Supplementary Materials for

Mitochondrial *Arabidopsis thaliana* Glyoxalase 2-1 Exhibits β-Lactamase Activity.

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EXPERIMENTAL PROCEDURES

Over-expression and purification of Arabidopsis Glx2-1. PCR was conducted on *A. thaliana* cDNA, with the primers CCTCCATGGTAAAAATCGAACTGGTGC and GAGTCGACTCGAGCTCTAGATCTTTTTTTTTT, which generated *Nde*I and *Hind*III sites at the 5' and 3' ends of the fragment. The 762 bp *glx2-1* PCR fragment was subcloned into *p*ET26b by using the *Nde*I and *Hind*III restriction sites, and the sequence of the resulting *Glx2-1/p*ET26b plasmid was confirmed by DNA sequencing using the T7 promoter and T7 terminator primers. Plasmid *Glx2-1/ p*ET26b was transformed into *E. coli* BL21(DE3) cells.

A 10 mL overnight culture of BL21(DE3) *E. coli* containing *Glx2-1/ p*ET26b was used to inoculate 1L of LB (Luria Bertani) medium containing 25 μg/mL kanamycin in the presence of 250 μM $Fe(NH_4)_2(SO_4)_2$ + 250 μM $Zn(SO_4)_2$. The cells were allowed to grow at 37 °C with shaking until the cells reached an optical density at 600 nm of 0.6-0.8. Protein production was induced by making the cultures 0.3 mM in isopropyl-β-*D*-thiogalactopyranoside (IPTG), and the cells were shaken at 15 °C for 24 h. The cells were collected by centrifugation (15 min at 7000 rpm), and the cell pellets were stored at -80 °C until further use.

The cell pellet was resuspended in 10 ml of 10 mM MOPS, pH 7.2, containing 0.1 mM phenylmethylsulfonyl fluoride. The cells were lysed by 3 passages through a French press at

16,000 p.s.i., and the cell debris was removed by centrifugation at 25,000 rpm for 30 min. The cleared supernatant was dialyzed versus 2L of 10 mM MOPS, pH 7.2, overnight at 4 °C and centrifuged for 20 min at 25,000 rpm to remove insoluble matter. The cleared supernatant was purified using a FPLC with a SP-Sepharose column (1.5 × 12 cm with a 25 mL bed volume) that was equilibrated with 10 mM MOPS, pH 7.2. Bound proteins were eluted with a 0-500 mM NaCl gradient in 10 mM MOPS, pH 7.2, at 2 mL/min. Protein purity was ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions (8 mL) containing Glx2-1 were pooled and concentrated by an Amicon ultrafiltration cell equipped with a YM-10 membrane (see Figure S2 for SDS-PAGE gel of the purified enzyme).

Metal Analyses. The metal content of Glx2-1 samples was determined using a Varian-Liberty 150 Inductively Coupled Plasma spectrometer with atomic emission spectroscopy detection (ICP-AES), as described previously *(1)*. Protein samples were diluted to 10 μM with 10 mM MOPS, pH 7.2. A calibration curve with four standards and a correlation coefficient of greater than 0.99 was generated using Fe, Zn, and Mn reference solutions. The following emission wavelengths were chosen to ensure the lowest detection limits possible: Fe, 259.940 nm; Zn, 213.856 nm; and Mn, 257.610 nm.

Merlin Micronaut TAXA PROFILE E assay. In an effort to identify a substrate for Glx2-1, Merlin Micronaut TAXA PROFILE E assay plates were used according to manufacturer's instructions. As-isolated Glx2-1 protein was used in these studies at a concentration of 5 μM. A protein solution, which was prepared by substituting *pGlx2-1*/*p*ET26b with empty vector *p*ET26b and following the same over-expression and purification protocol as described above, was used

as a control. For all positive reactions in the Merlin plates, activity assays were conducted using substrates purchased from Sigma and as-isolated Glx2-1 in 10 mM MOPS buffer, pH 7.2, using an Agilent 5483 Diode Array UV-visible spectrophotometer.

Steady-state kinetic assays. All kinetic studies were conducted on an Agilent 8453 UV-Vis diode array spectrophotometer at 25 °C. Steady-state kinetic parameters, the Michaelis constant, K_m, and the turnover number, k_{cat}, were determined by monitoring product formation at 485 nm using nitrocefin or substrate decay at 300 nm for imipenem or 260 nm for cefotaxime in 10 mM Chelex-treated, MOPS, pH 7.2. Absorbance changes were converted to concentration changes using Beer's law and the extinction coefficients (in $M^{-1}cm^{-1}$) of nitrocefin product (17,420), imipenem (-9,000), or cefotaxime (-7,036), respectively.

N-Acyl-homoserine lactonase assays. Using a previously described assay *(2)*, lactone hydrolysis was monitored using phenol red to detect net proton release upon ring opening. Briefly, purified Glx2-1 (0.27 and 2.1 μM) was added to Hepes buffer (1 mM), Na_2SO_4 (0.1M), phenol red (40 μM), and a lactone substrate [γ-butyrolactone (100 mM) or various *N*-acyl homoserine lactones (AHLs or *N*-acyl-HSLs) including *N*-butyl-HSL (20 mM), *N*-pentanoyl-HSL (10 mM), *N*-hexanoyl-(S)-HSL (10 mM), 3-oxo-hexanoyl-HSL (10 mM), *N*-octanoyl-(*S*)-HSL (4 mM), *N*-3-oxo-octanoyl-HSL (10 mM), *N*-decanoyl-(*S*)-HSL (0.7 mM) and *N*-*t*-butoxycarbonyl-HSL (10 mM)] at pH 7.5 and 25° C. The decrease in absorbance at 557 nm was monitored at over time and converted into product formation rates using a standard curve as described earlier. Under these conditions, the assay can detect catalysis by enzymes with k_{cat} values estimated to be > 0.02 s^{-1}, assuming substrate saturation. For comparison, the k_{cat} of AHL lactonase from *Bacillus thuringiensis* is approx 100 s^{-1} *(2)*.

Stopped-flow kinetic studies. Stopped-flow kinetic experiments were performed on an Applied Photophysics SX18MV spectrophotometer equipped with a constant temperature circulating water bath as previously described *(3, 4)*. All experiments were performed in 10 mM Chelex-treated, MOPS, pH 7.2, at 10 ºC. The substrate and enzyme concentrations were 94 and 15.5 μM, respectively.

Isolation of a Brassica oleracea Glx2-1 cDNA. The entire *B. oleracea* genomic sequence is not available; therefore, the *Brassica* Glx2-1 protein sequence could not be directly obtained from the database. The *A. thaliana* Glx2-1 protein sequence was used to query the *Brassica* DNA database to identify individual *Brassica Glx2-1* genomic sequences. A 774 bp *Brassica* sequence that corresponds to the C-terminal region of *A. thaliana Glx2-1* was initially obtained. This *Brassica* sequence was then used to further query the *Brassica* database to obtain overlapping sequences. These searches were repeated until two non-overlapping contigs containing an additional 3.0 kbp of *Brassica* sequence that corresponds to *A. thaliana Glx2-1* was obtained. The program Netgene 2 (http://www.cbs.dtu.dk/services/NetGene2/) was then used to predict the possible intron / exon junctions of the *Brassica Glx2-1* genomic DNA. The program predicted six splice sites, which correspond to six of the seven exons found in *Arabidopsis* Glx2-1. A sequence corresponding to exon four of the *Arabidopsis* Glx2-1was not obtained from the *Brassica* database. The *Brassica* genomic sequence was then used to design oligonucleotides to serve as primers to isolate a *Brassica Glx 2-1* cDNA.

The *Glx2-1* cDNA was synthesized from total RNA isolated from seven-day-old *B. oleracea* tissues by reverse transcription using an oligo dT-adaptor primer:

CGAGGATCCTCGAGTCGACGC followed by PCR with *Glx2-1* gene-specific primers using standard procedures. An 1100 bp PCR product was obtained, subcloned into a TA plasmid, and sequenced to obtain the *Glx2-1* cDNA sequence. The *Brassica Glx2-1* cDNA sequence obtained is 1272 bp with a start codon at position 148 and a stop codon at position 1171. The *Brassica* Glx2-1 protein is encoded by a 340 amino acid open reading frame (Figure S2). The deduced amino acid sequences of *B. oleracea* and *A. thaliana* Glx2-1 show 86% sequence identity. All of the predicted metal and substrate binding ligands including the arginine at position 246 of *A. thaliana* Glx2-1 are conserved in *B. oleracea* Glx2-1. Therefore, the cDNA clearly represents *Brassica Glx2-1*. The leader peptide of the two proteins is also highly conserved; however, there are eight asparagines in the *Brassica* Glx2-1 leader sequence that are not present in the *Arabidopsis* sequence. The asparagines are in both the genomic and cDNA sequences and therefore are not an artifact of PCR or cloning.

References

1. Crowder, M. W., Maiti, M. K., Banovic, L., and Makaroff, C. A. (1997) Glyoxalase II from A. thaliana Requires Zn(II) for Catalytic Activity, *FEBS Lett. 418*, 351-354.
2. Thomas, P. W., Stone, E. M., Costello, A., Tierney, D. L., and Fast, W. (2005) The quorum-quenching lactonase from *Bacillus thuringiensis* is a metalloprotein, *Biochemistry 44*, 7559-7569.
3. Garrity, J. D., Bennett, B., and Crowder, M. W. (2005) Direct evidence that reaction intermediate in metallo-β-lactamase is metal bound, *Biochemistry 44*, 1078-1087.
4. Carenbauer, A. L., Garrity, J. A., Periyannan, G., Yates, R. B., and Crowder, M. W. (2002) Probing Substrate Binding to Metallo-β-Lactamase L1 from Stenotrophomonas maltophilia by Using Site-Directed Mutagenesis, *BMC Biochemistry 3*, 4-10.

Table S1: Amino acid sequence identities of plant Glx2-1 enzymes with human Glx2-1 and metallo-β-lactamases L1 and CcrA

		% amino acid sequence identity[a]	
Enzyme	**Human Glx2**	**L1**	**CcrA**
A.t. Glx2-1	32	10	2
A.t. Glx2-1	53	7	7
A.t. Glx2-4	36	11	2
A.t. Glx2-5	33	11	2
Brassica Glx2-1	31	8	2

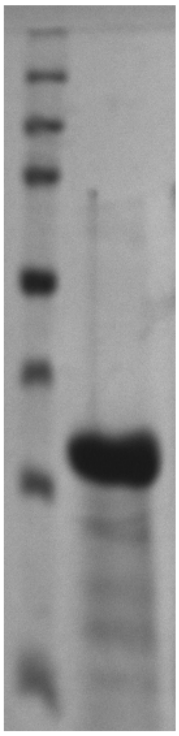
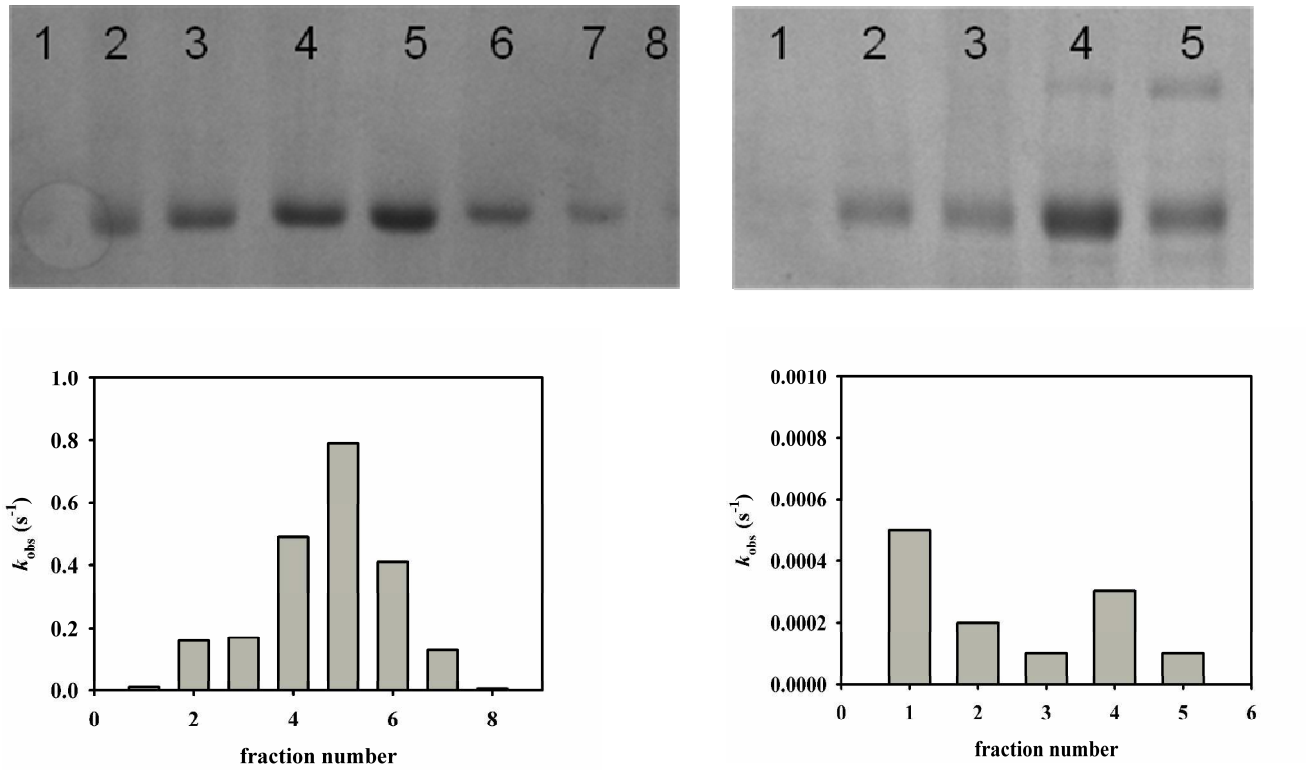


Figure S1: SDS-PAGE gel of Glx2-1 sample. Left lane is molecular weight markers, and right lane is the purified Glx2-1 sample.



Wild-type Glx2-1 **R172H/N174Y mutant**

Figure S2: Activity profiles of column fractions from preparation of wild-type Glx2-1 (LEFT) and the R172H/N174Y mutant of Glx2-1. The assays were conducted using 150 μM nitrocefin in 10 mM MOPS, pH 7.2. The values for k_{obs} were determined by dividing the observed rate (expressed in units of M/s) with the total protein concentration in each fraction.

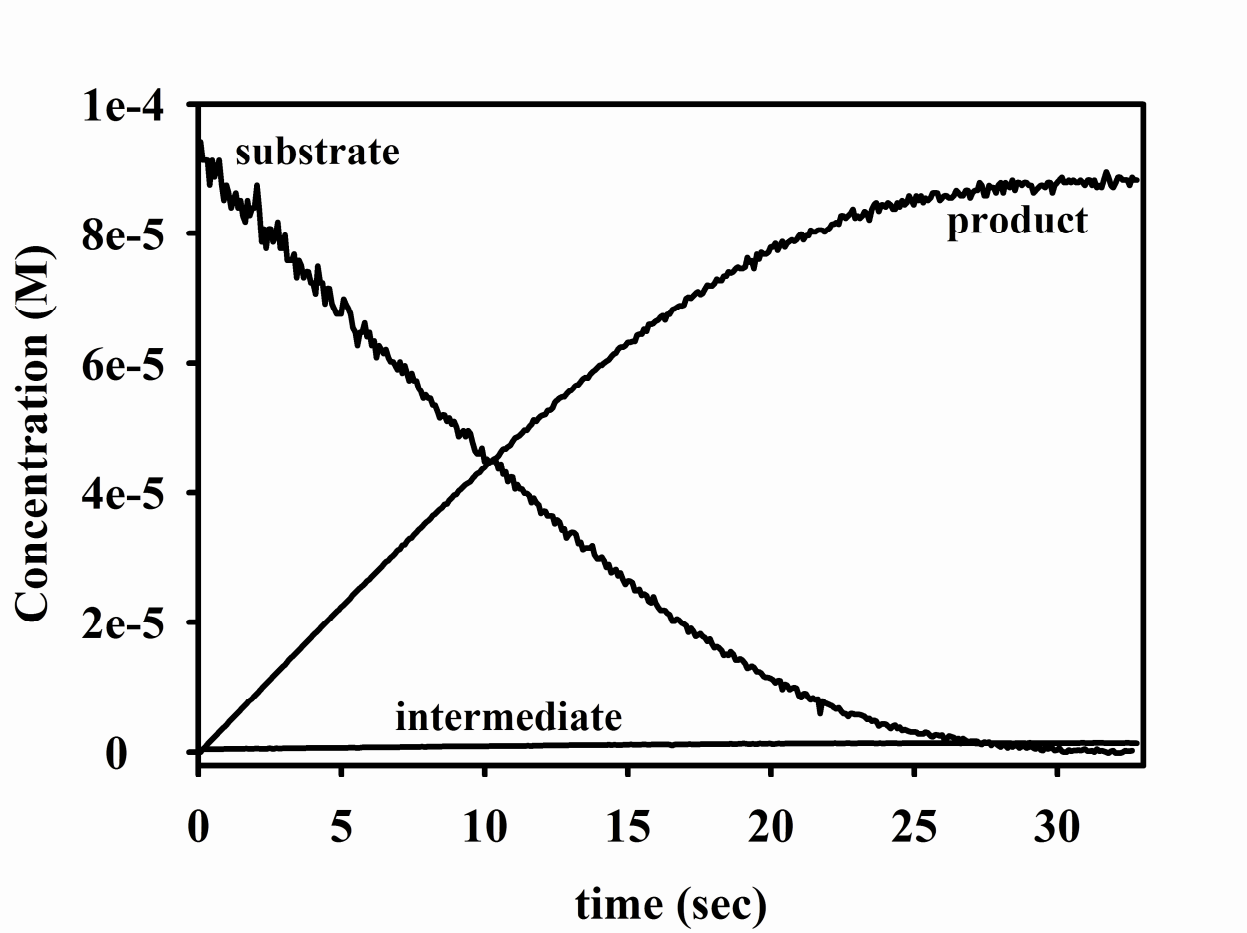


Figure S3: Stopped-flow UV-Vis studies with nitrocefin and Glx2-1. The substrate and enzyme concentrations were 94 and 15.5 μM, respectively, and the buffer was 10 mM MOPS, pH 7.2. Substrate depletion was monitored at 390 nm, product formation was monitored at 485 nm, and intermediate concentration was monitored at 665 nm, as previously reported.

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                ATCGGGNTAGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATTTTCCACCTGCTTCCTCCGC
GCAATTTCTCCTCTACTAGGTAAAGTCCTCTCGAATCAATCATCTCTCTGAAGAATCTAGAGAGAGAGAGAAGTAGAGGAT
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 M  P  I  I  S  K  A  S  S  S  T  N  N  N  N  N  N  N  S  S  F  P  S  S  S  S
ACGAAACAGATTGGAGGTAGGCTTTGTGTGTGGCCTGGTTTGAGACATCTTTGCCTGAGGAAAAGCTTGTTATACGGAGTT
 T  K  Q  I  G  G  R  L  C  V  W  P  G  L  R  H  L  C  L  R  K  S  L  L  Y  G  V
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 K  V  E  K  A  C  N  P  F  L  R  T  S  S  K  E  I  R  R  S  L  N  I  P  D  S  A
AACGAAGCAGAAGCACTGCGTCGTATACACAGAGCAAGAGACCGTTTCTAAAGAAGCTTGAGGGTTTGGAAGACAATCAC
 N  E  A  E  L  R  R  I  H  R  A  R  D  R  F  .
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Figure S4: Nucleotide and deduced amino acid sequence of *Brassica* Glx2-1.

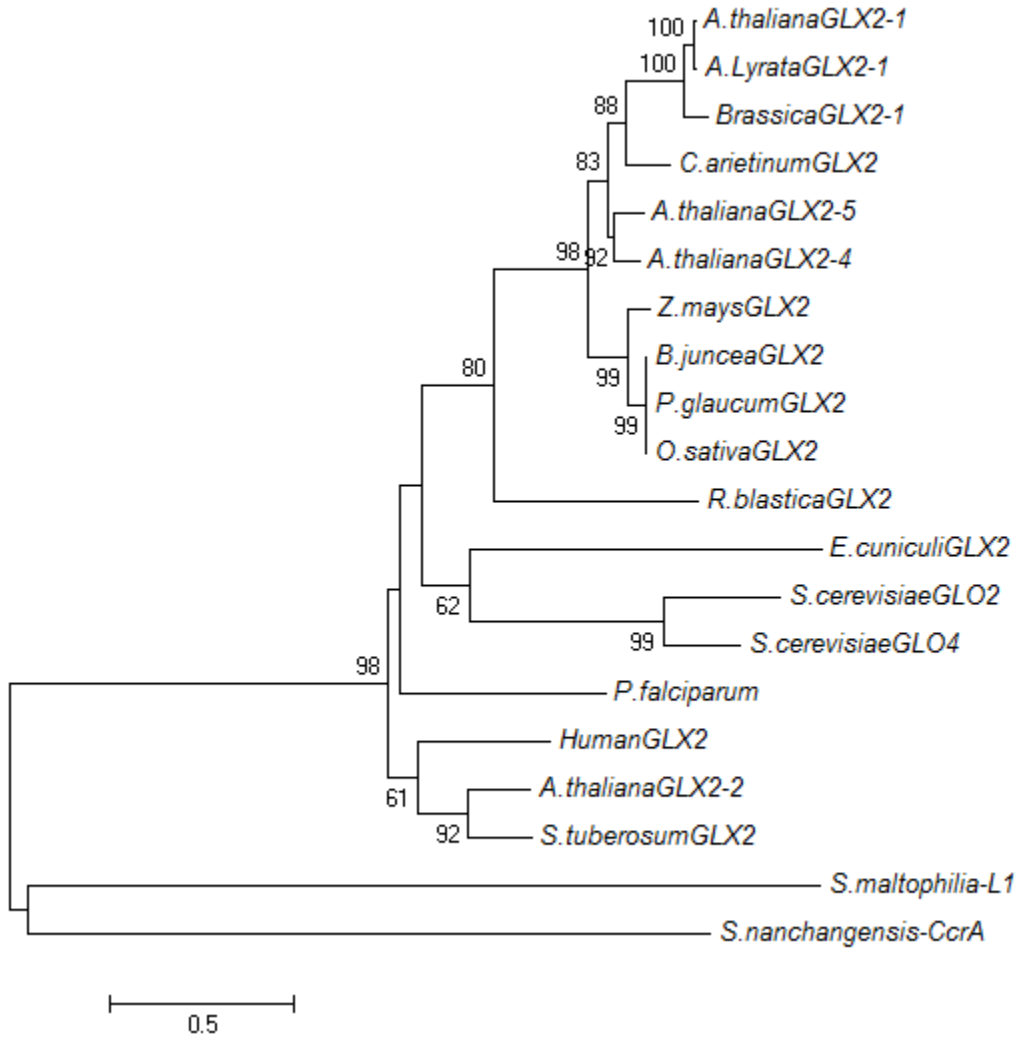

Figure S5: Phylogenetic tree of select putative and known GLX2 isozymes and L1/CcrA. The figure was generated using the amino acid sequences of the proteins and MEGA 4.0.2 software (neighbor joining method, Bootstrap test of inferred phylogeny of 1000, model: amino: JTT matrix-based, and complete deletion for Gaps/Missing data).