pWGH15 similarly cut, resulting in pJGJ369. Finally, a 2.6 Kb *Nco*I-*Xho*I (partial) fragment was cut from pJGJ369 and ligated into pET32a+ similarly cut, to yield pJGJ389. Plasmid pJGJ389 was transformed into the Rosetta *E. coli* strain (Novagen, Madison, WI) for expression of a recombinant TRX–His^6–MPO1 protein. Mid-log phase Rosetta/pJGJ389 cells were cultured overnight in LB media supplemented with 100 μg/ml Ampicillin, 30 μg/ml Chloramphenicol, and 0.2 mM isopropyl-β-D-thiogalactoside at 18 °C at 250 rpm. The cells were pelleted, lysed, and the native protein extract incubated with Ni-NTA superflow resin, and the TRX–His^6–MPO1 was eluted as per manufacture's instructions (Qiagen, Valencia, CA). The TRX–His^6–MPO1 enriched extract was mixed 1:1 (v:v) with 100% glycerol and stored at −20 °C. Prior to use, the recombinant protein extracts were buffer exchanged using a PD-10 column (GE Health Care Bio-Sciences AB, Uppsala, Sweden) into the same buffer used for the subsequent spectrophotometric-based amine oxidase assay (Kusche and Lorenz, 1983) on a Beckman DU-7400 spectrophotometer (Beckman Coulter Inc., Fullerton, CA). A variety of diamine substrates were assayed: putrescine, cadaverine, 1,3-diaminopropane (Sigma–Aldrich Co., St. Louis, MO), and *N*-methyl-1,4 diaminobutane (Toronto Research Chemicals Inc, North York, ON, Canada). Five to seven background-corrected diamine oxidase rates were graphed as Lineweaver–Burk plots in order to estimate the V_{max} and K_m of the recombinant TRX–His^6–MPO1 fusion protein using three independent enzyme preparations. A General Linear Model

(GLM) ANOVA with Tukey correction test was performed using Minitab version 14 for Windows (Minitab Inc., State College, PA) to determine whether the kinetic properties of each substrate were significantly different from those observed with *N*-methylputrescine (**2**) as substrate.

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