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Ascorbate biosynthesis and regulation in Chlamydomonas

Impact of oxidative stress on ascorbate biosynthesis in Chlamydomonas via regulation of the *VTC2* gene encoding a GDP-L-galactose phosphorylase

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Background: Ascorbate biosynthesis in plants occurs mainly via the L-galactose pathway. **Results**: *Chlamydomonas reinhardtii VTC2* encodes a GDP-L-galactose phosphorylase whose transcript levels are induced in response to oxidative stress concurrent with increased ascorbate accumulation.

Conclusion: Increased oxidative stress in *C. reinhardtii* results in an enzymatic and non-enzymatic antioxidant response.

Significance: First characterization of *C*. *reinhardtii* ascorbate biosynthesis and recycling pathways.

SUMMARY

The L-galactose (Smirnoff-Wheeler) pathway represents the major route to L-ascorbic acid (vitamin C) biosynthesis in higher plants. Arabidopsis thaliana VTC2 and its paralogue VTC5 function as **GDP-L-galactose** phosphorylases converting GDP-L-galactose to L-galactose-1-P, thus catalyzing the first committed step in the biosynthesis of Lascorbate. Here we report that the L-galactose pathway of ascorbate biosynthesis described in higher plants is conserved in green algae. The Chlamydomonas reinhardtii genome encodes all the enzymes required for vitamin C biosynthesis via the L-galactose pathway. We have characterized recombinant C. reinhardtii VTC2 as an active **GDP-L-galactose**

phosphorylase. C. reinhardtii cells exposed to oxidative stress show increased VTC2 mRNA and L-ascorbate levels. Genes encoding enzymatic components of the ascorbateglutathione system (e.g. ascorbate peroxidase, Mn superoxide dismutase, dehydroascorbate reductase) are also up-regulated in response to increased oxidative stress. These results indicate that C. reinhardtii VTC2, like its plant homologs, is a highly regulated enzyme in ascorbate biosynthesis in green algae and that, together with the ascorbate recycling system, the L-galactose pathway represents the major route for providing protective levels of ascorbate in oxidatively stressed algal cells.

L-ascorbic acid plays an essential role in plants by protecting cells against oxidative damage. In addition to its antioxidant role, Lascorbic acid is also an important enzyme cofactor, for example in violaxanthin deepoxidase, required for dissipation of excess excitation energy, and prolyl hydroxylases (1-3). In plants, several pathways have been proposed to function in L-ascorbic acid biosynthesis. The best described pathway, the Smirnoff-Wheeler pathway the L-galactose pathway, involves ten or enzymatic steps to convert D-glucose to L-ascorbic acid via intermediate formation of GDP-Dmannose, GDP-L-galactose, L-galactose-1-P, Lgalactose and L-galactono-1,4-lactone (4).

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Whereas the initial six steps are also involved in cell wall/glycoprotein biosynthesis, GDP-L-galactose phosphorylase (VTC2/VTC5) catalyzes the first committed step in L-ascorbic acid biosynthesis forming L-galactose-1-P (5,6). L-galactose-1-P phosphatase (VTC4), L-galactose dehydrogenase (L-Gal-DH) and L-galactono-1,4-lactone dehydrogenase (GLDH) catalyze the final steps in the Smirnoff-Wheeler pathway in higher plants such as *A. thaliana* (7-9).

The biosynthesis of L-ascorbic acid is not characterized in detail in the green algae. Unicellular green algae such as the chlorophytes Chlorella pyrenoidosa and Prototheca moriformis can synthesize L-ascorbate using the L-galactose pathway (10-12). Two other photosynthetic unicellular protists (Euglena gracilis and Ochromonas danica) (13,14) and a diatom (*Cyclotella cryptica*) utilize the inversion pathway commonly found in animals (Supplemental Figure S1) (15). Here we provide evidence that the Smirnoff-Wheeler pathway is completely conserved in the green alga C. reinhardtii. The VTC2 protein from C. reinhardtii is highly similar to higher plant VTC2/VTC5, containing the HxHxH motif characteristic of members of the HIT protein superfamily of nucleotide hydrolases and transferases (16).

Higher plants facing increased oxidative stress exhibit, in addition to increased VTC2 mRNA and activity levels, elevated transcript abundance for all the enzymes of the vitamin C recycling pathway (ascorbate-glutathione system) in the chloroplast including ascorbate peroxidase monodehydroascorbate (APX), reductase (MDAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GSHR) (2,17). In this work, we found that C. reinhardtii cells facing oxidative stress have increased abundance of VTC2 transcripts and all the enzymes of the ascorbate-glutathione system, as well as higher total ascorbate content. This suggests that C. reinhardtii cells respond to oxidative stress by producing more L-ascorbic acid both via de novo synthesis through the L-galactose pathway and via increased recycling.

EXPERIMENTAL PROCEDURES

Materials – ADP-D-Glc, GDP-D-Glc, GDP-D-Man, UDP-D-Gal, UDP-D-Glc (all in the

α-configuration), GDP-β-L-Fuc, and GDP were from Sigma. GDP-β-L-Gal, synthesized and purified as described (18) was provided by Prof. Shinichi Kitamura (Osaka Prefecture University). This preparation was further purified by the reversed-phase HPLC method described in (5). Fractions containing GDP-L-Gal were lyophilized, resuspended in H₂O, and stored at -20 °C. Hydrogen peroxide (30%) and *tert*-butyl hydroperoxide (tBuOOH) (70%) were purchased from Fisher and Lancaster Synthesis, Inc., respectively. Ascorbate oxidase from *Cucurbita sp.* (EC 1.10.3.3; A0157) was purchased from Sigma.

Strains and Culture Conditions – C. reinhardtii strains 2137 (CC1021) and CC425 were obtained from the Chlamydomonas culture collection (Duke University) and grown in Trisacetate-phosphate (TAP) medium (19) at 24 °C and 50-100 μ mol m⁻² s⁻¹ light intensity.

Sequencing of C. reinhardtii VTC2 – The VTC2 cDNA clone MXL096d05 (corresponding to EST BP098619) was completely sequenced. It contains the entire predicted VTC2 open reading frame, 1857 bp long, encoding a protein of 618 amino acids. The open reading frame is flanked by 499 nt of 5' untranslated region and a 3' untranslated region of 1396 nt followed by a 68 nt poly(A) tail. The complete VTC2 sequence has been deposited in NCBI (GenBank accession JQ246433).

VTC2 Cloning - The VTC2 expression construct was generated by nested PCR and the Gateway recombinational cloning system (Invitrogen, Carlsbad, CA) as described (20). Briefly, the coding sequence of VTC2 (amino acids D2-A618) was amplified with Phusion polymerase (New England Biolabs) from plasmid MXL096d05 using gene-specific primers with 5' extensions encoding a TEV protease cleavage site in the forward primer (VTC2.D2) and a C-terminal hexahistidine tag followed by a stop codon in the reverse primer (VTC2.A628) (Supplemental Table 4). The initial product was then amplified with a second set of primers to introduce AttB1 and AttB2 recombination sites (PE-277 and PE-278) (Supplemental Table 4). Amplification products were gel purified and recombined into the donor vector pDONR201 and subsequently into the expression vector pKM596 (20) to produce an Nterminal His-tagged maltose binding protein

(MBP) fusion using the Invitrogen protocol. DNA sequencing (Genewiz) was used to confirm the sequence of the expression construct.

VTC2 Expression and Purification – The expression plasmid was transformed into E. coli BL21-Gold (DE3) cells (Novagen). Cells were grown in LB medium at 37 °C to an OD_{600 nm} of 0.6 at which point the temperature was shifted to 18 °C and protein expression induced by the addition of IPTG to a concentration of 1 mM. Cell growth was continued overnight and the cells were collected by centrifugation the following day. The cell pellet was resuspended in wash buffer (20 mM Tris pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.2% NP40, 10% glycerol) supplemented with protease inhibitor cocktail (Sigma), PMSF (100 μ M), DNase (20 μ g mL⁻¹), a few crystals of lysozyme, and 10 mM β -mercaptoethanol. Cells were lysed using a French press. The lysate was clarified by centrifugation (30 min at $35,000 \times g$) and the supernatant incubated with Ni-NTA agarose beads (Qiagen) for 60 min at 4 °C. The beads were washed extensively with lysis buffer and bound protein eluted with elution buffer (lysis buffer containing 300 mM imidazole). VTC2 was further purified by size exclusion chromatography using a HiLoadSuperdex S-200 column (GE Life Sciences) equilibrated in 20 mM Tris pH 8.0, 300 mM NaCl, and 10% glycerol. Peak fractions were analyzed by SDS-PAGE and those containing VTC2 were pooled and concentrated. Two peaks containing VTC2 MBP fusion proteins were obtained by size exclusion chromatography. Both peaks contained pure MBP-VTC2 fusion protein and they were pooled separately and concentrated. The fraction showing the highest activity was used for enzymatic analyses.

Nucleic Acid Analysis – Total RNA was extracted from exponentially growing *C. reinhardtii* cells as previously described (21). RNA quality was assessed using an Agilent 2100 Bioanalyzer and by RNA blot hybridization for *CBLP* as described (22). The probe used for detection was a 915-bp EcoRI fragment from the cDNA insert (encoding *CBLP*) in plasmid pcf8-13 (23).

Quantitative Real-Time PCR on cDNA – cDNA synthesis and quantitative real-time PCR was performed on technical triplicates as described (22) using gene-specific primers listed in Supplemental Table 5. The data are presented as the fold change in mRNA abundance, normalized to an endogenous reference transcript (*CBLP* or *UBQ2*), relative to the sample grown before 1 mM H_2O_2 or 0.1 mM tBuOOH treatment (time zero). The abundance of the two reference transcripts did not change under the conditions tested.

Ascorbate Measurements – C. reinhardtii cells were grown in TAP medium to a density of 3 x 10^6 cells mL⁻¹, collected by centrifugation at 2,500 x g for 5 min, resuspended in extraction buffer containing 2% metaphosphoric acid, 2 mM EDTA and 5 mM DTT and stored at -80 °C. To prepare extracts for vitamin C analysis, cells were lysed by freeze/thaw cycling and the soluble fractions were separated by centrifugation (16,100 x g, 10 min at 4 °C). Vitamin C content was measured by reversed-phase HPLC on an Econosphere C-18 column (5 µm bead size, 4.6 x 250 mm; Alltech Associates, Deerfield, IL) using a Hewlett Packard Series II 1090 liquid chromatograph. A mobile-phase gradient of 0 to 40% acetonitrile in 20 mM triethyl ammonium acetate (TEAA), pH 6.0 was used at a flow rate of 1 mL min⁻¹. The injection volume was 50-100 μ L. Ascorbic acid was detected by monitoring the absorbance at 265 nm. The ascorbic acid peak was identified by comparison with the elution time of an L-ascorbate standard and by demonstrating decrease of the peak area after treatment of the samples with ascorbate oxidase. This treatment was performed by adding 2 U of ascorbate oxidase from Cucurbita sp. (EC 1.10.3.3) to 60 µL of the extract in a final concentration 0.12 M monosodium citrate for 1 h at 4 °C. The final pH of the reaction was about 5.6 using pH paper. The differences in the peak areas measured before and after addition of ascorbate oxidase were used to calculate ascorbic acid levels based on a standard curve. The cellular concentration of L-ascorbate was determined using a cell volume of 140 fL (24).

HPLC-based Nucleoside Diphosphate (NDP)-Hexose Phosphorylase Assay – NDPhexose phosphorylase activities of recombinant VTC2 enzyme were assayed by measuring NDP formation after incubation with NDP-hexose in a reaction mixture at pH 7.5 containing 50 mM Tris-HCl, 5 mM sodium phosphate, 10 mM NaCl and 1 mM DTT. Reactions (26 °C) were initiated by enzyme addition and stopped after 5 to 10 min by heating at 98 °C for 5 min. After removal of precipitated protein by centrifugation, supernatants were analyzed by anion-exchange HPLC as described in (5). NDP and NDP-hexose concentrations were calculated by comparing the integrated peak areas with those of standard NDP or NDP-hexose solutions. GraphPad Prism (La Jolla, CA) was used to calculate $K_{\rm m}$ and $V_{\rm max}$ values.

RNA-Seq - Total RNA samples prepared reinhardtii strain 2137 from С. grown photoheterotrophically in the presence of 1 mM H₂O₂ for 30 and 60 minutes were sequenced on a GAIIx platform. cDNA libraries were made using Illumina's protocol and sequenced as single-end 76mers. Raw and processed sequence files are available at the NCBI Gene Expression Omnibus (accession GSE34826). Sequence reads were aligned using Bowtie (25) in single-end mode and with a maximum tolerance of 3 mismatches to the Au10.2 transcript sequences corresponding to the version 4 assembly of the C. reinhardtii genome. Expression estimates were obtained for each individual run in units of RPKMs (reads per kilobase of mappable transcript length per million mapped reads) (26), after normalization by the number of aligned reads and transcript mappable length (27). Technical replicates were averaged to obtain per-sample expression estimates. Final expression estimates and fold changes were obtained for each of the biological replicate.

Sequence and Phylogenetic Analyses – To search for orthologues/homologues of the A. thaliana members of the L-galactose pathway in green algae, two BLAST searches were performed. In the first, protein sequences of the A. thaliana proteins were used as queries to search against algal genomes databases. Sequences of putative homologues were retrieved and used for the second query by performing BLASTp against the A. thaliana database. If in the second query the highest-scoring homologue in A. thaliana was exactly the original A. thaliana query sequence, that protein was considered an orthologue (mutual best hit). Phylogenetic relationships were inferred using the Maximum Likelihood method based on the Whelan and Goldman model (28). The tree with the highest log likelihood is shown. Branch lengths reflect the number of substitutions per site. All positions containing gaps and missing data were eliminated. Alignment of putative VTC2 homologs was performed using MUSCLE, and

evolutionary analyses were conducted in MEGA5 (29).

RESULTS

The C. reinhardtii genome encodes a homolog of plant GDP-L-galactose phosphorylase - Biosynthesis of vitamin C in higher plants occurs mainly via the L-galactose pathway (9). In A. thaliana, the first committed step in the sequence of ten enzymatic reactions from Dglucose to L-ascorbate is conversion of GDP-Lgalactose to L-galactose-1-P, a reaction catalyzed by the GDP-L-galactose phosphorylase VTC2. Therefore, we were interested in finding homologs of VTC2 in C. reinhardtii and other green algae such as Volvox carteri, Chlorella sp. NC64A, Coccomvxa sp. C169, Micromonas sp. RCC299, and Ostreococcus RCC809. BLASTp searches identified a VTC2 homolog (Cre13.g588150) in C. (Supplemental Figure reinhardtii S2). Cre13.g588150 exhibits 46% amino acid sequence identity to A. thaliana VTC2. The A. thaliana genome encodes a VTC2 paralog, VTC5, which shows enzymatic properties similar to those of VTC2 (30,31). The C. reinhardtii protein has 47% identity to VTC5 at the amino acid level. Since the C. reinhardtii genome encodes only a single protein highly similar to A. thaliana VTC2/VTC5, we termed Cre13.g588150 VTC2. The amino acid sequence of the VTC2 protein from C. reinhardtii does not contain any transmembrane domains. Several subcellular localization prediction programs (ChloroP, TargetP, Psort and PredSL) indicated that C. reinhardtii VTC2 does not possess obvious organellar targeting sequences, suggesting that, like the plant homologs, it is most likely a cytosolic protein. C. reinhardtii VTC2 contains a highly conserved histidine triad (HIT) motif (HxHxH, where x is a hydrophobic residue) (Supplemental Figure S2). C. reinhardtii VTC2 is more closely related to the Volvox VTC2 protein and among algal homologs it appears that the Micromonas sp. and Ostreococcus sp. proteins are more closely related to higher plant VTC2 proteins than to the animal VTC2 homologs (Figure 1).

Enzymatic components of the L-galactose pathway to vitamin C biosynthesis are conserved in green algae – Since higher plant VTC2 has orthologs in C. reinhardtii and other green algae, we investigated whether the green algae encode the rest of the components of the Smirnoff-Wheeler pathway. BLASTp and tBLASTn searches identified orthologs (defined as mutual best BLAST hit) for almost all L-galactose pathway enzymes in six green algae (C. reinhardtii, V. carteri, Chlorella sp. NC64A, Coccomyxa sp. C169, Micromonas sp. RCC299 and Ostreococcus lucimarinus). Orthologs of A. thaliana phosphomannose isomerase (PMI1), phosphomannomutase (PMM), GDP-D-mannose 3",5"-epimerase L-galactose-1-P (GME1), phosphatase (VTC4), L-galactose dehydrogenase (L-Gal-DH), and L-galactono-1,4-lactone dehydrogenase (GLDH) are present in all six species (Figure 2 and Supplemental Table 1). Interestingly, our sequence analysis identified orthologs of GDP-D-mannose pyrophosphorylase (VTC1) in C. reinhardtii, V. carteri, Chlorella sp. NC64A, and Coccomyxa sp. C169, but not in Micromonas sp. (RCC299 and M. pusilla) nor in Ostreococcus sp. (O. tauri, O. lucimarinus and O. RCC899) (Figure 2 and Supplemental Table 1). Micromonas sp. and Ostreococcus sp. both belong to the class Prasinophyceae, which diverged at the base of the algal lineage and are therefore more distantly related to the chlorophyte algae. It is possible that Micromonas spp. and Ostreococcus spp. synthesize GDP-D-mannose using a different pathway (see Discussion). Overall, we conclude that the L-galactose pathway to L-ascorbate biosynthesis is conserved in the green algae.

Since alternative L-ascorbate biosynthetic pathways have been proposed (7,32), we searched for orthologs/homologs of the enzymes catalyzing the proposed steps in these alternate pathways (Supplemental Figure S1). First, the proposed Lgulose pathway (33) involves the A. thaliana GDP-D-mannose 3",5"-epimerase (GME1) which is orthologous to C. reinhardtii SNE1. This enzyme can form GDP-L-gulose, which, if converted to L-gulono-1,4-lactone, would provide a substrate for an oxidase reaction leading directly to L-ascorbate. Although C. reinhardtii GLDH demonstrates 30% amino acid sequence identity to L-gulono-1,4-lactone dehydrogenase the rat (Gulo) (34), and two other similar /oxidase proteins are present (Cre14.g611650 with 26% amino acid identity and Cre03.g177600 with 22 % amino acid identity), these putative enzymes have not been characterized and no homologs of enzymes converting GDP-L-gulose to L-gulono-1,4-lactone have been found.

L-gulono-1,4-lactone could also be potentially formed from mvo-inositol via Dglucuronate (Supplemental Figure S1; animal-like pathway). C. reinhardtii codes for a potential myoinositol oxidase (Cre01.g025850) that might be responsible for the formation of D-glucuronate and shows 31% amino acid identity to A. thaliana MIOX4 (Supplemental Table 1). Conversion of Dglucuronate to L-gulonate would require the action of a glucuronate reductase, which has not been identified in plants. Formation of L-gulono-1,4requires from L-gulonate lactone an aldonolactonase (gulonolactonase) (35). SMP30 (senescence marker protein 30) has been recently identified to function as а glucono/ gulonolactonase (36). The C. reinhardtii genome does not encode a homolog to SMP30. Hence, it seems unlikely that C. reinhardtii would use this route as an alternate pathway to vitamin C biosynthesis.

It has also been proposed that biosynthesis of L-ascorbic acid could occur via the galacturonate or salvage pathway (7.9)(Supplemental Figure S1). This pathway would involve conversion of methyl-D-galacturonate to D-galacturonate. The enzyme catalyzing this reaction has not yet been identified. Formation of L-galactonate from D-galacturonate is catalyzed in ripening strawberry fruits by an aldo-keto reductase specific for D-galacturonate (GalUR) (37). The C. reinhardtii genome encodes several aldo-keto reductases with homology to strawberry D-galacturonate reductase (Supplemental Table 1), but none of them is an ortholog of the plant enzyme. On the other hand, orthologs of the strawberry GalUR are present in other algal species such as Chlorella sp. NC64A, Micromonas sp. RCC299, O. lucimarinus or V. carteri (Supplemental Table 1). The penultimate reaction in the galacturonate pathway (L-galactonate to Lgalactono-1,4-lactone conversion) would require the function of an aldonolactonase which has been recently characterized in the protist Euglena gracilis (38). BLASTp and tBLASTn searches did not identify any homologs to E. gracilis aldonolactonase in C. reinhardtii or V. carteri, but orthologs are present in Chlorella sp. NC64A, Coccomyxa sp. C169, Micromonas sp. RCC299, and O. lucimarinus (Supplemental Table 1).

We conclude that essential components of the alternate pathways to L-ascorbate biosynthesis are missing in *C. reinhardtii*. We could identify homologs of L-gulono-1,4-lactone dehydrogenase for the L-gulose pathway and of galacturonate reductase for the salvage pathway, but there are no orthologs to rat Gulo or strawberry GalUR and the sequence similarity is poor. On the other hand, we show that all the components of the plant Smirnoff-Wheeler pathway have orthologs in *C. reinhardtii* and in other algal species. These results point to a conserved L-galactose pathway to Lascorbate biosynthesis, which might represent the major route to L-ascorbate biosynthesis in algae, in particular *C. reinhardtii* and other Volvocales.

Recombinant C. reinhardtii VTC2 is a GDP-L-galactose/GDP-D-glucose phosphorylase - Previous studies demonstrated that A. thaliana are GDP-L-galactose VTC2 and VTC5 phosphorylases, converting GDP-L-galactose into L-galactose-1-P and GDP in the presence of Pi (5,30,31). To test whether C. reinhardtii VTC2 can catalyze this reaction, recombinant C. reinhardtii VTC2 was purified as a His- and MBPtagged protein. Since A. thaliana VTC2 shows GDP-L-galactose and **GDP-D-glucose** phosphorylase activities, we first determined the activity of the C. reinhardtii enzyme on various sugar nucleotides in the presence of inorganic phosphate (Table 1). Similar activity was seen with GDP-L-Gal and GDP-D-Glc, whereas a twofold lower activity was found with GDP-L-Fuc. No significant phosphorylase activity was measured with GDP-D-Man, UDP-D-Glc, UDP-D-Gal and ADP-D-Glc (Table 1). Thus, our data indicate that C. reinhardtii VTC2 possesses similar nucleotide sugar substrate specificity as A. thaliana VTC2.

The conserved HIT motif (HxHxH) in *C.* reinhardtii VTC2 is typical of HIT hydrolases, whereas plant VTC2 proteins contain a HIT motif typical for HIT transferases/phosphorylases (HxHxQ) (16). Therefore, we tested the acceptor specificity of *C. reinhardtii* VTC2 by measuring the GDP-L-Gal or GDP-D-Glc consumption after incubation of the recombinant VTC2 enzyme with different possible acceptors. When recombinant *C. reinhardtii* VTC2 was incubated in the absence of P_i , we detected no hydrolytic activity (Figure 3). However, in the presence of P_i , we observed a dramatic increase in GDP-L-Gal and GDP-D-Glc consumption (Figure 3). Incubation of the enzyme with pyrophosphate (PP_i), D-Glc-1-P (in the presence of GDP-L-Gal) or L-Gal-1-P (in the presence of GDP-D-Glc) did not result in any significant nucleotide sugar substrate consumption (Figure 3). Additionally, we did not detect the formation of GMP or GTP, the expected products of hydrolase or pyrophosphorylase activity, under any of these conditions (data not shown). These data clearly indicate that *C. reinhardtii* VTC2 is a phosphorylase like the Arabidopsis enzyme.

C. reinhardtii VTC2 has similar, low micromolar, Michaelis constants for both GDP-L-Gal and GDP-D-Glc (Table 2). Interestingly, with *C. reinhardtii* VTC2 we have found at least 10 times higher K_{cat} values for both substrates compared to the *A. thaliana* VTC2 recombinant enzyme, leading to about 10 times higher catalytic efficiencies for the former than for the latter enzyme (Table 2).

VTC2 transcript levels and ascorbate levels are increased in response to oxidative stress - Previous studies have indicated that A. thaliana VTC2 mRNA levels are increased in leaves subjected to high light (30) and in seedlings grown in the light compared to those grown in the dark (39). Therefore, we tested whether transcript levels of C. reinhardtii VTC2 respond to oxidative stress. C. reinhardtii was grown photoheterotrophically to 2 x 10^6 cells mL⁻¹, then challenged with 1 mM H₂O₂ or 0.1 and 0.2 mM tert-butyl-hydroperoxide (tBuOOH) for 30, 60, 120 and 240 min. Both H₂O₂ and tBuOOH (an organic peroxide capable of inducing lipid peroxidation) treatments enhance intracellular ROS production. The concentrations of H₂O₂ and tBuOOH and time points used in this study were previously shown to have no effect on cell growth in C. reinhardtii and yet were high enough to induce the antioxidant defense mechanisms (40-43). A lower concentration of tBuOOH was used because it was more stable than H₂O₂ under our culture conditions (Supplemental Figure S3). VTC2 transcript abundance was assessed by real-time PCR. We found that VTC2 mRNA transcript abundance increased 4-fold after 30 min and reached a maximum of 7-fold induction after 120 min exposure to 1 mM H₂O₂ (Figure 4 A). When C. reinhardtii cells were exposed to 0.1 mM tBuOOH, we observed a more dramatic induction in the VTC2 transcript levels (50-fold increase after 30 min with the highest induction of 155-fold after 240 min). Increasing the tBuOOH concentration to 0.2 mM resulted in an even higher increase in the *VTC2* mRNA abundance (150-fold after 30 min and 250-fold after 120 min) (Figure 4 A).

To assess the overall impact of peroxide stress on the Smirnoff-Wheeler pathway, we quantified the abundance of transcripts for each gene in the pathway in H₂O₂-treated vs. untreated cells. Changes in the VTC2 transcript levels after H₂O₂ exposure observed by RNA-Seq are very similar to those observed by real-time PCR (6.4fold induction after 30 min and 8.6-fold induction after 60 min) (Figure 4 B). Other components of the pathway including MPII, PMM and GMPI showed at best a 2-fold increase in their transcript abundance after 60 min of exposure to H_2O_2 . The transcript levels of SNE1, VTC4, L-GalDH and GLDH did not change significantly (Figure 4 B and Supplemental Table 2) in response to H_2O_2 treatment. Together, the combined real-time PCR and RNA-Seq analyses demonstrated that VTC2 mRNA levels are highly and selectively induced by oxidative stress, indicating that the GDP-Lgalactose phosphorylase step is potentially the key regulatory point of the L-ascorbate biosynthetic pathway in C. reinhardtii.

Next, we asked the question whether the increased VTC2 mRNA levels correlate with a change in ascorbate content. Total ascorbate levels were measured in cell extracts from C. reinhardtii grown under 1 mM H₂O₂ or 0.1 mM tBuOOH stress for 2, 4, 6 and 8 h. Total ascorbate content increased progressively after addition of H₂O₂, showing a slight increase after 2 h and reaching a maximum after 8 h, where we measured 7-fold higher ascorbate concentrations than in untreated cells (Figure 5 A). On the other hand, cells treated with 0.1 mM tBuOOH displayed a 4-fold higher ascorbate content 2 h after addition of tBuOOH, with a further increase after 4 h (5-fold). In contrast to C. reinhardtii cells exposed to H₂O₂, after 6 h of tBuOOH treatment we noticed a drop in the total ascorbate levels (3-fold more compared to untreated cells) which decreased even further after 8 h to levels similar to those observed for untreated cells (Figure 5 B). The observation that tBuOOH treatment depletes cellular ascorbate has been made previously in rat hepatocytes (44) and rat astrocytes (45). Altogether, our results indicate a correlation between the VTC2 mRNA levels and

L-ascorbic acid content in *C. reinhardtii* cells exposed to oxidative stress.

Genes encoding the components of the ascorbate-glutathione scavenging system are induced in response to oxidative stress - The ascorbate-glutathione cycle is a well known mechanism to scavenge H₂O₂ in various cell compartments (2), particularly in plants (46) (and see Fig. 7). Therefore, we were interested in expression profiles of the genes encoding the ascorbate-glutathione system components in C. reinhardtii cells exposed to 1 mM H₂O₂ or 0.1 -0.2 mM tBuOOH for 30, 60, 120 or 240 min. In plants, and most likely also in C. reinhardtii, Photosystem I is the major site for superoxide production (O_2) (17), anion which is disproportionated to H₂O₂ by the action of one or several superoxide dismutases. Here we found that in C. reinhardtii, MSD3 transcript levels (encoding plastid-localized MnSOD3) are highly induced in response to peroxide treatment (Figure 6 A). Treatment of C. reinhardtii cells with 1 mM H₂O₂ resulted in a 2-15 fold induction of this gene over the 4 h exposure period. An even higher level of up-regulation (100-fold after 60 min) was reached when C. reinhardtii cells were exposed to 0.1 mM tBuOOH. H₂O₂ produced by MnSOD3 is reduced to H₂O by ascorbate in a reaction catalyzed by ascorbate peroxidase (APX1). The mRNA abundance of C. reinhardtii APX1 was induced 2-4 fold after exposure to 1 mM H₂O₂, whereas 0.1 mM tBuOOH treatment resulted in a 10- to 15fold induction of APX1 transcript levels (Figure 6 A). Ascorbate peroxidase oxidizes ascorbate to monodehydroascorbate, which is either reduced to ascorbate by the action of monodehydroascorbate reductase (MDAR1), spontaneously or disproportionates to dehydroascorbate. MDAR1 mRNA abundance was induced in response to 1 mM H₂O₂ (5- to 6-fold after 120 min) whereas 0.1 mM tBuOOH treatment resulted in a more subtle to 3-fold up-regulation of this gene. 2-Dehydroascorbate can be reduced back to ascorbate by dehydroascorbate reductase (DHAR1). The reaction requires reduced glutathione (GSH). The resulting oxidized GSSG is converted back to GSH by glutathione reductases (GSHR1/2 in C. reinhardtii). DHAR1 transcript abundance was progressively upregulated after exposure to 1 mM H₂O₂ (from 2- to 3-fold after 30 minutes to 50-fold after 240 min).

A similar trend of DHAR1 overexpression was observed under tBuOOH treatment (Figure 6 A). The transcript levels of the key enzyme involved in glutathione synthesis, y-glutamylcysteine synthetase (GSH1), and of GSHR1 were induced only in response to 1 mM H_2O_2 (Figure 6 A). Interestingly, neither of those transcripts changed in abundance during the first 60 min after 0.1 or 0.2 mM tBuOOH addition and in fact they even decreased after 120 min (Figure 6 A). RNA-Seq analysis of C. reinhardtii cells exposed to 1 mM H₂O₂ for 30 and 60 min indicated up-regulation of all the genes encoding the enzymes of the ascorbate-glutathione cycle (Figure 6 B and Supplemental Table 3). The increase in their transcript abundance was higher after 60 minutes and, in agreement with the real-time PCR results, MSD3 and DHAR1 were the most highly induced genes.

We conclude, based on the transcript abundance changes observed in this study in response to peroxide stress, that the ascorbateglutathione system plays an important role in the oxidative stress response in *C. reinhardtii*.

DISCUSSION

Higher plants synthesize L-ascorbic acid using primarily the Smirnoff-Wheeler pathway (4,9), in which VTC2 catalyzes a rate-limiting step by converting GDP-L-galactose to L-galactose-1-P (5,8). Here we provide evidence that C. reinhardtii and other green algal genomes encode functional plant VTC2 homologs. Our sequence analyses identified orthologs of all the Smirnoff-Wheeler pathway enzymes in C. reinhardtii. Moreover, with the exception of GDP-D-mannose pyrophosphorylase (VTC1), which appears to be missing in Prasinophyceae like Micromonas spp. or Ostreococcus spp., all other enzymes of the Lgalactose pathway are conserved in divergent green algae. The absence of VTC1 in Prasinophyceae might be compensated by the operation, in those species, of VTC2 cycles such as those proposed by Laing et al. (2007) or Wolucka and Van Montagu (2007) (6,47), where L-galactose-1-P would be formed by a GDP-Lgalactose transferase activity of VTC2 (using D-Man-1-P or D-Glc-1-P as guanylyl acceptors instead of Pi) and GDP-D-mannose formation would be ensured by a hypothetical 2'-epimerase from GDP-D-glucose (8).

HIT proteins are members of a superfamily of nucleotide hydrolases and transferases, which, based on sequence, substrate specificity, structure, evolution and mechanism, are classified into the Hint, Fhit, Aprataxin, scavenger decapping protein, and GalT branches (16). The first four branches, characterized by a HxHxH motif, contain nucleotide hydrolases, branch members, generally whereas GalT possessing a HxHxQ motif, consist in nucleotide The bestphosphorylases or transferases. characterized member of the GalT branch is galactose-1-phosphate uridylyltransferase, which represents the second enzyme in the Leloir pathway of galactose utilization. The HIT motif in A. thaliana and other plant VTC2 proteins (HxHxQ) and the enzymatic properties of A. thaliana VTC2, which is a GDP-L-Gal/GDP-D-Glc phosphorylase, would place this protein in the GalT branch of the HIT superfamily. Interestingly, C. reinhardtii VTC2 possesses the HxHxH motif found in members of the hydrolase branches of the HIT superfamily (16). Animal homologs of plant VTC2 also have the HxHxH motif and have been shown to act as specific GDP-D-glucose phosphorylases needed for quality control of the nucleoside diphosphate sugar pool (48). This work provides an additional example to suggest that the HxHxH versus HxHxQ motifs do not always predict the biochemical reaction catalyzed by the corresponding HIT enzyme (48-50). In this study we indeed showed that the recombinant C. reinhardtii VTC2 enzyme has a GDP-L-Gal/GDP-D-Glc phosphorylase activity as do the land plant homologs. The algal enzyme can use both GDP-L-Gal and GDP-D-Glc as substrates and requires inorganic phosphate as acceptor. The recombinant purified enzyme displayed an about 10-fold higher catalytic efficiency with both nucleotide sugar substrates relative to A. thaliana VTC2. The latter was previously found to exhibit some transferase activity (31), and we also detected a minor GDP-L-galactose transferase activity with D-Glc-1-P as a guanylyl acceptor for recombinant C. reinhardtii VTC2. This transferase activity was at least 100fold lower than its phosphorylase activity (data not shown).

L-ascorbic acid is a major antioxidant in plants and animals (46). In plants, cellular L-

ascorbic levels are increased in response to environmental stresses such as high light (1,51), high temperature (52), and exposure to UV radiation (53,54) or ozone (55,56). L-ascorbic acid plays an important role in photosynthesis where it acts by scavenging superoxide and H₂O₂, participates in regeneration of α -tocopheryl radicals produced by α -tochopherol during reduction of lipid peroxyl radicals, and functions as cofactor for violaxanthin de-epoxidase (1) and prolyl hydroxylases (2,3).

Here we provide evidence suggesting a role of L-ascorbic acid in protecting C. reinhardtii cells against oxidative stress. ROS-inducing chemicals like H₂O₂ and tBuOOH resulted in increased VTC2 mRNA levels, which are 10- to 15-times more abundant after exposure to tBuOOH compared to H₂O₂ treatment. This might be explained by the fact that tBuOOH persists for a longer time than does H_2O_2 in liquid cultures. In addition, H₂O₂ can produce highly reactive whereas tBuOOH hydroxyl radicals, can decompose to other alkoxyl and peroxyl radicals. Pro-oxidant effects of H₂O₂ treatment resulted in persistent elevated levels of total ascorbate, whereas, after an initial increase, the total ascorbate levels dropped back to wild-type levels after exposure to tBuOOH for 8 h. This is not surprising since exposure of astrocytes, hepatocytes, or HepG2 cells to tBuOOH had previously been shown to lead to decreased levels of intracellular L-ascorbic acid and GSH (44,45,57). An A. thaliana line (ppr40-1) impaired in electron flow at complex III showed decreased levels of total ascorbate and enhanced activity of GLDH and ascorbate-glutathione cycle enzymes (58). Similarly, inhibition of mitochondrial respiratory electron transport at the levels of complex I, complex II or complex IV resulted in a 50% decrease in total ascorbate levels in A. thaliana (59). It is well known that plant mitochondria are the place where the last step of vitamin C biosynthesis occurs in plants. GLDH is an inner membrane mitochondrial flavin-enzyme that uses oxidized cytochrome c as an electron acceptor (60) and recently it has been shown to be required for accumulation of complex I in A. thaliana (61). On the other hand, tBuOOH has been shown to inhibit mitochondrial respiratory chain enzymes in rat hepatocytes (62). Therefore the higher VTC2 transcript levels and depletion of

intracellular ascorbate content in *C. reinhardtii* exposed for longer times to tBuOOH might at least in part be a result of oxidatively damaged mitochondria and impaired respiratory electron transport.

Our RNA-Seq analysis of H₂O₂ stressed C. reinhardtii cells indicates a significant increase in mRNA levels only for VTC2 and only a small increase (1.5-2 fold) for the other components of the L-galactose pathway. A similar pattern of expression for all the genes encoding the Lgalactose pathway enzymes has been observed in A. thaliana exposed to high light (30). Our results suggest that VTC2 might be the regulatory point controlling L-ascorbate biosynthesis in C. reinhardtii. Supporting evidence for this also comes from studies in A. thaliana where it has been demonstrated that supplementation with Lascorbate decreases VTC2 mRNA abundance, indicating possibly a feedback inhibition at transcriptional level (30). Moreover, the increased L-ascorbate content after exposure to high-light resulted in higher GDP-L-galactose phosphorylase activity (30).

The ascorbate-glutathione cycle is the major H₂O₂ scavenging system in photosynthetic organisms (2,17,46). In C. reinhardtii the superoxide anion (O_2^{-}) formed at the site of PSI is converted to H_2O_2 by the superoxide dismutases MnSOD3 and FeSOD. The H₂O₂ is reduced to water by ascorbate in a reaction catalyzed by APX1 (63). Oxidation of ascorbate produces monodehydroascorbate which either can be reduced to ascorbate by MDAR1 or can spontaneously disproportionate to dehydroascorbate. DHAR1 uses GSH to regenerate ascorbate from dehydroascorbate and GSHR1/2 regenerates GSH from GSSG. It has been demonstrated that overexpression of A. thaliana or tomato (Lycopersicon esculentum Mill.) monodehydroascorbate reductase (64,65) results in increased ascorbate levels. Similarly, overexpression of dehydroascorbate reductase had the same effect in enhancing the plant vitamin C conferring increased tolerance content. to oxidative stress (66,67). In this study, oxidatively stressed C. reinhardtii cells showed enhanced mRNA abundances for all the transcripts encoding the ascorbate-glutathione components. An interesting observation was that exposure of C. reinhardtii cells to tBuOOH did not induce

glutathione synthesis (*GSH1*) or GSSG reduction (*GSHR1*), suggesting that under these conditions, another (glutathione-independent) mechanism is required for dehydroascorbate reduction. A similar mechanism has been observed to be functional in rat liver where a selenoenzyme thioredoxin reductase reduces dehydroascorbate to ascorbate (68). *C. reinhardtii*, unlike land plants, has

selenoenzymes, including a thioredoxin reductase prototype (69,70).

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Phylogenetic tree of VTC2-like proteins. Protein sequences homologous to *C. reinhardtii* VTC2 were used to build the phylogenetic tree as described in the "Experimental Procedures" section. Bootstrap values are shown below the branches. Bar, 0.2 amino acid substitutions per site. *Chlamydomonas reinhardtii* (Cre), *Coccomyxa* sp. C-169 (Coc_C169), *Chlorella* sp. NC64A (ChlNC64A), *Volvox carteri f. nagariensis* (Vca), *Ostreococcus lucimarinus* (Ost9901), *Ostreococcus* sp. RCC809 (OstRCC809), *Micromonas pusilla* (MicpuC2), *Micromonas* sp. RCC299 (MicpuN3), *Arabidopsis thaliana* (Ath), *Physcomitrella patens* (Ppa), *Ricinus communis* (Rco), *Oryza sativa* (Osa), *Selaginella moellendorffii* (Selmo), *Drosophila melanogaster* (Dme), *Caenorhabditis elegans* (Cel) and *Homo sapiens* (Hsa).

FIGURE 2. The L-galactose pathway of ascorbic acid biosynthesis is conserved in green algae. Colored squares indicate the number of *A. thaliana* VTC2 orthologs present in each organism. The enzymes catalyzing the successive steps are hexokinase (HXK), phosphoglucose isomerase (PGI), phosphomannose isomerase (PIM), phosphomannomutase (PMM), GDP-D-mannose pyrophosphorylase (VTC1), GDP-D-mannose-3',5'-epimerase (GME), GDP-L-galactose phosphorylase (VTC2), L-galactose-1-P phosphatase (VTC4), L-galactose dehydrogenase (L-Gal-DH) and L-galactono-1,4-lactone dehydrogenase (GLDH). FIGURE 3. Acceptor specificity of *C. reinhardtii* recombinant VTC2. GDP-L-Gal and GDP-D-Glc consumption was measured by the HPLC assay described under "Experimental Procedures". GDP-L-Gal or GDP-D-Glc was added to the reaction mixtures at a final concentration of 50 μ M. The consumption of GDP-L-Gal (A) and GDP-D-Glc (B) was measured with a final enzyme concentration of 0.025 μ g/mL (light gray bars) or 0.25 μ g/mL (dark grey bars).

FIGURE 4. *VTC2* transcript levels are increased in response to oxidative stress. A) Fold change (log_{10}) of *VTC2* transcript assessed by real-time PCR (open circles) in *C. reinhardtii* cells grown photoheterotrophically in the presence of 1 mM H₂O₂ (open circles), 0.1 mM *tert*-butyl-hydroperoxide (tBuOOH) (open triangles), or 0.2 mM tBuOOH (filled triangles) for the indicated times. Each data point represents the average of three technical triplicates for the qPCR reaction from one biological replicate of treated cells. The symbol for the untreated cultures (time zero) represents the overlap of all three symbols used. B) Transcript abundance of genes encoding enzymes of the L-galactose pathway quantified by RNA-Seq in *C. reinhardtii* cells grown in the presence of 1 mM H₂O₂ for 30 or 60 minutes. Fold changes were calculated relative to the transcript abundances in untreated cells for both qPCR and RNA-Seq experiments.

FIGURE 5. Ascorbate levels are elevated in response to oxidative stress. Vitamin C concentration was measured in extracts of *C. reinhardtii* cells exposed to 1 mM H_2O_2 (A) or 0.1 mM tBuOOH (B) for 2, 4, 6 and 8 h. Error bars represent the standard deviation from three biological replicates. Error bars represent the standard deviation (SD), n = 3.

FIGURE 6. Oxidative stress conditions result in up-regulation of GSH-ascorbate cycle enzymes. A) Relative transcript levels of genes coding for ascorbate-glutathione cycle components were quantified by real-time PCR in *C. reinhardtii* cells grown in the presence of 1 mM H_2O_2 (open circles), 0.1 mM tBuOOH (open triangles) or 0.2 mM tBuOOH (filled triangles) for 30, 60, 120 and 240 min. Fold changes were calculated relative to the transcripts abundances in untreated cells and data represented in log_{10} scale. Relative transcript abundance was calculated as described in the legend of Figure 4A. Each data point represents the average of three technical triplicates from one biological replicate. B) RNA-Seq was also used to measure transcript levels of genes encoding for enzymes of the ascorbate-glutathione system in *C. reinhardtii* cells exposed to 1 mM H_2O_2 for 30 and 60 minutes.

FIGURE 7. Putative regulatory sites for ascorbate biosynthesis and ascorbate recycling in *C. reinhardtii.* Enzymes depicted in red indicate the proteins whose mRNA levels are increased in response to oxidative stress (1 mM H_2O_2 and 0.1 mM tBuOOH) and those in magenta that show changes in mRNA levels only in response to H_2O_2 treatment. APX1 (ascorbate peroxidase), MSD3 (Mn superoxide dismutase), MDAR1 (monodehydroascorbate reductase), DHAR1 (dehydroascorbate reductase), GSH1 (γ -glutamylcysteine synthetase), GSH2 (glutathione synthetase), GSHR1/2 (glutathione reductase), GLDH (L-galactono-1,4-lactone dehydrogenase), VTC2 (GDP-L-galactose phosphorylase), ASC (ascorbate), MDA (monodehydroascorbate) and DHA (dehydroascorbate).

TABLE 1. Substrate specificity of recombinant *C. reinhardtii* VTC2. Various sugar nucleotides (50 μ M) were incubated in the presence of *C. reinhardtii* VTC2 recombinant enzyme (0.025 μ g mL⁻¹) for 10 min at 26°C. With GDP-L-Gal as a substrate, the specific activity of the *C. reinhardtii* VTC2 enzyme was 25.4 ± 5.1 μ mol min⁻¹ mg protein⁻¹ (mean ± S.D., n = 3); this value was taken as 100%. The phosphorylase activities found with the other sugar nucleotides are given as a percentage of the activity found with GDP-L-Gal ± SD. Values represent the means of 2-3 individual experiments for each substrate.

Substrate	Relative activity $(\%) \pm SD$			
	Recombinant <i>C. reinhardtii</i> VTC2			
GDP-L-Gal	100			
GDP-D-Glc	87.4 ± 29.2			
GDP-L-Fuc	51.4 ± 15.1			
GDP-D-Man	0 ± 0			
UDP-D-Glc	0.5 ± 0.8			
UDP-D-Gal	2.5 ± 4.1			
ADP-D-Glc	3.7 ± 5.8			

ib., on March 18, 2012

TABLE 2. Characterization of the GDP-hexose phosphorylase activities of the recombinant *A. thaliana* and *C. geinhardtii* proteins. Values for the *A. thaliana* enzyme were taken from Linster *et al.* 2007 Table 1A (5). K_m and V_{max} values for the *C. reinfordtii* VTC2 homolog were obtained by fitting the initial rate data to the Michaelis-Menten equation using the K_m calculator of the GraphPad Prism program. Enzymatic turnover numbers were derived from the V_{max} values by using a molecular weight of 110 kDa for His-MBP-tagged C preinhardtii enzyme with the assumption that the enzyme preparations were pure. Incubation times and enzyme concentrations were adjusted to obtain initial velocity data. Enzymatic activities were measured by the HPLC assay described in the "Experimental Procedures" section. Values are the means \pm S.D. calculated from 2-3 individual experiments for each substrate.

Substrate	K _{cat}		K _m		$\frac{1}{\sum} K_{cat}/K_{m}$	
	S 1		mM		$-\frac{c}{s} s M'$	
	C. reinhardtii	A. thaliana	C. reinhardtii	A. thaliana	C. reinhard	A. thaliana
GDP-L-Galactose	615 ± 3	64 ± 8	0.008 ± 0.001	0.010 ± 0.001	$9.2 \pm 1.3 X Here ^{7}$	$6.3 \pm 0.9 \text{ X } 10^6$
GDP-D-Glucose	813 ± 277	23 ± 3	0.0088 ± 0.0029	0.0044 ± 0.0016	$8.0 \pm 1.3 \text{ X B}^{7}$	$5.7 \pm 2.3 \text{ X}10^6$

Figure 1





















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3

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2

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