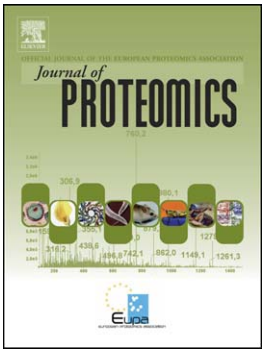

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Comparative shotgun proteomic analysis of wild and domesticated *Opuntia* spp. species shows a metabolic adaptation through domestication

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Abstract

The *Opuntia* genus is widely distributed in America, but the highest richness of wild species are found in Mexico, as well as the most domesticated *O. ficus-indica*, which is the most domesticated species and an important crop in agricultural economies of arid and semiarid areas worldwide. During domestication process, the *Opuntia* morphological characteristics were favoured, such as less and smaller spines in cladodes and less seeds in fruits, but changes at molecular level are almost unknown. To obtain more insights about the *Opuntia* molecular changes through domestication, a shotgun proteomic analysis and database-dependent searches by homology was carried out. More than 1000 protein species were identified and by using a label-free quantitation method, the *Opuntia* proteomes were compared in order to identify differentially accumulated proteins among wild and domesticated species. Most of the changes were observed in glucose, secondary, and 1C metabolism, which correlate with the observed protein, fiber and phenolic compounds accumulation in *Opuntia* cladodes. Regulatory proteins, ribosomal proteins, and proteins related with response to stress were also observed in differential accumulation. These results provide new valuable data that will help to the understanding of the molecular changes of *Opuntia* species through domestication.

Biological Significance

Opuntia species are well adapted to dry and warm conditions in arid and semiarid regions worldwide, they are highly productive plants showing considerable promises as an alternative food source. However, there is a gap regarding *Opuntia* molecular mechanisms that enable them to grow in extreme environmental conditions and how the domestication processes has changed them. In the

present study, a shotgun analysis was carried out to characterize the proteomes of five *Opuntia* species selected by its domestication degree. Our results will help to a better understanding of proteomic features underlying the selection and specialization under evolution and domestication of *Opuntia* and will provide a platform for basic biology research and gene discovery.

Keywords: Carbohydrate metabolism; domestication; 1C-metabolism; *Opuntia* spp.; LC-MS/MS; shotgun analysis

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1. Introduction

Domestication is an evolutionary process in which human selection or humanization is the crucial force causing genetic changes to favor the survival and reproduction of particular phenotypes of plant or animal species [1]. The resulting domesticated populations generally diverge from their parental wild populations in morphological, physiological and behavioral features [2-4]. Plant domestication is accompanied by shifts in resource allocation, as a result of farmer selection for genotypes that give higher yields in agricultural habitats. Plants favored are generally those with higher use, cultural and/or economic value and may involve artificial selection directed to increase abundance of favorable phenotypes [5].

Cacti are native of the New World, and their main distribution areas are arid and semiarid zones [6]. In Mexico, since prehistory, ancient indigenous peoples used cacti as food, medicines, tools, and for religious and magical practices [7].

Among the Cactaceae family, *Opuntia* is one of the most diverse and widely distributed genus in America; it includes 188 species, 78 of them being native of Mexico [8], where highest richness of wild variants and cultivars of the world can be found, and at least 126 with different degrees of domestication [9].

There are evidences that during the process of *Opuntia* domestication, the continuous and systematic gather of nopalitos (edible young cladodes) and fruit favored the development of exceptional features with the purpose to adapt plants to successfully live in human-made environment to maximize yield or any given selected feature [3]. The *Opuntia* fruit has been enhanced on flavor, size, shape, pulp texture, and decreased of seed hardness and seed quantity. In regard to nopalitos, changes occurred in shape, color, earliness, flavor, texture, and quantity and quality of mucilages [9].

A gradient of domestication can be appreciated in species of the *Opuntia* genus, wild species like *O. streptacantha* and *O. hyptiacantha*, others semi domesticated like *O. megacantha*, and *O. albicarpa* [9], and at the end of the gradient *O. ficus-indica*, which is a long-domesticated cactus crop that is important in agricultural economies throughout arid and semiarid parts of the world. Comparative studies of domesticated plants and their ancestors are a promising avenue to explore the molecular changes through evolution and to design strategies to improve plant breeding [10]. Since mass spectrometry (MS)-based proteomics requires the availability of a protein database, proteomic studies in plant systems have primarily been performed in fully sequenced model systems such as *Arabidopsis thaliana*, *Oryza sativa*, but also have been reported in plants with incomplete sequence information such as *Catharanthus roseus* [11], *Panax ginseng* [12], and *Amaranthus cruentus* [13]. Recently proteomics has been used

as a powerful tool to investigate the molecular changes of cotton fiber through domestication [14,15]. Proteomics is also a promise tool to obtain information about signatures of wild and domesticated plants, characteristics that have the potential to direct the crop improvement strategies directed to select traits of agronomic and nutritional importance [16].

The typical plant proteomic experiments utilize two-dimensional gel electrophoresis (2-DE) followed by protein identification by mass spectrometry. Although very powerful and offering a number of advantages, 2-DE yields relatively low number of identified proteins due to inherent limitations of this approach. For that, shotgun proteomic has been applied to increase the coverage of the proteome of plants such as wheat [17] and *Medicago truncatula* [18].

In this work, a comparative shotgun proteomic analysis of five *Opuntia* species with different domestication grade was carried out. More than 1000 protein species were detected in the *Opuntia* species and the differentially protein accumulation among wild and domesticated species was analyzed. Those proteins corresponded to metabolic pathways such glucose, secondary, and 1C-metabolism, which correlate with the observed protein, fiber and phenolic compounds accumulation in *Opuntia* cladodes among species. Interestingly a well-represented group of regulatory protein species such as proteasome and 14-3-3 were identified in all *Opuntia* samples. The present data shows the *Opuntia* molecular changes through domestication and will help to understand the agronomic and nutraceutical characteristics of wild and domesticated *Opuntia* species.

2. Materials and Methods

2.1 Plant Material

Opuntia young cladodes were collected from the CRUCEN-UACh *Opuntia spp*. germoplasm bank, an Agrobotanical garden located in southwest Zacatecas city, Mexico, in the “El Orito” locality (22° 44.7´ North latitude and 102° 36.4´ West length). Five species were selected from the wildest *O. streptacantha*, continuing with variants of *O. hyptacantha* and *O. megacantha*, following with *O. albicarpa* and the most domesticated variant, the *O. ficus-indica*. The donor *Opuntia* plants were grown under the same natural environmental conditions (temperature, precipitation and soil).

Opuntia young cladodes were collected on april 2010. The selection of 15–25 cm of length young cladodes was according to Ramirez-Tobias *et al*. [19]. Two young cladodes from each species were collected from three different plants (Supplementary Fig. S1). Cladodes samples were transferred in an ice-container to the laboratory where they were thoroughly rinsed with distilled water, dry in paper towels and stored at -80 ºC. Frozen samples were freeze-dried and grounded (KRUPS GX4100 grinder) to obtain a fine powder. Powder samples were stored at -80 °C until use.

2.2 Proximate composition and phenolic compounds quantification

Total protein content (Nx6.25) was determined by the micro-Kjeldhal method [20], fat was determined by the Soxhlet method [20], and crude fiber contents were determined following the AOAC methods [20]. Total soluble sugars were determined using the anthrone-H_2SO_4 reagent method [21]. Dextrose was used as a reference compound and results were expressed as mg of dextrose g^{-1} of sample. All determinations were analyzed at in triplicates.

The phenolic compounds extraction was carried out according to Guevara-Figueroa et al. [22]. Briefly 1 g of sample was mixed with 100 mL of absolute ethanol pre-chilled at -20 ºC. suspension was incubated under agitation at 4 ºC for 2.5 h and centrifuged at 13000 g at 4 ºC. Supernatants were filtered through 0.45 μm filter (Whatman). The ethanolic extract (20 μL) was added to 1.58 mL of MilliQ water and mixed. Then 300 mL of 20% Na_2CO_3 and 100 μL of Folin-Ciocalteu phenol reagent were added and the mixture was allowed to stand for 2 h at room temperature. Absorption of the solution was read at 765 nm in a UV-Vis spectrophotometer (Varian Cary, Palo Alto, CA, USA). Total phenolic content was expressed as μmol of Gallic acid equivalents g^{-1} of sample. All determinations were performed in triplicates. For flavonoid quantification, 15 μL of ethanolic extract were diluted 735 μL of MiiliQ water and mixed with 750 μL of 2% $AlCl_3$ solution. After 10 min, the absorbance of the mixture was read at 367 nm in a UV-Vis spectrophotometer (Varian Cary). Quercetin was used as a reference standard and results were expressed as μmol Quercetin equivalents g^{-1} of sample. All determinations were performed in triplicates.

2.3 Protein extraction and quantification

Opuntia samples (2 g) were washed with ethanol (twice) and then three times with acetone (containing 0.2% DTT). In each step the solution was eliminated by centrifugation at 4 °C for 15 min at 13000 g. Washed samples were homogenized, using a mortar and pestle on ice bath, with 15 mL of extraction buffer containing 100 mM Tris-HCl (pH 8.0), 1.5 mM potassium chloride, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1% SDS, followed by centrifugation at 4 °C for 20 min at 13000 g. Proteins were precipitated by the addition of three vol of 0.1 M

ammonium acetate (in methanol) per vol of extract; samples were kept at -20 °C overnight. Proteins pellets were recovered by centrifugation at 4 °C for 20 min at 13000 g and washed with 0.1 M ammonium acetate (once) and three times with acetone (containing 0.2% DTT), and centrifuged for 15 min as indicated above. All solvents were used pre-chilled at -20 °C. Pellets were allowed to dry and re-suspended in 0.1 M Tris-HCl (pH 8.0) and protein concentration was determined using the Protein Assay kit (Bio-Rad, Hercules, CA, USA), using BSA as standard. Triplicates of extractions were carried out.

2.4 Protein preparation by 1-DE and shotgun analysis

For the shotgun analysis, solubilized proteins (10 µg) were mixed with Laemmli sample buffer and loaded onto 12% SDS-PAGE. Electrophoretic migration was performed to fractionate the protein samples of each cultivar into gel lines of 40 mm long. Gels were colloidal coomassie blue stained and visualization was performed in a Pharos FX Plus (Bio-Rad). Each line was cut in 10 gel bands of similar size and distained, reduced and alkylated. Digestion of proteins by the trypsin and the extraction of resulting peptides were performed as in Gautier et al. [23].

The resulting peptides were analyzed by nanoLC-MS/MS using an Ultimate3000 system (Dionex, Amsterdam, The Netherlands) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Five µL of each sample were loaded on a C18 precolumn (300 µm inner diameter X 5 mm; Dionex) at 20 µL min^{-1} in 5% acetonitrile, 0.05% TFA. After 5 min of desalting, the precolumn was switched online with the analytical C18 column (75 µm inner diameter X 15 cm; PepMap 8, Dionex) equilibrated in 95% solvent A (5%

acetonitrile, 0.2% formic acid) and 5% solvent B (80% acetonitrile, 0.2% formic acid). The peptides were eluted using a 5 to 50% gradient of solvent B during 105 min at 300 nL min^{-1} flow rate. The LTQ-Orbitrap Velos was operated in data-dependent acquisition mode with the XCalibur software. Survey scan MS were acquired in the Orbitrap on the 300–2000 $m\ z^{-1}$ range with the resolution set to a value of 60,000. The 20 most intense ions per survey scan were selected for CID fragmentation, and the resulting fragments were analyzed in the linear trap (LTQ). Dynamic exclusion was employed within 60 s to prevent repetitive selection of the same peptide. Protein identification was carried out according to Stanislas et al. [24].

2.5 Database search and data validation

The Mascot Daemon software (version 2.3.2; Matrix Science, London, UK) was used to perform database searches, using the Extract_msn.exe macro provided with Xcalibur (version 2.0 SR2; Thermo Fisher Scientific) to generate peaklists. The following parameters were set for creation of the peaklists: parent ions in the mass range 400–4500, no grouping of MS/MS scans, and threshold at 1000. A peaklist was created for each analysed fraction (*i.e.*, gel slice), and individual Mascot (version 2.3.01) searches were performed for each fraction. The data were searched against *"Viridiplantae"* entries in Uniprot protein database.

Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine was specified as variable modification. Specificity of trypsin digestion was set for cleavage after Lys or Arg, and one missed trypsin cleavage site was allowed. The mass tolerances in MS and MS/MS were set to 5 ppm and 0.6 Da, respectively, and the instrument setting was specified as "ESI-Trap." To calculate the false discovery rate (FDR), the search was performed

using the “decoy” option in Mascot. Mascot results were parsed with the in-house developed software Mascot File Parsing and Quantification (MFPaQ) version 4.0 [25], thus peptide identifications extracted from Mascot result files were validated at a final peptide FDR of 1%. Peptide matches were validated if their score was greater than the Mascot homology threshold (when available, otherwise the Mascot identity threshold was used) for a given Mascot *p* value. The FDR at the peptide level was calculated as described in Navarro and Vazquez [26]. Using this method, the *p* value was automatically adjusted to obtain a FDR of 1% at the peptide level. Validated peptides were assembled into proteins groups following the principle of parsimony (Ocam’s razor), which involves the creation of the minimal list of protein groups explaining the list of peptide spectrum matches. Protein groups were then rescored for the protein validation process. For each peptide match belonging to a protein group, the difference between its Mascot score and its homology threshold (or identity threshold) was computed for a given *p* value (automatically adjusted to increase the discrimination between target and decoy matches), and these “score offsets” were then summed to obtain the protein group score. Protein groups were validated based on this score to obtain a FDR of 1% at the protein level (FDR = number of validated decoy hits/(number of validated target hits + number of validated decoy hits) X 100).

2.6 Protein grouping and data quantitation

Quantification of proteins and peptides was performed using the label-free module implemented in the MFPaQ v4.0.0 software (http://mfpaq.sourceforge.net/). General procedures of this software are detailed in Gautier et al. [23]. For each cultivar, quantification of peptide ions was performed in four replicates, two times for the first extraction and two times for the second

extraction. This method was based on calculated XIC area values and in the instance where the peptide was identified in several electrophoresis gel fractions; the XIC area values were summed. The similar peptide sequences with identical mass and containing the L, (leucine) or I (isoleucine) amino acid at the same position were not taken into consideration for the quantitation. To perform the normalization of peptide quantitation in the four replicates, the ratios of the XIC area values were calculated for all the extracted signals between one replicates and the three others and the median of the ratios was used as a normalization factor. The sum value of the peptide XIC area values of the four replicates was assigned at the corresponding protein as a protein abundance index (PAI).

Protein identifications, PAI and peptide quantitation were exported from MFPaQ and uploaded to the software Protein Center (Thermo Fisher Scientific, http://www.proxeon.com/productrange/data_interpretation/introduction/index.html) in the aim of the comparison of the five species. The proteins were assembled in a same group if they have at least one peptide in common. The anchors of the groups are the proteins, which have the best PAI and a maximal number of peptides. Only the groups were taken in consideration for the comparisons between the species.

2.7 Statistical analysis.

Data of total protein, fat, fiber ash, sugar, phenolic, and flavonoid compounds were expressed as mean values of triplicates. One-way analysis of variance (ANOVA) was performed with significant differences among means determined by the Tukey's test at $p \leq 0.05$.

3. Results

3.1 *Opuntia* morphological characteristics

It is known that within the *Opuntia* genus, the presence of spines, the number of spines/areole, and the number of areoles may differ drastically in different growing regions [27]. For this reason, all donor plants were collected from "Opuntario" located at southwest Zacatecas city, Mexico, where natural growing conditions (arid soil, low precipitation, high temperature), were the same for all species therefore molecular changes will be only for intrinsic characteristics of each species.

Morphological differences were observed among *Opuntia* species, the wildest *O. streptacantha* had more spines in cladodes and more circular shape while *O. ficus-indica*, the domesticated specie, presented few, small and soft spines, and elongated shape (Fig. 1 and Supplementary Table S1). However, it has been described that the presence of spines in the cladodes is an inadequate feature to discriminate *O. ficus-indica* from other arborescent *Opuntias* [28] suggesting that changes at molecular level may be a key for *Opuntia* classification.

The *Opuntia* cladodos macromolecular composition (Table 1) showed that *O. hyptiacantha* presented the lower total protein concentrations (10.5%) but has the higher fat (0.94%) contents, in regard to crude fiber contents the wildest *O. streptacantha* and *O. hyptiacantha* presented the highest values (6.52% and 6.58%, respectively) while domesticated *Opuntia ficus-indica* has the higher protein content but the lowest crude fiber. Fiber is an important characteristic due that some of medicinal effects claimed for *Opuntia* are attribute to the fiber content [29]. Synthesis of osmolytes is one important mechanism for plant tolerance to abiotic stress and soluble sugars content was determined. It was observed that *O.*

megacantha followed by *O. streptacantha* had the highest accumulation of total sugars (103.7 mg g^{-1} and 61.0 mg g^{-1}, respectively), while the lowest values were detected in *O. albicarpa*, the semi-domesticated species (Table 1). Phenolic and flavonoids compounds have been also claimed as the molecules responsible of the claimed *Opuntia* medicinal characteristics. Total phenolic compounds (Table 1) were higher in the wildest species *O. streptacantha* (65.1 μmol of GA g-1), while were not differences in flavonoids contents between the wildest *O. streptacantha* and the most domesticated *O. ficus-indica*. These results show that metabolism regulation among *Opuntia* species is different.

3.2 *Opuntia* shotgun analysis

Opuntia cladodes proteins were separated by SDS-PAGE, the gel was divided in 10 bands that were excised and digestion of each band was performed (Supplementary Fig. S2). The resulting tryptic-digested fragments were analyzed by nanoLC-MS/MS, database searches using MS/MS sequencing data were performed with MASCOT and the results files were parsed and validated based on target decoy calculated FDRs, set at 5% for peptides and 1% for proteins.

Into each cultivar, proteins with at least 60% in sequence identity were grouped. *Populus trichocarpa* proteome was used to compare the groups; this species was selected as reference because the most numbers of orthologs sequences were identified (Table 2). The protein family database (PFAM) allowed defining those proteins that were specific in the analyzed *Opuntia* species and those who were particularly abundant (Supplementary Files S1-S5).

3.3 Proteins overrepresented or underrepresented in the wild species compared to *O. ficus-indica*

Identified peptides were quantified using the MS signal in the MFPAQ software. The value assigned to each peptide for each variety and each replicate is the sum of the intensities detected in each region of the electrophoresis track. These values were normalized on the basis of the replicate where the sum of intensities of all peptides is the highest. The median ratios between replicates were used. The median was calculated only using the values of the quantified peptides in four replicates. The ratio of the average intensities of the replicates of the wild variety with that of *O. ficus-indica* was used to evaluate the up-accumulation or down-accumulation of the peptide. Student's T-test, taking into account the four replicates, was applied to define the *p*-value that must be less than 0.05 if the difference is significant. This test was applied after the missing values have been replaced. These missing values were calculated by taking into account the maintenance of the coefficient of variation of the population. Each wild species was compared to the *O. ficus-indica* variety and a quantitative ratio is calculated if the following criteria are in agree: a) at least two common peptides should be present on the same homologous protein, b) at least one of the peptides has been identified in three replicates in one of the five species, c) at least each peptide is quantified 2 times in the wild species and the *O. ficus-indica*.

The overrepresented proteins in the wild type variety proteins were selected if all quantified peptides had an intensity ratio greater than 1 between the wild type and *O. ficus-indica* and if at least two of them are significantly different (*p*-value < 0.05). The underrepresented proteins were selected if all peptides had a ratio less than 1, and if 2 of them were significantly different (*p-value* < 0.05). The differentially represented proteins have an abundance ratio index corresponding to the average ratio of all the quantified peptides. The lists of the overrepresented

proteins (excess proteins) and those of the underrepresented proteins (protein loss) were imported into Protein Center for functional analysis after grouping according to 60% of sequence identity.

Shotgun analysis revealed that the domestication greatly influences the protein composition in *Opuntia* cladodes. It was evident that the wildest species (*O. streptacantha* and *O. megacantha*) contain specific proteins or in excess versus *O. ficus-indica* (Supplementary Files S6-S10).

3.4 Specific proteins and peptides

Filtered proteins (60% identity) from *O. streptacantha*, *O. megacantha*, *O. hyptiacantha*, and *O. albicarpa* species were compared with the identified proteins from *O. ficus-indica*. A grouping was performed when the proteins have a common peptide at least between the two considered species. The groups of the wild variety that contain no identical peptide to *O. ficus-indica* were noted as specific. Then, these specific proteins were grouped again according to 60% sequence identity (Supplementary Files S11-S15). All the identified peptides of a wild species were compared to the total list of peptides from the *O. ficus-indica*. The list of peptides that were absent in the proteins of *O. ficus-indica* is show in the Supplementary Files S16-S19, these peptides may belong to specific or non-specific proteins.

4. Discussion

Classification of *Opuntia* proteins according to their biological processes (Fig. 2), it was observed that almost the half of the proteins corresponded to metabolic process, and the wildest species (*O. streptacantha, O. hyptiacantha, and O. megacantha)* contained the higher percentages (47.2% to 48.4%), while the

domesticated *O. albicarpa* and *O. ficus-indica,* had the lowest values (44.4% and 45.8%, respectively). Interestingly, proteins related with defense and responses to stimulus were more represented in *O. streptacantha* (13.1%) and *O. hyptiacantha* (13.9%). According to its molecular function, differences were observed in protein related with catalytic role (34.4% to 35.6%), followed by proteins with DNA, RNA, and nucleotide binding function (20.0% to 22.9%), metal binding proteins were also well represented in all *Opuntia* species (Supplementary Fig. S3).

4.1 Carbon metabolism is up-regulated in wild species

A considerable number of proteins involved in carbon metabolism were preferentially overrepresented in wild species, in particular the key enzymes involved in the regulation of glycolysis. Enolase showed significantly higher accumulation levels in *O. hyptiacantha*, while two orthologs of glycheraldehyde-3-phosphate dehydrogenase were detected in *O. streptacantha* (Table 3 and Supplementary Files S16 to S19). At least five orthologs of Fructose-1,6-bisphosphate aldolase (FBA), were detected in differential accumulation among *Opuntia* species, one FBA protein species was highly accumulated in *O. hyptiacantha*. FBA is another key enzyme in the glycolytic pathway that catalyses the reversible reaction by converting fructose-1,6-bisphosphate (FBP) into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P), two key intermediates for oil biosynthesis [30]. This finding correlates with the observation that *O. hyptiacantha* has the highest fat content (Table 1).

The accumulation of pyruvate kinase suggests higher energy consumption in wild species, especially in *O. hyptiacantha*. Aldehyde dehydrogenase (ADH) is an important enzyme that play important role in mitigating oxidative/electrophilic

stress when plants are exposed to unfavourable environmental conditions was over-represented in *O. hyptiacantha*. The overexpression in transgenic tobacco plants with *ADH* gene from *Syntrichia caninervis* (*ScALDH21*) enhanced drought and salt tolerance suggesting that could be a good candidate gene for molecular breeding of salt- and drought-tolerant plants [31].

Mitochondrial metabolic enzymes, as energetic generators and/or targets of signals, are important players in the distribution of intermediates between catabolic and anabolic pathways. The Krebs metabolism was less active in *O. megacantha* and *O. albicarpa* by the down-accumulation of aconitase, which catalyzes the reversible isomerization of citrate to isocitrate (Table 3). The 2- oxoglutarate dehydrogenase complex (OGDHC), which occupies an amphibolic branch point in the cycle, where the energy-producing reaction of the 2-oxoglutarate degradation competes with glutamate (Glu) synthesis was overrepresented in *O. streptacantha*. It has been shown that inhibition of *OGDHC* in tomato (*Solanum lycopersicum*) substantially reduces both photosynthetic and respiratory metabolism and plant development connecting carbon-nitrogen interactions [32,33].

4.2. Photosynthesis related-enzymes

The high *Opuntia* species productivity is related with their use of the crassulacean acid metabolism (CAM) photosynthetic pathway. CAM plants maximizes the water use efficiency by concentrating CO_2 around ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), favoring carboxylase activity. At least three isoforms of RuBisCO were detected, one ortholog was up-accumulated in *O. streptacantha* and another was down-accumulated in *O megacantha* (Table

3). The most distinctive features of CAM organisms are the nocturnal CO_2 uptake and fixation by phospho*enol*pyruvate carboxylase (PEPC) PEPC as well the pyruvate phosphate dikinase (PPDK) were up-accumulated in *O. streptacantha* suggesting an increase carbon fixation compared with the other *Opuntia* species. In darkness, also two energy-consuming enzymes of the Calvin cycle, the phosphoribulokinase (PRK) and glycerladeyde-3-phosphate dehydrogenase (GAPDH) are crucial enzymes for the global balance of the photosynthetic process under different environmental conditions [34]. Two protein species of PRK were detected, one up-accumulated in *O. streptacantha* and the second down-accumulated in *O. megacantha*.

Enzymes related with starch metabolism such as alpha1,4-glucan phosphorylase, UDP-G glucose 6-dehydrogenase and sucrose synthase (SUS) were also detected. Two orthologs of SUS were up-accumulated in *O. streptacantha*, while highest accumulation of alpha1,4-glucan was observed in *O. hyptiacantha* (Table 3). Studies on gene expression and evolution have provided new insights into the role of positive selection in the intra species divergence [35], and then the analysis of SUS could be a good candidate gene-protein to get information about the true domestication route of *Opuntia* species. An increase of SUS expression has been correlated with an increase in cellulose [36], this correlate with the detection of cellulose biosynthetic process protein in *O. hyptiacantha* and the higher fiber contents detected in this species (Table 1) as well with reports indicating that that wild species are higher in fiber contents [22,37].

In relation with the higher glucose metabolism in wild *Opuntia* species, enzymes related with the photosystem II (CP43, CP47, Lhcb, OEC, Cytb6, Cytf) and

photosystem I (PA700) were up-accumulated in wild species. Both CP43 and CP47, core antenna proteins of PSII, contain six trans-membrane helices, which binds 13 and 16 chlorophyll a molecules, respectively. The electron flow through Cytb6/f complex is considered to be a key rate-limiting step for RuBisCO regeneration [38], increasing CytB6/f content may be a useful biomolecular target for enhancing leaf photosynthesis for improved crop yield [39]. These results show that *Opuntia* wild species, the best adapted to environmental stresses, have higher accumulation of photosystem proteins subunits.

4.3. One-carbon and nitrogen metabolism

One carbon (C1) metabolism is essential to all organisms, in plants it supplies the C1 units to synthesize essential molecules biosynthetic and regulatory compounds, including proteins, nucleic acids, pantothenate, and methylated compounds such as lignin, alkaloids, and betaines [40]. It has been suggested that depletion of one-carbon tetahydrofolate (TFH) pools and methionine could effectively and specifically block protein translation, as well as alter DNA methylation and synthesis [41]. Serine hydroxymetil transferase (SHMT) is important for C1-metabolism and photorespiration in higher plants for tis participation in plant growth and development, and resistance to biotic and abiotic stresses [42]. S-adenosyl-L-methionine synthase (SAMS) catalyze the biosynthesis of SAM, which is a precursor for ethylene and polyamines, and a methyl donor for a number of biomolecules. SHMT and SAMS were up-accumulated in *O. streptacantha* (Table 3). S-adenosyl cysteine synthase (SACS) through the route of DNA methylation was also overrepresented in *O. streptacantha*, Transgenics Arabidopsis lines transformed with SAMS from *Solanum brevidens* (*SbSAMS*) exhibited higher salt and drought stress tolerance

[43]. The enzyme Δ-1-pyrolidine synthase was also over represented in wild species, suggesting a higher accumulation of proline. Thiamine thiazole (THI1) has been associated with heat tolerance in rice [44] and was up-accumulated in *O. megacantha*.

Glutamine synthetase (GS), found overrepresented in *O. streptacantha* and *O. hyptiacantha* (Table 3). GS plays a crucial role in plant growth by assimilating ammonium from the soil and in the re-assimilation of photorespiratory ammonium released during photorespiration [45]. GS has been shown to play role in water use efficiency by being responsive to drought and salt stress [46], its deficiency decline photorespiration and increase ammonium concentrations significantly, therefore, causing significant decrease in photosynthesis in barley [47,48], by contrary its overexpression increase photorespiratory capacity in wheat [49] and rice under osmotic stress [50].

4.4 Secondary metabolism

Plant geranylgeranyl hydrogenase (CHLP) reduces free geranylgeranyl diphosphate to phytyl diphosphate, which provides the side chain to chlorophylls, tocopherols, and plastoquinones [51]. CHLP and protochlorophyllide reductase (POR), enzymes related with chlorophyll synthesis were detected up-accumulated in wild *Opuntia* species (*O. streptacantha* and *O. hyptiacantha*) as well the was up-accumulated in the wild *Opuntia*, these results correlate with the observation that these species contain more chlorophyll-binding proteins such as Lcab (Table 3).

It is interesting to note that enzymes required for monlignol biosynthesis such as phenylalanine ammonia-lyase (PAL), caffeoyl-CoA O-methyltransferase, cinnamoyl alcohol dehydrogenase (CAD), and sinapyl alcohol dehydrogenase

[52], were detected in wildest *Opuntia* species (Supplementary Files S6-S10), which correlates with the observation that in this species was detected highest phenolic compounds accumulation (Table 1).

4.5. ROS Scavenging and oxide-reduction proteins

The SOD family of proteins are essential enzymes of ascorbate glutathione cycle and act as the first level of defense controlling concentrations of two important ROS, superoxide radicals and hydrogen peroxide. In the second line of defense, peroxidase-class enzymes, such as catalase (CAT), ascorbate peroxidase (APx) play key functions in the hydrogen peroxide detoxification reducing ROS to non-damaging concentration [53]. SOD was found accumulated in *O. streptacantha*, while CAT and two orthologs of APx were more representative in *O. hyptiacantha* (Table 3). Zhang et al. [54] reported that the more tolerance of Arabidopsis to severe salinity was due to the higher CAT, peroxidases as well proline accumulation.

Thioredoxin (Trx), redox-sensitive proteins, play crucial roles in the regulation of cellular processes in plants [55buchanan]. It has been reported that some of the Trx targets are PRK, GAPDH, and FBP [56]. Trx was up-accumulated in *O. hyptiacantha*, that correlate with the up-accumulation of FBP (Table 3).

Lipoxygenase (LOX) are enzymes related with the metabolism of several regulators named oxylipins, molecules that are directly associated with defense reactions in conditions of biotic and abiotic stresses as well as with the regulation of plant growth, propagation and senescence [57]. LOX was up-accumulated in *O. streptacantha*,

The glycolate oxidase (GO; S-2-hydroxyacid oxidase), a flavin mononucleotide (FMN)-dependent enzyme, was up-accumulated in *O. streptacantha* (Table 3). Transgenic tobacco plants with reduced levels of GO exhibited decrease photosynthetic electron transport rates in high light conditions [58].

4.6. Regulatory and Heat shock proteins

Protein ubiquitination, a critical post-translational regulatory mechanism, plays an important role in plant tolerance against various biotic and abiotic stresses [59-61]. Proteins related with the regulatory systems were detected in all *Opuntia* species (Fig. 3), However, *O. streptacantha* presented up-accumulation of several subunits of (Table 3). It has been reported that altering expression of 26S proteasome subunits may affect the amount as well as the activity of the 26S proteasome and could be related with flooding tolerance in soybean [62] and also in plant defense responses to pathogens during plant-pathogen interactions [63].

The study of 14-3-3 proteins is taking attention due to the increasing evidences that suggests that in plants they play important functions [64]. Some 14-3-3 subunits strongly interact with proteins involved in carbon and nitrogen metabolism, where nitrate reductase and SUS are some of the more extensively characterized 14-3-3 client proteins [65] and its overexpression has been related to the increased drought tolerance in rice [66]. 14-3-3 proteins also have been reported to be involved in the activation of plasma membrane H^+-ATPase [67]. Interestingly, in all *Opuntia* species were identified different protein species of 14-3-3 proteins (Supplementary Files S16-S19) suggesting the importance of these proteins in *Opuntia* growth and development.

Molecular chaperone/heat-shock proteins (HSPs) are responsible for protein stabilization, proper folding, assembly and translocation under both optimum and adverse growth conditions. A great variety of HSPs (90, 60, 70, 101, 902-1, 902-1), Hsc70, and HSP40s also known as DnaJ proteins were identified in all *Opuntia species*. DnaJ proteins as co-chaperones have critical functions in biotic and abiotic stress responses, but their biological functions remain largely un-investigated. The overexpression of DnaJ from *Solanum lycopersicum* (tomato) improved the resistance to the pathogen *Ralstonia solanacearum* in transgenic tobacco [68]. In wild *O. streptacantha* was detected the up-accumulation one cytosolic chaperonin and Hsp90, while in *O. hyptiacantha* one Hps was up-accumulated. Recently studies have indicated that *Hsp90* gene could be a novel candidate capable of influencing the chip colour of potato tubers [69].

4.7 Nuclear Proteins, transcription, and translation factors

Ribosomal proteins 60S (L7 and L12), 40S (S8, S14) were identified in wild species in higher concentrations, especially in *O. streptacantha* and *O. hyptiacantha* (Table 3). The increased production of ribosomal proteins, suggest an adaptation of wild species to survive in conditions of highest UV exposition as reported in *Zea mays* [70]. The elongation eF2 and translation initiation factor eIF4A were down-accumulated in *O. megacantha* (Table 3). eIF4F, which is composed of eIF4E, eIF4A, and eIF4G, is required to promote 40S subunit binding to an mRNA [71].

4.8. Membrane trafficking and transport

Under hypersaline conditions, several plant response which includes cell-wall hardening, osmoregulatory process, and the over-expression of the tonoplast specific intrinsic protein pyrophosphate-energized inorganic pyrophosphatase (H^+-PPase), suggesting that vacuoles are engaged in Na+ sequestration accordingly with a high capacity of proton pumping and Na+ uptake via the Na+/H+-antiporter in seagrasses and halophytes [72-74]. An up- accumulation of inorganic pyrophosphatase, V-type proton ATPase, and vacuolar H^+-ATPase were detected in wild *O. streptacantha* but down-accumulated in *O. megacantha* (Table 3).

Two isoforms of GTPase-sar1 were identified in *Opuntia*, one was up-accumulated in O*. hyptiacantha* and the second in *O. megacantha* (Table 3). Recently it has been reported that CPSAR1 protein, identified in chloroplast vesicles, play an important role for normal thylakoid formation [75]. In rice Sar acts as a molecular switch to regulate the assembly of coat protein complex II, which exports secretory protein form the ER to the Golgi apparatus [76]

Endocytosis involves the internalization or uptake of plasma membrane (PM) proteins, lipids, and extracellular molecules into the cell. Clathrin-mediated endocytosis (CME) is the major route in plants [77-78], and was observed up-accumulated in *O. streptacantha*. CME has been shown to be a major endocytic pathway in *Arabidopsis* and has been implicated in the signalling network associated with the recognition of microbes by plants [76].

5. CONCLUSIONS

This work represents the first large-scale proteomics analysis of *Opuntia* species, important plants that grows under extreme environmental conditions and with several medicinal properties. Although *Opuntia* genome has not been

reported, proteomics has proved to be a valuable tool to identify proteins and their differential accumulation along domestication. A brief schematic representation of protein changes, when comparing with the most domesticated *O. ficus-indica*, is shown in Fig. 4. Our data have shown that the wildest species *O. streptacantha* and *O. hyptiacantha* presented the most up-accumulation of proteins in cladodes in relation to the most domesticated *O. ficus-indica*. Accumulation of enolase in *O. hyptiacantha* could correlate with the observed higher fat content in cladodes. Accumulation of SUS in *O. megacantha* correlated with the higher fiber contents presented in this species. Enzymes of the 1C-metabolism were observed up accumulated in wild species suggesting that regulatory molecules such as polyamines, ethylene, and also methylation processes are involved as important mechanisms of plant responses to environmental conditions.

Finally our proteomic data indicates a correlation between domestication grade and protein abundance showing the *Opuntia* molecular changes through domestication, information that will help to understand the agronomic and nutraceutical characteristics of wild and domesticated *Opuntia* species.

Conflict of interest:

The authors declare that they do not have any conflict of interest.

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Fig. 1. A representative cladode from wild and domesticated *Opuntia* species. Upper arrow indicates the direction of domestication, from the wildest *O. streptacantha* to the most domesticated *O. ficus-indica*

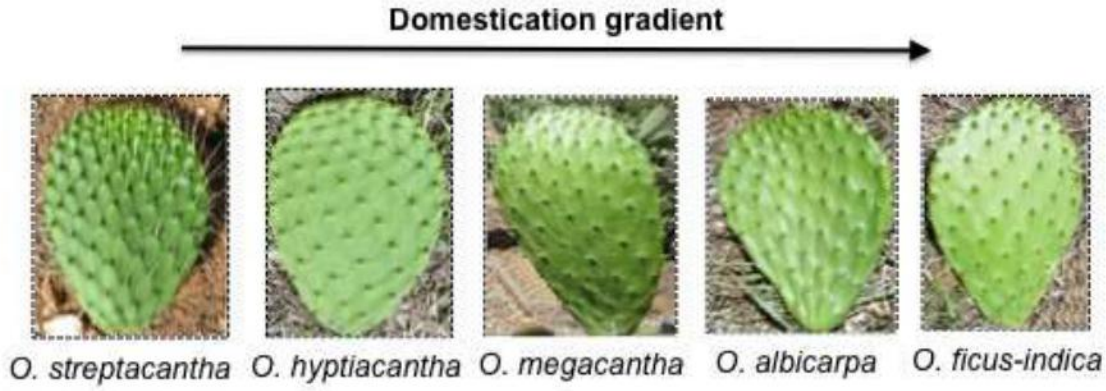


Fig. 2. Classification of the identified proteins species in *Opuntia* cladodes. The pay chart shows the distribution into their biological process in percentage according to Gen Ontology (http://www.geneontology.org/).

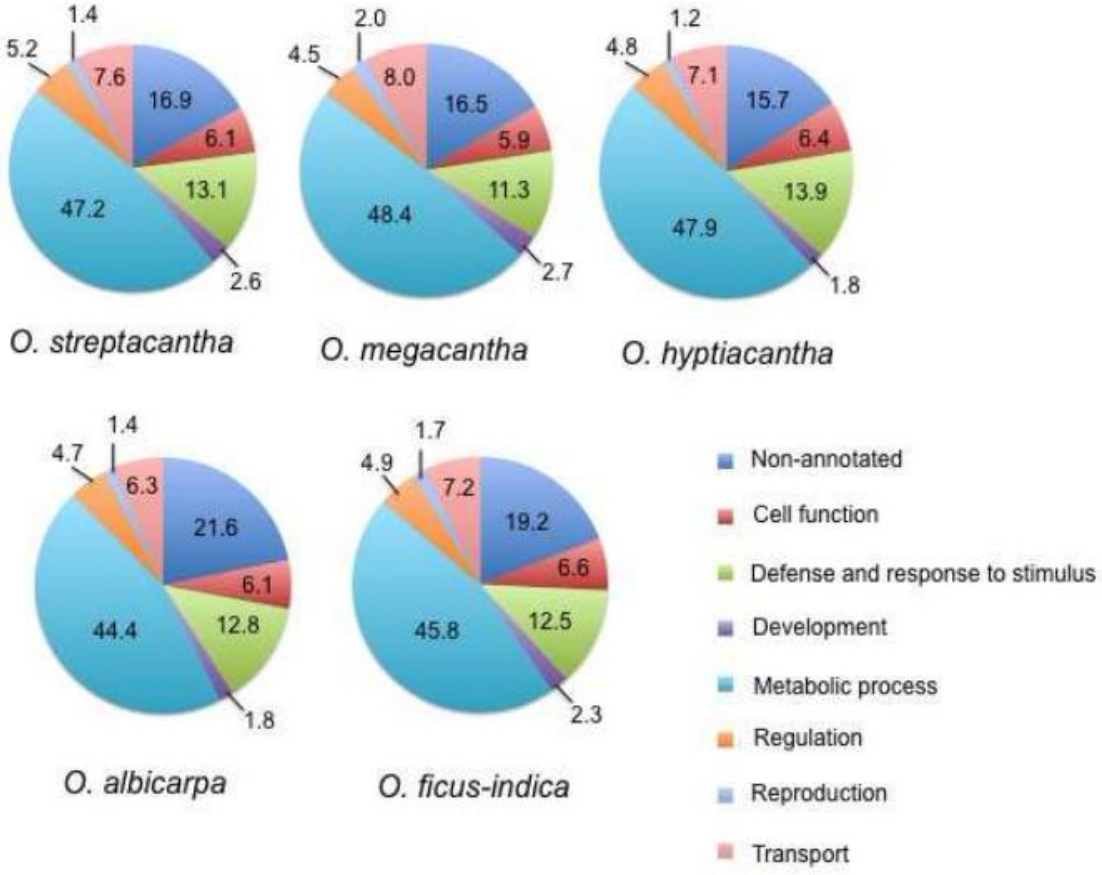


Fig. 3. Distribution of different protein species belonging to the proteasome complex identified in *Opuntia* cladodes.

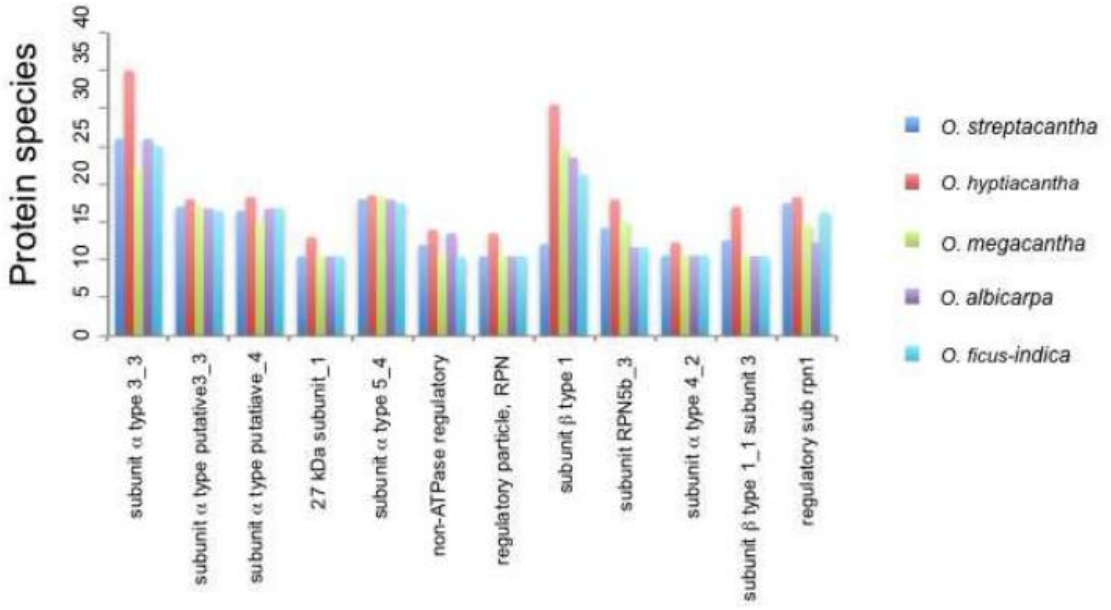


Fig. 4. Schematic representation of differentially accumulated proteins species in wild *Opuntia* cladodes when compared with the most domesticated *O. ficus-indica*. Up-accumulated proteins were detected in the wildest species *O. streptacantha* and *O. hyptiacantha* (blue letters A and B, respectively). Down-accumulated proteins were detected in domesticated *O. megacantha* and *O. albicarpa* (letters C and D, respectively). ADH=aldehyde dehydrogenase; AdoHcyase=Adenosyl-homocysteinase; FBP=fructose bisphosphate aldolase; GAPD=glyceraldehyde 3-phosphate dehydrogenase; GS=glutamine synthase; PEPC=phosphoenolpyruvate carboxylase; PK=pyruvate kinase; PPDK=pyruvate phosphate dikinase; PRK=phosphoribulokinase; RuBisCO=Ribulose-1,5-bisphosphate carboxylase/oxygenase; SHMT=S-adenosyl-L-homocystein hydrolase; SOD=superoxide dismutase; SUS=sucrose synthase; Trx=thioredoxin.

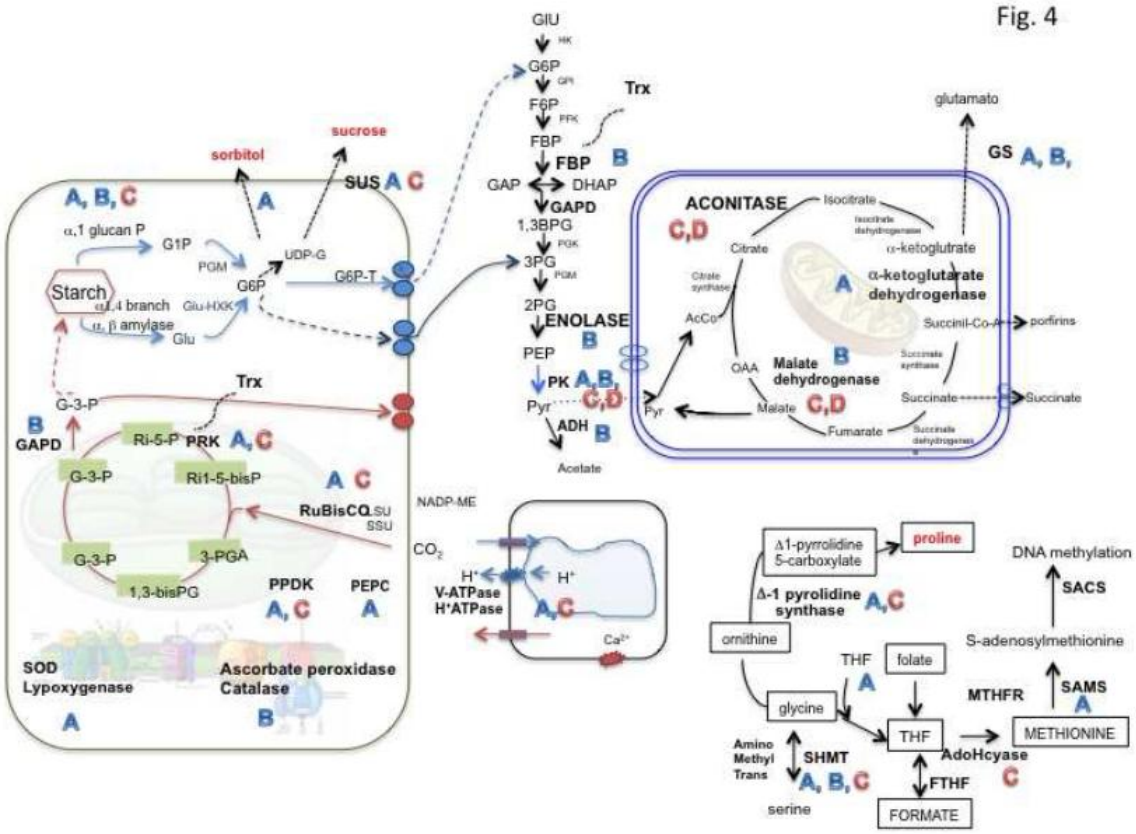


Table 1 Proximate composition and phenolic compounds of cladodes from *Opuntia* spp. with different domestication gradient.[1]

Species/ cultivar	Component					
	Protein[2]	Fat[2]	Crude fiber[2]	Total Sugars[3]	Phenolic acids[4]	Flavonoids[5]
O. streptacantha/ Tuna loca	11.0 ^{d} ±0.13	0.62 ^{c} ±0.02	6.52 ^{a} ±0.03	61.0 ^{b} ±7.1	65.1 ^{a} ±4.0	19.0 ^{a} ±1.0
O. hyptiacantha/ Memelo 1	10.5 ^{e} ±0.04	0.94 ^{a} ±0.06	6.58 ^{a} ±0.03	37.4 ^{d} ±3.4	36.1 ^{d} ±1.8	18.6 ^{a} ±0.2
O. megacantha/ Rubí reina	11.3 ^{b} ±0.03	0.63 ^{c} ±0.03	5.99 ^{b} ±0.22	103.7 ^{a} ±4.65	57.8 ^{b} ±10.4	16.5 ^{b} ±0.8
O. albicarpa/ Naranjón legítimo	11.2 ^{abc} ±0.41	0.77 ^{b} ±0.02	6.46 ^{a} ±0.02	36.9 ^{d} ±7.1	47.9 ^{c} ±7.1	16.8 ^{b} ±1.2
O. ficus-indica/ Rojo vigor	11.7 ^{ab} ±0.43	0.68 ^{c} ±0.04	5.63 ^{b} ±0.04	56.2 ^{bc} ±10.9	56.7 ^{b} ±4.4	20.4 ^{a} ±2.4

[1]values are the mean of triplicates expressed on dry weight basis ±standard deviation. [2]expressed as %. [3]expressed as mg dextrose g^{-1} of sample. [4]as μmol of gallic acid (GA) g^{-1} sample, [5]as μmol of quercetin (QE) g^{-1} sample. Different letter in the same column are statistically different (Tukey test $p \leq 0.05$).

Table 2 Total accessions containing at minimum one validated peptide.

Total numbers of proteins	*Opuntia*				
	streptacantha **(A)**	*hyptacanthia* **(B)**	*megacantha* **(C)**	*albicarpa* **(D)**	*ficus-indica* **(E)**
Identified (UNIPROT)[a]	13599	24463	12521	11428	14095
Selected	5169	5415	3737	3184	4491
Validated	1369	1506	684	590	1060
Protein groups (60% identity) in validated proteins	507	573	273	233	419
Orthologs proteins in five species[b]					
Popolus trichocarpa	679 (49.6)	704 (46.7)	305 (44.6)	253 (42.9)	511 (48.2)
Vitis vinifera	570 (41.6)	663 (44)	305 (44.6)	232 (39.3)	412 (38.9)
Arabidopsis thaliana	133 (9.7)	158 (10.5)	55 (8)	60 (10.2)	91 (8.5)
Zea mays	139 (10.2)	163 (10.8)	59 (8.6)	62 (10.5)	129 (12.2)
Glycine max	161 (11.8)	200 (13.3)	54 (7.9)	72 (12.2)	133 (12.5)

[a]*Viridiplante* database. [b]Numbers in parenthesis represents the percentage

Validation of each peptide is based on the comparison of the data between the UNIPROT database and a random database. Only the peptides with a *p*-value <0.01 were retained. Validated data were parsed and selected with the MFPAQ software.

Table 3 Abundance pattern of proteins present in wild *Opuntia* species compared with domesticated *Opuntia ficus-indica*

DESCRIPTION	TAXONOMY	ID UNIPROT	RATIO			
			A / E	B / E	C / E	D / E
Metabolism						
Enolase	*Gossypium hirsutum*	A8IMB0_GOSHI	-	644.8	-	-
Glyceraldehyde-3-phosphate dehydrogenase	*Solanum tuberosum*	F2Q9V9_SOLTU	5.77	-	-	-
Glyceraldehyde-3-phosphate dehydrogenase, predicted protein	*Populus trichocarpa*	B9H5U1_POPTR	2.28	-	0.57	-
Glucose-6-phosphate isomerase,	*Spinacia oleracea*	G6PI_SPIOL	3.28	-	0.53	-
Pyruvate kinase, predicted protein	*Populus trichocarpa*	A9P7U5_POPTR	2.91	14.57	-	-
Pyruvate kinase, putative	*Arabidopsis thaliana*	Q8LEY6_ARATH	-	-	0.35	-
Aldehyde dehydrogenase, putative	*Ricinus communis*	B9RB49_RICCO	-	7.53	-	-
Phosphoglucomutase, cytoplasmic	*Mesembryanthemum crystallinum*	PGMC_MESCR	2.99	2.86	-	-
Phosphoglycerate kinase	*Helianthus annuus*	A1Y2J9_HELAN	2.29	14.88	0.54	0.49
Fructose bisphosphate 1-phosphatase, hypothetical protein	*Vitis vinifera*	A5AYR7_VITVI	2.59	-	-	-
Fructose-bisphosphate aldolase, putative	*Ricinus communis*	B9RHD4_RICCO	1.87	-	0.62	-
Fructose-biphosphate aldolase,	*Mesembryanthemum crystallinum*	O04975_MESCR	-	128.79	-	-
Fructose-bisphosphate aldolase, class I	*Arabidopsis thaliana*	ALFC2_ARATH	-	21.13	-	-
Fructose-1,6-bisphosphatase, cytosolic	*Beta vulgaris*	F16P2_BETVU	-	-	0.63	0.64
Aconitase putative	*Capsicum chinense*	B1Q486_CAPCH	-	-	0.60	0.82
Malate dehydrogenase	*Vitis vinifera*	A5BEJ8_VITVI	-	-	0.37	
Malate dehydrogenase	*Perilla frutescens*	B0LF72_PERFR	-	-	-	0.35
Malate dehydrogenase,	*Oryza sativa Indica*	B8B9L3_ORYSI	-	6.31	-	-
2-oxoglutarate dehydrogenase E1 component, predicted protein	*Populus trichocarpa*	B9HTM3_POPTR	2.61	-	-	-
Predicted protein, 4-hydroxy-4-methyl-2-oxoglutarate aldolase	*Populus trichocarpa*	A9PFQ6_POPTR	-	27.24	-	-
Ribulose-1,5-bisphosphate carboxylase/oxygenase LSU	*Avonia papyracea*	A2VAZ6_9CARY	4.39	-	-	-
Ribulose bisphosphate carboxylase large chain	*Schlumbergera truncata*	RBL_SCHTR	-	-	0.48	-
Ribulose-phosphate 3-epimerase, chloroplastic	*Spinacia oleracea*	RPE_SPIOL	2.66	-	-	-
Phosphoenolpyruvate carboxylase (PEPC), hypothetical protein,	*Vitis vinifera*	A5AH72_VITVI	3.43	-	-	-
Pyruvate phosphate dikinase (PPDK), chloroplastic	*Flaveria brownii*	PPDK_FLABR	2.13	-	0.36	-
Chloroplast glyceraldehyde-3-phosphate dehydrogenase,	*Marchantia polymorpha*	A3QVW1_MARPO	-	15.25	-	-
UDP-glucose pyrophosphorylase	*Pinus taeda*	A6N839_PINTA	2.63	-	0.43	-
UDP-glucose pyrophosphorylase, predicted protein	*Populus trichocarpa*	B9MTE3_POPTR	-	-	0.43	-
Phosphoribulokinase, predicted protein	*Populus trichocarpa*	B9GZT5_POPTR	2.79	-	-	-
Phosphoribulokinase	*Zea mays*	B6TYM1_MAIZE	-	-	0.54	-
Sucrose synthase	*Chenopodium rubrum*	Q9LWB7_CHERU	4.21	-	-	-

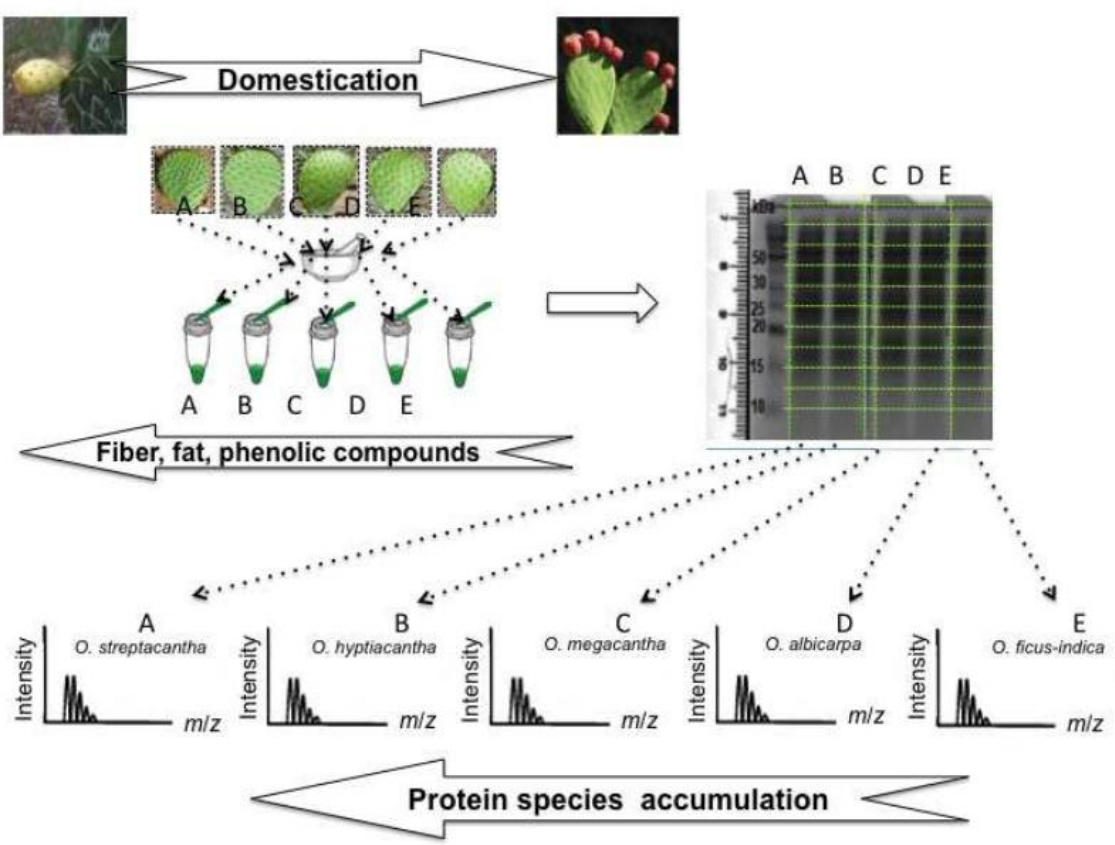
Sucrose synthase	*Dianthus caryophyllus*	D7US90_DIACA	3.73	-	0.45	-
Alpha-1,4-glucan phosphorylase L	*Cucurbita maxima*	B2DG13_CUCMA	3.49	20.41	0.47	-
DESCRIPTION	**TAXONOMY**	**ID UNIPROT**	**Ratio A / E**	**Ratio B / E**	**Ratio C / E**	**Ratio D / E**
Sorbitol related enzyme	*Solanum lycopersicum*	Q3C2L6_SOLLC	3.95	-	-	-
UDP-glucose 6-dehydrogenase, hypothetical protein	*Vitis vinifera*	A5AVX9_VITVI	2.43	-	0.32	-
Photosynthesis						
Photosystem II CP47 protein	*Trithuria submersa*	A4GGH0_9MAGN	7.11	-	-	-
Photosystem II CP43 chlorophyll apoprotein	*Smilax china*	E9L381_SMICH	6.13	26.48	-	-
Photosystem II stability/assembly factor HCF136	*Arabidopsis thaliana*	P2SAF_ARATH	3.47	-	-	-
Light-harvesting complex II protein Lhcb5	*Populus trichocarpa*	A9PGZ3_POPTR	2.69	53.16	-	-
PSI P700 apoprotein A2	*Cuscuta gronovii*	A7M900_CUSGR	5.39	-	-	-
Cytochrome b6	*Nymphaea alba*	CYB6_NYMAL	4.88	-	-	-
Cytochrome f	*Silene cryptoneura*	B0LNS1_9CARY	-	14.51	-	-
33 kDa precursor protein of the OEC	*Salicornia europaea*	B5BT06_SALEU	-	54.26	-	-
NADH-ubiquinone oxidoreductase, putative	*Ricinus communis*	B9T118_RICCO	2.07	-	-	-
One-carbon metabolism						
Serine hydroxymethyltransferase,	*Ricinus communis*	B9SMK7_RICCO	3.22	7.52	0.43	-
Serine hydroxymethyltransferase	*Zea mays*	B6T7J7_MAIZE	3.84	-	-	-
S-adenosyl-L-homocystein hydrolase	*Brassica oleracea var. alboglabra*	A7XB47_BRAOA	2.81	-	-	-
S-adenosylmethionine synthase	*Mesembryanthemum crystallinum*	METK_MESCR	2.46	-	-	-
Adenosyl-homocysteinase	*Picea sitchensis*	B8LNU2_PICSI	-	-	0.52	-
Glutamine synthetase	*Avicennia marina*	A5A7P7_AVIMR	1.57	4.56	-	-
Aminomethyltransferase, mitochondrial	*Mesembryanthemum crystallinum*	GCST_MESCR	4.31	-	-	-
Geranylgeranyl hydrogenase	*Mesembryanthemum crystallinum*	O81335_MESCR	4.05	-	-	-
Protochlorophyllide reductase, chloroplastic	*Daucus carota*	POR_DAUCA	3.22	5.27	-	-
Unnamed protein product, cysteine-synthase	*Vitis vinifera*	A5AFH5_VITVI	-	8.44	-	-
Unknown, cellulose biosynthetic process	*Picea sitchensis*	A9NKS5_PICSI	-	11.73	-	-
Formate--tetrahydrofolate ligase	*Spinacia oleracea*	FTHS_SPIOL	3.33	-	-	-
Tetrapyrrole biosynthetic processes,	*Glycine max*	C6TIB8_SOYBN	3.03	-	-	-
Delta 1-pyrroline-5-carboxylate synthetase	*Opuntia streptacantha*	B1NJ24_OPUST	2.43	-	0.67	-
Pyridoxal phosphate binding, hypothetical protein	*Vitis vinifera*	A5ACX0_VITVI	5.26	-	-	-
Flavin adenine dinucleotide binding unknown,	*Picea sitchensis*	A9NUH7_PICSI	2.94	-	-	-
Thiamine thiazole synthase, chloroplastic	*Citrus sinensis*	THI4_CITSI	-	-	0.37	-
ROS, oxide reduction related-pro						
SOD	*Citrus maxima*	Q38PL5_CITMA	1.90	-	-	-
Catalase	*Picea sitchensis*	A9NV82_PICSI	-	67.45	-	-
Chloroplast ascorbate peroxidase	*Gossypium hirsutum*	C6ZDB0_GOSHI	-	13.88	-	-
Cytosolic ascorbate peroxidase	*Arachis hypogaea*	A1Z1T1_ARAHY	-	115.31	-	-
Putative glutathione peroxidase	*Jatropha curcas*	D6BR59_9ROSI	-	7.31	-	-

Thioredoxin-dependent peroxidase	*Nelumbo nucifera*	A3FPF4_NELNU	-	66.55	-	-
Predicted protein, S-S bonds	*Populus trichocarpa*	B9H7F9_POPTR	-	19.65	-	-
Lipoxygenase, predicted	*Populus trichocarpa*	B9GMA4_POPTR	2.42	-	-	-
DESCRIPTION	**TAXONOMY**	**ID UNIPROT**	**Ratio A / E**	**Ratio B / E**	**Ratio C / E**	**Ratio D / E**
(S)-2-hydroxy-acid oxidase, putative. FMN binding	*Ricinus communis*	B9S0Y9_RICCO	2.82	-	-	-
Protein degradation						
Proteasome subunit alpha, hypothetical	*Vitis vinifera*	A5BF27_VITVI	2.75	-	-	-
Proteasome subunit alpha, hypothetical	*Vitis vinifera*	A5C3G9_VITVI	2.58	-	-	-
26S proteasome regulatory subunit rpn1,	*Ricinus communis*	B9RVT9_RICCO	2.96	-	0.50	-
26S proteasome non-ATPase regulatory subunit	*Medicago truncatula*	B7FL82_MEDTR	2.82	-	-	-
26S proteasome subunit 7, putative	*Ricinus communis*	B9SLJ3_RICCO	2.54	-	-	-
Presequence protease 2, chloroplastic/mitochondrial-like	*Vitis vinifera*	F6HQC5_VITVI	2.12	-	-	0.51
Gamma class glutathione transferase EF1Bgamma2	*Populus trichocarpa*	A9PE52_POPTR	4.28	-	-	-
Hypothetical protein, ubiquitin-dependent	*Arabidopsis lyrata subsp. lyrata*	D7MN09_ARALL	-	9.30	-	-
Heat Shock Proteins						
Chaperonin cytosolic	*Glycine max*	A7VJA5_SOYBN	2.79	-	-	-
Heat shock protein 90	*Vitis pseudoreticulata*	A8WEL7_9ROSI	2.25	-	-	-
Heat shock protein, putative	*Ricinus communis*	B9RYP6_RICCO	-	29.65	-	-
Ribosomal						
Rab11/RabA-family small GTPase	*Physcomitrella patens*	A9T2Z0_PHYPA	-	114.92	-	-
40S ribosomal S3a protein, unknown	*Populus trichocarpa*	A9PAH0_POPTR	3.64	-	-	-
60S ribosomal protein L7, putative	*Ricinus communis*	B9SDR8_RICCO	3.40	85.46	-	-
60S ribosomal protein L12, putative	*Ricinus communis*	B9SWB0_RICCO	-	88.82	-	-
Ribosomal protein S14, putative	*Wolffia arrhiza*	E7EDS9_WOLAR	3.18	6.43	-	-
40S ribosomal protein S8	*Zea mays*	B4FE90_MAIZE	3.18	10.20	-	-
Ribosomal protein L10	*Elaeis guineensis*	B3TLL9_ELAGV	2.78	-	-	-
Eukaryotic translation elongation factor, putative	*Ricinus communis*	B9RI35_RICCO	2.63	-	-	-
Splicing factor family protein, nucleic acid binding, predicted protein,	*Populus trichocarpa*	B9HM33_POPTR	2.51	-	-	-
Elongation factor predicted protein,	*Physcomitrella patens*	A9SJB4_PHYPA	2.47	140.2	0.43	-
Elongation factor 2 family, predicted protein,	*Populus trichocarpa*	B9HH11_POPTR	-	-	0.38	-
Translation initiation factor eIF-4A family, predicted protein	*Populus trichocarpa*	B9HDC5_POPTR	-	-	0.48	-
RNA-binding, unknown	*Medicago truncatula*	B7FMH6_MEDTR	2.35	-	-	-
RNA-binding protein	*Zea mays*	C0P6K6_MAIZE	-	11.52	-	-
Predicted Nascent polypeptide-associated complex	*Populus trichocarpa*	B9IDG2_POPTR	-	11.69	-	-
Unnamed protein product, Transcription DNA-templated	*Vitis vinifera*	D7TXR6_VITVI	-	5.70	-	-
Energy						
ATPase subunit 100 kDa subunit, putative vacuolar	*Mesembryanthemum crystallinum*	Q8GUB1_MESCR	5.07	-	-	-
predicted protein, phosphorylation	*Populus trichocarpa*	B9HE12_POPTR	4,6	-	-	-
ATP synthase	*Cucumis melo subsp. melo*	E5GC53_CUCME	4.33	21.16	-	-

ATPase, predicted protein	*Hordeum vulgare subsp. vulgare*	F2CRK6_HORVD	4.06	-	-	-
ATP synthase CF1 alpha subunit	*Scaevola aemula*	A9QC30_9ASTR	4.03	35.90	0.46	-
ATP-binding, hypothetical protein	*Sorghum bicolor*	C5YT23_SORBI	4.01	-	-	-
DESCRIPTION	**TAXONOMY**	**ID UNIPROT**	**Ratio A / E**	**Ratio B / E**	**Ratio C / E**	**Ratio D / E**
Hypothetical protein ATP binding	*Selaginella moellendorffii*	D8RV35_SELML	-	20.33	-	-
Unnamed protein, ATP-binding protein	*Vitis vinifera*	D7TI60_VITVI	-	16.43	-	-
ATP-binding protein	*Picea sitchensis*	A9NV22_PICSI	-	8.19	-	-
ADP glucose Pyrophosphorylase small subunit 1-like	*Malus x domestica*	D6PW21_MALDO	-	13.23	-	-
ATP synthase beta subunit	*Agrostistachys borneensis*	A0ZQ41_9ROSI	3.32	-	-	-
ATP synthase subunit beta	*Picea sitchensis*	A9NUR7_PICSI	-	-	0.49	-
ATP synthase subunit alpha, mitochondrial	*Glycine max*	ATPAM_SOYBN	3.16	-	-	-
ATPase subunit 1	*Cycas taitungensis*	E1CBG2_CYCTA	-	-	0.52	-
ATP-binding, predicted protein	*Oryza sativa Japonica Group*	B7EPR0_ORYSJ	3.05	-	-	-
Inorganic pyrophosphatase	*Chlamydomonas reinhardtii*	A8J0B0_CHLRE	6.32	20.30	-	-
Membrane trafficking and transport						
V-type proton ATPase catalytic subunit A-like	*Glycine max*	D7EYG6_SOYBN	2.51	-	0.42	-
Vacuolar H+-ATPase subunit B	*Zostera marina*	A0ZSE5_ZOSMR	2.42	-	0.51	-
Clathrin heavy chain, hypothetical	*Oryza sativa Japonica*	A3CE45_ORYSJ	4.08	-	-	-
Coatomer subunit alpha, unnamed	*Vitis vinifera*	D7TQ06_VITVI	3.83	-	-	-
Coatomer, hypothetical protein	*Vitis vinifera*	A5AHP0_VITVI	3.09	-	-	-
Chromosome region maintenance protein 1/exportin, putative	*Ricinus communis*	B9S1Z9_RICCO	2.36	-	-	-
GTP-binding protein sar1, putative	*Ricinus communis*	B9RNF8_RICCO	2.13	-	-	-
GTP-binding protein sar1, putative	*Populus trichocarpa*	B9IIE7_POPTR	-	-	0.31	-
Cytoeskeleton and Cell division						
GTP-binding, microtubules, hypothetical protein	*Oryza sativa Indica Group*	A2YG29_ORYSI	3.18	-	-	-
Actin	*Glycyrrhiza uralensis*	A8W4W6_9FABA	3.16	-	-	-
Actin-1	*Pisum sativum*	ACT1_PEA	-	-	0.58	-
Alpha-tubulin	*Medicago truncatula*	A2Q5W0_MEDTR	3.15	-	-	-
Cell division protease ftsh-like protein precursor	*Populus trichocarpa*	B9GQ31_POPTR	3.01	-	-	-
Cell division cycle protein 48, predicted protein	*Populus trichocarpa*	B9IFP5_POPTR	2.76	-	-	-

A=*Opuntia streptacantha;* B=*Opuntia hyptiacantha;* C=*Opuntia megacantha;* D=*Opuntia albicarpa;* E=*Opuntia ficus-indica.* Hyphen (-) indicates that no changes were observed.

Graphical abstract



Highlights

- Most of the protein changes were observed in the wildest *O. streptacantha*, however *O. megacantha* presented higher levels of accumulation in glycolysis key enzymes.
- Most of the changes in 1C-metabolism enzymes were detected in *O. streptacantha* followed by *O. hyptiacantha*.
- Superoxide dismutase and lypoxygenase were overrepresented in *O. streptacantha* but catalase and ascorbate peroxidase were up-accumulated in *O. hyptiacantha*.
- Regulatory 14-3-3 proteins were detected in all *Opuntia* species indicating its importance for plant growing and development.
- Most of the reported key targets for increase plant resistance to abiotic stresses were well represented at protein level in wild *Opuntia* species.