Molecular Definition of the Ascorbate-Glutathione Cycle in Arabidopsis Mitochondria Reveals Dual Targeting of Antioxidant Defenses in Plants*ο

Orinda Chew†, James Whelan§, and A. Harvey Millar¶
From the Plant Molecular Biology Group, School of Biomedical and Chemical Sciences, The University of Western Australia, Crawley 6009, Western Australia, Australia

Key components of the ascorbate-glutathione cycle in Arabidopsis cell organelles are encoded by single organelar targeted isoforms that are dual localized in the chloroplast stroma and the mitochondrion. We demonstrate the presence of the ascorbate-glutathione cycle in purified Arabidopsis mitochondria using enzymatic activity, proteomic and in vitro and in vivo subcellular targeting data that identify the gene products responsible. In vitro experiments using a dual import assay assessing mitochondrial and chloroplast import, simultaneously show dual targeting of ascorbate peroxidase, monodehydroascorbate reductase, and glutathione reductase gene products to mitochondria and chloroplasts, while a putative dehydroascorbate reductase protein is only imported into mitochondria. In vivo subcellular localization using green fluorescent protein fusion proteins shows clear targeting of all gene products to mitochondria. Transcript levels show these genes are induced by oxidative chemical stresses targeted to chloroplasts and/or mitochondria and are elevated during photosynthetic operation in the light. Together these data present a model of an integrated ascorbate-glutathione antioxidant defense common to plastids and mitochondria that is linked at the level of the genome in Arabidopsis.

The ascorbate-glutathione cycle is catalyzed by a set of four enzymes, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), glutathione-dependent dehydroascorbate reductase (DHAR), and glutathione reductase (GR). This cycle operates in chloroplasts of plants in order to remove the large amounts of H2O2 generated during photosynthetic operations in the light through thylakoid electron transport chain components with electrochemical potentials capable of direct reduction of O2 (1). The highest rates of O2 reduction occur on the reducing side of photosystem I (1). The high specific activity of these enzymes in plastids and extensive work on its operation clearly indicates that this is the major H2O2 metabolizing pathway in these photosynthetic organelles (2). The primary peroxidation of ascorbate (Asc) by APX yields the monodehydroascorbate radical (MDHA) that is either directly reduced back to Asc by MDHAR (3) or undergoes non-enzymatic disproportionation to ascorbate and dehydroascorbate (DHA). Recovery of the DHA produced occurs via the glutathione-dependent reaction catalyzed by DHAR, and the oxidized glutathione dimers are re-reduced by the NADPH-dependent GR (4).

The chloroplast Asc-glutathione cycle is housed primarily in the stroma (see review in Ref. 5). A stromal APX and a thylakoid-bound APX have been identified and purified from several plant species. An extended C-terminal sequence on the thylakoid APX facilitates binding to the membrane and makes this isoform ~5 kDa larger than the stromal APX (5). Sequence analysis in a variety of plants clearly delineates the two chloroplast APX classes from the cytosolic APX isoforms (6). The MDHA formed in the lumen disproportionates to DHA and penetrates the thylakoid membrane into the stroma. MDHA produced by both APX isoforms is reduced by stromal MDHAR. This enzyme is not present in the lumen, and chloroplast isoforms are differentiated from cytosolic isoforms by the presence of N-terminal targeting sequences (5, 7). DHAR and GR activities convert the DHA translocated from the lumen and the DHA generated in the stroma. Detailed enzymatic investigation and establishment of the stromal location of these enzymes have been reported (5, 8, 9).

A series of reports have also measured the activity of some of the enzymes of the Asc-glutathione cycle in mitochondrial preparations from plants (9–14). Evidence for a complete, chloroplast-like, Asc-glutathione cycle in plant mitochondria has also been presented. In green leaves, a mitochondrial cycle has been proposed to cope with photosynthetic and environmental stress-induced oxidative stress (15). In the microaerobic environment of N2-fixing legume root nodules, a mitochondrial Asc-glutathione cycle is proposed to remove mitochondrial-derived radicals to protect sensitive sites including the heme of leghemoglobin (16, 17). The latency of mitochondrial APX activities is generally low suggesting a location outside of the inner mitochondrial membrane (16, 17). APX is predominantly membrane-localized in plant mitochondria (12, 15, 17). The best collective evidence for MDHAR, DHAR, and GR presence is from Pisum sativum leaves (9, 14, 15) and Phaseolus vulgaris nodules (17). These biochemical data indicate much of the MDHAR, DHAR, and GR to be in the mitochondrial matrix (9, 14, 15). However the actual proteins and the associated genes...
that are responsible for these activities in plants have not been systematically investigated. As a result, it has been unclear to what degree these biochemical data represent true mitochondrial enzymes and to what degree they may be compromised by the presence of plastidic, peroxisomal, or cytosolic contaminations.

In mammalian mitochondria, glutathione peroxidases and glutaredoxins are found to function in the direct depletion of H₂O₂ (18). A mitochondria-localized thioredoxin-dependent peroxidase is also proposed to be responsible for H₂O₂ depletion in mammals (19). In yeast both an intermembrane space localized cytochrome c peroxidase and a maternally inherited thioredoxin-dependent peroxidase have been implicated in H₂O₂ removal (20). We have already identified a putative thioredoxin-dependent peroxidase in Arabidopsis mitochondria, but its role in H₂O₂ depletion is unknown (21).

If an Asc-glutathione cycle exists in plant mitochondria two possibilities can be entertained. First, a discrete set of genes encoding mitochondrial-specific components of this pathway could be responsible. Suggestions of mitochondrial-specific isoforms have been made in biochemical studies (12, 15–17). Second, a series of organelle-targeted genes could exist that are authentically multitargeted to chloroplasts and mitochondria. Evidence has been presented for the dual targeting of pea GR to both mitochondria and chloroplasts (9), and very recently the products of differential transcripts from a single Arabidopsis MDHAR gene have been proposed to be targeted to mitochondria and chloroplasts (7).

In this report we have demonstrated the activity of the Asc-glutathione cycle enzymes in purified mitochondria from the model plant Arabidopsis. A combination of a survey of the Arabidopsis genes encoding Asc-glutathione cycle enzymes, analysis of proteomic data in purified mitochondrial preparations, and in vitro import experiments showed dual targeting of APX, MDHAR, and GR proteins to mitochondria and chloroplasts, but only mitochondrial targeting of specific DHAR products. In vivo experiments using GFP chimeric proteins also revealed their mitochondrial subcellular localization. Transcript levels for these genes were induced by oxidative stresses imposed on chloroplasts and/or mitochondria and were elevated during photosynthetic operation in the light. Together these data were used to present a model of an integrated Asc-glutathione antioxidant defense complex to chloroplasts and mitochondria that is linked at the level of the genome in Arabidopsis.

EXPERIMENTAL PROCEDURES

Plant Materials—Soybean (Glycine max [L] Merr. cv. Stevens) and pea (Pisum sativum [L] Greenfeast) plants were grown in an environmentally controlled incubator at 28°C. The incubator was fitted with artificial lights of 600 μmol·m⁻²·s⁻¹ set to a 16-h light and 8-h dark period. Arabidopsis thaliana ecotype Columbia (Arabidopsis) were grown at 22°C under a 16-h light and 8-h dark period on solid Gamborg media. Cloning and Expression Analysis of Genes—Total RNA was isolated from whole Arabidopsis plants and expression analyzed using quantitative PCR as previously outlined (22). The following PCR primers were used to clone the genes: AtAPX-fwd, 5′-ATGGCAGAGGCTTGCTTCTC-3′; AtAPX-rev, 5′-TTAGATAACGATACCCTCC-3′; LC-APX-fwd, 5′-CTCATTTCTCTTACATGGGTG-3′; LC-APX-rev, 5′-GATCTGGCCGCAAACAGAAAAAC-3′; LC-MDHAR-fwd, 5′-GATTCGACTACAAAAAGGGGAAG-3′; LC-MDHAR-rev, 5′-CTGGGACCCTTTCTGAGAC-3′; LC-GR-rev, 5′-GATTAGACGCCATTACGAC-3′; LC-GR-fwd, 5′-5′-CAGAGTATTCTGCTGATTGGTCC-3′; LC-MDHAR-rev, 5′-5′-CATAGCGGACCATGACCGACCTT-3′; LC-APX-fwd, 5′-5′-CTCATTTCTCTTACATGGGTG-3′; LC-GR-rev, 5′-GATCTGGCCGCAAACAGAAAAAC-3′; LC-MDHAR-fwd, 5′-GATTCGACTACAAAAAGGGGAAG-3′; LC-MDHAR-rev, 5′-CTGGGACCCTTTCTGAGAC-3′; LC-GR-rev, 5′-GATTAGACGCCATTACGAC-3′; LC-GR-fwd, 5′-5′-CAGAGTATTCTGCTGATTGGTCC-3′;

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidopsis

Mitochondrial Asc-glutathione Cycle in Arabidop
Transient Expression of GFP Constructs in Vivo—The expression cassette containing the cauliflower mosaic virus (CaMV) 35S promoter, Arabidopsis ER chitinase signal peptide, m-gf5p and the nopaline synthase terminator was amplified by PCR using plasmid pBIN m-gp5-ER as a template and primers CaMV35S 5’ F (5’-CGCAAGGTCTCAGTGCTCAAGATTCG-3’) and 3’ R (5’-CGGTACGTTAAGACTGCTGC-3’). The PCR product was then cloned into a pGEM®-T cloning vector (Promega, Melbourne), generating construct pGEMGFp. The sequence corresponding to the AOX presequence, full-length TOC64, APX, MDHAR, AR, and GDHN was amplified with appropriate restriction sites by PCR using plasmid DNA as templates and primers listed as follows: AOXp(BamHI)-fwd, 5’-CGTACGATCCAACAATGGCGTCT-3’; TOC64(EcoRI)-rev, 5’-ATGACGAAATTCATCTTGGTTTCCGGAGAC-3’; APX(BamHI)-fwd, 5’-CGTACGATCCAACAATGGCGTCT-3’; APX(EcoRI)-rev, 5’-ATGACGAAATTCATCTTGGTTTCCGGAGAC-3’; MDHAR(BamHI)-fwd, 5’-CGTACGATCCAACAATGGCGTCT-3’; MDHAR(EcoRI)-rev, 5’-ATGACGAAATTCATCTTGGTTTCCGGAGAC-3’; GR(BamHI)-fwd, 5’-CGTACGATCCAACAATGGCGTCT-3’; GR(EcoRI)-rev, 5’-ATGACGAAATTCATCTTGGTTTCCGGAGAC-3’; GDXN(BamHI)-fwd, 5’-CGTACGATCCAACAATGGCGTCT-3’; GDXN(EcoRI)-rev, 5’-ATGACGAAATTCATCTTGGTTTCCGGAGAC-3’.

The ER signal peptide sequence was removed from the pGEMGFp construct by restriction digestion, PCR products were cloned upstream and in-frame of the m-gf5p gene and verified by sequencing.

Suspension cells developed from soybean (Glycine max cv. Stevens) leaf tissue were grown in 250 ml flasks containing Gamborg media agitated at 130 rpm under approx. 20 µEm-2 s-1 light at 28 °C. The cell stocks were maintained in an exponential growth phase by subculturing (1:6 dilution) at 7-day intervals. For transient expression analysis, 7-day-old soybean suspension cells were aseptically coated onto filter paper (Whatman No. 1 55 mm diameter, Maidstone, England), which was then placed onto solid media containing soybean culture media paper (Whatman No. 1 55 mm diameter, Maidstone, England), which stocks were maintained in an exponential growth phase by subculturing using helium pressure of 1400 kPa. The cells were then incubated at 130 rpm under approx. 20 °C. Leaf tissue were grown in 250 ml flasks containing Gamborg media.

Measurements before and after solubilization with 0.05% (w/v) Triton X-100. B, the subcompartmentation of these activities was measured in matrix (MA), inner membrane (IM) and intermembrane space plus outer membrane (IMS + OM) fractions and is expressed as % of the combined activities from all fractions. This sum of fraction activities is also noted in nmol min-1 mg-1 protein and is compared as % recovery with the + Triton rates in A. All data are from independent mitochondrial preparations, mean ± S.D. (n = 3).

|     | Intact | + Triton | Latency |
|-----|--------|----------|---------|
|     |        |          |         |
| AOX | 81 ± 5 | 110 ± 13 | 26      |
| MDHAR | 28 ± 6 | 117 ± 22 | 76      |
| DHAR | 33 ± 4 | 63 ± 11  | 48      |
| GR  | 2 ± 1  | 10 ± 2   | 80      |
| COX | 17 ± 4 | 520 ± 88 | 97      |

Mitochondrial Asc-glutathione Cycle in Arabidopsis

|     |        |          |         |
|-----|--------|----------|---------|
|     | B      | IMS      | Combined | Recovery |
|     |        | OM       |          |          |
|     |        | MA       | IM       |          |          |
| AOX | 10     | 89       | 109      | 82 ± 9   | 71       |
| MDHAR | 3   | 10       | 40       | 125 ± 31 | 107      |
| DHAR | 15     | 55       | 110      | 48 ± 9   | 76       |
| GR  | 27     | 58       | 85       | 11 ± 2   | 110      |
| COX | 0.5    | 0.9      | 1.4      | 402 ± 44 | 77       |

**Table I**

|     |        |          |         |
|-----|--------|----------|---------|
|     |        |          |         |
| AOX, MDHAR, DHAR, GR, and the mitochondrial marker cytochrome c oxidase (COX) were assayed according to the procedures outlined under “Experimental Procedures.” A, the latency of each enzyme (% activity dependent on solubilization) was calculated from measurements before and after solubilization with 0.05% (w/v) Triton X-100. B, the subcompartmentation of these activities was measured in matrix (MA), inner membrane (IM) and intermembrane space plus outer membrane (IMS + OM) fractions and is expressed as % of the combined activities from all fractions. This sum of fraction activities is also noted in nmol min-1 mg-1 protein and is compared as % recovery with the + Triton rates in A. All data are from independent mitochondrial preparations, mean ± S.D. (n = 3).

**RESULTS**

Asc-glutathione Cycle Enzyme Activities in Arabidopsis Mitochondria: Latency and Subcompartmentalization—Measurement of APX, GR, DHAR, and MDHAR activities revealed that each could be detected in Arabidopsis mitochondria (Table I). The two-Percoll gradient method for the high purity separation of mitochondria from plastid and peroxisomes, yields mitochondria only contaminated 1.5% by plastids, and essentially free from peroxysomal and cytosolic compartments, based on marker enzymes (26). Maximal activities for each enzyme of the Asc-glutathione cycle measured in the presence of 0.05% Triton X-100 (Table 1A) were 2–5-fold lower than those reported in mitochondrial preparations from other plant species (15, 17), but were clearly measurable above background levels of auto-oxidation. The latency of these activities suggested that GR and MDHAR activities were largely inaccessible in intact mitochondria, indicating a matrix location. In contrast more than half the APX and DHAR activities could be assayed by provision of substrates to intact mitochondria, indicating a significant portion of activity is probably on the intermembrane space side of the mitochondrial inner membrane (Table I).

Under the same conditions, cytochrome c oxidase latency was in excess of 95%, proving a high degree of membrane integrity in our preparations. To further assess compartmentalization of these activities, fractions enriched in matrix proteins (MA), intermembrane space and outer membrane vesicle proteins (IMS + OM) and inner membrane proteins (IM) were prepared by osmotic stock and centrifugation. Contamination between compartments prepared by this protocol are <5% for the MA and IM samples, and about 25% MA and <5% IM in the IMS + OM fraction (27, 32, 33). The IMS + OM fraction represented 10%, the matrix sample 40% and the membrane fraction 50% of total mitochondrial protein. Based on these proportions, the distribution of activity between the fractions is presented as a percentage of total activity in mitochondria (Table 1B). By comparing the combined activities rate (Table 1B) with the + Triton solubilization data (Table 1A), the percentage activity recovery (70–110%) during this subfractionation process was determined to be consistent. Nearly 90% of the APX and MDHAR activities were present in the inner membrane fraction, with about 10% in the matrix fraction. The low latency of APX (Table 1A) coupled to the subfractionation data for this enzyme (Table 1B) suggests that much of this activity is likely to be on the IMS side of the inner membrane. In contrast, the high latency of MDHAR (Table 1A) coupled to subfractionation data (Table 1B) suggests a predominantly matrix side localization. The GR and DHAR activities predominated in the matrix, but are also found in the other fractions, suggesting they are partitioned between compartments and can be partially membrane associated. These data are consistent with the view that the majority of the APX faces the outside of the inner membrane, while an Asc-glutathione generating system (MDHAR, DHAR, GR) operates in the matrix and to a lesser extent in the IMS to regenerate Asc.
are likely 9 APX genes, 5 MDHAR genes, 5 DHAR genes, and 2 GR genes in *Arabidopsis*. Further, there is an extensive set of glutaredoxins (GDXN) that are likely to catalyze DHAR activities in *Arabidopsis* (full data not shown in Table II). DHAR activities purified from mammalian tissues are found to be GDXN (34, 35), the mammalian mitochondrial GDXN has DHAR activity (36) and DHAR activity of plant GDXNs is widely reported (37). All of these genes, except the APX At1g43220, were represented in EST databases at the time of this analysis, suggesting they are all expressed genes (Table II).

**Predicted Organelle Targeting of Asc-glutathione Cycle Gene Products**—A simple comparison of apparent molecular masses of each predicted gene product within a family suggests that different classes of each protein are represented and that some have N- or C-terminal extensions that may represent targeting information for localization within the cell (Table II). Analysis of the predicted targeting of each gene product for APX, MDHAR, DAHR, and GR by three targeting prediction programs is presented in Table II. Based on the predictions by each program we have made a consensus prediction for each product as organellar targeted, cytosolic, or unclear (−). From this analysis we predict that only 6 of the 21 genes encode proteins that are likely to be organellar-targeted. In the case of MDHAR, GR, and the classical DHAR family, only one gene product of each class has a clearly predicted organellar-targeting presequence (Table II). The three organellar APXs have already been assigned to chloroplast locations in the lumen (At4g09010), thylakoid membrane (At1g77490) and the stroma (At4g08390) (6). The organellar GR and MDHAR are also assigned as the main chloroplast isoforms in *Arabidopsis* (7, 38, 39). A GDXN with strong mitochondrial targeting prediction is also included in Table II.

**Proteomic Evidence for Presence of Proteins in Mitochondria**—We have also been undertaking a comprehensive program of *Arabidopsis* mitochondrial proteome analysis (21, 33) using two-dimensional gel electrophoresis and direct analysis of whole mitochondrial tryptic digestions. During this analysis peptides have been identified for several Asc-glutathione cycle enzymes that are present in purified mitochondria isolated from *Arabidopsis* cell culture (Table I). A set of 3 peptides match to APX At4g08390, a series of 4 peptides match to the MDHAR At1g63940, one peptide from the GR At3g54660 has been identified, one peptide from the DHAR At1g19570 and a set of 3 peptides matched to a GDXN At3g15660 (Table III). Matching of these peptides by ProID software against the full *Arabidopsis* protein set proved that these identifications were highly significant with most scores of greater than 90 for matches of MS/MS spectra and delta masses of <0.1 Da for matches of the parent peptides masses to predicted peptide masses of the matched proteins. Thus with the exception of DHAR At1g19570, the proteins detected by proteome analysis are all predicted to be organelle-located.

**Dual Targeting of APX, MDHAR, and GR Precursor Proteins to Chloroplasts and Mitochondria**—Using the bioinformatic and proteomic analysis we decided that the most likely candidates for mitochondrial-targeted proteins were APX At4g08390, MDHAR At1g63940, and GR At3g54660. The situation with DHAR was not clear, as proteomic data pointed to either At1g19570 or the GDXN At3g15660, while targeting predictions pointed to At5g16710. To further resolve this situation all three proteins were used for import studies, further, the only other DHAR significantly expressed, At1g75270, was also included for completeness.

To ensure that import, rather than simply association of proteins with organelles was occurring, a dual import system established in our laboratory (31) was utilized for import of *in vitro* synthesized [35S]methionine-labeled precursors of the test proteins and two control proteins (Fig. 1A). Assessing the import properties of APX At4g08390, MDHAR At1g63940, and GR At3g54660 using this system indicated that they were all imported and cleaved to a mature product in both chloroplasts and mitochondria. The mature products were protected from digestion by addition of thermolysin. In the case of APX there was an additional methionine residue 25 residues from the initiator methionine that resulted in an additional band upon translation. In order to determine which protein was dual targeted we removed the first and second methionines independently. Removal of the first methionine (APXΔM1) resulted in a single precursor protein that was still dual targeted (Fig. 1B).
A mitochondrial protein was digested by trypsin and subjected to MS/MS analysis. Peptides from five Asc-glutathione cycle components were identified, and the mass differences between observed and predicted peptides (delta mass), the ProID match score for the MS/MS spectra (score), and the peptide molecular mass (MM) are shown.

| Enzyme | Matched sequence | Delta mass | Score | Peptide MM |
|--------|------------------|------------|-------|------------|
| APX    | At4g08390        | 0.081      | 99    | 2244.1649  |
|        | DEDLIVLPTDAIIFEDSSFK | 0.052      | 96    | 1517.9307  |
|        | HAANGLVNALNLIK   | 0.068      | 79    | 1427.8230  |
|        | LPDAGPSPATHLRL   | 0.086      | 99    | 1580.9822  |
|        | GIEVIYEDPVAGADFEK| 0.010      | 98    | 1003.3909  |
|        | VEHDVVAR         | 0.027      | 97    | 961.5022   |
|        | GVLVESGEPFQQLPK  | 0.058      | 94    | 1828.0278  |
| MDHAR  | At1g63940        | 0.086      | 99    | 1580.9822  |
|        | GIEVIYEDPVAGADFEK| 0.010      | 98    | 1003.3909  |
|        | VEHDVVAR         | 0.027      | 97    | 961.5022   |
|        | GVLVESGEPFQQLPK  | 0.058      | 94    | 1828.0278  |
| GR     | At3g54660        | 0.043      | 94    | 1402.8032  |
|        | TLTSYLSRPRALLSNHR| 0.005      | 93    | 2210.2760  |
|        | AVSAPDDILQDSQPSQR| 0.005      | 93    | 1739.8153  |
|        | VPPDSPDSLKL      | 0.031      | 92    | 1057.5662  |
|        | SFISHWPTFQIFIK   | 0.051      | 90    | 1733.4006  |

**Fig. 1. Targeting of precursor proteins for Asc-glutathione cycle enzymes to mitochondria and chloroplasts.** A, import of precursor proteins into dual organelle import system. Lane 1, precursor protein alone. Lane 2, precursor protein incubated with pea leaf chloroplasts and soybean cotyledon mitochondria and chloroplasts re-purified. Lane 3, as lane 2 but with addition of thermolysin. Lane 4, precursor protein alone. Lane 5 precursor protein incubated with pea leaf chloroplasts and soybean cotyledon mitochondria and chloroplasts re-purified. Lane 6, as lane 5 but with addition of thermolysin after import reaction. Lane 7, as lane 5 but with addition of valinomycin prior to import. Lane 8, as lane 7 but with addition of thermolysin after import reaction. B, import of precursor proteins into IMS-depleted mitochondria. Lane 1, precursor protein alone. Lane 2, precursor protein incubated with soybean cotyledon mitochondria. Lane 3, as lane 2 but with addition of protease K after import reaction. Lane 4, as lane 2 but with IMS-depleted mitochondria in place of mitochondria. Lane 5, as lane 4 but with proteinase K added. p and m refer to the precursor and mature forms of the protein, respectively. C, import of precursor proteins for Asc-glutathione cycle enzymes into *Arabidopsis* mitochondria. Lane 1, precursor protein alone. Lane 2, precursor protein incubated with *Arabidopsis* mitochondria. Lane 3, as lane 2 but with addition of valinomycin prior to import. Lane 4, as lane 2 but with addition of valinomycin after import reaction. Lane 5, as lane 4 but with proteinase K after the import reaction.
and rounded shapes of
in size. The TOC64 control produced a plastid pattern of oval
five Asc-glutathione cycle genes were expressed well in both
of transcript abundance in leaves and roots revealed that all
using GFP as a marker for
plastid targeting. Thus under the conditions used we could
ing. However only GR displayed a pattern that also indicated
full-length protein for APX
/H9004
/.

Upon rupture of the outer membrane these controls show that
while AOX is fully protected, TIM23 is fully converted to a
lower molecular mass (Fig. 1B). Upon rupture and salt washing
of mitochondria imported APX protein was completely lost even
without protease treatment indicating that it was removed in
this treatment (Fig. 1B, lane 4). We have previously reported
that this washing procedure also removed cytochrome c from
the inner membrane (27, 32). Imported MDHAR and GR mature
proteins were partially resistant to proteolysis in mito-
plasts further supporting a direct partitioning of these products
between the IMS and the matrix spaces during import. These
Arabidopsis proteins could also be imported into mitochondria
from Arabidopsis cell culture. In each case the same pattern of
import was observed into Arabidopsis mitochondria (Fig. 1C)
as had been seen in soybean mitochondria (Fig. 1A).

The DHAR At1g75270 and At1g19570 were not cleaved to a
mature product after incubation with either organelle and were
resistant to all the proteases tested in the presence and absence
of organelles, thus protease protection of these proteins could
not be assessed to determine if import was occurring (data not
shown). The DHAR At5g16710, predicted to be the main or-
ganellar targeted form in Arabidopsis (Table II), did not appear
to be imported into either chloroplasts or mitochondria in vitro
(data not shown). The GDXN At3g15660 was clearly imported
and cleaved in mitochondria in a valinomycin-sensitive man-
ner, but was not imported into chloroplasts (Fig. 1A).

Additionally, we have also conducted in vivo subcellular lo-
calization experiments using full-length APXA M1, MDHAR,
GR, and GDXN fused to green fluorescent protein (GFP). Con-
structs were introduced by biolistic transformation into non-
green plant cell cultures and fluorescence observed (Fig. 2).
Non-green cultures were used as green plant material yields
high chloroplast autofluorescence and chloroplast-targeted pro-
teins were likely to obscure subtle co-targeting to mitochondria.
We used the soybean AOX presequence fused to GFP as a
mitochondrial control and Arabidopsis TOC64 fused to GFP as
a plastid control. The AOX control produced the expected pat-
tern for mitochondria, reticulate, punctate spots less than 1 µm
in size. The TOC64 control produced a plastid pattern of oval
and rounded shapes of ∼5 µm in diameter. Fusion of the
full-length protein for APXA M1, MDHAR, GR, and GDXN to
GFP all produced patterns that indicated mitochondrial target-
ing. However only GR displayed a pattern that also indicated
plastid targeting. Thus under the conditions used we could
definitely confirm a mitochondrial location for all four proteins
using GFP as a marker for in vivo subcellular localization.

Transcript Abundances and Gene Regulation—Comparison
of transcript abundance in leaves and roots revealed that all
five Asc-glutathione cycle genes were expressed well in both
tissue types (Fig. 3). In contrast, nuclear-encoded ribosomal
transcripts for the plastidial rps1 dominated in leaves, while
transcripts for the nuclear-encoded mitochondrial rps13 were
more evenly distributed. These data provide further evidence
that this set of organellar-targeting gene products are un-
likely to be destined for photosynthetic roles in leaf tissue
alone but rather are being synthesized in both root and shoot
tissue, consistent with a dual location in chloroplasts and
mitochondria.

Further assessment of gene expression was undertaken dur-
ing oxidative stress treatments (Fig. 4). A wide variety of stress
conditions and photosynthetic operation are known to induce
transcripts of the cytosolic and chloroplast enzymes of the
Asc-glutathione cycle in plants (5, 41–43). When we recorded
transcript abundances in light grown and dark-adapted leaves,
a 2–6-fold increase in the light in 4 of 5 transcripts was re-
vealed. Notably, GDXN, the only transcript not considered to
be multitargeted, is the only transcript that does not respond to
light/dark changes. Transcript changes induced by specific
chemical stresses in lighted leaves were then assessed. Norflu-
razon and paraquat are both capable of inducing chloroplast
oxidative stress, by inhibiting carotenoid synthesis and by act-
ing as an artificial electron acceptor from photosystem I, re-
spectively. Menadione leads to oxygen activation and lipid per-
oxidation generally in membranes of the cell acting as a
quinone-like analogue in one-electron donation reactions. An-
timycin A is a specific inhibitor of complex III in the inner
mitochondrial membrane and induces superoxide formation
from a highly reduced ubiquinone pool in vitro and in vivo. The
significant transcript fold changes induced by treatments (Fig.
4, asterisks) reveal that both plastidial and mitochondrial tar-
geted stresses induce the MDHAR, GR and DHAR/GDXN tran-
scripts. APX was not readily inducible by chemical stresses.
Overall norflurazon and menadione yielded a later response
peaking 2–6 h, while antimycin A and paraquat yielded more
rapid changes in transcript abundance. However, even within a
stress, some transcript responses were rapid (∼1 h) and had
abated to near control levels by 6 h (such as MDHAR and GR with paraquat) while other transcripts were still increasing at 6 h (notably DHAR with paraquat). This complexity of response magnitude and timing hints at further levels of control on this antioxidant cycle, but its dual response to functional stresses emanating from the two organelles reveals a coordination of response consistent with transcriptional regulation of this gene set.

**DISCUSSION**

The Case for Molecular Definition of the Mitochondrial Cycle—We have used a variety of molecular approaches to identify the proteins involved in the Asc-glutathione cycle in Arabidopsis mitochondria. The defined set of proteins involved has dual activities in the intermembrane space and matrix. Further we show that many of these proteins are dual-targeted to plastochrons and mitochondria, thus not representing a specific mitochondrial antioxidant defense cycle at the level of the nuclear genome.

In virtually all cases of authentically dual targeted proteins between mitochondria and plastochrons, the plastochron target is the stroma (44). Of the three organellar targeted APXs predicted in Table II, it was the stromal APX (At4g08390) that was found to be dual targeted to mitochondria and plastochrons (Table III, Figs. 1 and 2). Orthologs of APX At4g08390 in tea, spinach and tobacco also have confirmed plastochron stroma locations (45–47). A stromal location for GR and MDHAR activity provides the necessary NAD(P)H for these reactions, as also well known in plastochrons (2, 5). Superoxide needs to be dealt with directly at the site of action as its charge prevents easy diffusion across membranes and its rapid reaction retards the veracity of spatially separated detoxification systems. The product of the SOD reaction, H$_2$O$_2$, can readily diffuse from the site of production and has a substantially longer half-life in cells than superoxide. The potential for the cytosol, peroxisomes or even chloroplasts to facilitate scavenging of plant mitochondrial synthesized H$_2$O$_2$ is therefore plausible. In yeast and mammalian systems, mitochondria are clearly the major source of active oxygen species (AOS), and it makes sense to deal with these radicals at the point of production. In yeast and mammals, cytochrome c peroxidase, thioredoxin peroxidase, and glutathione peroxidase play the dominant role (18–20, 53). In plants, chloroplasts and peroxisomes predominate in terms of both AOS formation and breakdown rates (2). The argument that plant mitochondria need their own defenses against H$_2$O$_2$ has been presented in...
plants, a mitochondrial Asc-glutathione antioxidant defense against H$_2$O$_2$ could be as much about protection from AOS generated by other organelles as about defense from self.

The cycle we present in *Arabidopsis* has very similar sub-compartmentation characteristics as those described in pea and bean mitochondria (15, 17). APX is located in the IMS in plant mitochondria, which is analogous to the location of cytosome peroxidase in yeast (20, 53). In plants this localization makes use of the production of Asc in the IMS by galactono-gamma-lactone dehydrogenase that is also attached to the inner membrane (56), while the yeast counterpart utilizes reduced cytosome c from the electron transport chain on the IMS side of the inner membrane. However, the MDHA and DHA that results from APX in plant mitochondria must move to the matrix for reduction back to Asc. The largely matrix location of MDHAR, DHAR, and GR are presumably required for the provision of redundant in the form of NADH and NADPH to fuel these reactions from the tricarboxylic acid cycle and/or photorespiratory glycine decarboxylation. Transporters for this function are not known in plant mitochondria to date, however, a diverse range of possible MDHA and DHA transport routes have been proposed in different plant compartments (2, 5). As the latency of both DHAR and MDHAR were not as high as cytosome c oxidase in both our studies and those in pea and bean mitochondria (15, 17), an unidentified route for mitochondrial inner membrane transport of these compounds seems likely.

**Dual Targeting between Plastids and Mitochondria in Plants and the Implications for Oxidative Stress Defense**—Almost twenty proteins have been reported to be dual targeted using an ambiguous targeting signal directing a mitochondrial and plastidic location (44, 51), and several other genes encode dual located proteins by means of alternative splicing or translation initiation defining an organelle specific targeting signal on the same protein (57–59). Almost half of these proteins play roles in DNA or RNA metabolism such as repair, binding, or amino-acyl-tRNA synthetases. The remainder fall into various groups such as biosynthetic pathways, phosphoribosyl aminomimidazole synthase and phosphatidyglycerophosphate synthase I (60, 61), protein modification functions such as methionine aminopeptidase (62), and anti-oxidant activities such as the first dual-targeted protein GR from pea (9) and now our evidence of 61), protein modification functions such as methionine aminopeptidase (62), and anti-oxidant activities such as the first dual-targeted protein GR from pea (9) and now our evidence of further Asc-glutathione cycle enzymes. This list is likely to expand with the identification of genes involved in various DNA functions clustered on chromosome 3 in *Arabidopsis*, many suggested to be dual-targeted (63). All these are common housekeeping functions required in mitochondria and plastids.

Thus it appears that many diverse functions may be linked by a single gene in the genome, which helps coordinate the function and biogenesis of mitochondria and plastids without the need for additional pathways to facilitate intra-organellar communication (64, 65). So if any independent regulation needs to occur it must be via a different mechanism at the level of protein import or post-translational modification. We (66) and others (67, 68) have previously discussed the need for signaling pathways to coordinate antioxidant defense in different cellular organelles in plants. But it appears that at least in the context of the mitochondrial and the chloroplast Asc-glutathione cycle, this coordination occurs by dual targeting rather than subtle retrograde signaling. Indeed in our stress induction experiments it appears transcripts respond to both mitochondria and chloroplast-localized stresses redundantly (Fig. 4). This makes most sense if mitochondrial H$_2$O$_2$ defense is significantly against stray chloroplast H$_2$O$_2$ rather than against moderate internal mitochondrial production, or at least that chloroplast-dependent mitochondrial functions, such as photo-

---

**Fig. 4.** Real-time PCR analysis of transcript abundance changes in response to five AOS producing treatments. Two independent preparations of total RNA (1 μg) from *Arabidopsis* tissue was reverse-transcribed, and each cDNA was assayed in duplicate using real-time quantitative PCR. Tissue was harvested 1 and 6 h following treatment. Treatments were light versus dark adapted (light), norflurazon (norf), menadione (mena), paraquat (para), and antimycin A (AA). Fold differences (ratio) between treatments and control at 1 h (white) or 6 h (black) are shown. For light treatment the fold difference is (light)/dark adapted as outlined in methods. Asterisks indicate significant changes (Student’s *t* test, *p* < 0.05). Transcripts for APX (At4g08390), MDHAR (At1g63940), GR (At3g54660), DHAR (At1g19570), and GDXN (At3g15660) were assessed.
respiration, are a primary requirement for a mitochondrial Asc-glutathione cycle in plants.

Acknowledgment—We thank Dr. Joshua Heazlewood, University of Western Australia for mass spectrometry expertise.

REFERENCES
1. Asada, K., and Takahashi, M. (1987) in Photoinhibition (Kyle, D., Osmond, C., and Arntzen, C., eds) pp. 227–287, Elsevier, Amsterdam.
2. Noctor, G., and Foyer, C. H. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 249–279.
3. Artigoni, O., Dipierro, S., and Borraccino, G. (1981) FEBS Lett. 125, 242–244.
4. Foyer, C. H., and Halliwell, B. (1976) Planta 133, 21–25.
5. Asada, K. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 601–639.
6. Jespersen, H. M., Kuennigard, I. V., Ostergaard, L., and Welinder, K. G. (1997) Biochem. J. 326, 305–310.
7. Obara, K., Sumi, K., and Fukuda, H. (2002) Plant Cell Physiol. 43, 697–705.
8. Obara, K., Sumi, K., and Fukuda, H. (2002) Plant Cell 15, 167–175.
9. Prasad, T. K., Anderson, M. D., and Stewart, C. R. (1995) Plant Physiol. 108, 1597–1605.
10. Jimenez, A., Hernandez, J. A., Ros Barcelo, A., Sandalio, L. M., del Rio, L. A., and Sevilla, F. (1998) Physiol. Plant. 104, 687–692.
11. De Leonardi, S., Dipierro, N., and Dipierro, S. (2000) Plant Physiol. Biochem. 38, 773–779.
12. De Leonardi, S., De Lorenzo, G., Borraccino, G., and Dipierro, S. (1995) Plant Physiol. 109, 847–851.
13. Edwards, E. A., Rawsthorne, S., and Mullineaux, P. M. (1990) Plant Physiol. 180, 278–284.
14. Jimenez, A., Hernandez, J. A., Del Rio, L. A., and Sevilla, F. (1997) Plant Physiol. 114, 275–284.
15. Day, D. A., Baird, L. M., Langeberg, L., Taugher, C. Y., Anyan, W. R., Vance, C. P., and Sarath, G. (1993) Plant Physiol. 102, 481–489.
16. Iturbe-Ormaetxe, I., Matamoros, M. A., Rubio, M. C., Dalton, D. A., and Leister, D., and Jarvis, P. (2003) Plant Cell 15, 1859–1871.
17. Miranda-Vizuete, A., Kamitani, T., Takada, T., and Shigeoka, S. (1997) Biochem. J. 328, 795–800.
18. Beardale, T. A., Bay-Chowdhury, S., Jaiswal, P., Buhot, L., Labbe-Mache, S., Stern, D. B., and Allison, L. A. (2002) Plant J. 31, 199–209.
19. Kubis, S., Baldwin, A., Patel, R., Bazzaz, R., Dupree, P., Lilley, K., Kurth, J., Leister, D., and Jarvis, P. (2003) Plant Cell 15, 1559–1570.
20. Dahlin, C., and Cline, K. (1998) Plant J. 13, 1131–1140.
21. Chev, O., and Whelan, J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 805–812.
22. Bienz, R., d. V., Lombardo, V. A., Ceccarelli, E. A., and Ottado, J. (2002) Eur. J. Biochem. 298, 541–549.
23. Charizanis, C., Nukane, H., Muro, B., and Eisinger, K. D. (1999) Mol. Gen. Genet. 262, 437–447.
24. Taylor, N. L., Day, D. A., and Millar, A. H. (2002) J. Biol. Chem. 277, 42663–42668.
25. Gomez, J. M., Hernandez, J. A., Jimenez, A., del Rio, L. A., and Sevilla, F. (1999) Free Radic. Res. 31, suppl. S1–S18.
26. Bartoli, C. G., Pastori, G. M., and Foyer, C. H. (2000) Plant Physiol. 123, 335–344.
27. Kobayashi, Y., Dokiya, Y., and Sugita, M. (2001) Biochim. Biophys. Res. Commun. 289, 1106–1113.
28. Watanabe, N., Che, F. S., Iwano, M., Takayama, S., Yoshida, S., and Ishiga, A. (2001) J. Biol. Chem. 276, 20474–20481.
29. Challegas, M. S., Luche, D. P., Van Sluys, M.-A., Menn, C. F. M., and Silva-Filho, M. C. (2003) J. Cell Sci. 116, 285–291.
30. Babich, M. C., and Shigeoka, S. (2003) Plant J. 20, 899–909.
31. Gottig, D. E., Lipscombe, R., Fedorova, E., Millar, A., Mann, A., Atkins, C. A., and Smith, P. M. (2003) Plant Physiol. 131, 1013–1041.
32. Giglione, C., Serero, A., Pierre, M., Boisson, B., and Meinnel, T. (2000) EMBO J. 19, 5916–5929.
33. Elo, A., Lyznik, A., Gonzalez, D. O., Kachman, S. D., and Mackenzie, S. A. (2003) Plant Cell 15, 1619–1631.
34. Larkin, R. M., Alonso, J. M., Ecker, J. R., and Chory, J. (2003) Science 299, 902–906.
35. Strand, A., Asami, T., Alonso, J., Ecker, J. R., and Chory, J. (2003) Nature 421, 79–83.
36. Millar, A. H., Considine, M. J., Day, D. A., and Whelan, J. (2001) IUBMB Life 51, 201–205.
37. Müller, M. I. (2001) Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 561–591.
38. Dutileille, C., Garmier, M., Noyer, G., Mathieu, C., Chretier, P., Foyer, C. H., and de Paep, R. (2003) Plant Cell 15, 1212–1226.
Molecular Definition of the Ascorbate-Glutathione Cycle in Arabidopsis
Mitochondria Reveals Dual Targeting of Antioxidant Defenses in Plants
Orinda Chew, James Whelan and A. Harvey Millar

J. Biol. Chem. 2003, 278:46869-46877.
doi: 10.1074/jbc.M307525200 originally published online September 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307525200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2003/09/15/M307525200.DC1

This article cites 66 references, 27 of which can be accessed free at
http://www.jbc.org/content/278/47/46869.full.html#ref-list-1