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The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions: proteomic and transcriptomic profiles

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Abstract

Key message **Proteomic and transcriptomic profiles of key enzymes were monitored in pineapple plants propagated under C3 and CAM-inducing metabolisms to obtain insight into the CAM-facultative metabolism and the relationship of CAM plants with oxidative stress.**

Abstract Pineapple is one of the most important tropical crops worldwide. The use of temporary immersion bioreactors for the first stages of pineapple propagation enables precise control of plant growth, increases the rate of plant multiplication, decreases space, energy and labor requirements for pineapple plants in commercial micropropagation. Once the plantlets are ready to be taken from the reactors, they are carefully acclimatized to natural environmental conditions, and a facultative C3/CAM metabolism in the first 2 months of growth is the characteristic of pineapple plants, depending on environmental conditions. We subjected two sets of micropropagated pineapple plants to C3 and CAM-inducing environmental conditions, determined by light intensity/relative humidity (respectively 40 µmol m^{-2} s^{-1}/85 % and 260 µmol m^{-2} s^{-1}/50 %). Leaves of pineapple plants grown under CAM-inducing conditions showed higher leaf thickness and more developed cuticles and hypodermic tissue. Proteomic profiles of several proteins, isoenzyme patterns and transcriptomic profiles were also measured. Five major spots were isolated and identified, two of them for the first time in *Ananas comosus* (OEE 1; OEE 2) and the other three corresponding to small fragments of the large subunit of Rubisco (LSU). PEPC and PEPCK were also detected by immunobloting of 2DE at the end of both *ex vitro* treatments (C3/CAM) during the dark period. Isoenzymes of SOD and CAT were identified by electrophoresis and the transcript levels of OEE 1 and CAT were associated with CAM metabolism in pineapple plants.

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
GR	Glutathione reductase
LSU	Rubisco large subunit
OEE	Oxygen Evolving Enhancer
PEPC	Phosphoenolpyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
SOD	Superoxide dismutase
SSU	Rubisco small subunit

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Introduction

Pineapple is the second most important harvest crop worldwide, after banana, contributing to over 20 % of the

production of tropical fruits (Coveca 2002). Nearly 70 % of the pineapple produced is consumed as fresh fruit in producing countries. Global production began at *circa* 1,500 tons when, in addition to tropical regions, pineapple propagation started in Europe (Coppens et al. 1993). In 2011, the international production surpassed two million tons with Brazil as the major producer, followed by The Philippines and Costa Rica (FAOSTAT 2011). The most widely used variety is Smooth Cayenne, which was first introduced in Europe from French Guyana. However, several new varieties have been introduced to improve the quality of the fruit that reaches international markets, such as MD2 (Golden ripe, Extra sweet and Maya gold) (Sopie et al. 2011).

The use of byproducts of pineapple culture in feed production, canning and juice extraction has been encouraged. In animal food, leaves can be used in three forms: fresh, dried and in silage (Coppens and Leal 2003). Pineapple may offer additional advantages, such as its relevance as fiber source. Pineapple fiber has numerous qualities, such as its texture, its length (up to 60 cm), high water and dye-holding capacity, high whiteness, brightness, resistance to salt and tension strength. Bromelin has been produced for use as meat tenderizer and as a component of pharmaceuticals. A major problem that both large scale commercial production of pineapple and the expansion of the existing small farms face is the difficulty in obtaining uniform planting material in large quantity due to the low rate of multiplication by conventional methods and the lack of high-quality propagules. Pineapple propagation is performed asexually, using shoots from different parts of the plant, such as bulbs, crowns and axillary buds. Recent advances in plant biotechnology methods applied to pineapple crops enhanced their potential application, both for basic studies and for direct application in agriculture (Read 2007; Aragón et al. 2009, 2010, 2012). The use of bioreactors enables more precise control of plant growth, gas exchange, illumination, medium agitation, temperature and pH than the conventional culture vessels (Escalona et al. 1999). TIB have been used since 1999 (Escalona et al. 1999) and considerably increase the number of plantlets produced in comparison with conventional vessels. In fact, this method not only increases the rate of plant multiplication, but also reduces space, energy and labor requirement, concerns that are paramount to commercial micropropagation.

Once the plantlets are ready for shifting outside the reactors, they have to be carefully acclimatized to natural environmental conditions. During hardening, the plantlets undergo physiological adaptations to changing external factors like water, temperature, relative humidity and nutrient supply. In the first 2 months of growth pineapple plants display a facultative C3/CAM (Crassulacean Acid Metabolism), depending on environmental conditions (Aragón et al. 2012). CAM metabolism developed as a response to a stressful environment and gave rise to more succulent leaves, higher starch concentration during the light period and higher photosynthetic rates than C3 metabolism (Aragón et al. 2012). However, direct results of the possible damage or other metabolic changes caused by these environmental conditions are still unclear.

CAM photosynthesis appears to have originated as a means to scavenge respiratory CO_2 under conditions where the carbon balance is restricted, in environments where water availability becomes temporarily or seasonally constrained, such as deserts or rock outcrops. CAM metabolism causes major changes to leaf structure, succulence being the most obvious innovation as it facilitates the capture of night-time CO_2 released by respiration (Guralnick et al. 2001; Sage 2002; Nelson et al. 2005). CAM metabolism is found in a wide range of species with distinct phylogenetic lineages and habitats. All these species developed diverse structures and adaptations to stressful environments but they all share anatomical and functional traits (Kore-eda et al. 2005; Nelson et al. 2005). Plants with CAM metabolism have a complex balance of carbon flux inside the cell. The soluble sugars resulting from the assimilation of CO_2 in the dark are essential for the light period that follows. PEPC catalyzes the reaction of carboxylation of phosphoenolpyruvate, with oxaloacetate and organic phosphate as products and has several functions in plants, such as an anapleurotic function in leaves and nonphotosynthetic tissues (Gehrig et al. 2005).

C3, C4 and CAM photosynthetic pathways are well characterized but there are still a few metabolic details not well understood (Willert et al. 2005). The interpretation of the C3/CAM transition as a complex interaction between environment and metabolism, in opposition to a basic molecular interpretation of the circadian cycle linked to carbon metabolism are elements still to be clarified on pineapple plants. In plants showing facultative C3/CAM metabolism the CAM state can be induced as a response to stressful conditions, showing different levels of transition from C3 to complete CAM metabolism (Luttge 2004). We have extensively investigated the ex vitro growth conditions needed to shift pineapple plants from C3 to CAM metabolism and performed a partial physiological characterization (Aragón et al. 2012), but more details of the molecular processes taking place will contribute to the interpretation of the events as a whole. Transport activities and the control of intracellular compartmentalization, organic acid and carbohydrate flux are some of the most challenging processes to consider on CAM metabolism. Their knowledge could be the key to understand the temporal separation of carbon metabolism and the control of enzymes such as pyruvate orthophosphate dikinase

(PPDK), phosphoenolpyruvate carboxylase (PEPC) and starch synthase (Kore-eda et al. 2005).

Previous studies with pineapple plants micropropagated in TIB assessed the environmental conditions that induce C3 and CAM metabolism and demonstrated that the CAM-inducing condition is the most stressful (Aragón et al. 2012). The main objective of the current research was to obtain more insight into the facultative metabolism of pineapple TIB grown plants under C3 and CAM-inducing conditions, with the main focus on the relationship with oxidative stress, more in evidence in the plants showing CAM metabolism.

Materials and methods

Plant material and ex vitro culture conditions

Pineapple plants (*Ananas comosus* (L.) Merr. var MD-2) micropropagated in temporary immersion bioreactors (TIB; Escalona et al. 1999) were grown in glass chambers as described in Aragón et al. (2012). Two sets of 120 plants each were placed in two glass chambers with different regulation of light intensity, relative humidity (RH) and irrigation (Table 1). Measurements were taken at the moment of transfer to acclimatization (week 0, w0), at w4 and at w8 of acclimatization. Samples were collected in the middle of the light period and in the middle of the dark period, always from leaf D, defined as the leaf forming a 45° angle with the vertical axis.

Histological analyses

To analyse the distribution of tissues in the leaf transverse cross sections of pineapple leaves were performed. The leaves were fixed in FAA reagent (solution of: 90 % ethanol (50 %), 5 % acetic acid, 5 % formaldehyde) and included in polyethylene glycol 20 % at 60 °C for 24 h. The inclusions were then cut by microtome (SM 2400, Leica Microsystems) and the cross sections were carefully stretched on glass slides for observation and measurements in an optical microscope (Axioskop 2, Zeiss®) with camera (AxioCam HRM, Zeiss®). Using the software AxioVision 4.8 (Zeiss®) the following parameters were measured: total leaf, mesophyll, epidermis and hypodermic tissue thickness.

Succulence and pH analyses

Succulence analyses were performed following the saturated water content (SWC) protocol, a dimensionless measure of water mass held by a given tissue or organ at full hydration normalized to the tissue's dry mass: SWC = (Leaf mass at full hydration (g)—dried leaf mass (g))/dried leaf mass (g). To obtain leaf mass at full hydration the leaves were soaked in distilled water overnight. When leaves gained <5 % in mass between subsequent weighings they were considered saturated. To obtain the dried leaf mass leaves were transferred to an oven at 60 °C until constant weight was attained.

To quantify pH in sap extracted from leaves, they were crushed until 0.5 mL of sap was obtained. The sap was clarified though the addition of activated charcoal to eliminate pigments and pH was immediately quantified with pH indicator strips (Merck KGaA 64271).

Protein extraction and two-dimensional electrophoresis (2-DE)

Frozen leaf material (0.5 g) previously collected in the middle of the light period was reduced to powder using a mortar and pestle in the presence of liquid nitrogen. Proteins were precipitated for 1 h at −20 °C with acetone containing 10 % (w/v) TCA (trichloroacetic acid) and 60 mM DTT (dithiothreitol), and centrifuged at $15,000 \times g$ for 15 min at 4 °C. The resulting pellet was washed in acetone with 60 mM DTT for 1 h at −20 °C and centrifuged again. This pellet was dried under vacuum and used as the crude extract, after being re-dissolved for 2 h at 25 °C in a buffer containing 7 M urea, 2 M thiourea, 0.4 % (v/v) Triton X-100, 4 % (w/v) CHAPS [(3-[(3-Cholami-dopropyl)-dimethylammonio]-1-propane sulfonate)], 60 mM DTT and 1 % (v/v) IPG (Immoblized pH gradient buffer; GE Healthcare Lifesciences). Protein was quantified by the method of Bradford (1976), modified by Ramagli (1999). Samples were incubated for 1 h at 25 °C in a re-hydration buffer containing 8 M urea, 4 % (w/v) CHAPS, 60 mM DTT and 1 % (v/v) IPG buffer.

Isoelectric focusing (IEF) was carried out using Ready-Strip-IPG-Strips (Bio-Rad, Hercules CA), 11 cm or 17 cm, with linear pH gradients 3–10 or 4–7 and 40 mg of each protein sample were loaded. The specific conditions of each IEF are specified in the respective Figure. IEFs were carried out using a Protean i12 IEF cell (Bio-Rad, Hercules CA), with rehydration at 50 V for 12 h, followed by four consecutive steps in the following conditions: 250 V/h,

Table 1 Environmental conditions applied in the growth chambers and that induced either C3 or CAM carbon metabolism

Treatments	C3	CAM
Light (µmol m^{-2} s^{-1})	40	260
Humidity (RH) (%)	85	50
Temperature (°C)	(22 ± 2/20 ± 2)	(28 ± 2/20 ± 2)
Irrigation	Saturation	Deficit

500 V/h, 8,000 V for 2.30 h and 8,000 V/h until 30,000 V were attained. After IEF, strips were equilibrated for 15 min in Tris–HCl 50 mM, pH 8.8, containing 6 M urea, 30 % (v/v) glycerol, 1 % (w/v) SDS (sodium dodecyl sulfate) and 65 mM DTT. To remove DTT in excess, strips were equilibrated for 15 min in the same buffer, replacing DTT with 135 mM iodoacetamide. SDS-PAGE in the second dimension was then performed in denaturing polyacrylamide gels according to the procedure of Laemmli (1970), without stacking gel.

Immunoblotting

Polyclonal antibodies against Rubisco were obtained as described in Esquível et al. (1998) and polyclonal antibodies against PEPC and PEPCK were purchased from Agrisera (Vännäs, Sweeden). Western blot analysis was performed after 2-DE through the transfer of proteins to a nitrocellulose membrane (Millipore). Membranes were probed with the respective antibodies using the procedure described by Ferreira et al. (1996) and staining was performed with AP Conjugate Substrate Kit (Bio-Rad, Hercules, CA).

Native PAGE and gel activity staining

Native polyacrylamide gel and gel activity staining isoforms of CAT, SOD, APX and GR were separated in nondenaturing polyacrylamide gels by the procedure of Laemmli (1970). Equal amounts of protein extracts (25 mg) were loaded on 7 % (CAT) or 10 % (SOD, GR and APX) polyacrylamide gels.

For SOD, the gel was stained according to Rao et al. (1996). Gels were incubated for 30 min in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA (ethylenediamine tetraacetic acid). To identify H_2O_2 and KCN (potassium cyanide) sensitive isoforms, this incubation solution contained 3 mM KCN or 5 mM H_2O_2, respectively. This step was followed by incubation in 50 mM potassium phosphate buffer (pH 7.8) containing 245 μM NBT (nitroblue tetrazolium), 33.2 mM riboflavin and 0.2 % TEMED (tetramethyl ethylene diamine) in darkness for 30 min before illumination to visualize SOD isoforms or bands (Donahue et al. 1997).

To visualize the CAT profile, gels were stained using the procedure described by Anderson et al. (1995). The gels were incubated in 3.27 mM H_2O_2 for 25 min, rinsed in distilled water, and then stained in a solution containing 1 % (w/v) potassium ferricyanide and 1 % (w/v) ferric chloride.

Isoforms of APX were visualized by incubating the gels for 30 min in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate, followed by an incubation in the same buffer containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min, as in Carvalho et al. (2005). Finally, gels were stained in 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT for 15 min.

GR was detected according to Carvalho et al. (2005), by incubating the gels for 60 min in darkness in 50 mM Tris–HCl buffer (pH 7.5) containing 0.5 mM 3-(4,5-dimethyl-thiazol-2-4)-2,5-diphenyl tetrazolium bromide, 0.7 mM 2,6-dichlorophenolindophenol, 3 mM GSSG and 0.5 mM NADPH. The staining reaction was stopped by adding 7.5 % (v/v) glacial acetic acid to the staining buffer.

RNA isolation and cDNA preparation

Total RNA from leaves was extracted by adapting the method described by Chang et al. (1993). Extraction buffer consisted of CTAB (2 %), complemented with PVPP (2 %, w/w), Trizma–HCl 100 mM, 25 mM Na_2EDTA, and 2 M NaCl; pH 8.0, heated to 85 °C prior to the addition of 400 μL 2-mercarptoethanol. Tissues were reduced to powder in liquid N_2 and 20 mL extraction buffer were added. The same volume of chlorophorm:isoamyl alcohol 24:1 was then added. This step was followed by a centrifugation at $12,000 \times g$ for 30 min at 20 °C and repeated once. The aqueous phase was transferred to a new tube, and a ¼ LiCl 10 M (v:v) was added. Samples were incubated at 0 °C overnight and after centrifugation at $12,000 \times g$ for 20 min at 4 °C the pellet was recovered and 1.5 mL of buffer was added (Trizma–HCl 10 mM, 1 mM Na_2EDTA, 1 M NaCl; 0.5 % SDS (w:v), pH 8.0, previously heated to 37 °C). Ethanol 100 % (2.5 volumes) was added and the samples were incubated for 1 h at −80 °C and then washed with ethanol 70 %. After drying, the RNA was resuspended in the desired volume of distilled water.

RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI). cDNA was synthesized from 2 μg of total RNA using oligo (dT) in a 20 μL reaction volume with RevertAid Reverse Transcriptase (Fermentas Life Science, Helsingborg, Sweden) according to the manufacturer's recommendations.

Real-time PCR

Primer pairs used for amplification of the genes studied are presented in Table 2. The genomic sequences for pineapple available in the GeneBank database (http://www.ncbi.nlm.nih.gov/genbank/) were used and when pineapple sequences were unavailable, the respective orthologs of *Arabidopsis thaliana*, *Agave tequilana* or *Leymus chinensis* were used.

The 20 μL reaction mixture was composed of cDNA, 0.5 μM gene-specific primers and master mix iQ EVA-Green Supermix (Bio-Rad, Hercules, CA) and an iQ5 Real-

Table 2 Real-time PCR primers used for evaluation of mRNA levels of the enzymes studied

Protein name Species	Gene bank accession	Primer sequences
Actin	CO731409.1	5′-GGCACCACACCTTCTACAAC-3′
Ananas comosus		3′-CTCACACCATCACCAGAATCC-5′
PEPC	AJ312631.1	5′-AGGTCATTGAGCAGTCTTTCG-3′
Ananas comosus		3′-CCACAACAGCGATCTCATCC-5′
Catalase	GU266543.1	5′-GACTAACCATAGCCACGCAACT-3′
Ananas comosus		3′-GCCAGGTCTTGGTCACATCA-5′
CuZnSOD	AJ250667.1	5′-CGCACCTGAAGATGAGACC-3′
Ananas comosus		3′-CCGATAATTCCACAAGCAACTC-5′
APX-thyl	GU266541.1	5′-CTCCTCCTCGTCTTCATCTTCC-3′
Ananas comosus		3′-ATGCCAGCCTAAGCGAACC-5′
SSU	GW667494.1	5′-GGGTCCAGTGCATGAAGG-3′
Arabidopsis thaliana		3′-TTCCACATCGTCCAGTACC-5′
PEPCK	NM_119948	5′-CGGAGCAGCAAAGAGAGGTAGC-3′
Agave tequilana		3′-CGGTGTGGTAGGAGCAGAACG-5′
OEE 1	ABQ52657.1	5′-CCAAGAGGCTGACCTTCGA-3′
Leymus chinensis		3′-CGGTGTGGTAGGAGCAGAACG-5′

Time PCR (Bio-Rad, Hercules, CA) was used. Amplification of PCR products was monitored via intercalation of EVAGreen (included in the master mix). The following program was applied: initial polymerase activation, 95 °C, 3 min, followed by 40 cycles at 95 °C 15 s (denaturation), 57 °C 30 s (annealing), 72 °C 20 s (extension) with a single fluorescence reading taken at the end of each cycle. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the lack of primer dimmers. Further, PCR products were resolved on 2 % (w/v) agarose gels run at 4 V cm^{-1} in Tris–acetate-EDTA buffer (TAE), together with a 50-bp DNA-standard ladder (Invitrogen Gmb H), to confirm the existence of a single product of the desired length.

To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔRn) versus cycle number, baseline data were collected between the cycles 5 and 17. All amplification plots were analyzed with an Rn threshold of 0.3 to obtain Cq (quantification cycle) and the data obtained were exported into a MS Excel workbook (Microsoft Inc.). Relative expressions were calculated in relation to w0 of acclimatization. To compare data from different PCR runs or cDNA samples, Cq values were normalized to the Cq value of *Act2*, a housekeeping gene expressed at a relatively high and constant level (Coito et al. 2012) and the $\Delta\Delta$Cq method was used for the quantification of gene expression.

Statistical analysis of the results

Statistical analyses were carried out using SPSS version 12 (Pérez 2005) and the treatments were analyzed using a non-parametric analysis Kruskall-Wallis H. and C-Dunnett and for gene expression Tukey's multiple range test, both at 5 % significance level.

Results

The present work compares the distinct features of pineapple plants when subjected to acclimatization under environmental conditions that promote the development of either CAM or C3 metabolism (Table 1). Leaf anatomy, proteomic analyses, patterns of in-gel isoenzyme activities and gene expression were assessed to characterize the two metabolism pathways and the moment of shifting from C3 to CAM, in CAM-inducing conditions.

Leaf anatomy, succulence and sap pH

Using leaf cross sections it was possible to measure total leaf, mesophyll, epidermis and hypodermic tissue thickness and identify the differences between the two acclimatization treatments (Table 3). Leaves of pineapple plants grown under CAM-inducing conditions showed higher leaf thickness and more developed epidermic and hypodermic tissue, especially the abaxial epidermal and hypodermal cell layers.

Succulence (S) was measured at the end of the in vitro phase (w0) and on w4 and w8 of acclimatization (Fig. 1). S values increased in leaves under both acclimatization treatments from w0 to w4 but on w8 they were significantly higher in plants grown in CAM-inducing metabolism when compared with C3 plants. The pH was measured in leaf sap of C3 and CAM-induced plants on the same time points, in the middle of the light or the dark period (Fig. 2). On w0,

Table 3 Morphological parameters measured on pineapple leaves

	Leaf	Mesophyll thickness (μm)	Cuticle (ax)	Cuticle (ab)	HT (ab)
C3	444.24 ± 30.21	315.64 ± 25.21	58.12 ± 2.85	64.82 ± 4.67	32.52 ± 2.61
CAM	550.49 ± 35.53	364.81 ± 28.12	52.43 ± 3.28	146.15 ± 13.39	133.79 ± 10.75
	*	–	–	*	*

HT hypodermic tissue, *ax* axial, *ab* abaxial

* Significant differences at 5 % level by C-Dunnett multiple range test ($n = 30$)
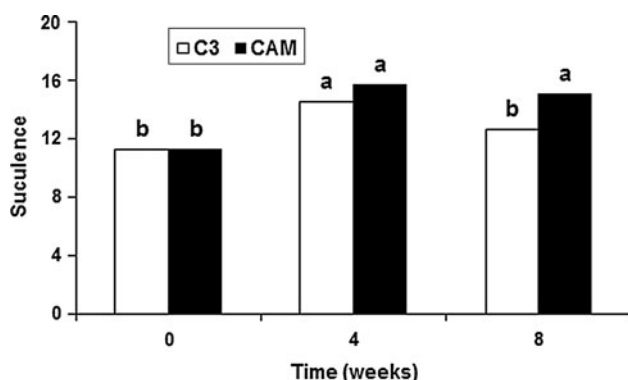
Fig. 1 Succulence index of leaves of pineapple plants grown under C3 and CAM-induced metabolism at transfer to acclimatization (w0), at 4 weeks (w4) and at the end of acclimatization (w8), estimated by the saturated water content (SWC). Significant differences at 5 % level by C-Dunnett multiple range test ($n = 5$)

the sap pH was significantly higher in the light period when compared to the dark and on w4 no significant difference could be detected. On w8, under CAM-inducing conditions the pH decreased significantly during the dark period, while plants under C3 inducing conditions had significantly higher pH in the dark period.

Protein analyses and immunobloting

Two-dimensional electrophoresis (2DE) applied to protein extracts put in evidence several differential spots. On w8 we focused on those that were absent or only vestigial in C3 conditions and that were present in high quantity in CAM grown plants (Fig. 3). Five spots were isolated and identified (Fig. 3; Table 4), the two isoforms of Oxygen Evolving Enhancers, OEE 1 and OEE 2, identified for the first time in *Ananas comosus* by comparison of peptide (mass/charge) profiles after digestion with trypsin (Spots 1, 2 and 3; sequences in Table 4) and small fragments of the large subunit of Rubisco (LSU, Spots 4 and 5), already annotated in Swiss-Prot, were also identified.

As LSU fragments were identified in CAM grown plants, immunoblotings with anti-LSU antibodies were performed following 2DE to identify all existing LSU fragments and assess the influence of the environmental
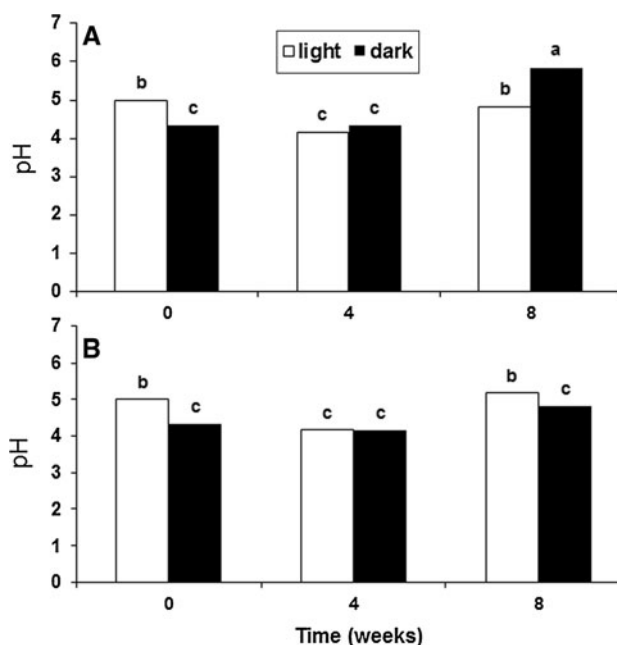

Fig. 2 Determination of pH of sap extracted from leaves of pineapple plants grown under C3 (**a**) and CAM (**b**)-induced metabolism at transfer to acclimatization (w0), at 4 weeks (w4) and at the end of acclimatization (w8), during the *light* and *dark periods*. Values on week 0 are the same on **a** and **b**, measured immediately before the beginning of acclimatization. Significant differences at 5 % level by C-Dunnett multiple range test ($n = 5$)

conditions over LSU degradation patterns (Fig. 4a, b). Under both inducing conditions the isoelectric focusing (IEF) separation revealed populations of spots at isoelectric point (pI) 4–5 and *circa* 9, at 50 kDa, and smaller spots corresponding to LSU degradation, with peptides of low molecular weight and several pI, all higher than 5, almost exclusively found in CAM-induced material (Fig. 4b).

PEPC was also detected by immunobloting of 2DE at the end of both ex vitro treatments (C3 or CAM-inducing) during the dark period (Fig. 4c, d). One single spot was visible in plants under CAM-inducing conditions, at *circa* pI 4.95 and 100 kDa (Fig. 4d), characteristics of the intact PEPC. In C3 conditions no corresponding spot was detected (Fig. 4c). Conversely to PEPC, PEPCK was also detected by immunobloting of 2DE in both ex vitro treatments, but during the light period (Fig. 4e, f). Two spots

Fig. 3 Detection of pineapple proteins by 2-dimentional electrophoresis. Soluble protein extracts of pineapple leaves at w8 of acclimatization under C3 (**a**) and CAM (**b**)-induced metabolism were run on pH 3–10 IPG strips (17 cm) and then separated on 18 × 20 cm SDS-PAGE. Gels were stained with *Coomassie* G. Spots 1–5 were isolated from **b** and sequenced; sequences are indicated in Table 4
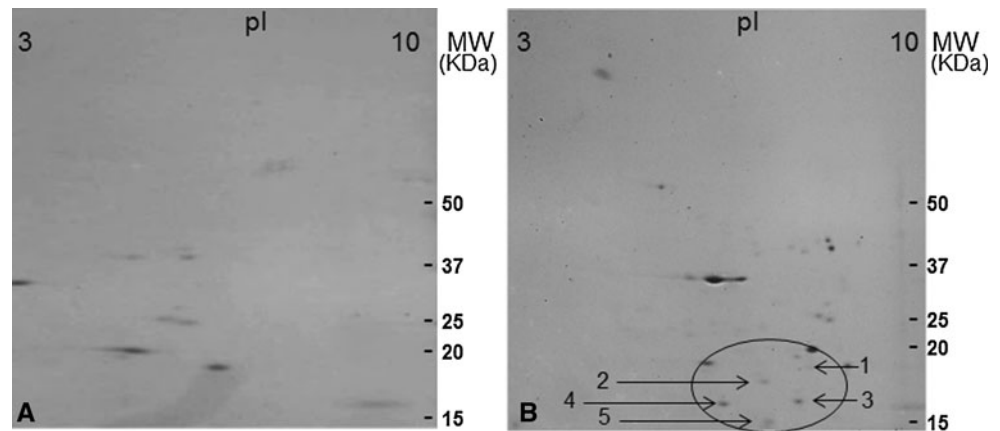
Table 4 Protein sequences obtained by mass spectrometry analysis of the spots represented in Fig. 3

Spot	Name	Annotation	Amino acid sequence (aa)
1, 2	OEE 2 *Ananas comosus*	P12302	——MASATPAIVFRSKSPSSSSQRYVSISK——LVCK————————TNTDFLPYNGDGF—— KWNPSKEVEYPGQVLRALG——IT————KDYGAPEEFLSKVDYLLGKQ——— YYFLSVLTRTADGDEGG——————-KAQAGDKR————————————————
3	OEE 1 *Ananas comosus*	P12359	–RLTYDEIQSKIGGRASSARPSSHVARAFGVDAG—ARELAQKCTDAAK MEGAAGPFKARLATS ALLVSGATAEGAPKRLTYDEIQSKTYMEVK———— KFCLEPTSFTVK———TKLMTRLTYTLDEMEGPLEVFGEE—— ERKDGIDYAAVTVQLPGGERVPFLFTIK———GSSFLDPK———
4	Rubisco *Ananas comosus*	P48683	RBL_ANACO
5	Rubisco *Ananas comosus*	P48683	RBL_ANACO

OEE oxygen evolving enhancer protein http://www.ebi.ac.uk/interpro/potm/2004_11/Table.htm

were visible in both treatments, one corresponding to the intact PEPCK, at pI 7.57 and 80 kDa and the other corresponding to an unspecific reaction with a lower molecular weight peptide (30 kDa), well described by the supplier of the antibody (Agrisera, Vännäs, Sweeden). Under CAM-inducing conditions (Fig. 4f), the spot corresponding to PEPCK was more intense than in C3 plants where the spot corresponding to the unspecific reaction was predominant (Fig. 4e).

In-gel enzyme activities

To assess the effects of the induced carbon metabolism on the antioxidative response, the activities of the different isoforms of four key enzymes of the antioxidative system, SOD, CAT, APX and GR were analyzed by in-gel activity staining (Fig. 5). It was possible to identify one CuZnSOD isoform, using specific reactions with inhibitors (Fig. 5a), one CAT (Fig. 5b), one APX (Fig. 5c) and two GRs (Fig. 5d). During the light period, SOD activity was present in C3 and CAM-induced material, the latter more

intensely all over the experiment except on w0, while in the dark period only CAM-induced plants showed SOD activity, mostly on w4. CAT was faintly detected on w0 and during the light in C3 plants had high activity on w4 that declined on w8 and in CAM plants was undetected. In the dark period, CAT activity was higher in C3 but was present in both treatments and time moments. In C3 plants, APX was active during the first part of the experiment while in CAM-induced plants the activities were much lower, even vestigial on w8 in the dark. The two isoforms of GR were only clearly visible on w0 both in the light and in the dark period.

Gene expression

The transcripts of genes coding for the proteins and enzymes referred above were quantified by RT-qPCR on w0 and w8 and their expression recorded on w8 in relation to w0, in C3 and CAM grown plants after light and dark periods (Fig. 6). *OEE 1 and SSU* were moderately down-regulated in C3 and CAM-induced plants in the light and
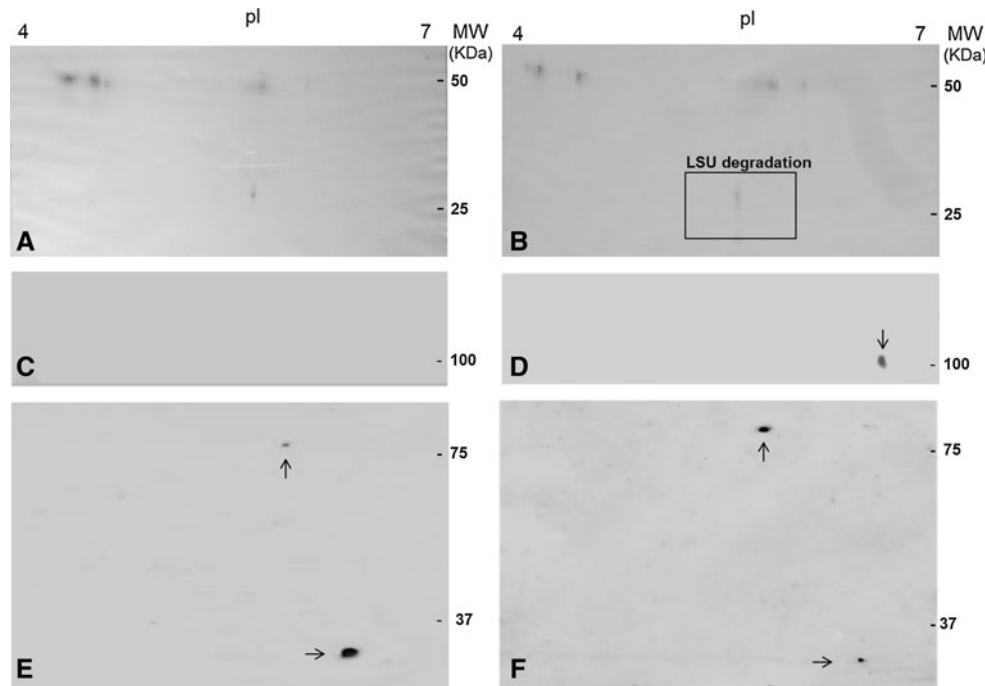
Fig. 4 Detection of LSU (large subunit of Rubisco; **a** and **b**), PEPC (phosphoenolpyruvate carboxylase; **c** and **d**) and PEPCK (phosphoenolpyruvate carboxykinase, **e** and **f**) by immunoblotting following 2-dimentional electrophoresis. Soluble protein extracts of pineapple leaves at w8 of acclimatization under C3 (**a**, **c** and **e**) and CAM (**b**, **d** and **f**)-induced metabolism were run on pH 4–7 IPG strips (7 cm) and then separated on 10 × 8 cm SDS-PAGE. In **b**, LSU degradation products are confined within a *rectangular box*; in **d**, PEPC is indicated by a *downward facing arrow*; in **e** and **f** PEPCK is indicated by an *upward facing arrow* and the unspecific product of 30 kDa described by the supplier of the antibody (Agrisera, Vännäs, Sweeden) is indicated by an *horizontal arrow*
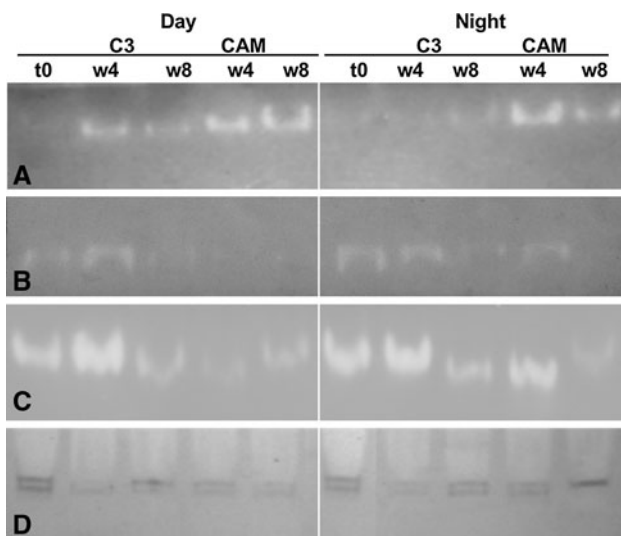

Fig. 5 SOD (**a**), CAT (**b**), APX (**c**) and GR (**d**) isoenzyme in-gel activities in leaves of pineapple plants on w0, w4 and w8 of acclimatization under C3 and CAM-induced metabolism, in the *light* and *dark periods*. Total protein extracts were subjected to native PAGE followed by activity staining for the four enzymes. Discrimination between SOD isoforms was revealed by inhibition with KCN and H2O2

slightly up-regulated in the dark. *APXt* was down-regulated during the light and the dark periods, in either C3 or CAM-induced plants. *PEPC* and *SOD* showed no changes in response to the inducing regimes and to the light conditions. *CAT* was significantly up-regulated in both C3 and CAM-inducing conditions in the dark while with significantly lower values in the latter.

Discussion

Although the designation Crassulacean Acid Metabolism (CAM) derives from the *Crassulaceae* family, the *Bromeliaceae Ananas comosus* is the CAM species with the highest commercial value. CAM physiological and biochemical features are deeply associated to leaf anatomy. In opposition to the majority of non CAM plants, the anatomy of adult pineapple leaves is characterized by the presence of aerating canals, fiber strands, water storage tissue and hypodermis as described in adult pineapple plants by D'Eeckenbrugge and Leal (2003) and more recently, by Pérez et al. (2011, 2012) in new pineapple somaclonal variants. Anatomy of in vitro and ex vitro pineapple leaves
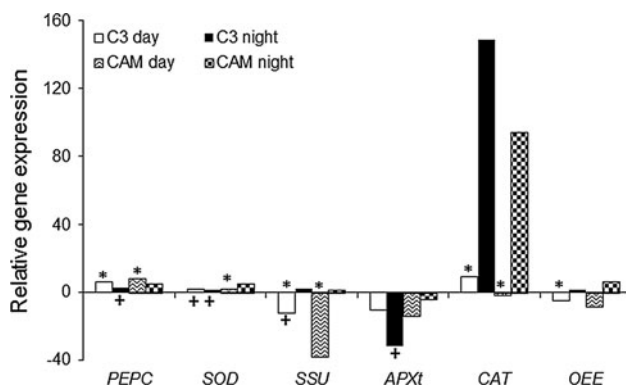
Fig. 6 Changes in the expression levels of *OEE 1, SSU, PEPC, SOD, CAT* and *APXt* in leaves of pineapple plants at the end of acclimatization (w8) under C3 and CAM-induced metabolism, in the *light* and *dark periods*. mRNA was isolated from leaves, converted to cDNA, and subjected to real-time quantitative PCR. Relative expressions in relation to w0 of acclimatization were calculated and values were normalized with respect to *Act2* mRNA. Comparisons of mean were performed between light and dark for each induced metabolism (* *above the column*) and between C3 and CAM in the light and in the dark period (+ *below the column*). The symbols * and + indicate significant differences at 5 % level by Tukey's multiple range test

has also been compared (Barboza et al. 2006). Leaf anatomy of in vitro propagated pineapple plants also shows canals, fibers, an aquifer parenchyma, a chlorenchyma and hypodermic tissue, mostly maintained during the initial ex vitro stages. In the current study, leaf sections were significantly thicker in CAM-induced ex vitro plants due to the thickness of the epidermis, hypodermis and water storage tissues. However, the leaf anatomy of CAM plants has a high degree of plasticity in response to the environmental conditions. For example, in different CAM plants the formation of water storage tissues could be observed after exposure to high light intensity, eventually as a heat dissipation strategy (Espírito and Pugialli 1998). The large vacuoles of the cells of those tissues are also associated to malic acid storage, an aspect described in a number of CAM species.

In agreement with previous results obtained in the same experimental system (Aragón et al. 2012), succulence, normally associated to leaf anatomy of CAM plants of different families (Madison 1977), was also verified. Mesophyll tight cells with highly enlarged vacuoles contribute not only to water but also to malic acid storage (Winter and Smith 1996). Tight packed large cells with less intercellular air space than in C3 plants (Nelson et al. 2005) can also be responsible for an enhanced CO_2 fixation efficiency due to the restriction of CO_2 efflux (Borland et al. 2000). The daily variations of mesophyll pH in CAM plants usually follow the trend of values decreasing in the dark period due to the accumulation of malic acid and increasing in the light period, when malic acid released

from the vacuole is decarboxylated by cytoplasmic malic enzyme (Antony et al. 2008). In fact, it is possible to establish a direct relationship between the low-pH values in the dark in in vitro and ex vitro CAM-induced plants and the high malic acid concentrations measured (Aragón et al. 2012; quantifications obtained in the same experimental system).

The differential protein profile of C3 and CAM grown plants revealed a prominent expression of two isoforms of the chloroplastic Oxygen Evolving Enhancer protein 1-1 (OEE1 and OEE2), a 33 kDa subunit of the oxygen evolving system of photosystem II (PSII), proteins that are responsible for the protection, repair and dissipation of excess energy in the PSII and that are essential for photosynthesis (Murakami et al. 2002). Due to their function of stabilizing the manganese cluster associated to the primary site of water splitting, these proteins are also known as Manganese-Stabilizing Proteins (MSP-1, Yi et al. 2005). In *Arabidopsis thaliana* MSP-1 is encoded by *psbO-1* and MSP-2 by *psbO-2* (Yi et al. 2005). It is interesting to note that, conversely to protein expression, the transcriptional rates of *Ananas comosus psbO-1* were higher in the dark period. CAM-inducing conditions, with high light and low water availability, are highly prone to oxidative stress (Aragón et al. 2012), and thus, CAM-induced plants appear to have developed mechanisms to avoid damage in the PSII.

Monitoring both the enzyme activities and the gene expression profiles of major ROS scavengers and enzymes of the Asc-glut cycle give insight into the overall response of the plants to oxidative stress and whether they are able to cope with the demanding environment of acclimatization (Aragón et al. 2009, 2010). A particular adaptation of pineapple CAM-induced plants to oxidative stress was revealed by their ROS (reactive oxygen species) scavenging profile, CuZnSOD and CAT activity during the whole photoperiod and SOD activity during the dark period. Among these two key players in the ROS detoxification pathways SOD is responsible for the superoxide radical dismutation, while CAT operates the rapid and efficient removal of hydrogen peroxide and its conversion to water in all cell compartments with the exception of chloroplasts (Shao et al. 2008). There seems to be a time shift between *CAT* expression and CAT enzyme activity in both C3 and CAM-induced plants, with high levels of *CAT* expression in the dark period and high CAT activity in the light period.

A strict regulation and coordination between the response to oxidative stress and carbon metabolism should exist in plants with CAM metabolism. The presence of several fragments of Rubisco's Large Subunit (LSU) in leaves of CAM-induced plants suggests a higher turnover of this protein than in C3 plants. Previous results showed polymerization patterns of LSU in plants under stress

conditions, namely under UV light (Ferreira et al. 1996; Wilson and Greenberg 1999). Rubisco is only able to fulfill its function of CO_2 fixation when LSU subunits are coupled with SSU subunits (Small Subunit of Rubisco), which also regulate enzyme activity (Ferreira et al. 2000). The significant down-regulation of *SSU* in the light period and its up-regulation in the dark period are not in agreement with the normal CO_2 fixation by Rubisco in CAM plants. Also, the differences between the accumulation of *APXt* transcripts and enzyme activity, either in C3 or CAM plants also point to the important role of circadian rhythm in the adaptation of this species to particular environmental conditions and in the regulation of mRNA transcription. The genes that are synchronized to the circadian-clock suggest a relationship between mRNA abundance and proteins associated to C3/CAM-facultative photosynthesis (Cushman 2005; Shao et al. 2006).

In CAM plants, PEPC catalyzes the fixation of atmospheric CO_2 in the dark and is also responsible, as in C3 plants, for anapleurotic roles in leaves and non-photosynthetic tissues, as well as for specific functions in stomatal guard cells (Kopka et al. 1997; Gehrig et al. 2005). Furthermore, PEPC is reported to be induced under forced stressing conditions in plants with CAM metabolism (Borland and Taybi 2004). In leaves of pineapple plants grown under CAM-inducing conditions PEPC had a lower isoelectric point (pI: 4.95), a characteristic usually reported in species displaying facultative C4/CAM metabolism (pI: 5.4; Lara et al. 2003). The differences in pI could be related to the patterns of regulation by phosphorylation in C4/CAM-facultative plants compared with C3/CAM ones (Vidal and Chollet 1997). Higher levels of PEPC phosphorylation reduce the sensitivity of the enzyme to its inhibitor, malate, and increase its sensitivity to its activator, Glucose-6-Phosphate. PEPC is phosphorylated in the dark in CAM plants and in response to light in C4 plants (Chollet et al. 1996; Nimmo 2000) and this post-translational modification decreases the pI of proteins (Lara et al. 2003). The only PEPC isoenzyme detected in CAM grown pineapple had a low value of pI (4.95) which can thus be associated with a high pattern of phosphorylation as well as a high functional capacity for CO_2 fixation. In our pineapple system, the expression of *PEPC* was continuously measured both in the light and in the dark and in C3 and CAM-induced plants. As so, the major role in the control of the physiological function of this protein must be assigned to the post-translational regulation by phosphorylation. In the CAM species *Kalanchoe pinnata*, PEPC is described as having seven isoforms (Gehrig et al. 2005). Two of these isoforms (A and D) are present in pineapple leaves, isoform D being the most abundant, suggesting that it is likely to serve a dedicated CAM-specific role. The systematic characterization of PEPC isoforms in families with species

that contain the full range of carbon assimilation patterns, from C3 photosynthesis to weak CAM and to strong CAM, as is the case of *Bromeliaceae,* could shed more light to PEPC regulation (Crayn et al. 2004; Gehrig et al. 2005).

PEPCK is responsible for a dual function, the decarboxylation of oxaloacetate with the concomitant phosphorylation of pyruvate to phosphoenolpyruvate and the release of a CO_2 molecule. In some CAM species including pineapple, PEPCK supplies CO_2 for the fixation by Rubisco during the light period (Weise et al. 2011). The presence of this enzyme in an active form in CAM grown plants confirms the typical CAM metabolism functioning of these plants, further supporting the results of succulence index, pH balance and PEPC behavior.

As a whole, pineapple plants grown under CAM-inducing conditions presented a typical CAM metabolism associated to oxidative stress resistance characteristics, as demonstrated by in-gel activities of antioxidative enzymes, protein degradation patterns and gene expression of key players in the oxidative stress network, mainly *CAT*.

The induction of C3 or CAM metabolisms by direct manipulation of environmental temperature has such high costs that it is almost impossible to be used commercially. In the present work, as in Aragón et al. (2012), the metabolism shift from C3 to CAM was achieved by temperature variation between day and night, by changing light intensity, relative humidity and water supply, whereas a difference of 2 °C in the C3 condition and of 8 °C in the CAM condition was obtained. This is a considerable advantage for commercial micropropagation and acclimatization procedures, as it is easy and cost-effective. Pineapple plants with the capacity to shift from C3 to CAM metabolism under specific environmental conditions become well-adapted for further growth under natural environmental conditions and are also an interesting model for plant biochemical studies concerning carbon metabolism and oxidative stress interactions.

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