Copper-Induced Activation of MAPKs, CDPKs and CaMKs Triggers Activation of Hexokinase and Inhibition of Pyruvate Kinase Leading to Increased Synthesis of ASC, GSH and NADPH in *Ulva compressa*

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In order to analyze whether copper induces activation of CaMK, CDPK and/or MAPK signaling pathways leading to carbon flux reprogramming and to the synthesis of ascorbate (ASC), glutathione (GSH) and NADPH in order to buffer copper-induced oxidative stress, *U. compressa* was initially cultivated with 10 µM copper for 0 to 10 days. The activities of hexokinase (HK), pyruvate kinase (PK), L-galactone 1,4 lactone dehydrogenase (L-GLDH) and glucose 6-P dehydrogenase (G6PDH) were analyzed. HK activity was increased whereas PK was inhibited, and L-GLDH and G6PDH activities were increased indicating a copper-induced modulation of glycolysis leading to carbon flux reprogramming. Then, the alga was cultivated with an inhibitor of CaMs and CaMKs, CDPKs and MAPKs, and with 10 µM of copper for 5 days and the activities of HK, PK, L-GLDH, G6PDH and glutathione synthase (GS), the levels of ASC/DHA, GSG/GSSG and NADPH/NADP, the levels of superoxide anions (SA) and hydrogen peroxide (HP) and the integrity of plasma membrane were determined. The activation of HK was dependent on MAPKs, the inhibition of PK on CDPKs/MAPKs, the activation of L-GLDH on MAPKs, the activation GS on CDPKs/MAPKs, and the activation of G6PDH on MAPKs. Increases in the level of ASC/DHA were dependent on activation of CaMKs/CDPKs/MAPKs, those of GSG/GSSG on MAPKs and those NADPH/NADP on CaMKs/CDPKs/MAPKs. The accumulation of superoxide anions and hydrogen peroxide and the integrity of plasma membrane were dependent on CaMKs/CDPKs/MAPKs. Thus, copper induced the activation of MAPKs, CDPKs and CaMKs leading to the modulation of glycolysis and carbon flux reprogramming which trigger an increase in ASC, GSH and NADPH syntheses and the activation of antioxidant enzymes in order to buffer copper-induced oxidative stress in *U. compressa*.

Keywords: ascorbate, CaMKs, CDPKs, copper, glutathione, MAPKs, NADPH, *Ulva compressa*
INTRODUCTION

Essential heavy metals are those required for proper function of proteins and enzymes and they are iron, copper, zinc, vanadium and nickel in animals and plants (Yadav, 2010). In addition, cadmium is an essential heavy metal for marine phytoplankton since it is required for the function of carbonic anhydrase (Xu and Morel, 2013). In particular, copper is an essential cofactor of several proteins and enzymes such as plastocyanin, citochrome c oxidase, laccase, Cu/Zn superoxide dismutase and several other oxidases and dehydrogenases (Yadav, 2010; Ali et al., 2019). However, copper in excess induces oxidative stress due to the accumulation of reactive oxygen species (ROS) that can oxidize biological macromolecules such as lipids, proteins, nucleic acids and sugars resulting harmful for cells (Yadav, 2010; Ali et al., 2019). Some heavy metals are not required for the function of proteins and enzymes and they are designated as non-essential heavy metals such as lead, arsenic, mercury, silver and others.

Essential and non-essential heavy metals can induce oxidative stress due to the synthesis of ROS such as superoxide anions and hydrogen peroxide in plants (Yadav, 2010; Ali et al., 2019). The increase in ROS can be mitigated by the activation of antioxidant enzymes such as superoxide dismutase (SOD), that dismutate superoxide anions to hydrogen peroxide, and catalase (CAT), ascorbate peroxidase (AP), glutathione peroxidase (GP) and peroxiredoxin (PRX) that reduce hydrogen peroxide. In addition, the increase in ROS induces the activation of enzymes that synthesize antioxidant molecules ASC, GSH and NADPH, thus, increasing the level of substrates used by AP, DHAR, GP and glutathione reductase (GR) (Foyer and Noctor, 2011). Important antioxidant enzymes are those that constitute the Halliwell–Asada–Foyer (HAF) cycle that operates in chloroplasts: AP, that oxidizes ASC to dehydroascorbate (DHA); dehydroascorbate reductase (DHAR), that reduces DHA to ASC by oxidizing GSH to glutathione (GSSG); and GR, that reduced GSSG to GSH using NADPH as reducing power (Foyer and Noctor, 2011).

ASC can also be directly oxidized by ROS such as superoxide anions and hydrogen peroxide producing dehydroascorbate (DHA) (Foyer and Noctor, 2011). In addition, ASC is also a cofactor of de-epoxidases in the xanthophyll cycle that protects thylakoid membranes from lipid oxidation (Smirnoff, 2011; Wheeler et al., 2015). Moreover, ASC prevent photoinhibition by donating electrons to electron transport chains in plants exposed to high light (Smirnoff, 2011; Wheeler et al., 2015). Thus, ASC has a triple function in plants acting as the substrate of AP and de-epoxidases, and as a direct electron donor. In plants, ASC is synthesized from glucose-6P (G6P) and the two last steps of ASC synthesis are displayed by L-galactono 1,4 lactone dehydrogenase (GuLDH) (Wheeler et al., 1998; Valpuesta and Botella, 2004). In animals, ASC is also synthesized from G6P but the two last steps are performed by aldono lactonase and gulono-lactone dehydrogenase (GuLDH) (Wheeler et al., 1998; Valpuesta and Botella, 2004). Interestingly, most animals can synthesize ASC in the liver but humans and primates present mutations in the gene encoding GuLDH (Nishikimi and Unfried, 1976; Nishikimi et al., 1994) and they need to acquire ASC from the diet.

GSH can also be directly oxidized by ROS producing GSSG (Foyer and Noctor, 2011; Lu, 2013). GSH is constituted by three amino acids, glutamate, cysteine and glycine and it is synthesized by the enzymes γ-glutamyl cysteinyl synthase (GCS) and glutathione synthase (GS), and both enzymes require ATP (Foyer and Noctor, 2011; Lu, 2013). Glutamate is formed by the amination of α-ketoglutarate produced in the Krebs cycle and it is the precursor of other amino acids such as glutamine, aspartate, proline, arginine and asparagine, and the neurotransmitter γ-aminobutyric acid (GABA) (Forde and Lea, 2007). Serine is synthesized from 3-phophoglycerate produced in glycolysis and it is the precursor of methionine, cysteine and glycine (Ros et al., 2014). In addition, GSH is the precursor of phytochelatins (PCs) that are formed by condensation of GSH units by the enzyme phytochelatin synthase (Cobbet and Goldsbrough, 2002; Yadav, 2010). PCs can also directly act as antioxidant molecules but their principal function is to sequester heavy metals ions, thus, reducing oxidative stress (Tsujii et al., 2002; Yadav, 2010). NAD and NADP are synthesized from aspartate and this amino acid is obtained from glutamate in plants (Noctor et al., 2006). In addition, NADPH is synthesized by the reduction of NADP by ferredoxin NADP-reductase in chloroplasts and/or by the enzyme glucose 6-P dehydrogenase (G6PDH) in the cytosol. NADPH is the final electron donor in antioxidant systems since it is the substrate of GR, the last enzyme of HAF cycle, and the substrate of NADPH-thioredoxin reductase that reduced thioredoxins that, in turn, reduced PRX that consumes hydrogen peroxide (Valderrama et al., 2006).

The marine macroalga Ulva compressa (Chlorophyta) is the dominant species in copper and heavy metal-contaminated coastal areas in northern Chile and all over the world (Villares et al., 2002; Ratkevicius et al., 2003). This marine macroalga has been extensively studied in regard to the mechanisms involved in copper tolerance (González et al., 2010; González et al., 2012; Mellado et al., 2012; Laporte et al., 2016; Moenne et al., 2016) and copper accumulation (Ratkevicius et al., 2003; Navarrete et al., 2019; Zuñiga et al., 2020). It has been shown that this alga presents exceptional mechanisms of copper tolerance that will be mentioned bellow (Laporte et al., 2020; Moenne et al., 2020). It was initially shown that the alga collected in copper-contaminated sites displayed and increase in intracellular copper level, AP activity and synthesis of ASC, which was accumulated as DHA (Ratkevicius et al., 2003). The alga cultivated in vitro with a sub-lethal concentration of copper (10 µM) for 7 days showed an increase in activities of antioxidant enzymes such as SOD, AP and GR (González et al., 2010; González et al., 2012). In addition, copper induced an increase in the level of antioxidant molecules ASC, GSH and PCs until day 7 (Mellado et al., 2012). Interestingly, an increase ASC is due to an increase in GDH and L-GLDH activities, whereas no GuLDH activity was detected (Mellado et al., 2012). In addition, the activities of GCS and GS involved in GSH synthesis were also increased until day 7 (Mellado et al., 2012). On the other hand, transcriptomic analyses were performed using
Illuminated in a mortar using a pestle. Three milliliters of the alga (1 g of FT) was frozen with liquid nitrogen and stored at −80°C. Extracts normally contained around 4 mg ml⁻¹ of proteins.

**Detection of Activities of Glycolysis Enzymes and Antioxidant Enzymes**

Hexokinase (HK) activity was determined as described in Kerve et al. (2008). HK activity was detected in 1 ml of 50 mM Tris–HCl (pH 8.5) containing 2 mM glucose, 5 mM MgCl₂, 2.5 mM ATP, 1 mM NAD, 15 mM KCl, 2 U of glucose 6-P dehydrogenase and 10 µg of protein extract. The increase in absorbance at 340 nm, due to synthesis of NADH, was monitored for 30 min. HK activity was determined using the extinction coefficient of NADH (ε = 6.2 mM⁻¹ cm⁻¹).

Pyruvate kinase (PK) activity was determined as described in Duggleby and Dennis (1973). PK activity was detected in 1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 2 mM ADP, 2 mM phospho-enol pyruvate, 0.15 mM NADH, 2 U of lactate dehydrogenase and 10 µg of protein extract. The decrease in absorbance at 340 nm, due to NADH oxidation, was monitored for 5 min. PK activity was determined using the extinction coefficient of NADH (ε = 6.2 mM⁻¹ cm⁻¹).

L-galactono 1,4 lactone dehydrogenase (L-GLDH) activity was detected as described in Mellado et al. (2012). L-GLDH activity was detected 1 ml of 100 mM phosphate buffer (pH 8) containing 3 mM L-galactono 1,4 lactone (Carbosynth, UK), 1 mg ml⁻¹ cytochrome c, 0.1 mM KCN, and 50 µg of protein extract. The increase in absorbance at 550 nm, due to cytochrome c reduction, was monitored for 20 min. L-GLDH activity was calculated using the extinction coefficient of reduced cytochrome c (ε = 21 mM⁻¹ cm⁻¹).

Glutathione synthase (GS) activity was determined as described in Mellado et al. (2012). GS activity was detected in 1 ml of 100 mM phosphate buffer (pH 7.5) containing 150 mM KCl, 20 mM MgCl₂, 2 mM γ-glutamyl-cysteine, 10 mM L-glycine, 5 mM ATP, 2 mM phospho-enol pyruvate, 5 U of pyruvate kinase, 5 U of lactate dehydrogenase, 150 µM NADH and 30 µg of protein extract. The decrease in absorbance at 340 nm, due to NADH oxidation, was monitored for 5 min. GS activity was calculated using the extinction coefficient of NADH (ε = 6.2 mM⁻¹ cm⁻¹).

Glucose 6-P dehydrogenase (G6PDH) activity was determined as described in González et al. (2010). G6PDH activity was determined in 1 ml of buffer 40 mM Tris–HCl (pH = 8.2) containing 4 mM glucose, 0.6 mM NADP, 5 mM MgCl₂ and 40 µg of protein extract. The increase in absorbance at 340 nm, due to synthesis of NADPH, was monitored for 5 min. G6PDH activity was calculated using the absorbance coefficient of NADPH (ε = 6.2 mM⁻¹ cm⁻¹).
Quantification of ASC and DHA
The alga (100 mg of FT) was frozen with liquid nitrogen and homogenized in a mortar using a pestle. One milliliter of 100 mM phosphate buffer (pH 7.0) was added and the homogenization was pursued until thawing. The mixture was centrifuged at 14,000 rpm for 15 min at 4°C and the supernatant was recovered. An aliquot of 10 μl was diluted 10 times and an aliquot of 10 μl of the dilution was mixed with 110 μl of ascorbate assay buffer of the Ascorbic Acid Assay Kit (Abcam, USA), 30 μl of catalysis buffer and 50 μl of the enzyme mix containing 2 μl of the probe. The absorbance was detected at 570 nm using a microplate spectrophotometer (Tecan, USA). To determine the amount of dehydroascorbate, 5 μl of 1 mM DTT were added to an aliquot of 500 μl of the supernatant and incubated at room temperature for 1 h and 5 μl of 5% N-ethylmalamide were added. Absorbance was determined at 570 nm as described previously.

Quantification of GSH and GSGG
The alga (200 mg of FT) was frozen with liquid nitrogen and homogenized in a mortar using a pestle and 1.2 ml of 0.1% trifluoroacetic acid (TFA)/6.3 mM dithiethyltriamine pentaacetic acid (DTPA) was added. The mixture was centrifuged at 14,000 rpm for 20 min and the supernatant was recovered. The supernatant was filtered through 0.22 μm PVDF membrane and an aliquot of 25 μl of the supernatant was mixed with 45 μl of 200 mM HEPES (pH 8)/6.3 mM DTPA and 1 μl of 25 mM monobromobimane and incubated for 30 min at room temperature in darkness. The reaction was stopped by addition of 30 μl⁻¹ mM metasulphonic acid. GSH and GSGG levels were analyzed by High Performance Liquid Chromatography (HPLC) using an Agilent 1260 Infinity and data was compiled using Chemidoc software. An aliquot of 20 μl was separated on a reverse phase C-18 column (5 μm particle size, 4.6 mm internal diameter and 15 cm length) at 24°C, eluted using solvent A 0.1% TFA in aqueous solution and solvent B (100% acetonitile) using a linear gradient (10 min from 0–20%, 30 min from 20–35% and 10 min from 35–100% of solvent B) and a flow rate of 1 ml min⁻¹. GSH and GSGG were detected by fluorescence using an excitation wavelength of 380 nm and an emission wavelength of 470 nm. Pure GSH and GSGG were dissolved in filtered water and used as standards. Retention times of GSH and GSGG were 9.25 and 8.9 min, respectively. The concentrations were calculated using a calibration curve of GSH or GSGG from 0 to 200 nM.

Quantification of NADPH and NADP
The alga (100 mg of FT) was frozen with liquid nitrogen and homogenized in a mortar using a pestle and 1 ml of lysis buffer at a concentration of 200 mg ml⁻¹ from the NAD/NADPH fluorimetric kit (Abcam, USA). The mixture was centrifuged at 14,000 rpm for 10 min and the supernatant was recovered. Two samples of 25 μl of the supernatant were added a 96-well microplate and incubated 15 min at room temperature. The first sample was treated with 25 μl of NADPH extraction solution and incubated 15 min at room temperature and 25 μl of NADP extraction solution were added. The second sample was treated with 25 μl of NADPH extraction solution and incubated 15 min at room temperature and 25 μl of NADPH extraction solution were added. Both samples were treated with 75 μl of NADPH reaction mixture and incubated at room temperature for 30 min in darkness. Fluorescence was measured using an excitation wavelength of 540 nm and an emission wavelength of 590 nm using a microplate spectrophotometer (Tecan, USA). The concentration of NADPH was calculated using a calibration curve of 0–10 μM of NADPH.

Visualization and Quantification of Superoxide Anions
To visualize superoxide anions, five laminae of the alga were incubated in 2 ml of 100 mM phosphate buffer (pH 7.0) containing 0.1 mM dehydroethyline (DHE) for 40 min in darkness. Algae were washed with 2 ml of filtered seawater to remove DHE excess. 2-hydroxyethyline (2OH-E) was visualized in a confocal microscope (Carl Zeiss model LSM800, Germany) using and excitation wavelength of 493 nm and emission wavelength of 590 nm. To quantify superoxide anions, the alga (100 mg of FT) was incubated in 2 ml of phosphate buffer (pH 7) containing 0.1 mM hydroethydine (DHE) for 40 min in darkness. Algae were washed with 2 ml of filtered seawater to remove the excess of DHE. The alga was homogenized in liquid nitrogen using a pestle. One milliliter of 40 mM Tris–HCL (pH 7.5) was added and the homogenization was pursued until thawing. The mixture was centrifuged at 14,000 for 15 min and the supernatant was recovered. An aliquot of 200 μl was added to a 96-well microplate and the fluorescence was detected using an excitation wavelength of 480 nm and an emission wavelength of 590 nm. The concentration was calculated using the extinction coefficient of 2OH-E (ε = 9.4 mM⁻¹ cm⁻¹).

Visualization and Quantification of Hydrogen Peroxide
To visualize hydrogen peroxide, five laminae of the alga were incubated in 2 ml of 100 mM phosphate buffer (pH 7.0) containing 0.1 mM 2′,7′-dichlorohydrofluoresceine diacetate (DCHF-DA) for 30 min in darkness. Algae were washed with 2 ml of filtered seawater to remove excess of DCH-DA. Dichlorofluoresceine (DCF) was visualized in a confocal microscope (Carl Zeiss model LSM800, Germany) using and excitation wavelength of 488 nm and emission wavelength of 517 nm. To quantify hydrogen peroxide, the alga (100 mg of FT) was incubated in 2 ml of phosphate buffer (pH 7) containing 0.1 mM dichlorofluoresceine (DCF) for 30 min in darkness. Algae were washed with 2 ml of filtered seawater to remove excess of DCF-DA. The alga was homogenized in liquid nitrogen using a pestle. One milliliter of 40 mM Tris–HCL (pH 7.5) was added and the homogenization was pursued until thawing. The mixture was centrifuged at 14,000 rpm for 15 min and the supernatant was recovered. An aliquot of 200 μl was added to a 96-well microplate and the fluorescence was detected using an excitation wavelength of 488 nm and an emission wavelength of 517 nm. The concentration was calculated using a calibration curve of 0–10 μM of DCF.
Detection of Ion Leakage and Plasma Membrane Damage

The alga (five laminae) were washed with 100 ml milliQ water, quickly transferred to well of a 12-well plate containing 2 ml of 700 mM manitol and incubated for 10 min at 22°C. An aliquot of 50 µl was recovered, conductivity was determined using a compact conductimeter and the aliquot was returned to manitol solution (Horiba Scientific, Japan). The mixture was transferred to 2 ml plastic tubes and treated at 121°C for 20 min and conductivity was measured again in order to determine 100% of conductivity.

Statistical Analyses

Significant differences were determined by two-way ANOVA followed by Tukey’s multiple comparison tests. Differences among mean values were considered to be significant at a probability of 5% (P < 0.05).

RESULTS

Copper-Induced HK Activation and PK Inhibition

The alga was cultivated in seawater without copper addition (control) and with 10 µM of copper for 0 to 10 days and the activities of hexokinase (HK), the first enzyme of glycolysis and pyruvate kinase (PK), the last enzyme of glycolysis, were determined (Figure 1). HK activity in the alga cultivated in control condition was 10 nmol min⁻¹ mg⁻¹ of protein at day 0 and it slightly increased to 13.7 nmol min⁻¹ mg⁻¹ of protein at day 10 (Figure 1A). HK activity in the alga cultivated with copper increased from 10 nmol min⁻¹ mg⁻¹ of protein to 33.1 nmol min⁻¹ mg⁻¹ of protein at day 5 and reached a maximal level of 43.3 nmol min⁻¹ mg⁻¹ of protein at day 10, which represents an increase of 3.2 times at day 10 (Figure 1A).

PK activity in algae cultivated in control condition was 75 nmol min⁻¹ mg⁻¹ of protein at day 0, it increased to 146 nmol min⁻¹ mg⁻¹ of protein at day 5 and decreased to 96 nmol min⁻¹ mg⁻¹ of protein at day 10 (Figure 1B). PK activity in the alga cultivated with copper increased from 75 nmol min⁻¹ mg⁻¹ of protein to 117 nmol min⁻¹ mg⁻¹ of protein at day 5 and decreased to 75 nmol min⁻¹ mg⁻¹ of protein at day 10, which represents a decrease of 38% at day 10 (Figure 1B). Thus, the increase in HK and the inhibition of PK indicate that glycolysis is inhibited in its final step that may lead to carbon flux reprogramming.

Copper-Induced L-GLDH and G6PDH Activation

Considering that glycolysis is inhibited in its final step, it is possible that carbon flux may be redirected to increase L-GLDH, that synthesize ASC from glucose 6-P, and G6PDH, that uses glucose 6-P as substrated and synthesize NADPH. The activity of L-GLDH in control algae was 2 nmol min⁻¹ mg⁻¹ of protein at day 0 and it increased to 6.6 nmol min⁻¹ mg⁻¹ of protein at day 10 (Figure 2A). L-GLDH activity in the alga cultivated with copper increased from 2 nmol min⁻¹ mg⁻¹ of protein to 45 nmol min⁻¹ mg⁻¹ of protein at day 5 and decreased to 22 nmol min⁻¹ mg⁻¹ of protein at day 10, which represents an increase of 33 times at day 10 (Figure 2A). The activity of G6PDH in control algae was 200 nmol min⁻¹ mg⁻¹ of protein at day 0 and it decreased to 143 at day 10 (Figure 2B). G6PDH activity in the alga cultivated with copper increased from 200 nmol min⁻¹ mg⁻¹ of protein at day 0 to 440 nmol min⁻¹ mg⁻¹ of protein at day 1, increased again at day 5 and reached a maximal level of 1,039 nmol min⁻¹ mg⁻¹ of protein at day 7 and then decreased to 871 nmol min⁻¹ mg⁻¹ of protein at day 10, which represents an increase of 6.1 times at day 10 (Figure 2B). Thus, there is a carbon flux reprogramming since G6-P is used to synthesize ASC, and NADPH due to G6PDH activation.
Copper-Induced HK Activation and PK Inhibition Is Dependent on Activation of CDPKs and MAPKs

The alga was cultivated in seawater without copper addition (control), with 10 µM of copper, and with an inhibitor of CaMKs, W-7, an inhibitor of CDPKs, staurosporine (St), and an inhibitor of MAPKs, PD-98059 (PD), and with 10 µM of copper for 5 d. HK activity in the alga cultivated in control condition was 100 nmol min$^{-1}$ mg$^{-1}$ of protein at day 5 (Figure 3A). HK activity in the alga treated with W-7 and (St) and copper was not significantly different from HK activity in control alga. In contrast, HK activity in the alga treated with PD and copper was 54 nmol min$^{-1}$ mg$^{-1}$ of protein, which is significantly different and represent an inhibition of 46% (Figure 3A). Thus, HK activation is dependent on activation of MAPKs.

The activity of PK in the alga cultivated in control condition was 229 nmol min$^{-1}$ mg$^{-1}$ of protein and in the alga treated only with copper it was 98 nmol min$^{-1}$ mg$^{-1}$ of protein (Figure 3B). PK activity in the alga treated with W-7 and copper was not significantly different to the activity found in the alga treated only with copper. In contrast, PK activity was 75 nmol min$^{-1}$ mg$^{-1}$ of protein in the alga treated with W-7 and copper, and it was 62 nmol min$^{-1}$ mg$^{-1}$ of protein and in the alga treated with PD and copper, which represents an inhibition of 23.4 and 36.7%, respectively (Figure 3B). Thus, the inhibition of PK activity is dependent on the activation of CDPKs and MAPKs.

Copper-Induced L-GLDH, GS and G6PDH Activation Is Dependent on Activation of CDPKs and MAPKs

The alga was cultivated in seawater without copper addition (control), with 10 µM of copper, and with inhibitors of CaMKs, CDPKs and MAPKs pathways and with 10 µM of copper for 5 d, and the activities of L-GLDH, GS and G6PDH were determined.

L-GLDH activity in control alga was 2.6 nmol min$^{-1}$ mg$^{-1}$ of protein and in the alga treated only with copper it was 0.5 nmol min$^{-1}$ mg$^{-1}$ of protein (Figure 2A). L-GLDH activity in the alga treated with W-7 and copper was not significantly different to the activity found in the alga treated only with copper. In contrast, L-GLDH activity was 1.1 nmol min$^{-1}$ mg$^{-1}$ of protein in the alga treated with W-7 and copper, and it was 0.6 nmol min$^{-1}$ mg$^{-1}$ of protein and in the alga treated with PD and copper, which represents an inhibition of 29% and 37%, respectively (Figure 2A). Thus, the inhibition of L-GLDH activity is dependent on the activation of CDPKs and MAPKs.

GS activity in control alga was 60 nmol min$^{-1}$ mg$^{-1}$ of protein and in the alga treated only with copper it was 10 nmol min$^{-1}$ mg$^{-1}$ of protein (Figure 2B). GS activity in the alga treated with W-7 and copper was not significantly different to the activity found in the alga treated only with copper. In contrast, GS activity was 30 nmol min$^{-1}$ mg$^{-1}$ of protein in the alga treated with W-7 and copper, and it was 15 nmol min$^{-1}$ mg$^{-1}$ of protein and in the alga treated with PD and copper, which represents an inhibition of 50% and 60%, respectively (Figure 2B). Thus, the inhibition of GS activity is dependent on the activation of CDPKs and MAPKs.

G6PDH activity in control alga was 500 nmol min$^{-1}$ mg$^{-1}$ of protein and in the alga treated only with copper it was 100 nmol min$^{-1}$ mg$^{-1}$ of protein (Figure 2B). G6PDH activity in the alga treated with W-7 and copper was not significantly different to the activity found in the alga treated only with copper. In contrast, G6PDH activity was 250 nmol min$^{-1}$ mg$^{-1}$ of protein in the alga treated with W-7 and copper, and it was 125 nmol min$^{-1}$ mg$^{-1}$ of protein and in the alga treated with PD and copper, which represents an inhibition of 50% and 60%, respectively (Figure 2B). Thus, the inhibition of G6PDH activity is dependent on the activation of CDPKs and MAPKs.
protein and in the alga treated only with copper and it was 186 nmol min\(^{-1}\) mg\(^{-1}\) of protein at day 5 (Figure 4A). L-GLDH activity was not significantly different to the activity in the alga treated with W-7 or St and with copper. In contrast, L-GLDH activity was 19.9 nmol min\(^{-1}\) mg\(^{-1}\) of protein in the alga treated with PD and copper, which represents an inhibition of 23%, at day 5 (Figure 4A). Thus, the increase in L-GLDH activity is due to activation of MAPKs.

GS activity in control alga was 152 nmol min\(^{-1}\) mg\(^{-1}\) of protein and it was 284 nmol min\(^{-1}\) mg\(^{-1}\) of protein in the alga treated only with copper (Figure 4B). GS activity was not significantly different to the activity in the alga treated with W-7 and copper whereas it was 218 nmol min\(^{-1}\) mg\(^{-1}\) of protein in the alga treated with St and copper and it was 202 nmol min\(^{-1}\) mg\(^{-1}\) of protein in the alga treated with PD and copper, which represent an inhibition of 23 and 29%, respectively, at day 5 (Figure 4B). Thus, the increase in GS activity is dependent on the activation of CDPKs and MAPKs.

G6PDH activity in the alga cultivated in control condition was 62 nmol min\(^{-1}\) mg\(^{-1}\) of protein and in the alga treated only with copper it was 186 nmol min\(^{-1}\) mg\(^{-1}\) of protein (Figure 4C). G6PDH activity was not significantly different in the alga treated with W-7 or St and with copper, whereas it was 90 nmol min\(^{-1}\) mg\(^{-1}\) of protein in algae treated with PD and copper, which represents an inhibition 51.6%, at day 5 (Figure 4C). Thus, the increase in G6PDH activity is dependent on the activation of MAPKs.

**Copper-Induced Increase in the Level of ASC and GSH, but not NADPH, is Dependent on the Activation of CaMK, CDPKs or MAPKs**

The alga was cultivated in seawater without copper addition (control), with 10 µM of copper, with inhibitors of CaMKs, CDPKs and MAPKs and with 10 µM of copper, for 5 d and the levels of ASC/DHA, GSH/GSSG and NADPH/NADP were determined. The levels of ASC and DHA in control alga were 4 and 2.2 µmoles g\(^{-1}\) of FT, respectively, and in the alga treated only with copper these levels were 0.6 and 8.5 µmoles g\(^{-1}\) of FT, respectively, at day 5. The level of total ASC (ASC + DHA) in control algae was 6.2 µmoles g\(^{-1}\) of FT and in the alga treated only with copper it was 9.1 µmoles g\(^{-1}\) of FT (Figure 5A). Total ASC significantly decreased in the alga treated with W-7, St or PD and with copper to 5.8, 4.4 and 0.7 µmoles g\(^{-1}\) of FT which represents a decrease of 56, 41 and 88%, respectively (Figure 5A). Thus, total ASC level is dependent on the activation of CaMKs, CDPKs and MAPKs.

The levels of GSH and GSSG in control alga were 1.9 and 0.5 µmoles g\(^{-1}\) of FT, respectively, and in the alga treated only with copper these levels were 2.5 and 0.5 µmoles g\(^{-1}\) of FT, respectively, at day 5 (Figure 3B). The level of total GSH (GSH + GSSG) in control alga was 2.4 µmoles g\(^{-1}\) of FT and in the alga treated only with copper it was 3 µmoles g\(^{-1}\) of FT which represents an inhibition of 23% (Figure 3B). Thus, total GSH level is dependent on the activation of MAPKs.
The levels of total GSH in algae treated with W-7 or St and with copper were 3.3 and 3.7 µmoles g⁻¹ of FT, respectively, but these levels were not significantly different compared to the level in the alga treated only with copper (Figure 5B). The level of total GSH in algae treated with PD and copper was 7.1 µmoles g⁻¹ of FT and this level was significantly higher than the level in algae treated only with copper (Figure 5B). Thus, the level of total GSH is dependent on activation of MAPKs.

The levels of NADPH and NADP in control alga were 8 and 19 nmoles g⁻¹ of FT, respectively, and in the alga treated only with copper these levels were 21 and 38 nmoles g⁻¹ of FT, respectively, at day 5 (Figure 5C). The level of total NADPH (NADPH + NADP) in control algae was 26 nmoles g⁻¹ of FT and in algae treated with copper it was 59 nmoles g⁻¹ of FT (Figure 5C). The levels of total NADPH in algae treated with W-7, St or PD and with copper were 59, 60 and 63 nmoles g⁻¹ of FT, respectively, but they were not significantly different compared to the level in the alga treated only with copper. Thus, the level of total NADPH is not dependent on the activation of CaMKs, CDPKs or MAPKs.

Copper-Induced Increased Levels of Superoxide Anions and Hydrogen Peroxide Are Dependent on Activation of CaMKs, CDPKs and MAPKs

The level of superoxide anion (SA) in control alga was 6 nmoles g⁻¹ of FT and it was 9 nmoles g⁻¹ of FT in the alga treated with copper at day 5, but these levels were not significantly different among them and the fluorescence was observed mainly in the chloroplast (Figures 6A, C). The level of SA in algae treated with W-7 and copper was 5 nmoles g⁻¹ of FT, which is lower than the level in the alga treated only with copper and the fluorescence is located mainly in the chloroplast of the cells (Figures 6A, C). The level of SA in the alga treated with St and copper was 12 nmoles g⁻¹ of FT which is higher than the level in the algae treated with copper and fluorescence is located mainly in the chloroplast of the cells (Figures 6A, C). The level of SA in the alga treated with PD and copper was 8 nmoles g⁻¹ of FT, this level is not significantly different from the level in the alga treated only with copper and the fluorescence is located mainly in the chloroplast. (Figures 6A, C) Thus, the level of SA is not different in control and copper-treated algae indicating that oxidative stress is efficiently buffered and that the increase in SA level is dependent on activation of CaMK and CDPKs.

The level of hydrogen peroxide (HP) in algae cultivated in control condition was 3 nmoles g⁻¹ of FT and it was 5 nmoles g⁻¹ of FT in the algae treated only with copper at day 5, but these levels were not significantly different and the fluorescence was located in the whole cell (Figures 6B, D). The level of HP in the algae treated with W-7 and copper was 5 nmoles g⁻¹ of FT and this level was significantly higher than the level in the algae treated only with copper and the fluorescence was located in the chloroplast (Figures 6B, D). The level of HP in the algae treated...
with St and copper was 4 nmoles g$^{-1}$ of FT and this level was not significantly higher than the level in the alga treated only with copper but the fluorescence was located mainly in the nucleus (Figures 6B, D). The level of HP in the alga treated with PD and copper was 5,700 nmoles g$^{-1}$ of FT and this level was significantly higher than the level in the alga treated only with copper and the fluorescence was located mainly in the chloroplast and the nucleus (Figures 6B, D). Thus, the level of HP is efficiently buffered in algae treated only with copper and the increase in HP level is dependent on the activation of CaMKs and more significantly on the activation of MAPKs.

**Copper-Induced Protection of Plasma Membrane Integrity Is Dependent on the Activation CaMKs, CDPKs and MAPKs**

The level of ion leakage (IL) in control algae at day 5 was 8.9% compare with algae subjected to 120°C for 20 min (100%). IL was 20.3% in the alga treated only with copper which is significantly higher than the level in control alga (Figure 7). IL in the alga treated with W-7 and copper was 26.6%, in the alga treated with St and copper it was 47.3% and in the alga treated with PD and copper it was 65% which and these percentages were significantly different compare to the percentage in the alga treated only with copper (Figure 7). Thus, plasma membrane integrity in the alga treated with copper is dependent on the activation of CaMKs, CDPKs and MAPKs.

**DISCUSSION**

**Copper-Induced Increase in HK, L-GLDH, GS and G6PDH Activities and Decrease in PK Activity Is Dependent on CDPKs and MAPKs**

In this work, we initially showed that copper induces the increase in HK activity through MAPKs activation whereas it inhibits PK
regulated by phosphorylation in the marine alga *U. compressa*. Thus, copper-induced HK activation may explain the increase in glutamate, cysteine and glycine levels as well as in G6PDH and GS activities may explain the increase in 3-phosphoglycerate that is produced by phospho-glycerate kinase, an enzyme located before PK in glycolysis. In addition, the increase in G6-P may enhance the synthesis of ASC and GSH, but Not NADPH, Is Dependent on the Activation of CaMK, CDPKs or MAPKs

Here, it was shown that ASC synthesis is dependent on CaMKs, CDPKs and MAPKs activation (see scheme in Figure 8) indicating that ASC synthesis is regulated by phosphorylation. Until now, it is not known how activation of CaMKs, MAPKs or CDPKs pathways can stimulate ASC synthesis in plants or algae. However, a direct phosphorylation of an enzyme involved in ASC synthesis may occur and/or the phosphorylation of transcription factor involved in the expression of an enzyme that participates in ASC synthesis, but the latter need to be further investigated. ASC is also the substrate of AP and this activity was strongly increased in the alga treated with copper (Mellado et al., 2012). Even if AP activity is increased, the level of ASC remained enhanced in the alga exposed to copper excess indicating that there is a strong synthesis of ASC in *U. compressa*. On the other hand, the level of GSH was dependent on activation of MAPKs. In this sense, it has been shown that inhibition of ERK, JNK or p38 MAPKs induced a decrease in the level of GSH in *U. compressa* exposed to copper excess (Rodríguez-Rojas et al., 2019). Thus, the activation of the three MAPK pathways are involved in the increase of GSH synthesis. In addition, GSH is the substrate of DHAR and this activity may also be increased in *U. compressa* since AP and GR are increased (González et al., 2010; Mellado et al., 2012). In addition, it has been shown that copper is released to the extracellular medium with GSH in equimolar amounts (Navarrete et al., 2019). Thus, GSH may be also required to extrude copper from *U. compressa* as...
well as to inhibit copper-induced ROS accumulation and oxidative stress (see scheme in Figure 8).

Surprisingly, the level of NADPH is not dependent on activation of CaMK, CDPKs and MAPKs. Considering that G6PDH is regulated by CaMKs and MAPKs and that the level of NADPH did not change in the alga treated with inhibitors and copper, it is possible that NADPH produced by G6PDH is used to increase GR activity in the cytoplasm (see scheme in Figure 8). In addition, NADPH produced by G6PDH may be consumed to increase C, N and S assimilation, which are processes requiring NADPH, in the cytoplasm (Kopriva et al., 1999; Kopriva et al., 2002). In this sense, it has been shown that C, N and S assimilation is increased in the alga exposed to copper excess since the level of transcripts and the activities of key enzymes involved in these processes are also increased (Laporte et al., 2020). The enhanced C, N and S assimilation may be required to the synthesis of new proteins and membranes that are damaged by copper-induced oxidative stress. In addition, the constant level of NADPH that did not change with inhibitors of CaMKs, CDPKs and MAPKs indicates that the constant NADPH level is due to photosynthesis. In this sense, it was shown that net photosynthesis is increased in the alga exposed to copper excess for 0 to 5 days (Laporte et al., 2020) and the increase in NADPH level is due to activation of ferredoxin-NADPH reductase (FNR) in chloroplasts. It has been shown FNR is not regulated by phosphorylation but instead by acetylation of a lysine residue located near the active site in A. thaliana (Lethimäki et al., 2014). The latter may explain the absence of regulation of FNR by phosphorylation due to CaMK, CDPKs or MAPKs activation.

Copper-Induced Levels of ROS and Membrane Damage Are Dependent on Activation of CaMKs, CDPKs and/or MAPKs

On the other hand, the level of SA and HP were not increased in the alga exposed only to copper excess (this work). The latter indicates that efficient antioxidant mechanisms were induced in the alga in response to copper that are able to cope with copper-induced oxidative stress. In addition, inhibitors of CaMKs, CDPKs, MAPKs lead to increased levels of SA and HP and to the damage of cellular membranes. Thus, the accumulation of SA and HP are the main responsible of cellular membrane damage observed in the alga treated with copper (González et al., 2010; González et al., 2012). It is interesting to mention that HP is located in the whole cell in alga treated only with copper whereas HP is located in the nucleus, and not the chloroplast or the cytoplasm, in the alga treated with CDPKs inhibitor and copper. The latter may indicate that CDPKs are involved in the inhibition of antioxidant enzymes in the chloroplast and the cytoplasm. In addition, the inhibition of MAPKs strongly increased the level of HP in the chloroplast and the nucleus suggesting that MAPKs are involved in the activation of antioxidant enzymes in the chloroplast and the nucleus. The latter is in accord with previous findings showing that the levels of transcripts encoding antioxidant enzymes such as SOD, CAT and AP are strongly decreased by inhibitors of ERK, JNK and p-38 MAPKs indicating that the decrease in activities of antioxidant enzymes is due to inhibition of gene expression (Rodríguez-Rojas et al., 2019). Thus, the mitigation of copper-induced oxidative stress is due mainly to the activation of antioxidant enzymes and the synthesis of ASC, GSH and NADPH. However, concentrations higher than 50 µM of copper can surpassed the mechanisms for buffering copper-induced oxidative stress leading to cell death in U. compressa (González et al., 2010). Thus, copper-induced oxidative stress is buffered by the increase in activities of antioxidant enzymes and in the synthesis of ASC, GSH and NADPH and the latter is dependent on the activation of CaMK, CDPKs and MAPKs signaling pathways in U. compressa.

CONCLUSION

Copper induced the activation of MAPKs, CDPKs and CaMKs leading to the modulation of glycolysis and induction of carbon flux reprogramming which trigger an increase in ASC, GSH and NADPH synthetics and the activation of antioxidant enzymes in order to buffer copper-induced oxidative stress and to inhibit cell membrane damage.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: 10.6084/m9.figshare.12142101.

AUTHOR CONTRIBUTIONS

DL did experimental work. AG helped with results analyses. AM helped with experimental design and wrote the manuscript.

FUNDING

This work was financed by Postdoctoral Fondecyt 3170511 to DL and by VRIDEI-USACH.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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