Trees (2018) 32:1481–1485
https://doi.org/10.1007/s00468-018-1750-8

SHORT COMMUNICATION

Carbon dioxide induces minor antioxidant responses in *Eucalyptus urophylla* chloroplasts

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Received: 2 February 2018 / Accepted: 10 August 2018 / Published online: 16 August 2018

Abstract

Key message **Minor effect on the chloroplast antioxidant proteins was detected in *Eucalyptus urophylla* cultivated in high-CO_2 atmosphere.**

Abstract Global climate change can significantly alter plant cell metabolism. A higher atmospheric CO_2 scenario may be beneficial for C3 plants through the stimulation of photosynthesis. This predicted increase in the rate of carbon assimilation may also increase the expression of enzymes involved in the antioxidant cellular defense. Here, we studied the responses of the chloroplastic antioxidant system of *Eucalyptus urophylla* plants cultivated in a high-CO_2 condition. Plants exposed to a high concentration (980 ppm) of CO_2 showed an increase in the H_2O_2 concentration and MDA content in relation to those cultivated at 410 and 680 ppm. With the discovery proteomics approach used herein, we identified 19 chloroplastic antioxidant proteoforms and pinpointed differentially regulated isoforms of an ascorbate peroxidase and a superoxidase dismutase upon cultivation in a high-CO_2 atmosphere. Our data indicate that the CO_2 stimulus induces only minor changes in the antioxidant metabolism of *E. urophylla* chloroplasts.

Keywords Antioxidants · Chloroplast · CO_2 · *Eucalyptus* · Proteomics

According to Earth System Research Laboratory (ESRL) Global Monitoring Division (2017), the atmospheric CO_2 concentration has increased considerably in the last decades: from 315 ppm in 1958 to 408 ppm in 2017, the highest CO_2 concentration ever recorded at the Mauna Loa Observatory. If CO_2 emissions continue to increase, the Earth's atmospheric CO_2 concentration could reach the remarkable concentration of 985 ppm in the year 2100 (IPCC Panel 2014). Although there is no current consensus on the impact of this global prediction, the increasing CO_2 concentration scenario may be beneficial for most plant species, especially for those employing the C3 photosynthetic pathway, as they depend on a high CO_2:O_2 ratio to counterbalance losses due to the photorespiration process.

It is generally accepted that, in addition to the direct modulation of plant growth though photosynthesis, CO_2 stimulus may also impact abiotic stress responses. Currently, there are two parallel and potentially complementary hypotheses that sustain the idea that CO_2 could alleviate stress effects: the antioxidant hypothesis and the relaxation hypothesis (reviewed by AbdElgawad et al. 2016). While the former relies on the hypothesis that an increase in the CO_2 concentration would promote a fast defense response due to the higher availability of carbon molecules for antioxidant molecule biosynthesis, the latter relies on a decreased production of reactive oxygen species (ROS) and antioxidant molecules due to the stimulation of the carboxylase activity of RuBisCO over the oxygenation process. As there are plenty of reports that give support to both hypotheses (AbdElgawad et al. 2016), it is important to evaluate the effect of CO_2 on the antioxidant system on a case by case basis.

Although not limited to them, plant antioxidant enzymes play a fundamental role in the control of ROS homeostasis. Such enzymes can be identified and their expression quantified by large-scale analysis, such as proteomics, which

Communicated by Heckathorn.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00468-018-1750-8) contains supplementary material, which is available to authorized users.

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combines advanced separation techniques, mass spectrometry and bioinformatic tools to characterize proteins in complex mixtures. This technology becomes even more powerful if targeted to a specific cell compartment, such as in subcellular proteomics, which explores a specific cell compartment and the processes that occur within it. Here, we evaluated the changes in the abundance of chloroplast antioxidant proteins of *Eucalyptus urophylla* caused by a CO_2-enriched atmosphere (980 vs 410 ppm).

Under abiotic stress, ROS production in plants is elevated, which may lead to an imbalance in the cellular homeostasis if not regulated by antioxidant metabolism. To evaluate the oxidative status of *E. urophylla* leaves under CO_2 stimulus, we cultivated young plants (90 days old) in plant growth chambers with controlled conditions (temperature adjusted at 27 °C and photoperiod of 12 h) and CO_2 concentrations at 410, 680 and 980 ppm. After 30 days of growth, plants presented small but significant differences in the H_2O_2 and MDA concentrations (Fig. 1). The H_2O_2 concentration and the MDA content reached the highest levels in the plants grown at 980 ppm CO_2 concentration. As the levels observed at the concentration of 680 ppm were similar to the control, the results indicate that the plants showed a high oxidative stress only when submitted to a 980-ppm condition. Although the CO_2 effect on oxidative metabolism has already been predicted in several plant species (Kumari et al. 2013; Farfan-Vignolo and Asard 2012; Liu et al. 2016; Singh and Agrawal 2015), our data suggest the existence of a CO_2 concentration threshold within the range of 680 and 980 ppm for triggering ROS stress in *E. urophylla* plants.

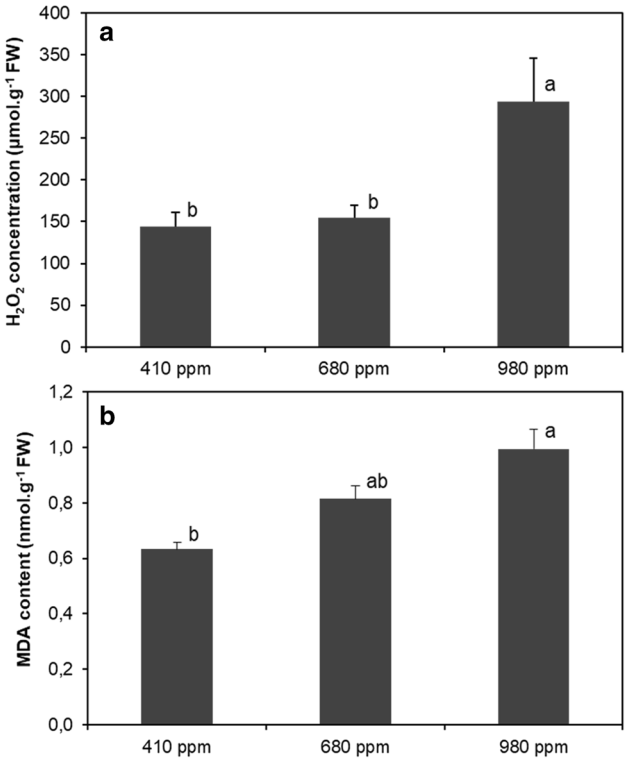

Fig. 1 Concentrations of H_2O_2 (**a**) and malondialdehyde (MDA) (**b**) of *E. urophylla* leaves from plants cultivated for 30 days in growth chambers with controlled CO_2 concentrations (410, 680 and 980 ppm). Different letters indicate significant differences according to Tukey's test ($p < 0.05$). Standard errors of the mean from three biological replicates are also indicated

Finding the key players in antioxidant response requires a multi-step investigation that usually starts with an untargeted, discovery-driven approach. If CO_2 acts as an elicitor for the biosynthesis of antioxidant molecules, an increase in the abundance of those metabolites would be expected when cells are challenged by an abiotic stress. In the absence of an environmental stressor, CO_2 stimulus alone could lead to an increase in oxidative damage, acting itself as a stressor, or alleviate ROS production by inhibition of photorespiration. We used large-scale proteomics from isolated chloroplast extracts (Fig. 1S) to identify and relatively quantify the abundance of chloroplast antioxidant proteins from plants exposed to two CO_2 concentrations: 410 and 980 ppm. In addition to mass spectrometry data analyses, we carried out a stringent data mining approach for plastid localization of antioxidant proteins identified through database searches. From a total of 30 identifications involved in the antioxidant defense, a small sub-set of 19 proteins met the criteria of having high sequence homology with at least one plant plastid database, and having a primary sequence indicating chloroplast localization (Table 1). It is worth noting that of the 19 chloroplastic antioxidant proteins identified here, 15 were thioredoxin-related (TRX) proteoforms. This high fraction of TRX identifications reflect the abundant representation of genes coding this enzyme in the genome of the reference species (*Eucalyptus grandis*) used here and the key role of this class of enzymes in controlling the redox status of a myriad of protein species. Although TRX forms were the most abundant molecules identified in the present study, two copies of ascorbate peroxidases (Eucgr.F00373.1, Eucgr.F04344.1), one copy of Fe-superoxide dismutase (Eucgr.K00110.6) and one copy of dehydroascorbate reductase (Eucgr.J01595.1) were also confidently identified in the *E. urophylla* chloroplastic protein extracts (Tables 1, 1S).

An up-regulation, induced by CO_2 stimulus, within the antioxidant sub-proteome described here was only detected for two proteins: a thylakoid ascorbate peroxidase (Eucgr.F04344.1) and the Fe-superoxide dismutase 2 (Eucgr.K00110.6) (Fig. 2). Both protein species presented a statistically significant difference in the relative abundance criteria used here (Normalized Spectral Abundance Factor-NSAF, Paoletti et al. 2006) according to the Kruskal–Wallis test ($p < 0.05$). The fact that APX and SOD levels were significantly higher when plants were exposed to 980 ppm treatment may be an indication of a high production of the

Table 1 Non-redundant list of all antioxidant proteins identified in *E. urophylla* chloroplasts. Chloroplast proteins were isolated by Percoll gradient centrifugation, identified by mass spectrometry and only reported if the sequence met two in silico criterias: (1) predicted to have a plastid location and (2) have a high sequence homology with an accession deposited in a plant subcellular database

Accession[a]	Description[b]	Unique Peptides[c]	PSMs[d]	Coverage (%)[e]	Highest ID Score[f]		Expression (NSAF)[g]		Predictors[h]	Databases[i]
					MS Amanda	Sequest HT	410 ppm	980 ppm		
Chloroplastic										
Eucgr.C00774.1	Thioredoxin superfamily protein	16	231	39.19	589.457	5.679	0.00418	0.00692	2	3
Eucgr.K02606.1	Thioredoxin superfamily protein	9	228	42.24	815.504	7.987	0.00499	0.00685	4	3
Eucgr.F00373.1	Ascorbate peroxidase 4	14	221	54.21	587.886	5.929	0.00421	0.00461	4	3
Eucgr.F04344.1	Thylakoidal ascorbate peroxidase	13	118	42.60	632.028	7.513	0.00106	0.00271	4	3
Eucgr.F02754.1	Thioredoxin superfamily protein	5	46	44.68	449.503	3.908	0.00136	0.00201	4	2
Eucgr.L03049.1	Thioredoxin M-type 4	4	25	43.10	242.658	2.253	0.00109	0.00183	1	3
Eucgr.A01555.1	Thioredoxin superfamily protein	2	21	32.62	282.981	3.043	0.00075	0.00124	1	3
Eucgr.K02032.1	Thioredoxin family protein	3	19	24.01	508.146	3.675	0.00042	0.00040	3	3
Eucgr.C03812.2	Thioredoxin-dependent peroxidase 1	3	14	40.58	577.730	3.469	0.00071	0.00079	1	2
Eucgr.K00110.6	Fe-superoxide dismutase 2	4	14	16.23	464.857	4.198	0.00020	0.00096	4	3
Eucgr.H01941.1	Thioredoxin superfamily protein	3	12	20.61	273.230	2.807	0.00033	0.00039	3	3
Eucgr.F02729.1	Thioredoxin superfamily protein	1	11	7.03	328.769	3.143	0.00024	0.00040	4	2
Eucgr.H02333.1	Thioredoxin superfamily protein	1	11	8.53	165.928	2.329	0.00027	0.00035	4	3
Eucgr.A01813.1	Thioredoxin superfamily protein	1	4	12.95	176.848	-	0.00020	0.00019	4	3
Eucgr.H01629.1	Thioredoxin F2	1	4	9.73	321.699	3.088	0.00025	0.00013	4	2
Eucgr.B01424.1	Thioredoxin F2	1	3	3.61	218.518	-	0.00010	0.00011	2	2
Eucgr.J01595.1	Dehydroascorbate reductase 1	2	3	14.18	248.261	2.333	0.00010	0.00020	4	3
Eucgr.G03224.2	Thioredoxin z	1	2	6.11	235.729	-	0.00018	0.00010	4	3
Eucgr.A02464.1	WCRKC thioredoxin 1	1	1	4.59	157.742	-	0.00010	0.00013	1	1

[a]Identifier according to the *Eucalyptus grandis* annotation v2.0

[b]Functional annotation

[c]Number of peptides that are unique to only one proteoform

[d]Number of peptide-spectrum matches

[e]Sum of identified peptides lengths in relation to total proteoform length

[f]Highest PSM score from all identified peptides

[g]Mean data of the relative abundance from three replicates

[h]Number of prediction tools suggesting a plastid localization—Predictors: TargetP, ChloroP, WoLF and Predotar

[i]Number of proteomic databases where a plastid homologuous sequence was found—databases: PPDB, AT-Chloro and SUBA3

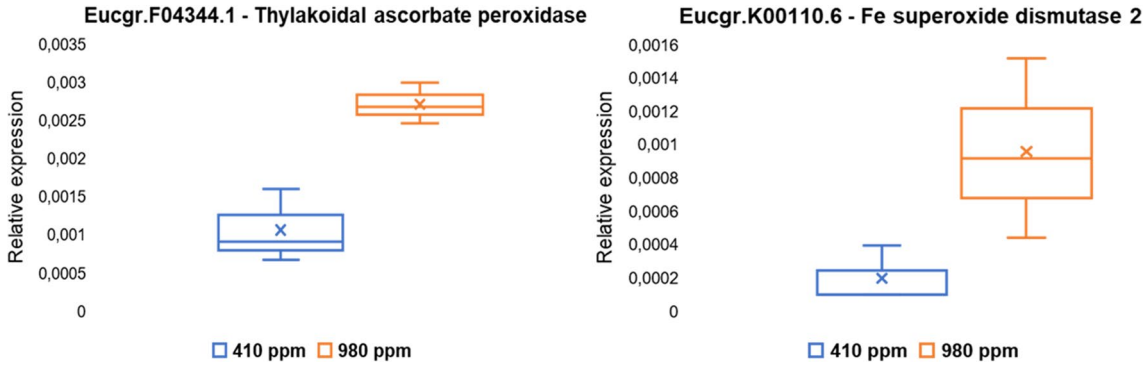

Fig. 2 Expression profiles of the antioxidant proteins Eucgr.F0344.1 and Eucgr.K00110.6 identified as differentially regulated, according to the Kruskal–Wallis test ($p < 0.05$), in young leaves of *E. urophylla* grown at CO_2 concentration of 410 and 980 ppm. Distribution of data is illustrated as color-coded boxplots: blue for plants cultivated at 410 ppm and orange for plants cultivated at 980 ppm

superoxide radical ($O_2^{\cdot-}$) within the cells upon CO_2 stimulus, leading to the higher expression of the SOD enzyme, and consequently generating a large amount of H_2O_2. One of the main ROS neutralization pathways in plants is the ascorbate–glutathione cycle, in which APX is the enzyme responsible for neutralizing H_2O_2 into H_2O. Overproduction of H_2O_2 by SOD may also lead to overexpression of the APX protein under this stress condition. These data corroborate with the antioxidant responses observed for other plant species (Zlatev et al. 2006; Hu et al. 2008; Mishra et al. 2013) and may be used as a possible indicator of stress for *E. urophylla* plants cultivated in high atmospheric CO_2 concentration. However, it is worth mentioning that the low number of differentially regulated proteins from *E. urophylla* chloroplasts may also indicate that other cellular compartments are playing an equal or a major role in the antioxidant response against CO_2 stimulus, as an increase in H_2O_2 and MDA levels was seen in plants exposed to 980 ppm concentration (Fig. 1). Additionally, the minor effect of the high CO_2 cultivation on the chloroplast antioxidant proteins identified in the present study indicates that it is very unlikely that CO_2 induces a standby antioxidant defense that could rapidly respond to oxidative damage induced by other stressors.

In the present communication, we described a differential response in the oxidative status of young *E. urophylla* plants cultivated in controlled conditions with variations in CO_2 concentration according to the last IPCC predictions. Using a proteomics-driven approach, we qualitatively and quantitatively reported what are probably the most abundant antioxidant proteoforms found in *E. urophylla* chloroplasts. We are aware that the stringent identification criteria used here penalized the chloroplast proteome coverage and, thus, a holistic overview of the CO_2-induced alterations. Conversely, protein identifications and quantitative data reported here are of high confidence and strict enough to preferentially consider only major changes induced by the atmospheric CO_2. Our data suggest that CO_2 may contribute to a higher tolerance to abiotic stress through metabolic means other than a direct induction of the biosynthesis of antioxidant proteins. However, this still needs to be tested in *E. urophylla* plants grown in high CO_2 conditions and challenged by a broad spectrum of abiotic stressors.

Author contribution statement ACB carried out the experiment, processed the experimental data, analyzed the data and wrote the manuscript with support from TSB. BMS carried out the experiment and verified the analytical methods. TSB designed the study, supervised the project and contributed to the interpretation of the results. All authors read and approved the final manuscript.

Acknowledgements We thank Dra. Priscila Lupino Gratão from the São Paulo State University for providing the laboratory infrastructure for the quantification of H_2O_2 and MDA content analysis. This work had financial support from São Paulo Research Foundation—FAPESP (Grant number 2015/23354-8) and Coordination for the Improvement of Higher Education Personnel—CAPES.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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