Molecular and Structural Comparisons of C3 Cotyledons with C4 Leaves in Species of Salsoloideae (Chenopodiaceae)

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Research Article

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Abstract

C_4 plants had evolved from C_3 as a response to decreasing atmospheric CO_2 levels and conditions promoting photorespiration. C_4 plants evolved from C_3 ancestors at least in more than 60 independent lineages of angiosperms for suppressing of photorespiration. *Salsola*, *Petrosimonia* and *Cyathobasis* genera of Salsoloideae subfamily contain some species with C_3 cotyledons followed by C_4 leaves. The aim of this study was to compare the biochemical and structural differences between C_3 cotyledons and C_4 leaves in these genera. The results showed that there were dorsiventral C_3 cotyledons in *Salsola grandis* and *Cyathobasis fruticulosa*, while salsoloid type C_4 Kranz anatomy was present in mature leaves. *Petrosimonia nigdeensis* had isobilateral C_3 cotyledons and a salsoloid type C_4 leaves. Phosphoenolpyruvate carboxylase (PEPC) and pyruvate orthophosphate dikinase (PPDK) enzymes were absent or sparse in cotyledons of these species, whereas they were abundant in their C_4 leaves. Glycolate oxidase (GOX) and glycine decarboxylase- H subunit (GDC-H) were generally higher in cotyledons than leaves. Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme content was lower in C_4 leaves compared to C_3 cotyledons. Transcript levels of these enzymes were generally consistent with their protein content except for GOX in *S. grandis* and *S. tragus*, and glycine decarboxylase complex (GDC) in *S. tragus*. As a result, we demonstrate that not only the protein amounts and transcript levels of the enzymes required in C_4 pathway increased but also the levels of C_3 and photorespiratory enzymes were lowered during transition from C_3 cotyledons into C_4 leaves. These results are important in terms of shedding light on understanding of evolutionary transition from C_3 to C_4 biochemical pathway in a single plant and contributing to C_4 engineering.

Introduction

Photosynthesis is a series of biochemical reactions, where photosynthetic organisms assimilate atmospheric CO_2 and convert it to organic substances using ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) enzyme. This enzyme is inefficient due to its second nature of oxygenation reaction as a result of unavoidable phenomenon of its structure, in which the two monomeric catalytic subunits interact to form the active site required for both carboxylation of CO_2 and the fixation of molecular O_2 (Leegood, 2013). The oxygenation reaction, commonly referred as photorespiration, yields 3-phosphoglyceric acid (3-PGA) and 2-phosphoglycollate (2-PG). PGA is recycled back to ribulose-1,5-bisphosphate (RuBP) by Calvin-Benson cycle. PG has no use for C_3 plants and excessive amount is detrimental (Gowik and Westhoff, 2011; Sage et al., 2012). Under non-stressed conditions in C_3 plants photorespiration can cause up to 25% photosynthetic inhibition at temperatures below 30°C (Sage et al., 2012). Under stressful conditions such as drought and high temperatures, however, photorespiration can result in a loss of up to 50% of the carbon fixed in C_3 plants (Ogren, 1984). This phenomenon was not a problem earlier when C_3 plants evolved at the time when atmospheric CO_2 concentrations were very high and O_2 concentrations very low or absent (Sage, 1999; Sage, 2004). However, starting from recent geological times some 35 million years ago and onward, atmospheric conditions, mainly lowering of

atmospheric CO_2 and deterioration of the climate with frequent drought events favoured photorespiration significantly in plants (Sage, 2004). Therefore, C_4 plants had evolved from C_3 ancestors as a response to decreasing atmospheric CO_2 levels and conditions promoting photorespiration.

C_4 plants evolved from C_3 ancestors at least in more than 60 independent lineages of angiosperms for suppressing of photorespiration during the late miocene (Sage 2016). Nine of all C_4 lineages are included in Chenopodiaceae family. Salsoloideae subfamily in Chenopodiaceae has five clades comprised in two lineages of *Caroxyloneae* and *Salsoleae s.s* (Akhani et al. 2007; Sage 2016; Schussler et al. 2017). Salsoloideae has a great photosynthetic diversity, which includes C_3, C_4 and C_3-C_4 intermediate species as well as species with a phenomenon known as "C_3 cotyledons and C_4 leaves". This phenomenon was firstly discovered in *Haloxylon aphyllum* and *H. persicum* species (Pyankov et al., 1999). These species possess isobilateral C_3 anatomy in their cotyledons and salsoloid type C_4 Kranz anatomy in their leaves. After this discovery, the same phenomenon was observed in more species from various genera of Salsoloideae (Pyankov et al. 2001; Pyankov et al. 2000).

In other two studies, C_3 cotyledons and C_4 leaves of the same individuals of *Salsola soda* and *Haloxylon ammodendron* species were studied for their gene regulations in photosynthesis (Lauterbach et al. 2016; Li et al. 2015). In these species, the increasing expression levels of genes coding the enzymes specific to C_4 photosynthesis and decreasing levels of the enzymes specific to C_3 in transition from C_3 cotyledons to C_4 leaves were determined. In *S. soda* studied for PEPC levels in transition from C_3 to C_4, while no PEPC was detected in cotyledons, it was highly generated in mature C_4 leaves (Lauterbach et al. 2016). Apart from PEPC, there has been no study comparing translation levels of photosynthetic and photorespiratory enzymes functioning in C_3 cotyledons and C_4 leaves in Salsoloideae subfamily.

In this study, we aimed to determine structural and biochemical differences between C_3 cotyledons and C_4 leaves of *Salsola grandis*, *Cyathobasis fruticulosa*, *Petrosimonia nigdeensis* as well as C_4 cotyledons and C_4 leaves of *S. tragus*. C_3 cotyledons followed by C_4 leaves phenomenon in some species, besides transcript levels, were discovered for the first time in this study. We determined the protein levels of PPDK, PEPC, GDC, GOX and Rubisco enzymes through Western Blotting, while the transcription levels of the genes encoding these proteins were detected by qRT-PCR. Results showed that, when the plants were transited from C_3 to C_4, not only did the amounts of enzymes related with C_4 photosynthesis increased, but also the protein levels of enzymes functioning in photorespiration and C_3 cycle decreased during this transition. In general, there was a consistency between mRNA levels of the genes coding photosynthetic and photorespiratory enzymes and the proteins produced from these genes.

Materials And Methods

Seed Collection, Growth Conditions and Sampling

Seeds of *Salsola grandis* Freitag, Vural & N. Adıgüzel and *Cyathobasis fruticulosa* (Bunge) Aellen were collected from Nallıhan, Ankara, Turkey, while *Petrosimonia nigdeensis* Aellen *and S. tragus* L. were obtained from Aksaray and Mersin, Turkey, respectively. All samples were gathered in November 2017. Before sown, all seeds were soaked in tap water for five hours to break dormancy. Seeds were then sowed in vials containing 50% sand and 50% peat mixture. Plants were grown in a growth chamber set at 24 °C/22 °C (day/night temperature), 40% relative humidity under 100 µmol quanta m^{-2} s^{-1} PPFD (photosynthetic photon flux density) at the Faculty of Agriculture, Kahramanmaras Sutcu Imam University. After germination, ten-day-old mature cotyledons as well as the first, the second and approximately 30-day-old mature leaf pairs of five to eight individuals of *S. grandis* and *S. tragus* were pooled for RNA and protein isolations as described by Lauterbach et al. (2016). In *C. fruticulosa* and *P. nigdeensis*, mature cotyledons and mature leaves were used for the isolation of RNA and proteins. For all species, mature leaves were preferred for the isolation of DNA, while mature cotyledons and leaves were used for the anatomical investigations.

DNA Isolation and Internal Transcribed Spacer (ITS) **Phylogeny**

DNAs were isolated from 100 mg of fresh leaf material ground in liquid nitrogen using DNA isolation kit (ref: 740770.50, Macherey-Nagel, Germany). Quantity and purity of isolated DNAs were verified by using spectrophotometry “Thermo Scientific™ NanoDrop 2000” and their integrities were determined by running 1 µg of each DNA sample through 1.5% agarose gel via electrophoresis. Polymerase chain reactions (PCR) were set up with these DNA samples by using *ITS1*, Internal transcribed spacer 1, (O'Kane et al. 1996) and *ITS4*, Internal transcribed spacer 4, (White et al. 1990) primers. The programme of PCR was set as 1 min. at 95 ^{o}C, 35 cycles of 1 min. at 94 ^{o}C, 1 min. at 57 ^{o}C, 2 min. at 72 ^{o}C and finally 5 min. at 72 ^{o}C. 100 ng of PCR products were run in 1.5% agarose gel and the gels were photographed by an imaging system. PCR products were sent to sequencing facility for sequencing nuclear ribosomal DNA *ITS* regions. Results of sequencing were blasted with *ITS* sequences of related species at NCBI (National Center for Biotechnology Information, USA) as described by Morgulis et al. (2008) and a phylogenetic tree was created via “Seaview6” using the method of Gouy et al. (2010).

Light Microscopy

Anatomical practices were developed with some modifications to the protocol described by Kulahoglu et al. (2014). Briefly, cotyledon and leaf samples were cut into 1X2 mm size and incubated in fixative solution of 2% paraformaldehyde, 2% glutaraldehyde for 24 hours at 4 ^{o}C. Samples were then washed with phosphate buffer saline (PBS) solution once and with dH_2O twice. They were dehydrated with acetone series from 30–96%. After that, they were treated with ascending resin (mixture of araldite M, Sigma, ref: 10951, DDSA, Merck, ref: 45346 and araldite M accelerator, Sigma, ref: 10952; 20 ml, 22 ml and 1.1 ml, respectively) series (25%, 50%, 75%, 100%) and finally cured in mold at 65 ^{o}C for 72 hours. Cross sections in 5µm thickness from polymerized resins were cut using a rotary microtome, then, toluidine-blue dye was dropped on cross sections for 20–30 seconds. After rinsing the samples with

water, permanent slides were made by dropping some “DPX, Slide Mounting Medium” (Sigma, ref: 06522). Cross sections were examined and photographed using a light microscope (Nikon, ECLIPSE 80i).

RNA Isolation and qRT-PCR

Total RNA of three biological replicates were isolated from 100 mg of freshly ground cotyledons and leaves in liquid nitrogen by using GF-1 Total RNA Extraction Kit (Vivantis, ref: GF-TR-050) according to the manufacturer’s instructions. Quantity and purity of isolated RNAs were verified by using spectrophotometry “Thermo Scientific™ NanoDrop 2000” and their integrities were determined by running through 1.5% agarose gel. cDNAs were synthesized from proper RNA by using 2-Step RT-PCR Kit (ref: RTPL12; Vivantis, Malaysia) according to the manufacturer’s instructions. qRT-PCR for these cDNAs were performed using an oligo (dT) primer and Bright Green 2X qPCR MasterMix-No Dye (ABM, Canada) in 25 µl reaction volume included 5 µl cDNA. PPDK, Rubisco, glycine decarboxylase-P subunit (GDC-P), GOX and EF-1 primer pairs, in accordance with previous work of Lara et al. (2008). qRT-PCR cycle parameters were set up as 95 ^{o}C for 10 min; 95 ^{o}C for 15 s, 60 ^{o}C for 1 min at 45 cycle. Melting curves were determined from 45 ^{o}C to 95 ^{o}C. Each cDNA sample was run in duplicates with targeted primers along with duplicates of the EF-1 primer containing tubes. Relative transcript abundance of genes were normalized to the abundance of reference gene *EF-1* and calculated by comparative $2^{-\Delta\Delta Ct}$ method as described by Schmittgen and Livak (2008). Finally, as Lara et al. (2008) and Lauterbach et al. (2016) defined, mature cotyledons were assumed 1.00 as control group and first, second and old mature leaf pairs were statistically compared to mature cotyledons by one-way-ANOVA (for more than two tissues) or t-test (two tissues) using “Graphpad Prism” (CA, USA) statistical programme.

Protein Extraction and Western Blot

500 mg of fresh material was ground in liquid nitrogen for each sample. Total protein of cotyledons and leaves were isolated by adding 500 µl protein extraction buffer [100 mM Tris-HCl (pH 7.5), 10 mM (w/v) dithiothreitol (DTT), 5 mM (w/v) ethylenediaminetetraacetic acid (EDTA), 2% β-mercaptoethanol, 0.5 M sodium dodecyl sulfate (SDS), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF)] to each sample. This mixture was vortexed and centrifuged at 4 ^{o}C, 15000 rpm for 15 minutes. Supernatants were collected as total proteins and kept at -20 ^{o}C until use. Concentrations of proteins were measured by Bradford assay. For this purpose, 2.5 µl protein samples were added to 50 µl Bradford solution (Alfa Aesar, J61522) and 5 minutes later measured at 595 nm. Measurements without proteins were used as blanks. For western blot, protein samples and ladder (Abcam, USA) were separated through a SDS gel by their molecular weights at 100 V and were transferred to a PVDF membrane (Thermo Scientific, ref: 88520, USA) at 350 mA. Membranes were treated with PEPC (Agrisera-AS09458, Sweden), PPDK (Agrisera-AS132647, Sweden), GOX (Agrisera-AS142772, Sweden), GDC-H (Agrisera-AS05074, Sweden) and Rubisco (Agrisera-AS07259, Sweden) primer antibodies overnight at 4 ºC, following mouse anti-rabbit IgG-HRP (Santa Cruz Biotechnology, SC-2357, USA) secondary antibody for two hours at room temperature. Actin (Agrisera-AS132640, Sweden) antibody was used as the loading control. After that, membranes were treated with ECL substrate (Biorad, ref: 1705061, USA) to obtain chemiluminescence and the bands were visualized by UVP Imaging System (CA, USA). The intensity of the bands obtained for

each protein was measured by Image J software (Public domain). The values measured for PEPC, PPDK, GOX, GDC-H and Rubisco bands were normalized to actin value of the same lane for adequate comparison.

Results

Verification of Species

S. grandis, *S. tragus*, *C. fruticulosa* and *P. nigdeensis* plants were grown in a climate chamber and DNAs from their leaves were isolated. After PCR amplifications of their *ITS* regions, the products were sequenced. Sequence results were compared with the sequences from the NCBI. Percent identity of *S. grandis*, *S. tragus*, *C. fruticulosa* and *P. nigdeensis* were determined as 100%, 98%, 98%, 99%, respectively. A phylogenetic tree was created using sequences and the comparison of sequences verified the species identity used in this study (Fig. 1). Results from the phylogenetic tree showed that our sequence of *S. grandis* was closer to *S. grandis* from the NCBI rather than to closely related species, *S. soda*. Furthermore, we were able to successfully distinguish *P. nigdeensis*, *C. fruticulosa and S. tragus* from *P. brachiata*, *Haloxylon ammodendron* and *S. kali*, respectively (Fig. 1).

Anatomy of Cotyledons and Leaves

Cross sections were taken from cotyledons and leaves of *S. grandis*, *S. tragus*, *C. fruticulosa* and *P. nigdeensis* after embedding in resin. Anatomical features clearly indicated that there was a dorsiventral C_3 anatomy in cotyledons and salsoloid type C_4 Kranz anatomy in leaves of *S. grandis* (Fig. 2A-B) and *C. fruticulosa* (Fig. 2E-F). Cotyledons of *P. nigdeensis* had isobilateral C_3 anatomy and leaves had salsoloid type C_4 Kranz anatomy (Fig. 2G-H). Large intercellular spaces and a lack of Kranz anatomy of bundle sheath cells were obvious in cotyledons of *S. grandis*, *P. nigdeensis* and *C. fruticulosa*. In C_4 leaves of all the species studied, bundle sheath cells were in close contact with outer mesophyll cells and completely surrounding the inner water storage tissue of the leaf. This was the first anatomical evidence for the fact that *S. grandis*, *P. nigdeensis* and *C. fruticulosa* have C_3 photosynthetic pathway in cotyledons and C_4 pathway in leaves. *S. tragus* had salsoloid type C_4 Kranz anatomy both in cotyledons and leaves (Fig. 2C-D). It was observed that chloroplasts of bundle sheath cells were localized in close proximity to peripheral vascular bundles in C_4 organs of all of the species (Fig. 2B-D, F, H). Although water storage (WS) tissue was present in C_3 cotyledons and C_4 leaves, cells of this tissue were highly enlarged in C_4 leaves. Furthermore, a hypodermis layer was observed in only C_4 leaves of *C. fruticulosa* among all species (Fig. 2F).

Comparison of mRNA Levels of C_3, C_4 and Photorespiratory Proteins Among Photosynthetic Organs

RNAs were isolated from cotyledons and developmental stages of leaves of *S. grandis* (C_3 cotyledons, C_4 leaves) and *S. tragus* (C_4 cotyledons, C_4 leaves), and only C_3 cotyledons and C_4 mature leaves of *P. nigdeensis* and *C. fruticulosa*. qRT-PCRs were set up with cDNAs generated after RNA isolation and the RNA levels of PPDK, Rubisco, GDC-P and GOX enzymes between cotyledons and leaves were compared.

mRNA levels of PPDK enzyme in first leaf pairs, second leaf pairs and mature leaves of *S. grandis* were measured as 5.71, 5.07 and 4.39 fold higher, respectively, than mRNA level of C_3 cotyledons (Fig. 3A). Rubisco mRNA levels, on the other hand, were calculated to be 18.86, 23.25 and 100 fold lower, respectively, in first leaf pairs, second leaf pairs and mature leaves of *S. grandis* compared with the levels in cotyledons (Fig. 3B). During the transition from C_3 cotyledons to C_4 in three developmental stages of leaves of *S. grandis*, GDC-P mRNA levels were found 2.32, 2.0 and 1.88 fold lower, respectively, than the mature cotyledons of *S. grandis* (Fig. 3C). GOX mRNA levels in the first leaf pairs, second leaf pairs and mature leaves of *S. grandis* were 3.22, 3.22 and 2.94 fold lower, respectively, compared with the levels in cotyledons (Fig. 3D).

PPDK mRNA level in C_4 leaves of *P. nigdeensis* was 23.59 fold higher than C_3 cotyledons (Fig. 3M). Rubisco mRNA in leaves was half of the cotyledons (Fig. 3N). On the other hand, GDC-P mRNA level in C_4 leaves was 2.27 times lower (Fig. 3O) and GOX mRNA level was 1.81 fold lower than in C_3 cotyledons (Fig. 3P). mRNA level of PPDK increased 5.02 fold in C_4 leaves of *C. fruticulosa* compared with C_3 cotyledons (Fig. 3I). Although Rubisco mRNA was approximately 3 times higher in C_4 leaves relative to C_3 cotyledons of *C. fruticulosa*, the difference was not statistically significant (Fig. 3J). However, during the transition from C_3 cotyledons to C_4 leaves, mRNA levels of GDC-P and GOX decreased significantly in *C. fruticulosa* (Fig. 3K-L). On the other hand, while the levels of PPDK and GOX mRNAs of leaf developmental stages of *S. tragus* were not significantly different from the cotyledons (Fig. 3E and H), mRNA levels of Rubisco and GDC-P were significantly lower in first and second leaf pairs and mature leaves (Fig. 3F and G).

Protein Quantities of C_3, C_4 and Photorespiratory Enzymes in Cotyledons and Leaves

PEPC enzyme was completely absent in C_3 cotyledons of *S. grandis* (Fig. 4A). However, its levels gradually increased from the first leaf pairs to mature leaves (Fig. 4A, B). For example, PEPC was 18% and 44% higher in the second and mature leaf pairs compared to the first leaf pairs, respectively (Fig. 4B). A similar trend was observed for PPDK enzyme (Fig. 4A, B). The first leaf pairs, second leaf pairs and mature leaves had 1.97, 2.80 and 2.74 fold higher PPDK enzyme, respectively, than mature cotyledons (Fig. 4B). GOX enzyme was significantly higher in the first leaf pairs, but lower in the second and mature leaves compared to the cotyledons of *S. grandis* (Fig. 4A-B). Another major photorespiratory enzyme, GDC-H, and Rubisco levels were significantly lower in the C_4 first and second leaf pairs and mature leaves compared to the C_3 cotyledons (Fig. 4A-B).

While no PEPC enzyme was detected in both *C. fruticulosa* and *P. nigdeensis* C_3 cotyledons (Fig. 4E and G), PEPC in mature C_4 leaf pairs of these species was highly expressed. Similarly to PEPC, there was no PPDK enzyme in cotyledons of *P. nigdeensis* while it was highly observable at mature leaf pairs (Fig. 4G). On the other hand, PPDK enzyme in mature leaf pairs of *C. fruticulosa* was 1.73 fold higher than the levels in cotyledons (Fig. 4F). Moreover, GOX enzyme levels were found to be 2.94 and 2.77 fold lower at mature leaf pairs of *C. fruticulosa* and *P. nigdeensis*, respectively, compared to their C_3 cotyledons (Fig. 4F and H). Similar trend was observed for GDC-H. GDC-H enzyme levels were significantly decreased from C_3 cotyledon to C_4 mature leaves in *C. fruticulosa* and *P. nigdeensis* (Fig. 4E, F, G and H). In these species, Rubisco enzyme levels were also significantly less in C_4 mature leaf pairs compared to C_3 cotyledons (Fig. 4F and H).

S. tragus was proven to have the same C_4 photosynthetic pathway in both photosynthetic organs, cotyledons and mature leaves. Although some general reductions were observed for most of the enzymes studied, no significant differences in quantities of PEPC, PPDK, GDC-H and Rubisco proteins were observed between the C_4 cotyledons and developmental leaf stages of *S. tragus* (Fig. 4C and D). GOX enzyme level in old leaf pairs of *S. tragus*, on the other hand, was determined to decrease dramatically compared to cotyledons, first and second leaf pairs (Fig. 4C and D).

Discussion

In this study, it was hypothesized that if a plant switches its photosynthetic pathway from C_3 to C_4 during maturation then C_4 enzymes and their transcript levels should increase from C_3 cotyledons to C_4 leaves, while photorespiratory and C_3-specific enzymes and their transcript levels are expected to decrease at the same time. In an effort to investigate this hypothesis, two different photosynthetic organs; cotyledons and three developmental stages of leaves of *S. grandis* and *S. tragus* and only mature leaves of *P. nigdeensis* and *C. fruticulosa* were compared for their structural and biochemical differences. For this comparison, leaf anatomical cross sections, mRNA levels of genes coding C_3, C_4 and photorespiratory enzymes and quantities of the proteins translated from these mRNAs were investigated.

Determination of Photosynthetic Pathways by Leaf Anatomy

After the discovery of "C_3 cotyledons followed by C_4 leaves" phenomenon in *Haloxylon* by Pyankov et al. (1999), especially Salsoloideae subfamily gained interest in a search for more species or genera with this transition. *Petrosimonia*, *Halimocnemis* and *Halocharis* were some of them in *Caroxyloneae* lineage and *Salsola* was another genus from *Salsoleae s.s.* lineage (Akhani et al. 2009). In our study, for the first time, we were able to determine *Cyathobasis* as a new genus having this phenomenon in *Salsoleae s.s.* lineage. Besides, *P. nigdeensis* and *C. fruticulosa* were shown to carry this phenomenon as well. Previously, Freitag et al. (1999) demonstrated that *S. grandis* exhibited C_3 cotyledons and C_4 leaves by

using hand drawn anatomy pictures. In the present study, this observation was confirmed by anatomical features captured from light microscopy (Fig. 2A-B). All C_4 leaves from anatomical observations exhibited typical Kranz anatomy with highly packed bundle sheath cells inner to and in close contact with parenchymal cells and surrounding large water storage cells. All C_3 cotyledons had one or two layers of palisade mesophyll cells with large intercellular spaces and relatively smaller inner water storage cells (Fig. 2).

Gene Expression and Protein Levels of Photosynthetic Enzymes

It is well known that C_4 species have evolved from ancestral C_3 species and genes recruited for C_4 function in C_4 plants exist in C_3 species having unrelated functions (Li et al. 2017). There are many reports through which C_4 species were shown to have more C_4 photosynthetic key enzymes compared to C_3 enzymes than C_3 species in the same genus such as *Salsola*, *Cleome* and *Suaeda* (Koteyeva et al. 2011; Smith et al. 2009; Voznesenskaya et al. 2013). As expected, in our study PPDK mRNA levels were measured higher in C_4 mature leaves than C_3 cotyledons in *S. grandis*, *C. fruticulosa* and *P. nigdeensis*. Moreover, relative quantities of PPDK were detected to be significantly higher in C_4 mature leaves of these species compared to C_3 cotyledons. This observation indicated that mRNA and enzyme levels of PPDK were consistent with each other in these species. In addition, another C_4 cycle enzyme PEPC, which has a key enzymatic role of initial carbon dioxide (CO_2) fixation reaction in C_4 pathway (Reeves et al. 2017), was absent in C_3 cotyledons but highly expressed in C_4 leaves of *S. grandis*, *C. fruticulosa* and *P. nigdeensis*. We were also able to demonstrate that mRNA levels of PPDK enzyme were similar in C_4 cotyledons and C_4 leaves of *S. tragus*. Consistent with mRNA levels, relative amounts of PPDK enzyme were detected as similar in C_4 cotyledons and C_4 leaves of *S. tragus*, as well.

As a consequence of its kinetic properties, Rubisco is required 60–80% in less amount in C_4 than C_3 species of similar life forms (Sage 2002). In this study, we observed that mRNA levels and protein quantities of Rubisco decreased from C_3 cotyledons to C_4 leaves in *S. grandis* and *P. nigdeensis* with the exception of *C. fruticulosa*. While the difference in Rubisco mRNA levels of *C. fruticulosa* between leaves and cotyledons was not statistically significant, a decrease in the Rubisco protein level was apparent. In agreement with our observations, Covshoff et al. (2016) revealed that the difference of Rubisco mRNA levels between C_4 *Echinochloa glabrescens* and C_3 rice was similar to the difference between C_3 cotyledons and C_4 leaves of *S. grandis* and *P. nigdeensis*. Smith et al. (2009) also reported that some C_3 *Salsola* species have more Rubisco content than C_4 *Salsola* species. Interestingly, a decrease in Rubisco mRNA and protein levels was also observed in C_4 leaves of *S. tragus* compared to its C_4 cotyledons, but the decrease especially in protein level was not as significant relative to the decreased level in *S. grandis* (Fig. 4).

Photorespiratory enzymes were shown to be more abundant in C_3 and C_3-C_4 intermediate species compared to C_4 species, due to the lack or the suppression of photorespiratory cycle in C_4 species (Mallmann et al., 2014). In this study, we observed that GOX enzyme, which functions in photorespiration cycle, significantly decreased from C_3 cotyledons to C_4 leaves in *C. fruticulosa* and *P. nigdeensis*. Additionally, another important photorespiratory enzyme, GDC-H enzyme, was measured significantly lower in C_4 leaves of *S. grandis*, *C. fruticulosa* and *P. nigdeensis* compared to their C_3 cotyledons. GOX mRNA levels in C_4 leaves of *S. grandis*, *C. fruticulosa* and *P. nigdeensis* were seen to be lower than their C_3 cotyledons. However, GOX transcript abundances were the same in C_4 cotyledons and in C_4 first, second and old leaf pairs of *S. tragus* (Fig. 3). Besides, it was determined that mRNA levels of GOX enzyme were consistent with the protein levels in those species with the exception of first leaf pairs in *S. grandis* where GOX was slightly higher. On the other hand, mRNA levels of GDC-P enzyme were measured lower in C_4 leaves of *S. grandis*, *C. fruticulosa* and *P. nigdeensis* compared to their C_3 cotyledons. Although GDC-P and GDC-H were two different enzymes in GDC enzyme complex of photorespiration, it was shown that there was a consistency between mRNA levels of GDC-P and enzyme amounts of GDC-H in these species. These results may support earlier reports by Palmieri et al. (2010) and Timm et al. (2015) claiming that GDC enzyme complex is more essential in C_3 than C_4 species.

In our study, we observed that the first leaf pair of *S. grandis* had more GOX enzyme than its cotyledons., GOX enzyme levels of the second and the mature leaves were found to decrease gradually below the levels of cotyledons. On the other hand, GOX mRNA levels in all C_4 leaf pairs of *S. grandis* were measured as significantly lower than C_3 cotyledons. All of these results showed that GOX mRNA and enzyme levels in *S. grandis* were not consistent. A possible explanation for this inconsistency between mRNA and protein levels may be found in a report by Fortelny et al. (2017). They showed that predictions of protein quantities were not accurately supported by mRNA levels for most genes in human genome. In addition, Somerville and Ogren (1982) and Levey et al. (2019) also indicated that GOX could have more essential functions than expected in plants. For example, cellular H_2O_2 homeostasis and some other metabolic cycles such as glyoxylate cycle could result in variation of GOX levels of first leaf pairs in this study.

An unexpected observation of our study was the fact that C_4 mature leaf pairs of *S. tragus* had lower levels of GOX enzyme compared to C_4 cotyledons. This tendency was not seen at GOX mRNA levels in this species. In *S. tragus*, while mRNA levels of GDC-P enzyme decreased during the transition from C_4 cotyledon to C_4 leaves, similar to what was observed in *S. grandis*, GDC-H enzyme amount of *S. tragus* remained virtually constant in both photosynthetic parts.

In previous studies, it was reported that Calvin-Benson cycle (CBC) proteins, their mRNA levels and C_4 key proteins increased during leaf development in C_4 *Bienertia sinuspersici* (Lara et al. 2008; Offermann et al. 2015). Similarly, different leaf stages of C_4 *Gynandropsis gynandra* had an increase in transcript levels of CBC, photosystem I (PSI) and photosystem II (PSII) enzymes such as AspAT (Kulahoglu et al. 2014). Moreover, Li et al. (2015) clearly demonstrated that there was an increase in the transcript levels of C_4

genes and a decrease in photorespiratory and C_3 genes during transition from C_3 cotyledons to C_4 leaves in *Haloxylon ammodendron*. Lauterbach et al. (2016) also showed that *S. soda* had gradual photosynthetic development from C_3 cotyledons to C_4 leaves with an increase in the transcript levels of C_4 genes and a decrease in the transcript levels of C_3 and photorespiratory genes. Our current study supports the results by Li et. al. (2015) and Lauterbach et. al. (2016) at the gene expression level.

The differences measured between cotyledons and leaves of *S. grandis*, *C. fruticulosa* and *P. nigdeensis* for PEPC enzyme were similar to what was observed for *S. soda* by Lauterbach et. al. (2016). In addition, our western blot results showed that not only was an increase in C_4 enzymes, but also a decrease in C_3 and photorespiratory enzymes during the transition. Finally, as investigated before in C_4 *Bienertia sinuspersici*, C_4 *Zea mays*, C_3 *Picum sativum* and other species at proteomics level (Offermann et al. 2015; Majeran et al. 2010; Brautigam et al. 2008), whole protein contents of *S. grandis*, *C. fruticulosa* and *P. nigdeensis* are needed to be detected in future studies to discover more C_3-, C_4- and photorespiratory pathway-specific enzymes in Salsoloideae subfamily.

Conclusions

In this study, we compared anatomical structures and mRNA and protein levels of cotyledons and leaves of *Salsola grandis*, *S. tragus*, *Cyathobasis fruticulosa* and *Petrosimonia nigdeensis* species. Anatomical investigations clearly showed, for the first time, that *S. grandis*, *C. fruticulosa* and *P. nigdeensis* have C_3 pathway in their cotyledons and C_4 pathway in their mature leaves. In these species, PPDK mRNA levels, which is one of the markers of the C_4 pathway, was higher in leaves than in cotyledons, and GOX and GDC-P mRNA levels of photorespiratory enzymes were higher in cotyledons than in leaves. It was observed that the Rubisco mRNA levels were lower in leaves of *S. grandis* and *P. nigdeensis* than in their cotyledons, although there was no significant change in *Cyathobasis fruticulosa*. On the other hand, there was no significant change in mRNA levels of PPDK and GOX enzymes of *S. tragus*, which were determined to have a C_4 pathway in cotyledons and leaves, while a decrease was detected in Rubisco and GDC-P mRNA levels in leaves compared to cotyledons. In *S. grandis*, *C. fruticulosa* and *P. nigdeensis* species, PEPC and PPDK enzymes were higher in leaves than in cotyledons, while GOX, GDC-H and Rubisco enzymes were observed more in cotyledons than in leaves. In summary, with some exceptions, mRNA and protein levels of C_4 pathway enzymes were increased, mRNA and protein levels of C_3 and photorespiration enzymes were decreased during the transition from C_3 cotyledons to C_4 leaves in *Salsola grandis*, *Cyathobasis fruticulosa* and *Petrosimonia nigdeensis* species.

Declarations

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Author contributions

FC and FK designed the experiments, FC performed the lab experiments, FC, FK and SC analysed the data, FC and SC led the drafting of the manuscript and all authors contributed and approved the final version.

Conflict of interest

The authors declare no competing interests.

Data availability

ITS region sequences of *S. grandis*, *S. tragus*, *C. fruticulosa* and *P. nigdeensis* are available in NCBI GeneBank (https://www.ncbi.nlm.nih.gov/) with the accession numbers of MT321628, MT321631, MT321630 and MT321629, respectively.

References

1. Akhani H, Edwards EJ, Roalson EH (2007) Diversification of the old world Salsoleae s.l. (Chenopodiaceae) molecular phylogenetic analysis of nuclear and chloroplastic data sets and revised classification. Int J Plant Sci 168:931-956
2. Akhani H, Lara MV, Ghasemkhani M, Ziegler H, Edwards GE (2009) Does Bienertia cycloptera with the single-cell system of C_4 photosynthesis exhibit a seasonal pattern of delta $\delta^{13}C$ values in nature similar to co-existing C_4 Chenopodiaceae having the dual-cell (Kranz) system?. Photosynth Res 99:23-36
3. Brautigam A, Hoffmann-Benning S, Weber, APM (2008) Comparative Proteomics of Chloroplast Envelopes from C_3 and C_4 Plants Reveals Specific Adaptations of the Plastid Envelope to C_4 Photosynthesis and Candidate Proteins Required for Maintaining C_4 Metabolite Fluxes. Plant Physiology 148:568-579
4. Covshoff S, Szecowka M, Hughes TE, Smith-Unna R, Kelly S, Bailey KJ, Sage TL, Pachebat JA, Leegood R, Hibberd JM (2016) C_4 Photosynthesis in the Rice Paddy: Insights from the Noxious Weed Echinochloa glabrescens. Plant Physiol 170:57-73
5. Fortelny N, Overall CM, Pavlidis P, Freue GVC (2017) Can we predict protein from mRNA levels? Nature 547:E19-E20
6. Freitag H, Vural M, Adigüzel N (1999) A remarkable new Salsola and Some new records of Chenopodiaceae from Central Anatolia, Turkey. Willdenowia 29:123-139
7. Gouy M, Guindon S, Gascuel O (2010) SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27:221-224
8. Gowik U, Westhoff P (2011) The path from C_3 to C_4 photosynthesis. *Plant Physiol*. 155: 56-63

9. Koteyeva NK, Voznesenskaya EV, Roalson EH, Edwards GE (2011) Diversity in forms of C_4 in the genus Cleome (Cleomaceae). Ann Bot 107:269-283
10. Kulahoglu C, Denton AK, Sommer M, Mass J, Schliesky S, Wrobel TJ, Berckmans B, Gongora-Castillo E, Buell CR, Simon R, De Veylder L, Brautigam A, Weber AP (2014) Comparative transcriptome atlases reveal altered gene expression modules between two Cleomaceae C_3 and C_4 plant species. Plant Cell 26:3243-3260
11. Lara MV, Offermann S, Smith M, Okita TW, Andreo CS, Edwards GE (2008) Leaf development in the single-cell C_4 system in *Bienertia sinuspersici*: expression of genes and peptide levels for C_4 metabolism in relation to chlorenchyma structure under different light conditions. Plant Physiol 148:593-610
12. Lauterbach M, Billakurthi K, Kadereit G, Ludwig M, Westhoff P, Gowik U (2016) C_3 cotyledons are followed by C_4 leaves: intra-individual transcriptome analysis of *Salsola soda* (Chenopodiaceae). J Exp Bot 68:161-176
13. Leegood RC (2013) Strategies for engineering C_4 photosynthesis. *J. Plant Physiol*. 170: 378-388
14. Levey M, Timm S, Mettler-Altmann T, Luca Borghi G, Koczor M, Arrivault S, Pm Weber A, Bauwe H, Gowik U, Westhoff P (2019) Efficient 2-phosphoglycolate degradation is required to maintain carbon assimilation and allocation in the C_4 plant *Flaveria bidentis*. J Exp Bot 70:575-587
15. Li Y, Heckmann D, Lercher MJ, Maurino VG (2017) Combining genetic and evolutionary engineering to establish C_4 metabolism in C_3 plants. J Exp Bot 68:117-125
16. Li Y, Ma X, Zhao J, Xu J, Shi J, Zhu XG, Zhao Y, Zhang H (2015) Developmental genetic mechanisms of C_4 syndrome based on transcriptome analysis of C_3 cotyledons and C_4 assimilating shoots in *Haloxylon ammodendron*. PLoS One 10(7):e0117175
17. Mallmann J, Weber APM, Heckmann D, Westhoff P, Bräutigam A, Gowik U (2014) The role of photorespiration during the evolution of C_4 photosynthesis in the genus Flaveria. Elife
18. Majeran W, Friso G, Ponnala L, Connolly B, Huang M, Reidel E, Zhang C, Asakura Y, Bhuiyan NH, Sun Q, Turgeon R, van Wijka KJ (2010) Structural and Metabolic Transitions of C_4 Leaf Development and Differentiation Defined by Microscopy and Quantitative Proteomics in Maize. The Plant Cell 22:3509-3542
19. Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schaffer AA (2008) Database indexing for production MegaBLAST searches. Bioinformatics 24:1757-1764
20. O'Kane SL, Schaal BA, Al-Shehbaz IA (1996) The Origins of Arabidopsis suecica (Brassicaceae) as Indicated by Nuclear rDNA Sequences. Systematic Botany 21:559-566
21. Offermann S, Friso G, Doroshenk KA, Sun Q, Sharpe RM, Okita TW, Wimmer D, Edwards GE, van Wijk KJ (2015) Developmental and Subcellular Organization of Single-Cell C_4 Photosynthesis in Bienertia sinuspersici Determined by Large-Scale Proteomics and cDNA Assembly from 454 DNA Sequencing. J Proteome Res 14:2090-2108

22. Ogren, WL (1984) Photorespiration: Pathways, Regulation and Modification. *Ann. Rev. Plant Biol.* 35: 415-442
23. Palmieri MC, Lindermayr C, Bauwe H, Steinhauser C, Durner J (2010) Regulation of Plant Glycine Decarboxylase byS-Nitrosylation and Glutathionylation. Plant Physiology 152:1514-1528
24. Pyankov VI, Black CC, Artyusheva EG, Voznesenskaya EV, Maurice SBK, Edwards GE (1999) Features of Photosynthesis in *Haloxylon* species of Chenopodiaceae that are dominant plants in central Asian deserts. Plant Cell Physiology 2:125-134
25. Pyankov VI, Voznesenskaya EV, Kuz'min AN, Ku MSB, Ganko E, Franceschi VR, Black CCJ, Edwards GE (2000) Occurrence of C_3 and C_4 photosynthesis in cotyledons and leaves of *Salsola* species (Chenopodiaceae). Photosynthesis Research 63:69-84
26. Pyankov VI, Ziegler H, Kuz'min AN, Edwards G (2001) Origin and evolution of C_4 photosynthesis in tribe salsoleae (Chenopodiaceae) based on anatomical biochemical types in leaves and cotyledons. Plant Syst Evol 230:43-74
27. Reeves G, Grange-Guermente MJ, Hibberd JM (2017) Regulatory gateways for cell-specific gene expression in C_4 leaves with Kranz anatomy. J Exp Bot 68:107-116
28. Sage RF (1999) "Why C_4 photosynthesis?" in *C_4 plant biology*, Eds: RF Sage, RK
29. Monson (San Diego, CA, USA: Academic Press): 3–16
30. Sage RF (2002) Variation in the kcat of Rubisco in C_3 and C_4 plants and some implications for photosynthetic performance at high and low temperature. Journal of Experimental Botany 53:609-620
31. Sage RF (2004) The evolution of C_4 species. *New Phytol.* 161: 341-370
32. Sage RF (2016) A portrait of the C_4 photosynthetic family on the 50th anniversary of its discovery: species number, evolutionary lineages, and Hall of Fame. J Exp Bot 67:4039-4056
33. Sage RF, Sage, TL, Kocacinar, F (2012) Photorespiration and the Evolution of C_4 photosynthesis. *Ann. Rev. Plant Biol.* 63: 19-47
34. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101-1108
35. Schussler C, Freitag H, Koteyeva N, Schmidt D, Edwards G, Voznesenskaya E, Kadereit G (2017) Molecular phylogeny and forms of photosynthesis in tribe Salsoleae (Chenopodiaceae). J Exp Bot 68:207-223
36. Smith ME, Koteyeva NK, Voznesenskaya EV, Okita TW, Edwards GE (2009) Photosynthetic features of non-Kranz type C_4 versus Kranz type C_4 and C_3 species in subfamily Suaedoideae (Chenopodiaceae). Functional Plant Biology 36:770-782
37. Somerville CR, Ogren WL (1982) Mutants of the cruciferous plant *Arabidopsis thaliana* lacking glycine decarboxylase activity. Biochem. J. 202:373-380

38. Timm S, Wittmiß M, Gamlien S, Ewald R, Florian A, Frank M, Wirtz M, Hell R, Fernie AR, Bauwe H (2015) Mitochondrial Dihydrolipoyl Dehydrogenase Activity Shapes Photosynthesis and Photorespiration of *Arabidopsis thaliana*. The Plant Cell 27:1968-1984
39. Voznesenskaya EV, Koteyeva NK, Akhani H, Roalson EH, Edwards GE (2013) Structural and physiological analyses in Salsoleae (Chenopodiaceae) indicate multiple transitions among C_3, intermediate, and C_4 photosynthesis. J Exp Bot 64:3583-3604
40. White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds.) PCR Protocols A Guide to Methods and Applications. Academic Press, New York, pp 315-322

Figures

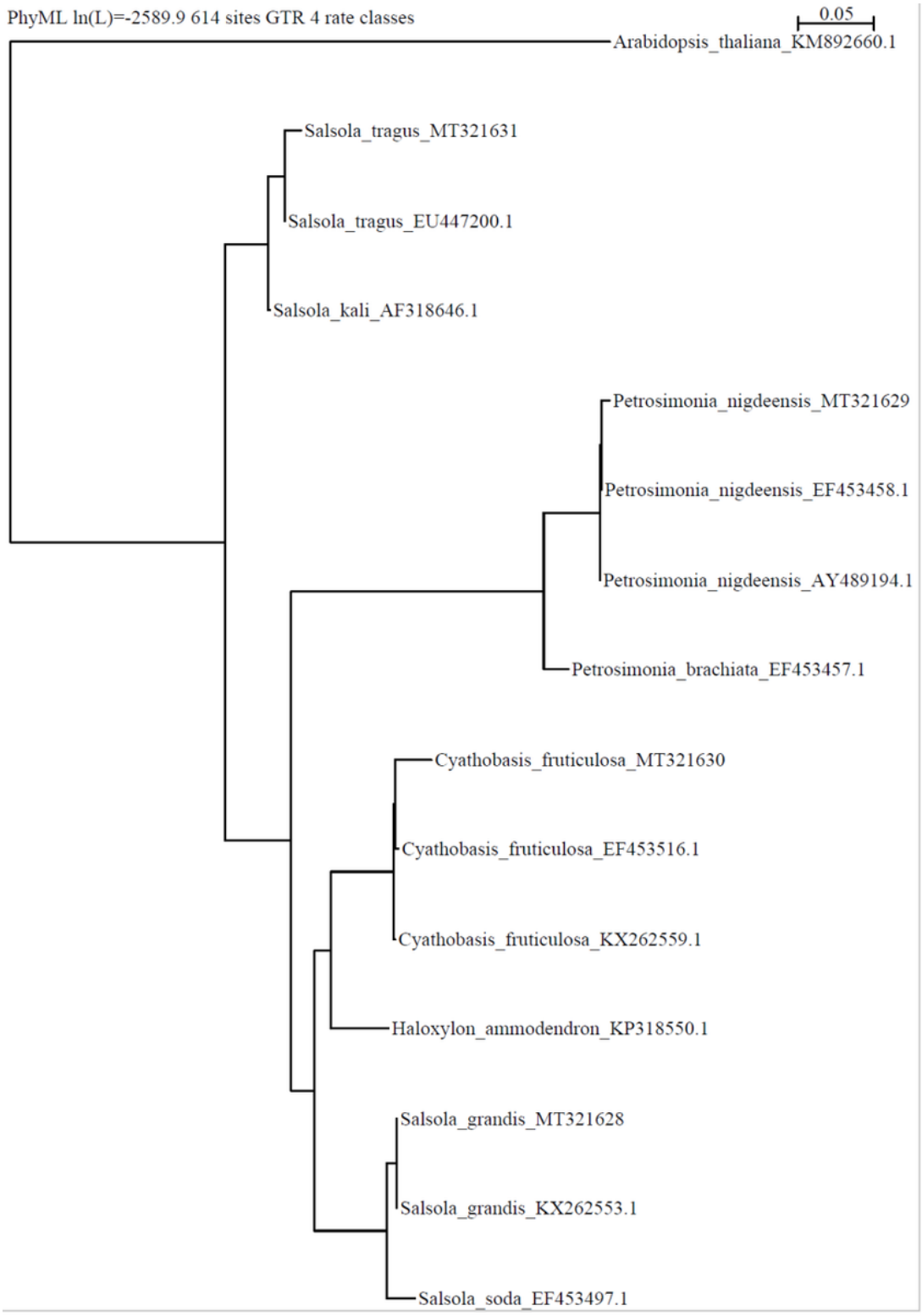

Figure 1

Phylogenetic tree created from internal transcribed spacer (ITS) regions of *Salsola grandis*, *S. tragus*, *Cyathobasis fruticulosa* and *Petrosimonia nigdeensis*. Sequences marked with MT are from current study.

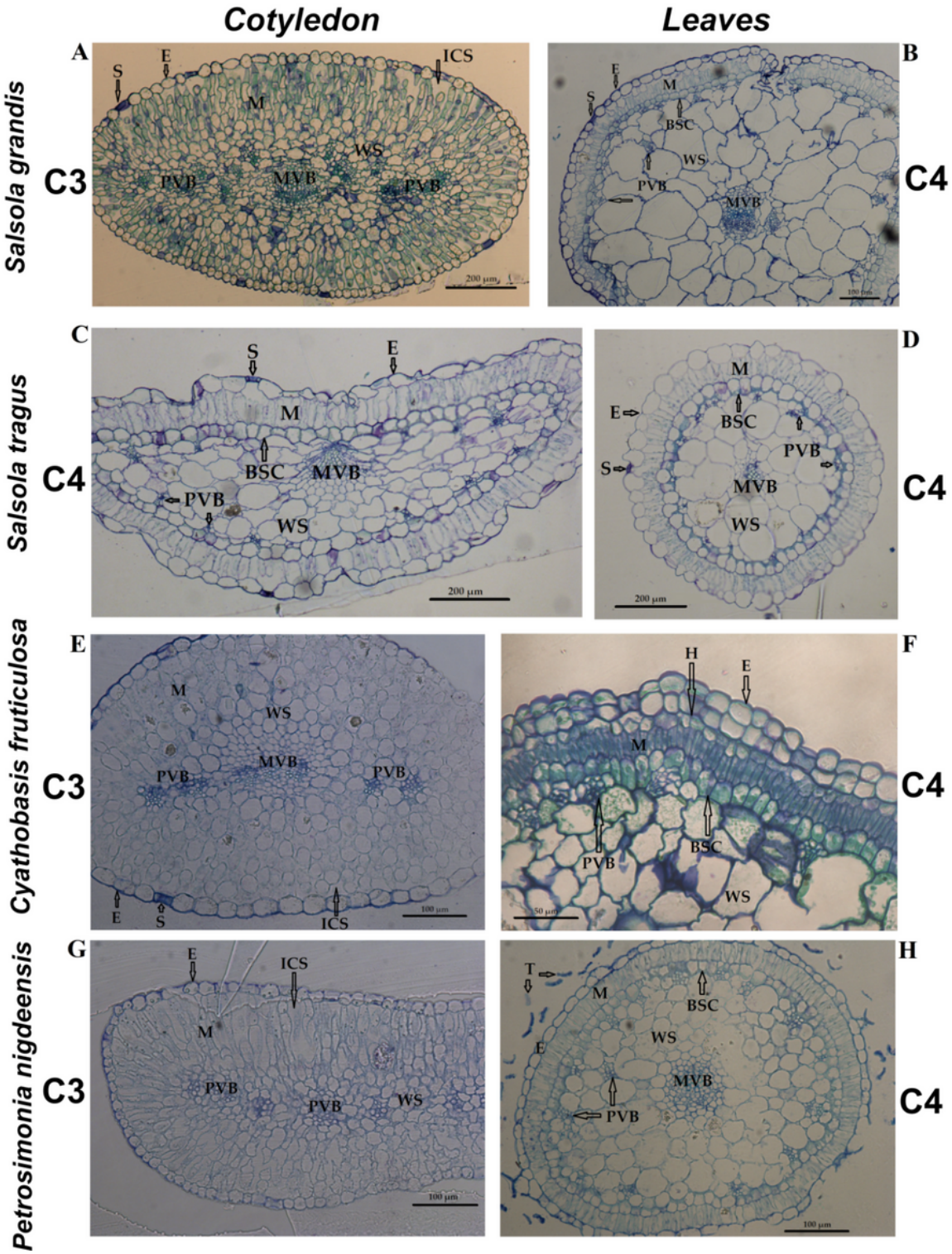

Figure 2

General anatomy of cotyledons (A, C, E, G) and leaves (B, D, F, H) of *Salsola grandis*, *S. tragus*, *Cyathobasis fruticulosa* and *Petrosimonia nigdeensis*. E: epidermis, ICS: intercellular space, M: mesophyll, MVB: main vascular bundle, PVB: peripheral vascular bundle, S: stoma, T: trichomes, WS: water storage tissue.

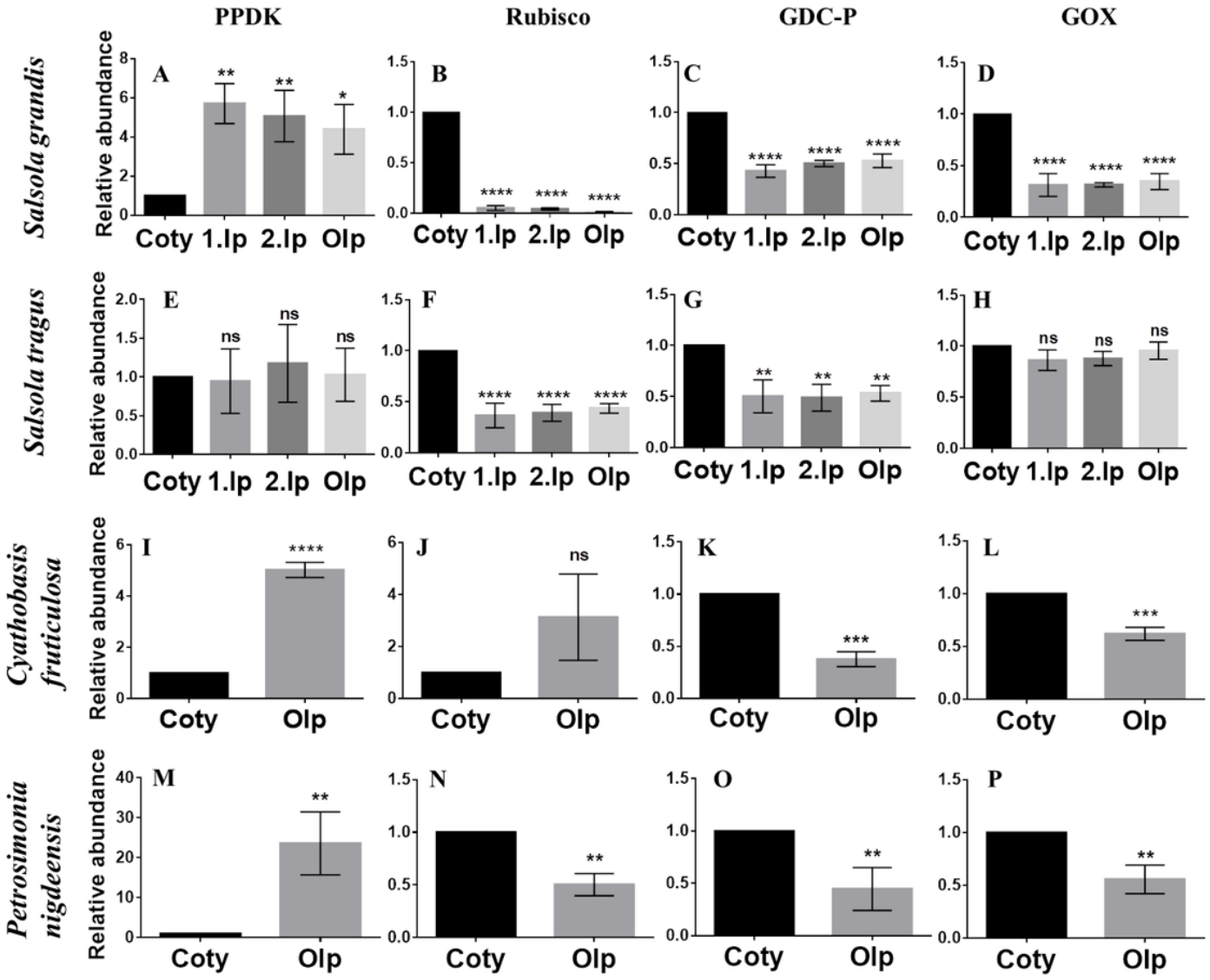

Figure 3

Comparisons of relative transcript abundances of cotyledons and developmental leaf stages in *Salsola grandis*, *S. tragus*, *Cyathobasis fruticulosa* and *Petrosimonia nigdeensis*. PPDK: pyruvate orthophosphate dikinase, Rubisco: ribulose 1,5-bisphosphate carboxylase/oxygenase, GDC-P: glycine decarboxylase-P subunit, GOX: glycolate oxidase, 1. lp: first leaf pairs, 2. lp: second leaf pairs, O lp: old leaf pairs, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****), ns: non-significant.

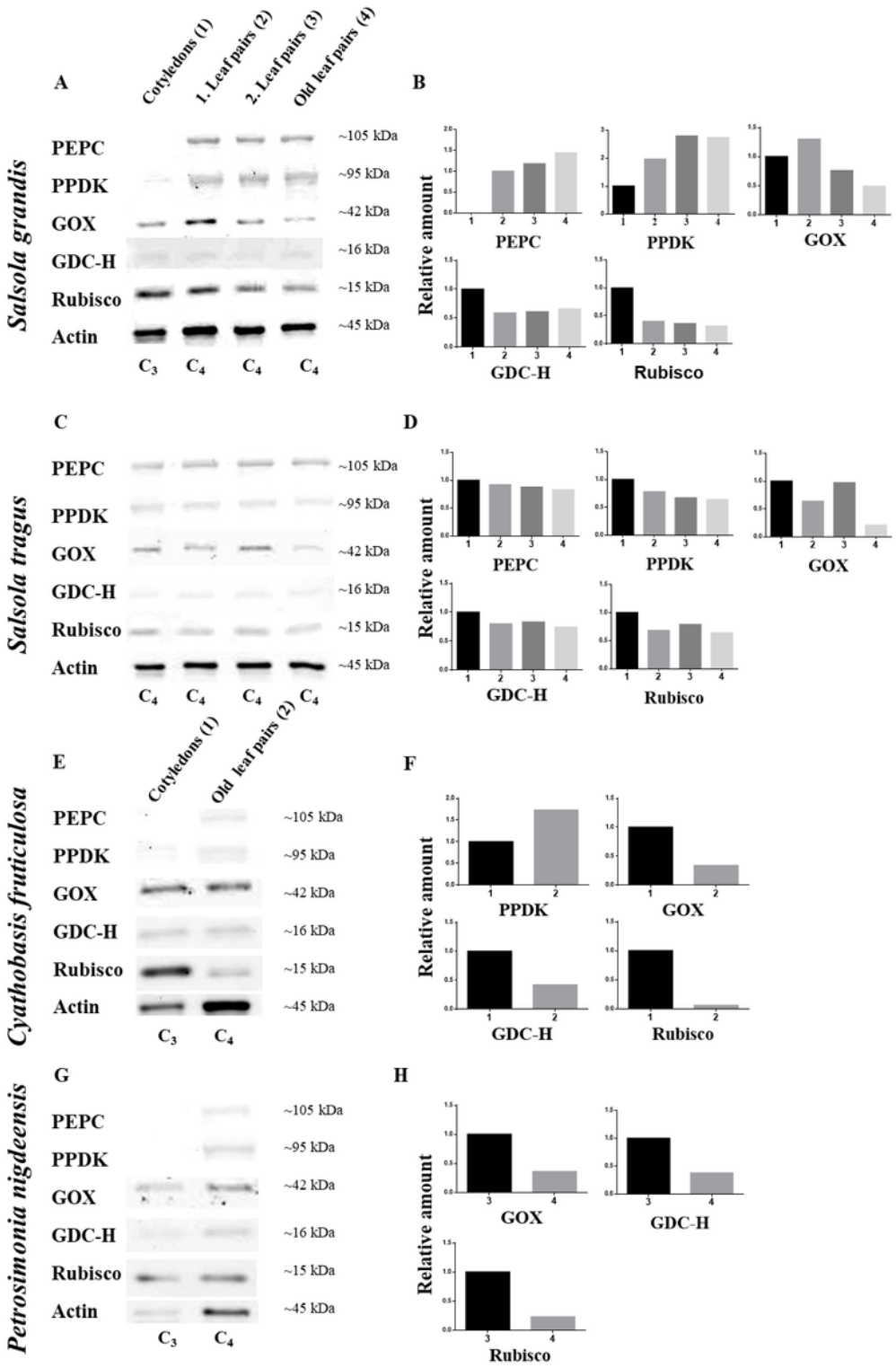

Figure 4

Western blot results of PEPC, PPDK, GOX, GDC-H and Rubisco enzymes extracted from cotyledons and developmental leaf stages in *Salsola grandis* (A) and *S. tragus* (C). Western blot results of PEPC, PPDK, GOX, GDC-H and Rubisco enzymes extracted from cotyledons and old leaves in *Cyathobasis fruticulosa* (E) and *Petrosimonia nigdeensis* (G). Comparisons of relative amounts of PEPC, PPDK, GOX, GDC-H and Rubisco enzymes in *S. grandis* (B), *S. tragus* (D), *Cyathobasis fruticulosa* (F) and *Petrosimonia*

nigdeensis (H). PEPC: phosphoenolpyruvate carboxylase, PPDK: pyruvate orthophosphate dikinase, Rubisco: ribulose 1,5-bisphosphate carboxylase/oxygenase, GDC-H: glycine decarboxylase-H subunit, GOX: glycolate oxidase, Actin is a protein for loading control.