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ORIGINAL ARTICLE

Proteomic changes may lead to yield alteration in maize under carbon dioxide enriched condition

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Abstract

In the present study, the effect of elevated CO_2 on growth, physiology, yield and proteome was studied on two maize (*Zea mays* L.) varieties grown under Free-air CO_2 enrichment. Growth in high CO_2 (530 ppm) did not affect either photosynthesis or pigment contents in both varieties. Reduced MDA content, antioxidant and antioxidative enzymes levels were observed in both varieties in response to high CO_2. PEHM-5 accumulated more biomass than SMH-3031 under eCO_2. PEHM-5 also had more seed starch and total soluble sugar than SMH-3031. However, SMH-3031 had increased number of seed per cob than PEHM-5. Interestingly, thousand seed weight was significantly increased in PEHM-5 only, while it was decreased in SMH-3031 under eCO_2. We observed increased seed size in PEHM-5, while the size of the SMH-3031 seeds remained unaltered. Leaf proteomics revealed more abundance of proteins related to Calvin cycle, protein synthesis assembly and degradation, defense and redox homeostasis in PEHM-5 that contributed to better growth and yield in elevated CO_2. While in SMH-3031 leaf, proteins related to Calvin cycle, defense and redox homeostasis were less abundant in elevated CO_2 resulting in average growth and yield. The results showed a differential response of two maize varieties to eCO_2.

Keywords Elevated CO_2 · FACE · Maize · Yield · Antioxidative enzymes · Protein abundance · Mass spectrometry

Introduction

Anthropogenic activities have caused accumulation of greenhouse gases (GHGs) in the atmosphere, leading to the potential hazards of climate change. High carbon dioxide (CO_2) and other GHGs tend to warm up the atmosphere, besides affecting other meteorological variables. The pre-industrial CO_2 concentration was 278 parts per million (ppm) and it has reached up to 411.77 ppm in July 2019 (https://www.esrl.noaa.gov/gmd/ccgg/trends/) and there are further predictions for atmospheric [CO_2] reaching to 936 ppm (RCP8.5) by the year 2100 (IPCC 2013). The rise in atmospheric CO_2 together with warming will likely cause a significant impact on agricultural systems (Abebe et al. 2016; Mina et al. 2019; Qiao et al. 2019).

Plants with different photosynthesis type (C_3, C_4, and CAM) show variable response to elevated [CO_2]. Elevated atmospheric CO_2 normally promotes growth and yield of C_3 plants through fertilization effect and enhanced photosynthesis. However, there are conflicting reports on the effect of elevated CO_2 on maize crop ranging from no effect (Erbs et al. 2015) to a significantly positive effect on yield (Xie et al. 2018). In a meta-analysis study, Taub et al. (2008) reported that elevated CO_2 tend to decline protein concentrations in several major crop plants viz; wheat, rice, barley, soybean and many more. For accurate prediction of future food supply and fully adapting crops to exploit the additional atmospheric resource, it is important to understand crop response to elevated [CO_2] (Ainsworth et al. 2008; Leakey 2009; Leakey et al. 2009; Leakey and Lau 2012; Ruiz-Vera et al. 2015). About 18% of global primary productivity is comprised of C_4 plants and contribute up about 40% of the world grain harvest (Ehleringer et al. 1997). Therefore, it is

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important to understand the responses of economically valuable C_4 crop plants to future rises in atmospheric CO_2. The Indo-Gangetic plains region of India is the most intensively farmed zone of the country (Erenstein et al. 2007).

In India, maize (*Zea mays*) is among the three largest food crops after rice and wheat. Apart from being a good food for human being and excellent feed for animals, maize is also the raw material for various industrial products that include food sweeteners, starch, oil, protein, alcoholic beverages, pharmaceutical, cosmetic, textile, package and paper industries etc. (Parihar et al. 2011). An agricultural estimate suggests it was cultivated in 8.7 m ha (2010–2011) during Kharif season (July–October) in India. Maize contributes nearly 1400 million USD to the national agricultural gross domestic product of India besides generating employment of over 100 million man-days. (https://agrigoaexpert.res.in/icar/category/agriculture/fieldcrops/cere-als/maize.php).

Current knowledge on growth stimulation through elevated CO_2 was obtained by growing crops in various controlled environments such as leaf chambers (Valle et al. 1985), sunlight-controlled environment facilities (Jones et al. 1985a, b, c), greenhouses (Kimball 1983), and open-top chambers (Rogers et al. 1983, 1984a, b). But all these studies had many technical drawbacks mainly the chamber effect. Advancement of scientific understanding leads to the development of Free Air Carbon Dioxide Enrichment (Leakey 2009; Leakey et al. 2009). Free Air Carbon Dioxide Enrichment (FACE) experiments provide realistic future field conditions allowing the researchers to elucidate the mechanism of crop response closest to natural conditions.

There are very limited studies in India on the impact of elevated CO_2 on maize. Abebe et al. (2016) reported a positive impact of elevated CO_2 on maize in north-western India. Whereas Mina et al. (2019) reported better crop heath index of two maize varieties exposed to elevated CO_2. However, both these studies were done in open-top chambers. Till date, there are no studies on maize under FACE in India. Moreover, there is little published information on maize response to elevated CO_2 at the proteomic level. The objectives of the current study were to evaluate the impact of elevated CO_2 on growth, yield and proteome of two maize varieties grown under FACE condition.

Materials and methods

Carbon dioxide exposure

FACE facility is established at CSIR-National Botanical Research Institute, Lucknow (80,059′ E, 26,055′ N, 123 m above sea level), Uttar Pradesh, India (Pandey et al. 2017). FACE consists of six hexagonal rings having a diameter of 10 m, fitted with horizontal and vertical GI pipes with nozzles for the release of CO_2. Compressors are used to pump CO_2 into the rings and its flow was controlled via a solenoid valve. Out of six, three rings were used as control (ambient) and rest three were serves as CO_2 enrichment (elevated) ring. Monitoring of CO_2 concentration in the ring was achieved by an infra-red gas analyzer. Other sensors for the monitoring of temperature (resistance temperature detectors), humidity (humidity sensor), light intensity (PAR sensor), Wind speed (anemometer) and Wind direction (potentiometer) were towered at the centre of each ring. Microprocessor-based supervisory control and data acquisition (SCADA) system were used to harmonize, control and data acquisition from the installed sensors.

Experimental material, plot preparation and crop cultivation

Two commonly grown Indian maize (*Zea mays*) varieties: PEHM-5 and SMH-3010 were selected for the study. PEHM-5 (pusa early hybrid makka-5) an early maturing hybrid, is tolerant to waterlogging and with the life span of 80–90 days and average seed yield are 4.5–5.5 tonnes hectare^{-1}. SMH-3031 (short mid drought hybrid) is a late variety with the life cycle of 90–100 days, suitable for all conditions and seasons, having average seed yield 6.0–6.5 tonnes hectare^{-1}. Out of six FACE rings three were used for ambient carbon dioxide (control) and remaining three for carbon dioxide enrichment (treatment). Each ring was divided into six subplots of 4.5 m^{-2} area. In randomized way seeds of each variety (spacing of 20 × 25 cm) were sown during mid-June. There were approximately 50–60 plants ring^{-1} grown till final harvest. NPK (nitrogen: phosphorus: potassium) in the ratio of 100:60:40 kg per hectare was applied in the form of urea, diammonium phosphate (DAP), and muriate of potash, respectively. Phosphorus and Potassium were applied at the time of seedbed preparation while nitrogen fertilizer was applied in three splits doses. One-third of nitrogen was applied at the time of seedbed preparation. The second dose (1/3) of nitrogen was applied 30 days after germination (DAG) while the final dose 60 DAG. Irrigation and removal of weeds were done regularly throughout the experiment.

Meteorological condition and free air carbon dioxide enrichment data

During the experimental period, daily 24-h weather data (temperature and humidity) of Lucknow city was obtained from an online database (https://www.wunderground.com). The recorded average temperature varied between 35 and 22 °C and humidity varied between 97 and 56% (Supplementary Table S1). On-site field data were obtained from the SCADA system, comprising CO_2 concentration,

temperature, humidity, PAR, wind speed and wind direction. Plants of elevated rings were regularly (8 h per day from 9:00 AM to 5:00 PM) exposed to the elevated level of CO_2 (550 ppm). However, at the end of season average elevated CO_2 concentration was 530 ppm (+ 130 ppm above ambient; ambient average 398 ppm) (Supplementary figure S1).

Plant photosynthesis, growth and yield

Rate of photosynthesis, stomatal conductance, respiration, maximal photochemical efficiency of PSII was measured from Li-COR model 6400 (Lincoln, Nebraska, USA). The youngest fully mature leaf from three randomly selected plants within each cultivar and ring was selected for measurement.

Plants were harvested for biomass at initial grain filling stage (70 days after germination; DAG) and final harvest (full maturity; 100 DAG) in five replicates from each ring ($n = 15$). Up-rooted plants were immediately washed in running water to remove the soil. Leaf samples ($n = 5$) were frozen in liquid N_2 and kept at − 80 °C for biochemical and proteomic study. Grains were also stored at − 20 °C for biochemical and proteomic analysis after harvesting. Root, shoots and sprout were separated and oven-dried at 70 °C until a constant weight was achieved and biomass were taken from Sartorius (CP 124S) balance. To study the yield parameters root weight, shoot weight and sprout weight, the number of seed per cob, thousand seed weight and harvest index were considered.

Pigments, antioxidant enzymes and antioxidant estimation

Pigments, antioxidant and antioxidant enzymes were estimated from leaf samples of initial grain filling stage (70 DAG). Total chlorophyll, chlorophyll a and chlorophyll b content were measured using Arnon (1949) 80% acetone method. Lichtenthaler (1987) method was used for the estimation of carotenoid. Lipid peroxidation in terms of malondialdehyde (MDA) content was measured, using thiobarbituric acid (Heath and Packer 1968). Photochemical NBT reduction method was used for Superoxide dismutase (SOD) activity assay (Beyer and Fridovich 1987). Catalase (CAT) activity was measured in terms of decrease in absorbance at 240 nm as H_2O_2 was consumed (Rao et al. 1996). Rate of hydrogen peroxide dependent oxidation of ascorbic acid (Chen and Asada 1989) was used to estimate Ascorbate peroxidase (APX). Glutathione reductase (GR) activity was measured by the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) via glutathione to form TNB (Smith et al. 1988). Bradford (1976) method was used to estimate protein content using the Bradford reagent and bovine serum albumin (BSA, Sigma) as a standard. The activity of SOD, CAT, APX and GR were calculated based on protein content. Enzyme recycling assay given by Griffith (1980) was used to determine reduced glutathione (GSH) and oxidised glutathione (GSSG) content.

Starch, total soluble sugar of seed

Starch and total soluble sugar were estimated by colorimetric method using soluble starch and glucose as standard. Perchloric acid was used for starch extraction (Whelan 1955) and estimated using iodine (Nguyen et al. 2002). Ethanolic extraction of total soluble sugar (Cross et al. 2006) was performed and estimation was done by anthrone method (Yemm and Willis 1954).

Yield parameters

Yield parameters were studied at final harvesting and comprised of root weight, shoot weight, sprout weight per plant, number of sprouts per plant, number of seed per cob, thousand seed weight and harvest index (Hay 1995).

$$\text{Harvest Index (HI)} = \frac{\text{Grain weight per plant}}{(\text{Shoot weight} + \text{sprout weight})}$$

To obtain the size of the seed, images were captured using a stereo fluorescence microscope (LEICA M205 FCA) with an inbuilt camera (LEICA DFC7000 T) without image enhancement and saved in TIFF (tagged image file format) files. Fifteen mature dry seeds were scanned and images were captured at a pixel density of 1920 × 1440. Using LEICA application suite version 4.2 (LAS V4.2) image analysis software, the length (major) and the width were measured.

Proteomic studies

Leaf protein extraction

Leaf samples (70 DAG) were powdered in liquid N_2 and homogenized in extraction buffer (50 mM Tris–HCl, pH 8.0 + 25 mM EDTA + 500 mM thiourea) and 0.5% β-mercaptoethanol (BME). Homogenate was kept at − 20 °C for overnight precipitation after adding 10% TCA in acetone + 0.07% BME. Overnight samples were pelleted and washed with chilled acetone containing 2% BME and vacuum dried. Dried pellet was resuspended in 5 ml buffer containing 0.1 M Tris–HCl pH 8.0, 50 mM EDTA and 2% BME. Tris-saturated phenol (2.5 ml) was added in above homogenate and mixed by gentle inversion. Lower phenol phase was collected and proteins were precipitated in methanol containing 0.1 M ammonium acetate and 1% β-ME. The precipitate was air-dried and washed with chilled acetone.

Dried pellet was solubilized in solubilization buffer (7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS; w/v), 25 mM DTT and 0.5% immobilized pH gradients buffers (v/v) at room temperature for 2–3 h. Protein concentration was quantified by Bradford assay using BSA as the standard.

Two-dimensional gel electrophoresis and image analysis

Two-dimensional gel electrophoresis was performed as described by Sharma et al. (2017). Briefly, passive rehydration on immobilized pH gradient (IPG) strips of 7 cm, pH 4–7 (GE Healthcare) was performed for 12 h in triplicates. The cocktail (135 µl) of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, 0.5% v/v immobilized pH gradients buffers, 0.001% Bromophenol blue) and protein (120 µg) was prepared. IPG strip rehydration was performed in the reswelling tray (Amersham Biosciences, Uppsala, Sweden). Ettan IPGphore-3 (GE Healthcare) system was used for Isoelectric focusing (IEF) at a fixed temperature (20 °C) in 5 step program comprises of: step 1—250 V for 1 h, step 2—500 V for 1 h, step 3—1500 V for 2 h (all step and hold), step 4—4000 V for 2 h (Gradient) and step 5—6000–12000 V (Sharma et al. 2017). Reduction (by 1% w/v DTT) then alkylation (by 2.5% w/v Iodoacetamide) of focused strip was performed in 5 ml equilibration solution (6 M urea, 30% w/v glycerol, 2% w/v sodium dodecyl sulfate (SDS) and 50 mM Tris–HCl buffer, pH 8.8) for 15 min separately. Equilibrated strips were placed on stacking gel and sealed with 0.5% agarose.

Polyacrylamide gels electrophoresis (PAGE) was carried out using Mini Protein tetra cell (Bio-Rad) in Tris–Glycine running buffer and 12% homogeneous SDS–polyacrylamide gel (acrylamide: Bis ratio of 29:1 supplied by Sigma-Aldrich) at 70 V for 30 min after that 120 V till dye front moves out from the gel. Gels were stained with coomassie brilliant blue G250 (0.5%), destained (methanol: water: glacial acetic acid = 10: 50: 40) and gel images were captured.

Protein abundance analysis was performed using Image Master 2D Platinum (IMP) 7.0 software (Amersham Bioscience) based on relative % volume of protein spots in all triplicate images. Change in % volume ≤ 1.5-fold was taken as increased abundance and decreased ≥ 1.5-fold was taken as decreased abundance.

Seed protein extraction

Total seed proteins were extracted using a method described by Guo et al. (2012) with some modifications. 500 mg seeds were powdered in liquid N_2 and homogenized in 1 ml extraction buffer-A (400 mM Sucrose, 35 mM Tris–HCl pH 7.00, 200 mM EDTA, 1 mM DTT, 1 mM Phenyl-methane-sulfonyl fluoride (PMSF)). Further 2 ml of extraction buffer-B (extraction buffer-A + 400 µl Triton X-100) was added in above homogenate and mixed properly for 3–5 min and then shaken vigorously. The supernatant was collected after twice centrifugation. Proteins from the supernatant were precipitated overnight at − 20 °C by adding ¼ volume 10% TCA-acetone. After that likewise leaf protein, seed proteins were extracted by phenol precipitation, purified, solubilized and quantified by Bradford method.

Seed SDS-PAGE and image analysis

Seed protein samples were loaded on stacking gel with 2X gel loading buffer (125 mM Tris–HCL pH 6.8, 2% (w/v) SDS, 20% [v/v] glycerol, 100 mM DTT and 0.01% (w/v) bromophenol blue). Electrophoresis was carried out on slab gels (60 mm × 80 mm × 1 mm), using the discontinuous buffer system. The 5% SDS–polyacrylamide stacking gel was overlaid upon the 12% SDS–polyacrylamide separating gel (acrylamide: Bis ratio of 29:1 supplied by Sigma-Aldrich). Electrophoresis was performed in a standard Tris–glycine running buffer in Mini Protein tetra cell (Bio-Rad) at a constant 70 V using a Power PAC300 (Bio-Rad) till dye front move out. Finally, gels were stained with coomassie brilliant blue G250 (0.5%), destained (Methanol: Water: Glacial Acetic acid = 45: 45: 10) and gel images were captured. Image analysis was performed using Image Quant TL 7.0 software (GE Healthcare). Again, change in % volume ≤ 1.5-fold was taken as increased abundance and decreased ≥ 1.5-fold was taken as decreased abundance.

Mass spectrometry and protein identification

Differentially expressed spots/bands were excised from the gel, sample preparation and tryptic digestion were performed (Shevchenko et al. 2007). Cut gel pieces were chopped and destained with 50 mM ammonium bicarbonate (ABC) in 50% methanol by incubation with occasional vortexing, depending on the staining intensity. Destained pieces were rehydrated and dehydrated with 25 mM ABC and 2:1 (v/v) solution of ACN: 50 mM ABC, respectively. After three cycles of rehydration and dehydration, samples were lyophilized (Labconco, CentriVap Concentrator, USA) and again rehydrated with trypsin solution (10–20 µl from 20 ng/µl stock) for digestion. The above gel slices were emerged in 25 mM ABC after 30 min incubation upon the ice and kept overnight at 37 °C in a circulating water bath (Multitemp II Thermostatic Circulator). Peptides were extracted in 50% acetonitrile (ACN) and 1% trifluoroacetic acid (TFA) twice and concentrated to a final volume of 10-15 µl. The concentrated samples were spotted on Opti-TOF TM384 well target plate (ABSCIEX, USA) in equal volume with the matrix solution (5 mg/ml a-Cyano-4-hydroxycinnamic acid in 50% ACN containing

0.1% TFA). Mass spectrometry (MS) was performed from 4800 Plus MALDI TOF/TOF Analyzer (ABSCIEX, USA), that generates monoisotopic peptide masses and were further analyzed using 4000 Series Explorer software version 3.5 (ABI). Protein identification was performed using mass signals from Mascot software (https://www.matrixscience.com) against the protein database (NCBInr/Swiss Prot). Search parameter includes; taxonomy- viridiplantae, fixed modification- carbamidomethylation, variable modification-oxidation, peptide tolerance- ± 100 ppm or 1.2 Da (Dalton), MS/MS tolerance- ± 0.2 Da or 0.6 Da, respectively and peptide charge + 1.

Statistical analysis

Effect of elevated level of carbon dioxide upon two maize varieties was tested with two-tailed Student's *t* test having 95% significance for all physiological, biochemical and yield parameters individually. Principal component analysis (PCA) using PAST-3 [Paleontological Statistics, Version 3.11] was performed for morphological, physiological, biochemical and harvest parameters collectively. The protein spot intensities of differentially expressed proteins in a 2D-gel were calculated from three biological replicate gels. Venn diagram analysis was performed to distinguish variety-specific differentially abundant proteins.

Results

Physiological, biochemical and yield response

All physiological, biochemical and yield response of the maize varieties against elevated carbon dioxide were analyzed via principal component analysis (PCA). A clear separation was observed between ambient and elevated CO_2 treatment and between the two tested varieties (Fig. 1).

The PCA bi-plot explained two principal components, together explaining 72% of the observed variations. PC1 disclosed 47% of the variability having a positive influence with most of the harvesting parameters including root, leaf and grain starch, root and leaf TSS. Biomass parameters at the early grain filling stage also contributed positively for PC1 (Supplementary Figure S2A). PC2, the second principal component disclosed 25% of the variability and the contribution was mainly due to antioxidative enzymes and antioxidants (such as SOD, APX, GR, glutathione) and most of the gas exchange parameters (Supplementary Figure S2B).

In yield attributes, PEHM-5 showed significantly increased root weight (50%), shoot weight (29%) and sprout weight (39%) and above-ground biomass (34%) due to eCO_2 treatment (Fig. 2, Table 1). Similarly, in SMH-3031 significant increment in root weight (35%), sprout weight (32%)

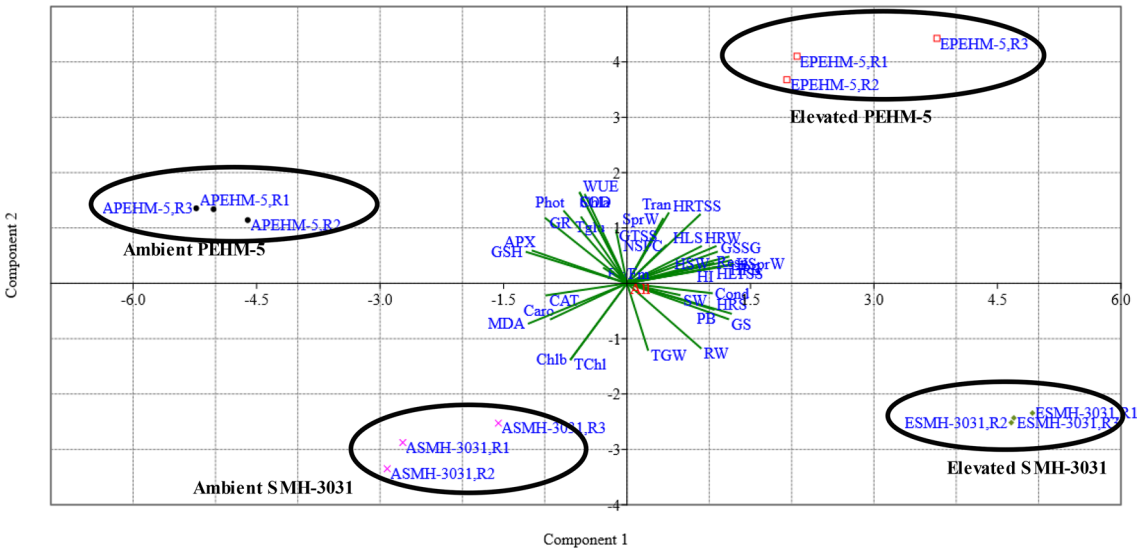

Fig. 1 PCA bi-plot showing two principal components with loadings of different variables (morphological, physiological and yield) and factor loadings of ambient (A) and elevated (E) CO_2 treatment for two maize varieties (PEHM-5, SMH-3031). Variables: *RW* root weight, *SW* shoot weight, *SprW* sprout weight, *PB* plant biomass, *Phot* photosynthetic rate, *Cond* stomatal conductance, *Trns* transpiration, *WUE* water use efficiency, *Resp* respiration, Fv/Fm-PSII maximal efficiency, *APX* ascorbate peroxidase, *GR* glutathione reductase, *CAT* catalase, *SOD* superoxide dismutase, *TGlu* total glutathione; *GSSG* oxidized glutathione; *GSH* reduced glutathione, *MDA* Malondialdehyde, HLS Harvesting stage leaf starch, *HRS* harvesting stage root starch, *GS* seed starch, *HLTSS* harvesting stage leaf total soluble sugars, *HRTSS* harvesting stage root total soluble sugars, *GTSS* seed total soluble sugars, *Chla* chlorophyll a, *Chlb* chlorophyll b, *TChl* total chlorophyll, *Caro* carotenoids, *HRW* harvesting stage root weight, *HSW* harvesting stage shoot weight, *HSprW* harvesting stage sprout weight, *HPB* harvesting stage plant biomass, *NSPC* number of seed per cob, *TGW* thousand seed weight, *HI* harvest index

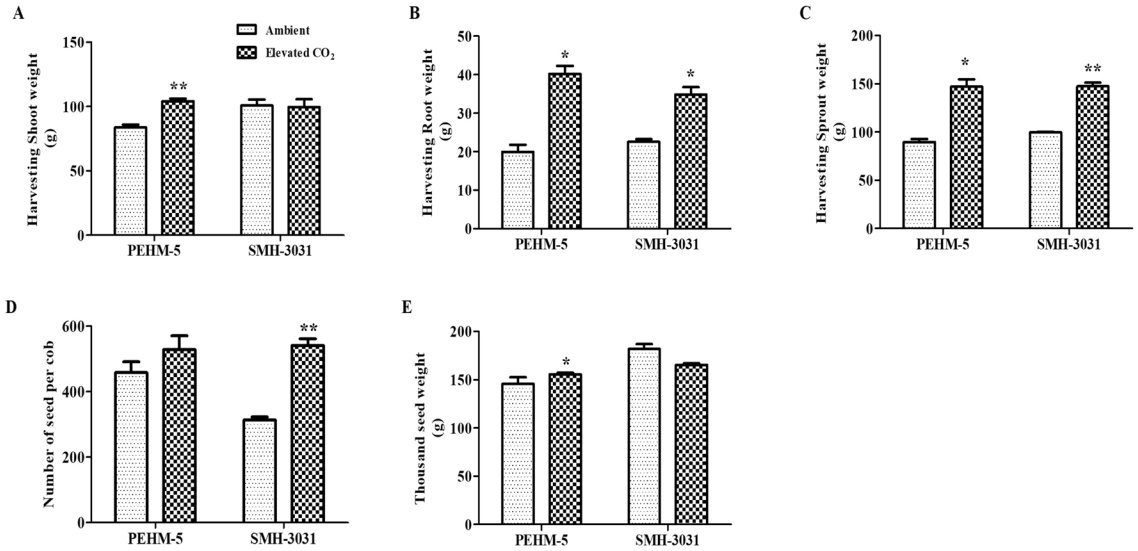

Fig. 2 Yield attributes of two *Zea mays* cultivars (PEHM-5 and SMH-3031) in ambient conditions (serving as a control) and with carbon dioxide concentrations enrichment (530 ppm) (mean ± standard error) ($n = 15$). **a** Shoot weight; **b** root weight; **c** sprout weight; **d** number of seed per cob; **e** thousand seed weight. Values represent average ± standard error. Significant results of student's *t* test are marked with asterisks (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$)

and above-ground biomass (21%) were found while shoot weight showed some insignificant increase of 5% in response to eCO_2 (Fig. 2, Table 1). There was a 13% increase in the number of seed per cob in PEHM-5 and 42% in SMH-3031 ($p < 0.05$). Interestingly, thousand seed weight was significantly increased (6%) in PEHM-5 only and decreased by (10%) in SMH-3031 (Fig. 2, Table 1). We observed increased seed size in PEHM-5 (15% and 5% more seed length and width, respectively), while the size of the SMH-3031 seeds remained unaltered (3.5% more length and 1.6% decreased width) (Fig. 3, Supplementary Table S2).

Seed Starch and total soluble sugar content

PEHM-5 showed 6% increment in seed starch and total soluble sugar content in eCO_2. On the other hand, SMH-3031 showed 5% increased seed starch content and 8% decreased total soluble sugar content in eCO_2 (Table 1). The increase in seed starch content was significant while total soluble sugar content was insignificant in both the varieties in response to eCO_2.

Leaf protein profiling and functional classification

The image analysis of leaf protein 2D-gels showed the differential abundance of proteins due to elevated carbon dioxide in both the varieties. PEHM-5 showed 55 differentially abundant proteins from 275 reproducibly detected protein spots. Similarly, SMH-3031 had 63 differentially abundant proteins out of 320 reproducibly detected spots (Fig. 4, Supplementary Tables S3). Of the total identified spots (48 from PEHM-5 and 52 from SMH-3031) via mass spectrometry, 60% were more abundant and 40% were less abundant in eCO_2 condition.

Venn diagram analysis of identified proteins showed the distribution of differentially abundant proteins between the two tested maize varieties. PEHM-5 and SMH-3031 had 29% and 28% unique proteins, respectively. About 43% of proteins were common in both the varieties (Fig. 5a, b; Supplementary Table S4a, b). More abundant proteins included Fructose-bisphosphate aldolase (FBA), Chlorophyll *a-b* bp, Oxygen-evolving enhancer (OEE), 70 kDa Heat shock cognate (HSP-70), Harpin binding protein, Actin-7 in PEHM-5. Similarly, SMH-3031 showed increased abundance of Ferredoxin-NADP reductase (FNR), Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Rubisco-LSU), Ribulose-phosphate 3-epimerase (RPE), Glyoxylase, Chaperonin, Pro-resilin. Lower abundance of Adenylate kinase, ATP synthase subunit α, Chlorophyll *a-b* bp, OEE (in PEHM-5) and Ascorbate peroxidase (APx), Ascorbate peroxidase-like protein, β-D-glucosidase (in SMH-3031) were found in eCO_2.

Functional classification of the identified proteins showed that most proteins belonged to carbon metabolism (CM) followed by protein synthesis, assembly and degradation (PSAD); photosynthesis (PHO); energy metabolism (EM); defense (DEF); Cell redox homeostasis (CRH), amino acid metabolism (AAM) and cytoskeleton (CYT) (Fig. 6). Out of total differentially abundant proteins 56% in PEHM-5 (34% + 22%) and 54% proteins in SMH-3031 (29% + 25%) collectively belonged to CM and PSAD category. In

Table 1 Effect of elevated CO_2 on antioxidant; antioxidative enzymes; pigments; biomass; yield; harvesting stage total soluble sugar content (leaf, root and seed); harvesting stage starch content (leaf, root and seed) in two *Zea mays* varieties (PEHM-5 and SMH-3031)

S. no	Parameter	PEHM-5				SMH-3031			
		Ambient	Elevated	Level of Significance	Percentage change	Ambient	Elevated	Level of significance	Percentage change
1	APX (μ mol $mg^{-1}protein^{-1}$ min^{-1})	(1.11 ± 0.01)	(0.89 ± 0.05)	ns	− 24.40	(1.01 ± 0.02)	(0.56 ± 0.03)	**	− 79.29
2	CAT (n mol $mg^{-1}protein^{-1}$ min^{-1})	(11.07 ± 0.62)	10.04 ± 0.62)	*	− 10.20	(14.18 ± 0.75)	(7.75 ± 0.25)	**	− 82.91
3	SOD (n mol g^{-1}fresh wt.)	(8.30 ± 0.22)	(8.58 ± 0.28)	ns	3.26	(4.52 ± 0.07)	(3.49 ± 0.05)	***	− 29.51
4	GR (μ mol $mg^{-1}protein^{-1}$ min^{-1})	(0.21 ± 0.00)	(0.18 ± 0.01)	*	− 16.14	(0.16 ± 0.00)	(0.12 ± 0.00)	**	− 29.19
5	MDA (n mol g^{-1}fresh wt.)	(90.26 ± 1.09)	(52.89 ± 0.71)	**	− 70.66	(101.33 ± 5.33)	(55.17 ± 1.54)	**	− 83.66
6	Total GSH (μ mol g^{-1}fresh wt.)	(27.57 ± 0.90)	(23.93 ± 0.41)	*	− 15.23	(20.27 ± 0.50)	(21.64 ± 0.69)	ns	6.32
7	GSSG (μ mol g^{-1}fresh wt.)	(3.49 ± 0.09)	(8.06 ± 0.38)	**	56.74	(2.93 ± 0.02)	(9.29 ± 0.10)	***	68.48
8	GSH (μ mol g^{-1}fresh wt.)	(24.09 ± 0.95)	(15.87 ± 0.70)	**	− 51.79	(17.34 ± 0.22)	(12.35 ± 0.60)	*	− 40.41
9	Chl *a* (mg g^{-1}fresh wt.)	(20.26 ± 0.28)	(17.60 ± 0.19)	**	− 15.1	(20.64 ± 0.49)	(19.50 ± 0.26)	ns	− 5.84
10	Chl *b* (mg g^{-1}fresh wt.)	(11.90 ± 0.19)	(9.66 ± 0.11)	*	− 23.20	(11.95 ± 0.29)	(11.49 ± 0.18)	ns	− 4.01
11	Total chl (mg g^{-1}fresh wt.)	(11.84 ± 0.19)	(9.61 ± 0.11)	*	− 23.19	(11.89 ± 0.29)	(11.43 ± 0.18)	ns	− 4.01
12	Carotenoids (mg g^{-1}fresh wt.)	(7.37 ± 0.06)	(6.28 ± 0.07)	**	− 17.52	(6.87 ± 0.19)	(6.78 ± 0.10)	ns	− 1.42
13	Root weight (g)	(18.75 ± 0.68)	(23.54 ± 1.66)	*	20.32	(30.96 ± 1.18)	(39.75 ± 0.64)	**	22.11
14	Shoot weight (g)	(103.42 ± 0.61)	(115.11 ± 2.35)	*	10.16	(112.98 ± 4.09)	(119.17 ± 3.34)	ns	5.19
15	Sprout weight (g)	(30.91 ± 0.44)	(37.68 ± 1.23)	*	17.98	(27.53 ± 0.47)	(31.73 ± 1.14)	*	13.25
16	Total Biomass (g)	(153.08 ± 0.34)	(176.33 ± 1.58)	**	13.19	(171.47 ± 3.45)	(190.65 ± 2.30)	*	10.06
17	Photosynthesis (μ mol CO_2 m^{-2} s^{-1})	(32.42 ± 0.26)	(32.21 ± 0.21)	ns	− 0.65	(31.43 ± 0.26)	(30.54 ± 0.18)	ns	− 2.93
18	Conductance (m^{-2} s^{-1})	(0.19 ± 0.01)	(0.14 ± 0.01)	*	− 33.51	(0.22 ± 0.02)	(0.13 ± 0.01)	*	− 78.42
19	Respiration (m mol CO_2 m^{-2} s^{-1})	(2.89 ± 0.09)	(1.75 ± 0.05)	**	− 64–83	(3.84 ± 0.09)	(2.26 ± 0.04)	**	− 70.03
20	Transpiration (m mol m^{-2} s^{-1})	(4.34 ± 0.06)	(2.98 ± 0.11)	**	− 45.68	(3.47 ± 0.29)	(2.74 ± 0.16)	ns	− 26.71
21	Fv/Fm	(0.77 ± 0.01)	(0.76 ± 0.00)	ns	− 0.26	(0.76 ± 0.00)	(0.76 ± 0.01)	ns	0.32
22	HLeaf TSS (mg g^{-1}dry wt.)	(20.00 ± 0.31)	(25.27 ± 0.41)	**	20.86	(21.15 ± 0.30)	(25.27 ± 0.41)	*	16.31
23	HRoot TSS (mg g^{-1}dry wt.)	(24.53 ± 0.51)	(30.25 ± 0.12)	**	18.90	(22.43 ± 0.65)	(26.78 ± 0.53)	ns	16.23
24	HSeed TSS (mg g^{-1}dry wt.)	(10.57 ± 0.12)	(11.31 ± 0.51)	ns	6.58	(10.82 ± 0.20)	(9.99 ± 0.21)	ns	− 8.41
25	HLeaf Starch (mg g^{-1}dry wt.)	(137.50 ± 0.57)	(151.66 ± 6.40)	ns	9.33	(141.28 ± 0.98)	(145.17 ± 0.85)	ns	2.68
26	HRoot Starch (mg g^{-1}dry wt.)	(20.35 ± 0.13)	(24.32 ± 0.15)	**	16.35	(22.79 ± 0.08)	(28.44 ± 0.14)	***	19.86
27	HSeed Starch (mg g^{-1}dry wt.)	(677.11 ± 0.68)	(722.97 ± 1.71)	**	6.34	(714.44 ± 1.70)	(757.49 ± 0.68)	**	5.68
28	HRoot weight (g)	(19.95 ± 1.80)	(40.18 ± 2.29)	*	50.34	(22.52 ± 0.77)	(34.81 ± 1.95)	*	35.31

Table 1 (continued)

S. no	Parameter	PEHM-5				SMH-3031			
		Ambient	Elevated	Level of Significance	Percentage change	Ambient	Elevated	Level of significance	Percentage change
29	HShoot weight (g)	(83.66 ± 2.26)	(117.50 ± 0.68)	**	28.80	(100.72 ± 4.74)	(106.05 ± 2.58)	ns	5.03
30	HSprout weight (g)	(89.50 ± 3.17)	(146.95 ± 7.82)	*	39.09	(99.51 ± 0.73)	(147.47 ± 3.58)	**	32.52
31	HAbove ground biomass (g)	(173.16 ± 4.06)	(264.45 ± 7.81)	ns	34.52	(200.22 ± 4.39)	(253.52 ± 6.02)	**	21.02
32	HTotal biomass (g)	(193.11 ± 5.58)	(304.63 ± 9.64)	**	36.61	(222.74 ± 5.05)	(288.34 ± 4.52)	**	22.75
33	Number of seed per cob	(458.00 ± 33.29)	(528.00 ± 42.33)	**	13.26	(312.67 ± 9.33)	(541.33 ± 20.34)	**	42.24
34	Thousand seed weight (g)	(145.75 ± 6.79)	(155.57 ± 1.58)	ns	6.31	(181.89 ± 5.00)	(165.47 ± 1.40)	ns	−9.92
35	Harvest Index	(0.52 ± 0.01)	(0.55 ± 0.01)	ns	6.88	(0.50 ± 0.01)	(0.58 ± 0.00)	*	14.47

Values represent mean ± standard error

*Indicate significant differences between control and treatment (eCO_2) within each cultivar ($p < 0.05$) by student's *t* test

PEHM-5, proteins of other functional groups belonged 12% to PHO, 14% to EM and 18% to DEF, CRH, AAM and CYT collectively. Similarly, in SMH-3031 proteins of other functional group included PHO (13%), EM (8%) and DEF + CRH + AAM (25%). The detailed information of differentially abundant proteins of both maize varieties is summarized in Table 2; Supplementary Figure S3, Supplementary Table S5.

Seed protein profiling and functional classification

Image Quant TL analysis showed a difference between eCO_2 treatment and control as well as within cultivars. In PEHM-5, 27 bands were analyzed ($p < 0.05$); eight bands were more abundant and were identified as Globulin-2, Glycosyl hydrolase family 31 protein, Lipid body-associated protein L2, Chitin-binding, type 1 and Chitinase. Similarly, in SMH-3031, 26 bands were analyzed ($p < 0.05$), eleven bands were found differentially abundant (4 more abundant, 7 less abundant) (Fig. 7) and seven bands were identified as Globulin-1 S allele, 50kD gamma zein, Hydroxyproline-rich glycoprotein family protein, Oleosin Zm-II and Receptor protein kinase-like protein ZAR1. Identified seed protein names and their detailed information are given in Table 3.

Discussion

The free air carbon dioxide enrichment study was conducted in natural field condition to understand the response of two commonly grown maize varieties concerning biochemical, yield and proteomics changes. Elevated CO_2 modulate various biological process including respiration, photosynthesis, antioxidant metabolism (Penuelas et al. 2013; Singh and Agrawal 2015; Xu et al. 2015). Results obtained from the present study showed unaltered photosynthesis (*A*) in both maize varieties under eCO_2 condition (Table 1). Significantly decreased transpiration (46%), respiration (65%) and water use efficiency (33%) were observed in PEHM-5 under eCO_2. Decreased transpiration under high CO_2 may be due to stomatal closure (increasing internal CO_2 concentration tend to close stomata). Similarly, SMH-3031 demonstrated eCO_2 induced reduction in respiration (70%) and water use efficiency (78%). These findings support the results obtained in previous studies on maize plants (Leakey et al. 2004, 2006; Markelz et al. 2011; Mina et al. 2019; Ruiz-Vera et al. 2015) and other C_4 plants (Hatfield et al. 2011). One of the possible reasons for unaltered *A* could be the lack of photorespiration or higher CO_2 concentration in bundle sheath cell, where Rubisco resides. Another reason might be *A* at high photosynthetic photon flux density could be reduced by reduced Rubisco carboxylation capacity (Bunce 2014). Ghannoum et al. (2000) also reported unaffected *A* in C_4

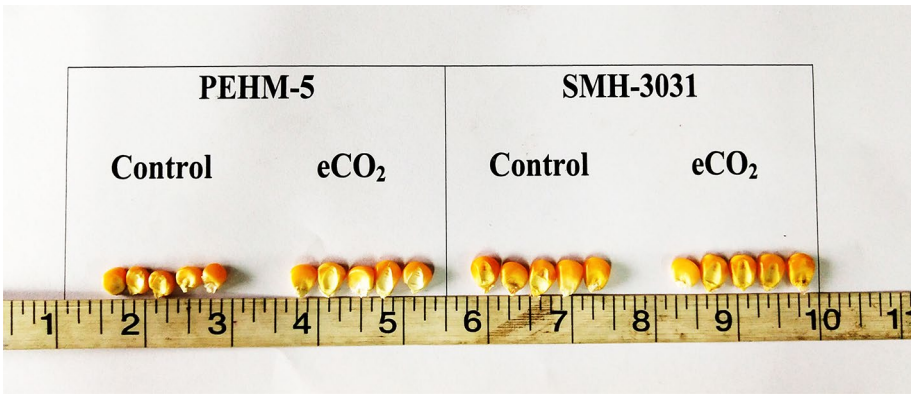

Fig. 3 Effect of eCO_2 (mean ± standard error) on seed size of two *Zea mays* varieties (PEHM-5 and SMH-3031)

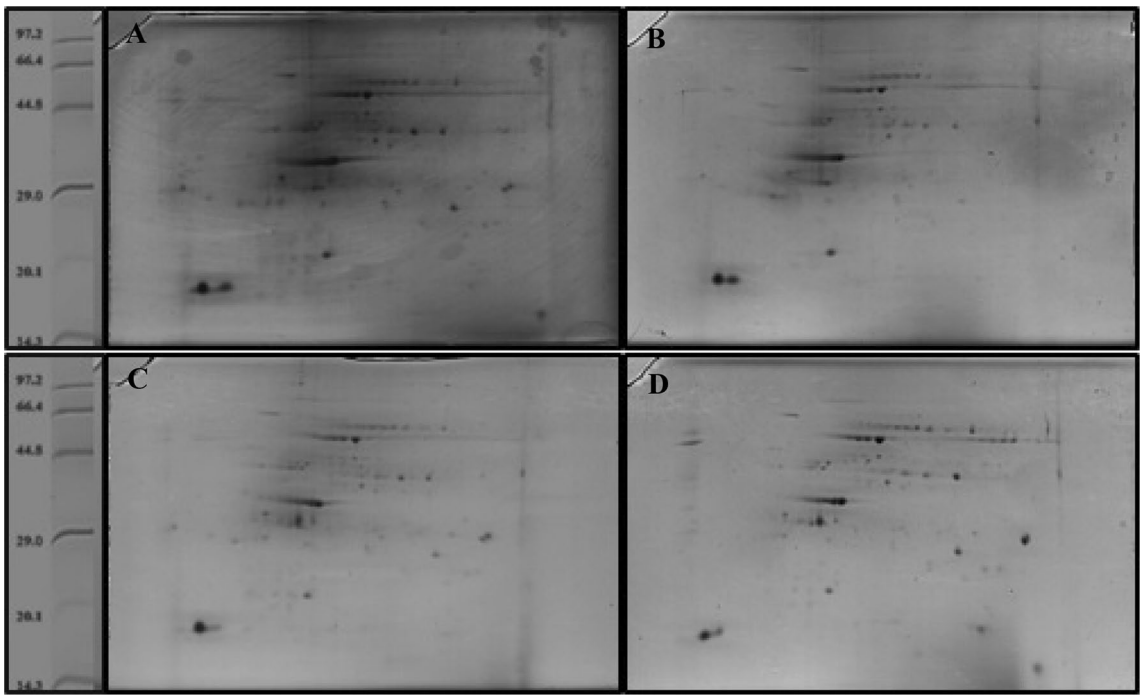

Fig. 4 Effect of eCO_2 on leaf protein expression pattern of two *Zea mays* varieties; PEHM-5 (**a**, **b**) and SMH-3031 (**c**, **d**). 12% gel, 120 µg protein loading, pH range 4–7, Brilliant blue G stained two-dimensional gel representing control (**a**, **c**), 530 ppm eCO_2 (**b**, **d**)

plants under optimal growth condition (water and nitrogen fertilizer).

Photosynthetic pigments such as total chlorophyll, chlorophyll *a* and *b* and carotenoid content decreased in both the varieties and decrease was significant in PEHM-5 (Table 1). Reduced concentration of photosynthetic pigments under high CO_2 may reflect feedback inhibition of photosynthesis when C availability exceeds the utilization. Similar results were reported for many plant species grown under eCO_2 (Mishra et al. 2013 and reviewed by Dong et al. 2018). Decreased carotenoid content could lead to damage to photosynthetic assembly and ultimately chlorophyll destruction.

Elevated CO_2 protects the plant from membrane damage and was reflected in the form of significantly decreased MDA content in both maize varieties (Table 1). Reduced MDA content in plants grown under high CO_2 might be due to reduced ROS generation (Mishra et al. 2013; Mishra and Agrawal 2014). Elevated CO_2 promotes carboxylation over oxygenation at rubisco, reducing reactive oxygen species (ROS) formation (relaxation hypothesis; AbdElgawad et al. 2015; Ainsworth et al. 2008; Booker et al. 1997; Long and Drake 1991; Zinta et al. 2014). This indicates that the stress-mitigating effect of elevated CO_2 cannot be universally attributed to increased antioxidant defenses. Moreover, Elevated CO_2 may or may not enhance the antioxidant system.

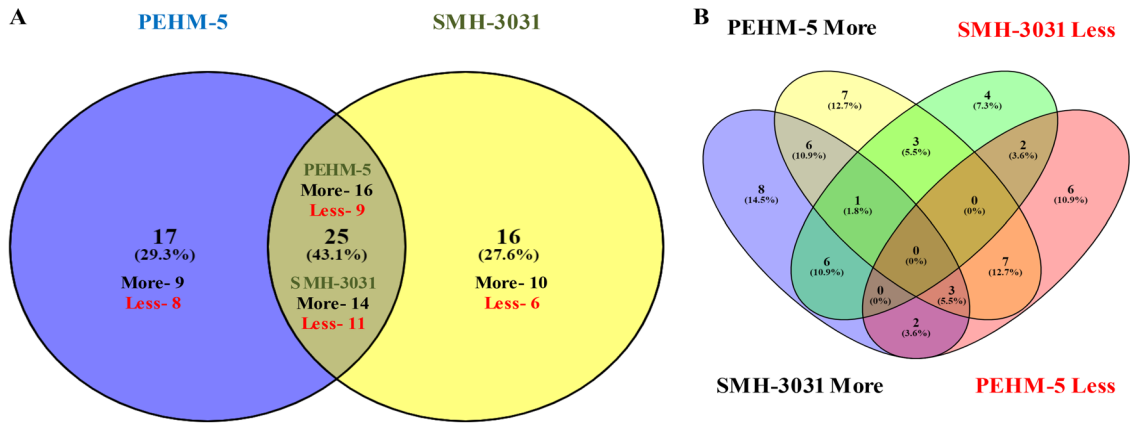

Fig. 5 Venn diagram of differentially abundant leaf proteins of two maize varieties (PEHM-5 and SMH-3031). **a** Variety specific overall differentially abundant proteins, **b** Abundance specific distribution of proteins. The overlapping areas suggest common proteins between the two groups (for detail see supplementary tables S4a, S4b)

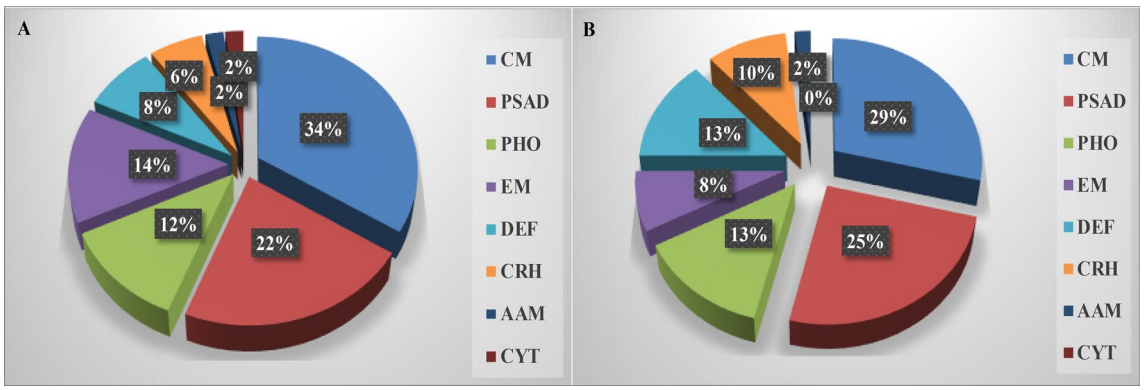

Fig. 6 Functional classification of eCO_2 responsive differentially abundant leaf proteins in two maize varieties. **a** PEHM-5; **b** SMH-3031. Carbon metabolism (CM); Photosynthesis (PHO); Protein synthesis, assembly and degradation (PSAD); Energy metabolism (EM); Defense (DEF); Cell redox homeostasis (CRH), Amino Acid metabolism (AAM) and Cytoskeleton (CYT)

It depends on plants species, crop variety, developmental stage or other abiotic stresses (reviewed in Xu et al. 2015). There are reports in which elevated CO_2 had little or no effect on antioxidants, or even decreased their levels (Erice et al. 2007; Farfan-Vignolo and Asard 2012; Mishra et al. 2013; Perez-Lopez et al. 2009). A key alternative process probably involved in the effect of elevated CO_2 on oxidative stress is photorespiration. When measured simultaneously, reduced photorespiration correlates well with the decrease in H_2O_2 and lower oxidative damage levels under high CO_2 in some studies (Aranjuelo et al. 2008; Mishra et al. 2013). Although we did not measure photorespiration in the present study, reduced level of antioxidant (total and reduced glutathione) and antioxidative enzymes in both the varieties may reflect reduced photorespiration and decreased ROS level due to eCO_2. Previous elevated CO_2 studies suggest maize crops do not respond to elevated carbon dioxide in the absence of drought (Leakey et al. 2006; Erbs et al. 2015). Similar results in eCO_2 grown plants were also reported in C_3–C_4 grasses- *Caragana microphylla*, *Stipa grandis*, and *Cleistogenes squarrosa* (Xu et al. 2014).

Total plant biomass increased under eCO_2 condition in comparison to the ambient condition in both maize varieties (Table 1). Under enriched CO_2 condition, PEHM-5 showed a more positive response in above-ground biomass than SMH-3031, probably because of enhanced allocation of photosynthetic assimilates towards shoot and sprout growth. This could be a reason for significantly increased thousand seed weight in PEHM-5 despite small increment in seeds number per cob. Despite this PEHM-5 had better seed quality in terms of significantly improved total soluble sugar and starch content of grains (Table 1). However, it has been shown that larger seeds showed higher germination rate and a better yield (Liu et al. 2012; Nik et al. 2011). So, there seems to be a trade-off in PEHM-5. Increased total soluble sugars and starch content under elevated CO_2 is previously

Table 2 List of eCO_2 treatment responsive leaf proteins in two maize (*Zea mays*) varieties (PEHM-5 and SMH-3031) analyzed from two-dimensional gel electrophoresis (2-DGE) and identified by mass spectrometry (MS)

Serial number	Match ID (PEHM-5/ SMH-3031)	Protein name (accession number)	Molecular mass theoretical (Da)	Molecular mass calculated (Da)	Theoretical (pI)	Calculated (pI)	Mascot score	emPAI	Fold change in PEHM-5	Fold change in SMH-3031
Carbon metabolism										
1	47/64	Malate dehydrogenase 1 [*Zea mays*] ACG28169.1	47.39	48.67	6.39	5.49	639	1.05	**2.20**↑	**1.57**↓
2	71/–	Pyruvate orthophosphate dikinase 1 [*Zea mays*] ADW27481.1	105.94	93.00	5.71	5.08	1018	0.66	**1.75**↓	–
	102/–	Pyruvate orthophosphate dikinase 1 [*Zea mays*] ADW27481.1	105.94	93.67	5.71	5.22	1018	0.66	**1.53**↑	–
3	85/40	Adenylate kinase, chloroplastic P43188.1	24.91	24.33	4.95	4.91	705	–	**1.68**↓	**1.52**↑
	105/–	Adenylate kinase [*Zea mays*] ACG27659.1	31.18	11.67	6.79	5.03	136	0.37	**1.59**↓	–
4	–/81	NADP-dependent malic enzyme [*Zea mays*] ADW27478.1	70.3	74.00	6.28	6.05	141	–	–	**1.52**↑
	–/134	NADP-dependent malic enzyme [*Zea mays*] ADW27478.1	70.3	73.67	6.28	5.63	175	0.15	–	**1.71**↓
5	2/98	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, [*Poranopsis paniculata*] AAM56020.1	48.59	65.00	8.41	7.07	567	1.08	**1.82**↓	**1.69**↓
	32/8	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial [*Aporosa frutescens*] CAB00003.1	52.22	60.00	6.2	6.1	162	0.21	**1.52**↑	**1.62**↑
	39/102	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [*Zea mays*] NP_043033.1	53.3	65.67	6.33	6.82	692	–	**1.51**↓	**1.54**↓
	72/120	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [*Zea mays*] NP_043033.1	53.3	75.67	6.71	6.33	692	–	**3.21**↓	**1.96**↑
6	42/114	Phosphoglycerate kinase [*Zea mays*] NP_001147628.1	50.00	47.67	6.07	5.16	1192	–	**1.55**↑	**1.55**↓
7	89/3	Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic [*Zea mays*] AQK78435.1	37.99	37.00	6.41	7.17	554	0.92	**1.71**↑	**1.70**↑

Table 2 (continued)

Serial number	Match ID (PEHM-5/ SMH-3031)	Protein name (accession number)	Molecular mass theoretical (Da)	Molecular mass calculated (Da)	Theoretical (pI)	Calculated (pI)	Mascot score	emPAI	Fold change in PEHM-5	Fold change in SMH-3031
8	30/–	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic [*Zea mays*] ONM16940.1	43.17	35.67	7.00	6.05	526	1.41	**2.31**↓	–
	143/–	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic [*Zea m*ays] ONM16940.1	43.17	33.00	7.00	7.03	813	2.5	**2.37**↑	–
9	34/–	Fructose-bisphosphate aldolase [*Zea mays*] ACG36798.1	41.92	38.33	7.63	5.41	1412	–	**1.59**↑	–
10	66/87	Transketolase, chloroplastic Q7SIC9.1	73.35	85.67	5.47	5.97	407	0.51	**2.17**↑	**2.03**↑
	–/95	Transketolase, chloroplastic Q7SIC9.1	73.35	80.67	5.47	5.89	591	0.59	–	**1.88**↑
11	99/63	Sedoheptulose-1,7-bisphosphatase chloroplastic [*Zea mays*] 2::ONM36378.1	41.65	45.67	6.38	5.01	654	1.58	**1.71**↑	**1.65**↓
12	–/43	Ribulose-phosphate 3-epimerase [*Zea mays*] AQL09393.1	33.82	27.00	6.24	6.65	167	–	–	**2.64**↑
13	80/101	Phosphoenolpyruvate carboxykinase homolog 2 [*Zea mays*] NP_001146178.1	72.97	66.00	6.28	6.69	64	–	**2.04**↓	**1.52**↑
Photosynthesis										
14	15/22	Thylakoid lumenal 19 kDa protein [*Zea mays*] NP_001148434.1	27.36	23.33	5.48	5.21	396	1.07	**1.63**↑	**1.51**↓
	–/88	Thylakoid lumenal 19 kDa protein [Zea mays] NP_001148434.1	27.36	85.00	5.48	4.94	59	–	–	**1.59**↑
15	18/10	Cytochrome b6-f complex iron-sulfur subunit [Zea mays] ACG28186.1	24.32	23.33	8.52	6.42	193	–	**2.30**↓	**1.81**↑
16	76/51	Uncharacterized LOC100272890 isoform X1 [Zea mays] (OEE) XP_008647514.1	34.78	35.00	5.59	5.11	442	1.02	**1.66**↓	**1.63**↑

Table 2 (continued)

Serial number	Match ID (PEHM-5/ SMH-3031)	Protein name (accession number)	Molecular mass theoretical (Da)	Molecular mass calculated (Da)	Theoretical (pI)	Calculated (pI)	Mascot score	emPAI	Fold change in PEHM-5	Fold change in SMH-3031
17	81/–	Oxygen-evolving enhancer protein 2–1 chloroplastic [Zea mays] ONM51277.1	32.07	22.00	9.5	6.8	1037	–	**1.62**↑	–
	83/–	Oxygen-evolving enhancer protein 1–1 chloroplastic [Zea mays] AQK66518.1	39.34	31.00	7.57	5.28	617	1.17	**1.57**↓	–
	106/–	Oxygen-evolving enhancer protein 2–1 chloroplastic [Zea mays] ONM51277.1	32.07	22.67	9.50	6.5	383	0.86	**2.00**↑	–
18	–/5	Ferredoxin-NADP reductase, leaf isozyme, chloroplastic OS = Oryza sativa subsp. japonica FENR1_ORYSJ	40.38	35.67	8.72	6.06	43	0.14	–	**3.77**↑
	–/7	Ferredoxin-NADP reductase, leaf isozyme [Zea mays] ACG35645.1	37.88	37.00	8.37	5.53	847	2.74	–	**1.52**↓
	–/126	Ferredoxin-NADP reductase, chloroplastic OS = Mesembryanthemum crystallinum FENR_MESCR	41.23	35.67	8.54	6.97	42	–	–	**1.51**↑
Protein synthesis, assembly and degradation										
19	05/10	Chlorophyll a-b binding protein 1, chloroplastic OS = *Zea mays* CB21_MAIZE	27.91	32.00	5.14	3.98	46	–	**2.30**↑	**2.43**↓
	126/38	Chlorophyll *a-b* binding protein 8, chloroplastic OS = Solanum lycopersicumCB13_SOLLC	29.34	24.67	8.96	5.52	44	–	**1.97**↓	**2.02**↓
	22/–	Chlorophyll *a-b* binding protein CP24 10B, chloroplastic [*Dichanthelium oligosanthes*] OEL19177.1	26.83	19.67	5.15	7.9	58	0.21	**1.52**↑	–
	75/–	Chlorophyll *a-b* binding protein, chloroplastic [*Zea mays*] P06671.1	28.15	24.33	5.29	5.37	99	0.19	**1.53**↓	–
	–/12	Chlorophyll *a-b* binding protein 1, chloroplastic OS = *Zea mays* CB21_MAIZE	27.91	29.00	5.14	3.97	39	–	–	**1.69**↓

Table 2 (continued)

Serial number	Match ID (PEHM-5/ SMH-3031)	Protein name (accession number)	Molecular mass theoretical (Da)	Molecular mass calculated (Da)	Theoretical (pI)	Calculated (pI)	Mascot score	emPAI	Fold change in PEHM-5	Fold change in SMH-3031
20	58/78	RuBisCO large subunit-binding protein subunit beta, chloroplastic [*Zea mays*] NP_001306697.1	64.28	66.00	5.47	5.26	253	–	**1.66↑**	**1.50↑**
	–/133	RuBisCO large subunit-binding protein subunit beta, chloroplastic [*Zea mays*] NP_001306697.1	64.28	74.67	5.47	5.35	253	–	**–**	**1.90↑**
21	–/94	Chloroplast protein synthesis2 [*Zea mays*] ONM62310.1	61.94	84.33	5.04	5.72	57	0.16	**–**	**1.86↓**
	–/117	Chloroplast protein synthesis2 [*Zea mays*] PCKA_MAIZE	73.78	80.33	6.57	4.99	73	–	**–**	**1.51↑**
22	–/127	Eukaryotic translation initiation factor 5A Q9AXQ7.1	17.61	15.33	5.55	6.07	87	–	**–**	**3.17↓**
23	70/89	Elongation factor G-2, chloroplastic [*Zea mays*] XP_008663416.1	84.53	102.33	5.33	5.60	264	–	**1.75↑**	**1.79↑**
24	129/–	Elongation factor Tu [*Zea mays*] NP_001150410.1	50.79	49.00	6.07	5.83	360	–	**3.36↑**	**–**
25	63/–	Heat shock cognate 70 kDa protein OS = Petunia hybrida2::HSP7C_PETHY	71.58	70.67	5.11	5.40	117	–	**3.25↑**	**–**
	100/116	Heat shock cognate 70 kDa protein [*Glycine soja*] KHN13879.1	75.7	85.33	5.96	4.88	66	0.07	**1.57↑**	**2.17↑**
26	57/75	Chaperonin 60 subunit beta 2 chloroplastic [*Zea mays*] AQL02522.1	63.36	64.67	5.35	5.40	253	0.26	**1.70↓**	**1.58↑**
27	–/17	20 kDa chaperonin chloroplastic [*Zea mays*] AQL01343.1	12.96	10.67	10.03	5.53	72	–	**–**	**1.50↑**
28	124/70	Peptidyl-prolyl cis–trans isomerase [*Zea mays*] NP_001151852.1	46.49	57.33	4.91	4.74	69	–	**1.50↑**	**1.56↑**
Energy metabolism										
29	3/121	ATP synthase subunit alpha, chloroplastic Q6L3A1.2	55.77	65.33	5.87	5.85	930	1.3	**1.61↑**	**1.60↑**
	27/112	ATP synthase subunit alpha, chloroplastic Q6L3A1.2	55.77	24.00	5.87	6.28	655	0.88	**1.59↓**	**1.56↑**
	–/14	ATP synthase subunit alpha, chloroplastic Q6L3A1.2	55.77	66.33	5.87	6.21	1610	2.86	**–**	**1.50↑**
	132/–	ATP synthase CF1 alpha subunit (plastid) [*Zea diploperennis*] YP_009259176.1	55.33	35.67	5.87	5.80	1124	1.48	**1.50↓**	**–**

Table 2 (continued)

Serial number	Match ID (PEHM-5/ SMH-3031)	Protein name (accession number)	Molecular mass theoretical (Da)	Molecular mass calculated (Da)	Theoretical (pI)	Calculated (pI)	Mascot score	emPAI	Fold change in PEHM-5	Fold change in SMH-3031
30	49/–	ATP synthase CF1 beta subunit (chloroplast) [*Zea mays*] NP_043032.1	54.06	50.67	5.31	5.47	1053	1.48	**1.51↑**	–
31	38/55	ATP synthase subunit gamma, chloroplastic precursor [*Zea mays*] NP_001150872.1	40.1	41.67	8.44	6.28	447	–	**1.51↑**	**1.51↓**
Defense										
32	16/15	Germin-like protein precursor [*Zea mays*] NP_001140857.1	22.1	17.33	6.02	6.5	424	1.07	**1.69↑**	**1.56↑**
	86/28	Germin-like protein precursor [*Zea mays*] NP_001140857.1	22.1	20.00	6.02	5.54	138	–	**2.04↑**	**3.10↓**
	114/–	Germin-like protein precursor [*Zea mays*] NP_001140857.1	22.1	18.00	6.02	6.52	424	1.07	**1.50↑**	–
33	145/–	Harpin binding protein 1 [*Zea mays*] NP_001169754.1	29.41	22.35	9.57	5.86	218	0.66	**1.71↑**	–
34	–/37	Ascorbate peroxidase-like protein [*Zea mays*] AQL07484.1	26.13	24.33	8.26	5.70	573	2.06	–	**2.33↓**
35	–/39	APx1—Cytosolic Ascorbate Peroxidase [*Zea mays*] NP_001152249.1	27.48	24.33	5.65	6.08	368	1.61	–	**1.58↓**
36	–/90	Beta-D-glucosidase precursor [*Zea mays*] AEM17055.1	63.46	74.67	6.75	6.30	365	0.36	–	**1.61↓**
37	–/62	Pro-resilin precursor [*Zea mays*] ACG32088.1	42.77	47.00	5.55	4.67	220	0.52	–	**1.50↑**
38	–/9	Glyoxylase1 [*Zea mays*] AQK40899.1	36.51	33.67	5.11	6.29	58	–	–	**1.99↑**
Cell redox homeostasis										
39	16/10	Thioredoxin M-type [*Zea mays*] ACG30704.1	18.59	9.00	8.70	5.00	123	–	**1.64↑**	**2.02↓**
40	13/21	Peroxiredoxin-5 [*Zea mays*] AQL04842.1	23.86	12.67	8.58	4.87	70	0.23	**2.07↑**	**1.62↓**
	–/20	Peroxiredoxin-5 [*Zea mays*] AQL04842.1	23.86	12.33	8.58	5.08	100	1.89	–	**1.51↓**
41	127/32	2-cys peroxiredoxin BAS1 [*Zea mays*] ACG35092.1	28.03	20.67	5.81	4.69	544	1.89	**3.15↑**	**1.66↑**
	–/31	2-cys peroxiredoxin BAS1 [*Zea mays*] ACG35092.1	28.03	21.00	5.81	4.54	58	0.2	–	**1.82↓**

Table 2 (continued)

Serial number	Match ID (PEHM-5/SMH-3031)	Protein name (accession number)	Molecular mass theoretical (Da)	Molecular mass calculated (Da)	Theoretical (pI)	Calculated (pI)	Mascot score	emPAI	Fold change in PEHM-5	Fold change in SMH-3031
Amino Acid Metabolism										
42	36/103	Aspartate aminotransferase chloroplastic [*Zea mays*] AQK75827.1	45.65	43.67	6.06	6.69	81	–	**2.46**↑	**1.52**↓
Cytoskeleton										
43	41/–	Actin-7 [*Zea mays*] AQK95630.1	50.54	50.00	5.94	5.11	369	–	**2.03**↑	–

Values (mean of three replicates within each treatment) represent fold changes with threshold of ≥ 1.5-fold increased/more abundance (↑) or decreased/less abundance (↓)

reported (Drake et al. 2011; Huang and Xu 2015; Pandey et al. 2017) and reviewed (Dong et al. 2018).

Leaf proteomics

Carbon metabolism and photosynthesis-related proteins

Malate dehydrogenase 1 (MDH1; S.No. 1) was found more abundant in PEHM-5 and less abundant in SMH-3031 under eCO_2. In PEHM-5 two protein spots were identified as Pyruvate orthophosphate dikinase 1 (PPDK1; S.No. 2) showing mixed abundance. In C_4 plants these proteins play an important role to fix atmospheric CO_2 into four carbon-containing oxaloacetates (via PEPC) and malate (via MDH) inside mesophyll cells. More abundance of MDH and PPDK showed better CO_2 incorporation in PEHM-5 over SMH-3031. The SMH-3031 showed the mixed (mostly decreased) abundance of NADP-ME (S.No. 4) that lead to decreased decarboxylation rate and reduced malate consumption. The lower decarboxylation rate in the bundle sheath cells may also negatively influence the functioning of Calvin-Cycle. Though most of the Rubisco (S.No. 5) proteins were less abundant in PEHM-5, there was more abundance of Calvin cycle enzymes such as phosphoglycerate kinase (PGK), glyceraldehyde 3 phosphate dehydrogenase (G3P), fructose-bisphosphate aldolase (FBA), transketolase (TK), sedoheptulose bisphosphatase (SBPsae) (S.No. 6–11). The increased abundance of these enzymes can be explained by the availability of sufficient CO_2 in the bundle sheath cell that would be incorporated in Calvin cycle into 3-phosphoglycerate and further requirement of downstream proteins in PEHM-5, leading to better biomass and yield. The SMH-3031 variety showed increased abundance of Rubisco but due to the limited availability of CO_2 (from reduced NADP-ME), Calvin cycle slowed down. Decreased abundance of PGK in SMH-3031 strengthens the above argument. In bundle sheath cytoplasm, Phosphoenolpyruvate carboxykinase (PEPCK) converts OAA into phosphoenolpyruvate (PEP) and liberate CO_2. This CO_2 is then transported to bundle sheath chloroplast for the incorporation in the Calvin cycle. Increased abundance of PEPCK (S.No. 13) in SMH-3031 and decreased abundance in PEHM-5 supports the requirement of CO_2 in SMH-3031 to attain proper growth.

Among photosynthesis-related proteins, more abundance of 19 kDa thylakoid lumenal proteins (S.No. 14), Oxygen-evolving enhancer proteins (S.No. 17) and less abundant Cytochrome b6-f complex iron-sulfur subunit (S.No. 15) was observed in PEHM-5. The mixed abundance of photosynthesis-related structural proteins in PEHM-5 could be a possible explanation for unaltered photosynthetic activity (Table 1). OEE proteins play a crucial role in photosynthesis by controlling the O_2 evolution from photolysis of water and conserve the stability of photosystem II

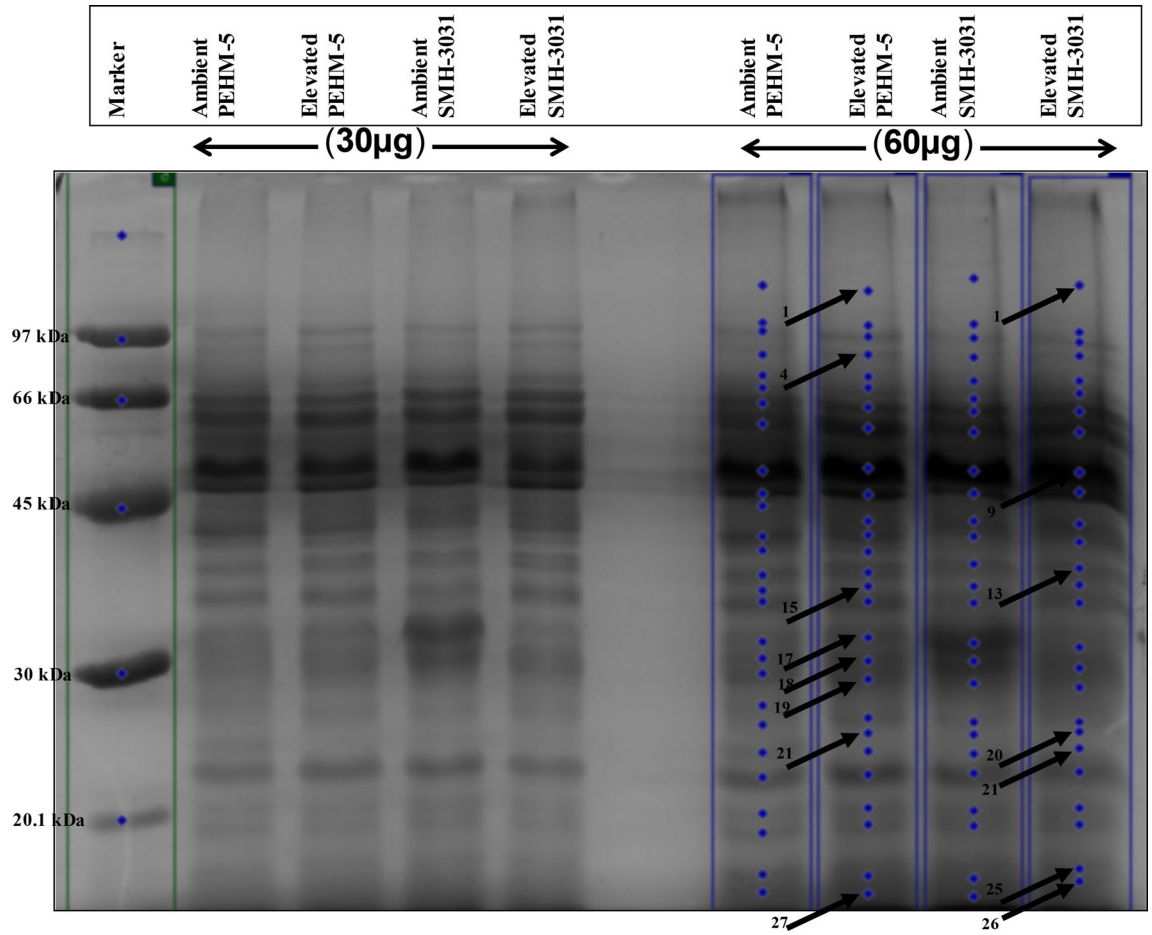

Fig. 7 Effect of eCO_2 on seed protein expression pattern of two *Zea mays* varieties PEHM-5 and SMH-3031. 5% Stacking and 10% Resolving gel, 30 µg and 60 µg protein loading, Brilliant blue G stained SDS-PAGE gel

(PSII). In PEHM-5, more OEE showed its requirement for repairing protein damage and maintenance of OEE protein pool. SMH-3031 showed more abundant thylakoid lumenal proteins, Cytochrome b6-f complex iron-sulfur subunit, OEE and Ferredoxin-NADP reductase (S.No. 14–18) still photosynthetic activity remained unaltered in SMH-3031. The enhanced abundance of Cytochrome b6-f complex iron-sulfur subunit and lower abundance of OEE was earlier reported in maize under drought (Benevenuto et al. 2017). In another drought study, Zhang and Shi (2018) in alfalfa and Zhao et al. (2016) in maize reported the role of enhanced OEE with SOD in ROS detoxification. Benevenuto et al. (2017) correlated more abundance of FNR with high energy requirement to counter stress while studying the abiotic stress response of maize.

Protein synthesis, assembly and degradation-related proteins

In eCO_2, Chlorophyll *a-b* bp and Rubisco-LSU bp- beta (S.No. 19, 20) were more abundant in PEHM-5. Similarly, SMH-3031 showed more abundance of Rubisco bp and less abundance of chlorophyll *a-b* bp. The increased abundance of these proteins could strengthen the photosynthetic machinery by promoting their respective protein assembly. Chloroplast protein synthesis 2 (cps2), Eukaryotic translation initiation factor (eIF), elongation factor (eEF) (S.No. 21–24) are proteins which play important role in transferring genetic information from mRNA to proteins. Chloroplast protein synthesis 2 helps in ATP binding, cysteine t-RNA ligation and also involved in carbohydrate biosynthesis. More abundance of translation-related protein was observed in PEHM-5 while SMH-3031 showed the decreased abundance of these proteins. Dworak et al. (2016) suggested drought causes a decreased abundance of cps2 and protein synthesis impairment in maize. Increased abundance of

Table 3 List of eCO_2 treatment responsive seed proteins in two maize (*Zea mays*) varieties (PEHM-5 and SMH-3031) analyzed from SDS—PAGE and identified by mass spectrometry (MS)

Serial number	Band identification number	Protein name (Accession Number)	emPAI	MASCOT Score	Molecular mass Obs./Cal. (kDa)	Peptide sequences matched	Expected score	Sequence coverage (%)	Function	Fold change due to eCO_2
PEHM-5										
1	MP1	Globulin-2 [*Zea mays*] (ONM10181.1)	0.28	44	20.88/84.14	R.FTHELLEDAVGNYR.V	0.045	7	Nutrient reservoir activity	**2.34**↑
2	MP15	Globulin-2 [*Zea mays*] (ONM10181.1)		72	20.88/24.79	R.FTHELLEDAVGNYR.V	0.0014	7	Nutrient reservoir activity	**8.35**↑
3	MP19	Globulin-2 [*Zea mays*] (ONM10183.1)	1.28	229	26.11/20.03	R.LLDMDVGLANIAR.G R.LLDMDVGLANIAR.G + Oxidation (M) K.LLAFGADEEQQVDR.V	0.00049 0.011 0.000065	16	Nutrient reservoir activity	**1.53**↑
4	MP21	Globulin-2 [*Zea mays*] (ONM10181.1)	0.3	80	20.88/17.72	R.FTHELLEDAVGNYR.V	0.00019	7	Nutrient reservoir activity	**13.68**↑
5	MP4	Glycosyl hydrolase family 31 protein [*Zea mays*] (AQK81481.1)	0.25	86	23.45/63.00	R.GVLVSPVLEPGATTVEAYFPAGR.W	0.000034	10	Carbohydrate metabolism	**1.70**↑
6	MP17	Chitin-binding, type 1 [*Cynara cardunculus*] (KVI08093.1)	0.2	85	29.66/22.07	R.GPIQLSWNYNYGPAGR.S	0.000063	5	Carbohydrate metabolism	**1.89**↑
7	MP18	Chitinase [*Zea mays* subsp. parviglumis] (AAT39991.1)	0.45	164	29.61/20.88	R.GPLQISWNYNYGPAGR.E R.AIDGALECNGNNPAQMNAR.V	0.00017 0.0000026	12	Carbohydrate metabolism	**1.77**↑
8	MP27	Lipid body-associated protein L2[*Zea mays*] (P21641.1)	0.33	53	18.55/11.52	R.TPDYVEEAR.R	0.011	4	Structural role	**1.52**↑
SMH-3031										
9	MS20	Globulin-2 [*Zea mays*] (ONM10183.1)	0.24	98	26.11/17.69	R.LLDMDVGLANIAR.G	0.0000059	5	Nutrient reservoir activity	**1.57**↑
10	MS26	Globulin-2 [*Zea mays*] (ONM10181.1)		67	20.88/24.79	K.LLAFGADEEQQVDR.V	0.0047	7	Nutrient reservoir activity	**3.42**↓

Table 3 (continued)

Serial number	Band identification number	Protein name (Accession Number)	emPAI	MASCOT Score	Molecular mass Obs./Cal. (kDa)	Peptide sequences matched	Expected score	Sequence coverage (%)	Function	Fold change due to eCO_2
11	MS1	Globulin-1 S allele [*Zea mays*] (GLB1_MAIZE)	0.08	42	65.45/85.43	R.VAVLEANPR.S	0.01	1	Nutrient reservoir activity	**3.64**↓
12	MS9	50kD gamma zein [*Zea mays*] (AAL16979.1)	0.34	180	35.4/32.16	K.SQQQQCHCQEQQQTTR.C R.LIQPSSCQVLQQQCCHDLR.Q	0.0046 0.000000058	11	Nutrient reservoir activity	**1.67**↓
13	MS13	Hydroxyproline-rich glycoprotein family protein [*Zea mays*] (ONL97562.1)		97	41.04/25.52	K.ILPWGDEAYAAGGSSAANAPR.F	0.0000041	5	Structural role	**1.73**↓
14	MS25	Oleosin Zm-II [*Zea mays* (OLEO3_MAIZE)		43	18.55/12.27	R.TPDYVEEAR.R	0.012	4	Structural role	**3.34**↓
15	MS21	Receptor protein kinase-like protein ZAR1 [*Arabidopsis thaliana*] (ZAR1_ARATH)	0.07	36	79.28/16.97	R.VVSLSIPR.K	0.031	1	Signal transduction	**1.87**↑

Values represent fold changes with threshold of ≥ 1.5-fold increased/more abundance (↑) or decreased/less abundance (↓)

proteins that assists in protein folding, assembly and transport such as HSP-70 kDa, chaperonin 60 and 20 kDa (S.No. 25–27) were observed in both the varieties. Their roles in quality control of proteins are well documented (Gupta et al. 2018; Sharma et al. 2017; Wang et al. 2010). Peptidyl-prolyl cis–trans isomerase or cyclophilin TLP40 (S.No. 28) was more abundant in both the varieties exposed to elevated CO_2 condition helping in better growth and yield. Cyclophilin mediates various cellular functions including organogenesis, photosynthetic and hormonal signalling, stress adaptation, and defense response. TLP40 regulate a PSII specific phosphatase in the thylakoid membrane and helps in proteolysis and integration of new D1 protein subunits and regulates PSII protein turnover (Fulgosi et al. 1998; Romano et al. 2004; Vener et al. 1999). Cyclophilins increase oxidative stress tolerance in rice (Lee et al. 2015a) and drought tolerance in rice and *Arabidopsis* (Lee et al. 2015b).

Energy metabolism and defense-related proteins

Subunits of ATP synthase (α, β and γ) provide proton pump (H^+) to generate ATP from ADP. As CO_2 concentration increases carbon fixation increases and there is an increased demand of ATP (required for Rubisco regeneration) which leads to the shifting of photosynthesis control towards Rubisco regeneration (Ainsworth and Roger 2007; Aranjuelo et al. 2011). Elevated CO_2 grown PEHM-5 showed increased abundance of ATP synthase (α, β and γ) subunits (S.No. 29–31). Moreover, Rubisco regeneration/ recycling enzymes; fructose 1,6-bisphosphate aldolase, transketolase and sedoheptulose 1,7-bisphosphatase (S.No. 10–12) were more abundant in PEHM-5 (Rosenthal et al. 2011). Benevenuto et al. (2017) demonstrated the increased abundance of ATP synthase γ subunit to fulfil high energy demand in maize to overcome herbicide stress. More abundant Rubisco recycling enzymes were also demonstrated in wheat leaves exposed to eCO_2 (Pandey et al. 2017). SMH-3031 showed more abundance of α- subunit and decreased abundance of γ-subunit leading to a reduction in ATP production and thereby affecting the Calvin cycle. Decreased or impaired abundance of Rubisco recycling enzymes (more abundant TK and less abundant SBPase) also lead to reduced activity of the Calvin cycle (Agrawal et al. 2002) resulting in decreased yield. The germin like protein (GLP) exerts SOD-like activity due to the presence of three histidine and one glutamate residue responsible for metal ion (Mn) binding (Rietz et al. 2012; Zhang et al. 2017). Harpin binding proteins are specific RNA binding proteins that bind to the hairpin loop of mRNA and helps in folding and protection of mRNA, determine interaction in a ribozyme and can also act as a substrate for the various enzymatic reaction. The increased abundance of GLP precursor (S.No. 32) and harpin binding protein (S.No. 33) was found in PEHM-5 showing their better stability towards abiotic response. On the other hand, SMH-3031 showed the decreased abundance of GLP precursor, APx and APx like proteins and D-glycosidase precursor (S.No. 32, 34–36) showing diminished defense response. However, more abundance of pro-resilin and glyoxylase 1 (S.No. 37 and 38) was found in SMH-3031. Glyoxylase 1 help in the maintenance of glutathione homeostasis via recycling of reduced glutathione (GSH). The role of glyoxylase 1 is well documented in drought and other abiotic stresses induced defense (Dworak et al. 2016; Zadraznik et al. 2013).

Cell redox homeostasis, amino acid metabolism and cytoskeleton-related proteins

Thioredoxin M-type, peroxiredoxin-5 and 2-cys peroxiredoxin (S.No. 39–41) proteins help to maintain cellular redox homeostasis. These proteins were more abundant in PEHM-5 and less abundant in SMH-3031 under elevated CO_2. Thioredoxins (Trxs) are small thiol: disulfide oxidoreductases, peroxiredoxin-5 (Prxs) and 2-cys peroxiredoxin are dithiol-disulfide peroxidase and are essential redox regulatory elements in plant metabolism. They play a crucial role in CO_2 assimilation and redox homeostasis (Cheng et al. 2014) and are major regulators of Calvin cycle enzymes (Okegawa and Motohashi 2015). Many other cellular processes require Trx/Prx-mediated regulation including photosynthetic electron transport, oxidative stress response, starch and nitrogen metabolism, lipid biosynthesis, Ca^+ mediated signalling, hormone signalling, protein homeostasis and many more (reviewed by Dietz 2011; Liebthal et al. 2018; Serrato et al. 2013). More abundance of Trx, Prx and 2-cys Prx proteins suggest improved redox homeostasis and defense response in PEHM-5.

Aspartate aminotransferase (S.No. 42) belongs to amino acid metabolism and actin-7 (S.No. 43) is an integral part of Cytoskeleton. More abundance of these proteins was found in PEHM-5 leaves while SMH-3031 showed the decreased abundance of aspartate aminotransferase in eCO_2 condition. More abundance of actin protein was previously reported for better phytohormone response in *Arabidopsis* (Gilliland et al. 1998) and improved drought tolerance in barley (Sniegowska-Swierk et al. 2015). In agreement, eCO_2 induced abundance of actin-7 maintained the cytoskeleton and intracellular organelles positioning, maintaining their function for better growth.

Seed proteomics

Globulin-2 (GLB2, nutrient reservoir activity), a salt soluble protein was more abundant in PEHM-5 (S.No. 1–4) and with mixed abundance in SMH-3031 (S.No. 9–11). GLB2 is the second most abundant seed storage protein in maize embryo

after GLB1. GLB2 is encoded by the single-copy, highly polymorphic with several different alleles *Glb2* genes (Fasoli et al. 2009; Ning et al. 2017; Xiong et al. 2014). Thus, multi-proteoforms of GLB2 have obtained from gene polymorphism, post-translational modification and instance of degradation. It can be a probable explanation for the identification of different GLB2 proteins in our study. In PEHM-5 seeds, Glycosyl hydrolase family 31 proteins, chitin-binding type 1 and chitinase proteins (S.No. 5–7) were induced under eCO_2 and these are involved in carbohydrate metabolism. Glycosyl hydrolase family 31 protein comprises of enzymes with several known activities and play an important role in the hydrolysis of *O*- or *S*-glycosidic bond, affecting cellulose biosynthesis and therefore, biomass and yield (Lopez-Casado et al. 2008; Szyjanowicz et al. 2004; Xie et al. 2013). Chitin-binding type 1 and chitinase proteins induced under eCO_2 also play an important role in defense. SMH-3031 showed less abundance of 50kD gamma zein (S.No. 12) under eCO_2. Lipid body-associated protein L2 (S.No. 8) play an important structural role to stabilize the lipid body and was more abundant in PEHM-5. Hydroxyproline-rich glycoprotein (S.No. 13), oleosin Zm-II (Band No.14); having a structural role to stabilize cell shape were less abundant in SMH-3031. More abundance of Receptor protein kinase-like protein ZAR1 (S.No. 15), involved in signal transduction, was found in SMH-3031. The increased abundance of proteins in PEHM-5 can be linked with improved grain weight and TSS in PEHM-5 under eCO_2. Similarly, decreased abundance of proteins in SMH-3031 can be linked with reduced grain weight and TSS in SMH-3031.

Conclusion

The present study showed that maize, a C_4 crop, responded positively to elevated CO_2. However, two varieties responded differently to eCO_2. On one hand, PEHM-5 responded to eCO_2 by higher biomass accumulation, larger seeds, and more seed starch and soluble sugars. On the other hand, SMH 3031 had less biomass accumulation, less 1000 grain weight and smaller seeds. Leaf proteins which played an important role in better growth and yield of PEHM-5 included G3P, FBA, TK, SBPase, OEE, EF, HSP 70, GLP, peroxiredoxin and Actin. Furthermore, PEHM-5 seed proteomics also revealed the higher abundance of Globulin-2, Glycosyl hydrolase family 31 protein, Chitin-binding and Chitinase protein resulting in better growth in this maize variety. However, protein activity measurements should be done to check whether the changes in protein abundance observed in this study could really be correlated with changes in maize traits observed. Moreover, there could be some post-translational modifications that regulate protein functioning. Further work should be done to evaluate the response of elevated CO_2 along with elevated temperature and/or ozone on different varieties of maize.

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Author contributions VP, NP and VKM designed the experiment. Yield and physiological analysis were performed by VKM, SKG, BM and MS. VKM and BM did the enzymatic and biochemical analysis. VK, SKG, MS and FD did proteomic work. VKM and VP analyzed the data. VKM wrote and VP reviewed the manuscript. All the authors approved the paper.

Compliance with ethical standards

Conflict of interest Authors declare that there is no conflict of interest.

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