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.

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 parologue 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 \(L\)-ascorbate. 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 showed increased VTC2 mRNA and \(L\)-ascorbate levels. Genes encoding enzymatic components of the ascorbate-glutathione system (e.g. ascorbate peroxidase, manganese superoxide dismutase, and 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, \(L\)-ascorbic acid is also an important enzyme cofactor, for example, in violaxanthin de-epoxidase, 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 or the \(L\)-galactose pathway, involves 10 enzymatic steps to convert D-glucose to \(L\)-ascorbic acid via intermediate formation of GDP-D-mannose, GDP-\(L\)-galactose, \(L\)-galactose-1-P, \(L\)-galactose, and \(L\)-galactono-1,4-lactone (4). 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), \(^3\) \(L\)-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 *Arabidopsis 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 crypta*) utilize the inversion pathway commonly found in animals (supplemental Fig. 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

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\(^2\) This article contains supplemental Figs. S1–S3 and Tables S1–S5.

\(^3\) The abbreviations used are: VTC4, \(L\)-galactose-1-P phosphatase; \(L\)-Gal-DH, \(L\)-galactose dehydrogenase; GLDH, \(L\)-galactono-1,4-lactone dehydrogenase; APX, ascorbate peroxidase; MDA, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GSHR, glutathione reductase; MBP, maltose-binding protein; NDP, nucleoside diphosphate; HIT, histidine triad; tBuOOH, tert-butyl-hydroperoxide.
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 (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GSHR) (2, 17). In this work, we found that Chlamydomonas 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 a-configuration), GDP-b-L-Fuc, and GDP were from Sigma. GDP-b-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 as described in Ref. 5. Fractions containing GDP-b-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 Tris acetate-phosphate (TAP) medium (19) at 24 °C and 50–100 μmol 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 MXL096d05 sequence of VTC2 (amino acids D2-A618) was amplified with the Gateway recombinational cloning system (Invitrogen) as described (20). Briefly, the coding region and the untranslated region of 1396 nt followed by a stop codon in the reverse primer (VTC2.D2) and a C-terminal hexahistidine tag followed by a TEV protease cleavage site in the forward primer (VTC2.A628) (supplemental Table S5). 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 H2O2, or 0.1 mM tBuOOH treatment (time 0). 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 × 10^6 cells ml-1, collected by centrifugation at 2,500 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 × 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 × 250 mm; Alltech Associates, Deerfield, IL) using a Hewlett Packard Series II 1090 liquid chromatograph. A 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 Escherichia coli BL21-Gold (DE3) cells (Novagen). Cells were grown in LB medium at 37 °C to an A560 nm of 0.6 at which point the temperature was shifted to 18 °C and protein expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside 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% Nonidet P-40, 10% glycerol) supplemented with protease inhibitor mixture (Sigma), PMSF (100 μM), DNase (20 μg 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 × g) and the supernatant was incubated with nickel-nitrilotriacetic acid agarose beads (Qiagen) for 60 min at 4 °C. The beads were washed extensively with wash buffer and bound protein was eluted with elution buffer (wash buffer containing 300 mM imidazole). VTC2 was further purified by size exclusion chromatography using a HiLoad Superdex 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 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 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 the gene-specific primers listed in supplemental Table S5. 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 H2O2, or 0.1 mM tBuOOH treatment (time 0). The abundance of the two reference transcripts did not change under the conditions tested.

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mobile-phase gradient of 0–40% acetonitrile in 20 mM triethyl ammonium acetate, pH 6.0, was used at a flow rate of 1 ml min⁻¹. The injection volume was 50–100 μl. 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 a decrease of the peak area after the samples were treated with ascorbate oxidase. This treatment was performed by adding 2 units 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. 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 femtoliters (24).

HPLC-based Nucleoside Diphosphate (NDP)-Hexose Phosphorylase Assay—NDP-hexose 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 5–10 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 Ref. 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 Kₘ and Vₘₐₓ values.

RNA-Seq—Total RNA samples prepared from C. reinhardtii strain 2137 grown phototrophically in the presence of 1 mM H₂O₂ for 30 and 60 min were sequenced on a GAIIx platform. cDNA libraries were made using the protocol from Illumina and sequenced as single-end 76-mers. Raw and processed sequence files are available at the NCBI Gene Expression Omnibus (accession number 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 (GenBank accession number GGA0100766). This treatment was performed by adding 2 units 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. 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 femtoliters (24).

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 10 enzymatic reactions from D-glucose to L-ascorbate is conversion of GDP-L-galactose 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, Coccomyxa sp. C169, Micromonas sp. RCC299, and Ostreococcus RCC809. BLASTp searches identified a VTC2 homolog (Cre13.g588150) in C. reinhardtii (supplemental Fig. 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. Because the C. reinhardtii genome encodes only a single protein highly similar to A. thaliana VTC2/VTC5, we termed this protein 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 (HXXHX, where X is a hydrophobic residue) (supplemental Fig. 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 (Fig. 1).

Enzymatic Components of L-Galactose Pathway to Vitamin C Biosynthesis Are Conserved in Green Algae—Because 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 (GE1), L-galactose-1-P phosphatase (VTC4), L-Gal-DH, and GLDH are present in all six species (Fig. 2 and supplemental Table S1). 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.
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Figure 1. Phylogenetic tree of VTC2-like proteins. Protein sequences homologous to C. reinhardtii VTC2 were used to build the phylogenetic tree as described under “Experimental Procedures.” Bootstrap values are shown below the branches. Bar, 0.2 amino acid substitutions per site. C. reinhardtii (Cre), Coccomyxa sp. C-169 (Coc_C169), Chlorella sp. NC64A (ChIN64A), Volvox carteri f. nagariensis (Vca), O. lucimarinus (Osa9901), Ostreococcus sp. RCC809 (OstRCC809), M. pusilla (MicpuC2), Micromonas sp. RCC299 (MicpuN3), A. thaliana (Ath), Physcomitrella patens (Ppa), Ricinus communis (Rco), Oryza sativa (Osa), Selaginella moellendorfii (Selmo), D. melanogaster (Dme), C. elegans (Cel), and H. sapiens (Hsa).

(RCC299 and Micromonas pusilla) nor in Ostreococcus sp. (Ostreococcus tauri, Ostreococcus lucimarinus, and Ostreococcus RCC899) (Fig. 2 and supplemental Table S1). 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.

Because 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 Fig. S1). First, the proposed L-gulose pathway (33) involves the A. thaliana GDP-D-mannose 3’;5’-epimerase, 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 the rat L-gulono-1,4-lactone dehydrogenase/oxidase (Gulo) (34), and two other similar proteins are present (Cre14.g011650 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 myo-inositol via D-glucurionate (supplemental Fig. S1, animal-like pathway). C. reinhardtii codes for a potential myo-inositol oxidase (Cre01.g025850) that might be responsible for the formation of D-glucurionate and shows 31% amino acid identity to A. thaliana MIOX4 (supplemental Table S1). Conversion of D-glucurionate to L-gulonate would require the action of a glucuronate reductase, which has not been identified in plants. Formation of L-gulono-1,4-lactone from L-gulonate requires an aldolactonase (gulonolactonase) (35). SMP30 (senescence marker protein 30) has been recently identified to function as a 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 Fig. S1). This pathway would involve conversion of methyl-D-galacturionate to D-galacturionate. 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 S1), 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 S1). The penultimate reaction in the galacturonate pathway (L-galactonate to L-galactono-1,4-lactone conversion) would require the function of an aldolactonase, which has been recently characterized in the protist E. gracilis (38). BLASTp and tBLASTn searches did not identify any homologs to E. gracilis aldolactonase 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 S1).

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 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 components of the
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Recombinant C. reinhardtii VTC2 Is a GDP-1-galactose/ GDP-D-glucose Phosphorylase—Previous studies demonstrated that A. thaliana VTC2 and VTC5 are GDP-1-galactose phosphorlyases, converting GDP-1-galactose into 1-galactose-1-P and GDP in the presence of P_i (5, 30, 31). To test whether C. reinhardtii VTC2 can catalyze this reaction, recombinant C. reinhardtii VTC2 was purified as a His- and MBP-tagged protein. Because A. thaliana VTC2 shows GDP-1-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 2-fold 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 a 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 (HXHAQ) (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 (Fig. 3). However, in the presence of P_i, we observed a dramatic increase in GDP-L-Gal and GDP-D-Glc consumption (Fig. 3). Incubation of the enzyme with pyrophosphate (P_i), GDP-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 (Fig. 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 with 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 light compared with those grown in the dark (39). Therefore, we tested whether transcript levels of C. reinhardtii VTC2 respond to oxidative stress. C. reinhardtii was grown phototrophically to 2 × 10^9 cells ml^-1, then challenged with 1 mM H_2O_2 or 0.1 and 0.2 mM tBuOOH for 30, 60, 120, and 240 min. Both H_2O_2 and tBuOOH (an organic peroxide capable of inducing lipid peroxidation) treatments enhance intracellular reactive oxygen species production. The concentrations of H_2O_2 and tBuOOH and time points used in this study were

| Substrate | Relative activity of recombinant C. reinhardtii 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                                            |

Table 1: Substrate specificity of recombinant C. reinhardtii VTC2

Plant Smirnoff-Wheeler pathway have orthologs in C. reinhardtii and in other algal species. These results point to a conserved 1-galactose pathway to 1-ascorbate biosynthesis, which might represent the major route to 1-ascorbate biosynthesis in algae, in particular C. reinhardtii and other Volvocales.

FIGURE 2. The 1-galactose pathway of ascorbic acid biosynthesis is conserved in green algae. Colored squares indicate the number of A. thaliana orthologs present in each organism. The enzymes catalyzing the successive steps are hexokinase (HXK), phosphoglucose isomerase (PGI), phosphomannose isomerase (PMI), phosphomannomutase (PMM), GDP-L-mannose pyrophosphorylase (VTC1), GDP-D-mannose-3-phosphomutase (GME), GDP-D-mannose phosphorylase (VTC2), L-galactose-1-P phosphatase (VTC4), L-Gal-DH, and GLDH.

TABLE 1: Substrate specificity of recombinant C. reinhardtii VTC2
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FIGURE 3. Acceptor specificity of C. reinhardtii recombinant VTC2. GDP-\(\beta\)-Gal and GDP-\(\gamma\)-Glc consumption was measured by the HPLC assay described under "Experimental Procedures." GDP-\(\beta\)-Gal or GDP-\(\gamma\)-Glc were added to the reaction mixtures at a final concentration of 50 \(\mu\)M. The consumption of GDP-\(\beta\)-Gal (A) and GDP-\(\gamma\)-Glc (B) was measured with a final enzyme concentration of 0.025 \(\mu\)g/ml (light gray bars) or 0.25 \(\mu\)g/ml (dark gray bars).

| Substrate          | \(k_{\text{cat}}\) \(\text{s}^{-1}\) | \(k_{\text{m}}\) \(\text{mM}\) | \(k_{\text{cat}}/k_{\text{m}}\) |
|--------------------|---------------------------------|----------------------------|-----------------------------|
| GDP-\(\beta\)-galactose | 615 ± 3                        | 0.008 ± 0.001               | 9.2 ± 1.3 × 10^7            |
| GDP-\(\gamma\)-glucose   | 813 ± 277                      | 0.0088 ± 0.0029             | 6.3 ± 0.9 × 10^6            |

TABLE 2
Characterization of the GDP-hexose phosphorylase activities of the recombinant A. thaliana and C. reinhardtii proteins

Values for the A. thaliana enzyme were taken from Linster et al. (5). \(K_{\text{m}}\) and \(V_{\text{max}}\) values for the C. reinhardtii VTC2 homolog were obtained by fitting the initial rate data to the Michaelis-Menten equation using the GraphPad Prism program. Enzymatic turnover numbers were derived from the \(V_{\text{max}}\) values by using a molecular mass of 110 kDa for His-MBP-tagged C. reinhardtii 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 as described under "Experimental Procedures." Values are the mean ± S.D. calculated from 2–3 individual experiments for each substrate.

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 Fig. 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 (Fig. 4A). 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) (Fig. 4A).

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 versus 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 (6.4-fold induction after 30 min and 8.6-fold induction after 60 min) (Fig. 4B). Other components of the pathway including MP1, PMM, and GMPI showed at best a 2-fold increase in their transcript abundance after 60 min of exposure to H2O2. The transcript levels of SNE1, VTC4, \(\alpha\)-GalDH, and GLDH did not change significantly (Fig. 4B and supplemental Table S2) in response to H2O2 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-\(\alpha\)-galactose phosphorylase step is potentially the key regulatory point of the \(\alpha\)-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 (Fig. 5A). 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 with untreated cells), which decreased even further after 8 h to levels similar to those observed for untreated cells (Fig. 5B).

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 \(\alpha\)-ascorbic acid content in C. reinhardtii cells exposed to oxidative stress.

Genes Encoding Components of 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 H$_2$O$_2$ 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 anion production (O$_2$.), which is disproportionated to H$_2$O$_2$ 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 (Fig. 6A). Treatment of C. reinhardtii cells with 1 mM H$_2$O$_2$ 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. H$_2$O$_2$ produced by MnSOD3 is reduced to H$_2$O by ascorbate in a reaction catalyzed by APX1. The mRNA abundance of C. reinhardtii APX1 was induced 2–4-fold after exposure to 1 mM H$_2$O$_2$, whereas 0.1 mM tBuOOH treatment resulted in a 10–15-fold induction of APX1 transcript levels (Fig. 6A). Ascorbate peroxidase oxidizes ascorbate to monodehydroascorbate, which is either reduced to ascorbate by the action of MDAR1, or spontaneously disproportionates to dehydroascorbate. MDAR1 mRNA abundance was induced in response to 1 mM H$_2$O$_2$ (5–6-fold after 120 min), whereas 0.1 mM tBuOOH treatment resulted in a more subtle 2–3-fold up-regulation of this gene. Dehydroascorbate can be reduced back to ascorbate by DHAR1. The reaction requires reduced GSH.

The resulting oxidized GSSG is converted back to GSH by glutathione reductases (GSHR1/2 in C. reinhardtii). DHAR1 transcript abundance was progressively up-regulated after exposure to 1 mM H$_2$O$_2$ (from 2–3-fold after 30 min to 50-fold after 240 min). A similar trend of DHAR1 overexpression was observed under tBuOOH treatment (Fig. 6A). The transcript levels of the key enzyme involved in glutathione synthesis, γ-glutamylcysteine synthetase (GSH1), and GSHR1 were induced only in response to 1 mM H$_2$O$_2$ (Fig. 6A). 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 (Fig. 6A). RNA-Seq analysis of C. reinhardtii cells exposed to 1 mM H$_2$O$_2$ for 30 and 60 min indicated up-regulation of all the genes encoding the enzymes of the ascorbate-glutathione cycle (Fig. 6B and supplemental Table S3). The increase in their transcript abundance was higher after 60 min 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 ascorbate-glutathione 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...
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like *Micromonas* spp. or *Ostreococcus* spp., all other enzymes of the L-galactose 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.* (6) or Wolucka and Van Montagu (47), where L-galactose-1-P would be formed by a GDP-L-galactose transferase activity of VTC2 (using D-Man-1-P or D-Glc-1-P as guanylyl acceptors instead of P) 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 decaptose oxidoreductases, whereas GaIT branch members, generally possessing a HXHXQ motif, are nucleotide phosphorylases or transferases. The best-characterized member of the GaIT 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 GaIT 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 100-fold 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-tocopherol radicals produced by α-tocopherol during reduction of lipid peroxyl radicals, and functions as cofactor for violanthanin 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. Reactive oxygen species-inducing chemicals like H₂O₂ and tBuOOH resulted in increased VTC2 mRNA levels, which are 10–15 times more abundant after exposure to tBuOOH compared with H₂O₂ treatment. This might be explained by the fact that tBuOOH persists for a longer time than does H₂O₂ in liquid cultures. In addition, H₂O₂ can produce highly reactive hydroxyl radicals, whereas tBuOOH can decompose to other alkoxyl and peroxy radicals. Prooxidant 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 because exposure of astrocytes, hepatocytes, or Hep2G 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) that has impaired 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 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 genes encoding L-galactose 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-ascorbic biosynthesis in *C. reinhardtii*. Supporting evidence for this also comes from studies in *A. thaliana* where it has been demonstrated that supplementation with L-ascorbic decreases VTC2 mRNA abundance, possibly indicating a feedback inhibition at the 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) (Fig. 7). In *C. reinhardtii* the superoxide anion (O₂⁻) formed at the site of Photosystem I is converted to H₂O₂ by 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 content, conferring increased tolerance to oxidative stress (66, 67). In this study, oxidatively stressed *C. reinhardtii* cells showed enhanced mRNA abundance for all 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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