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ORIGINAL ARTICLE

miRNA expression during prickly pear cactus fruit development

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Abstract miRNAs are a class of small non-coding RNAs that regulate gene expression. They are involved in the control of many developmental processes, including fruit development. The increasing amount of information on miRNAs, on their expression, abundance, and conservation between various species, provides a new opportunity to study the role of miRNAs in non-model plant species. In this work, we used a combination of Northern blot and tissue print hybridization analysis to identify conserved miRNAs expressed during prickly pear cactus (*Opuntia ficus indica*) fruit development. Comparative profiling detected the expression of 34 miRNAs, which were clustered in three different groups that were associated with the different phases of fruit development. Variation in the level of miRNA expression was observed. Gradual expression increase of several miRNAs was observed during fruit development, including miR164. miR164 was selected for stem-loop RT-PCR and for a detailed spatial–temporal expression analysis. At early floral stages, miR164 was mainly localized in meristematic tissues, boundaries and fusion zones, while it was more homogenously expressed in fruit tissues. Our results provide the first evidence of miRNA expression in the prickly pear cactus and provide the basis for future research on miRNAs in *Opuntia*. Moreover, our analyses suggest that miR164 plays different roles during prickly pear cactus fruit development.

Keywords Fruit · miRNAs · miR164 · *Opuntia* · Prickly pear cactus

Abbreviations

sRNA	Small RNA
miRNA	microRNA
LMW	Low molecular weight
HMW	High molecular weight
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

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Introduction

Gene expression can be regulated in many ways and one of them is mediated by microRNAs (miRNAs), which are short single-stranded RNAs of 21–24 nt in length that regulate gene expression in eukaryotes at the post-transcriptional level (Brodersen and Voinnet 2006). The understanding of gene regulatory mechanisms that involve miRNAs has had a significant impact on the knowledge about the regulation of plant developmental processes such as leaf morphogenesis and polarity, floral organ identity and differentiation, organ development, signaling pathways, and the response to stresses (Válóczi et al. 2006; Pulido and Laufs 2010; Rubio-Somoza and Weigel 2011).

miRNAs are grouped into families according to their sequence and they regulate their target genes through mRNA cleavage (Dugas and Bartel 2004) or translational repression (Brodersen et al. 2008). In plants, miRNAs perfectly or near-perfectly bind to their target mRNAs, and mRNA cleavage is the most common mechanism (Mallory et al. 2004). Based on the diversity and abundance of plant miRNAs, it may be possible that the regulation of all physiological and biochemical processes in plants involve the action of miRNAs (Jones-Rhoades et al. 2006; Chen 2009). Fruit development is a complex process and many genes are under dynamic control to ensure the proper establishment of the different fruit tissues and structures (McAtee et al. 2013). There is an impressive diversity of fruit developmental programs across the plant kingdom and it is to be expected that miRNAs also play important roles in these. miRNA studies in fruits are relatively scarce still. Currently, miRNAs were identified in fruits such as tomato (Pilcher et al. 2007; Din et al. 2014), grape (Carra et al. 2009; Wang et al. 2012, 2014), citrus (Song et al. 2009; Xu et al. 2010), melon (Gonzalez-Ibeas et al. 2011), apple (Xia et al. 2012), and strawberry (Xu et al. 2013; Ge et al. 2013), among others. Highly conserved miRNAs such as miR156, miR159, miR164, miR166, and miR172 have been identified in fruit tissues. Several studies demonstrated that conserved and specific miRNAs regulate genes involved in fleshy fruit development (e.g., Moxon et al. 2008; Karlova et al. 2011; Mohorianu et al. 2011).

For the prickly pear cactus (*Opuntia ficus indica*), no information on miRNAs is available. Currently, the genome sequence is unavailable; however, the use of the knowledge on conserved miRNAs of other plant species (Chavez-Montes et al. 2014) could be an approach to identify miRNAs in prickly pear cactus. The prickly pear cactus fruit is a simple berry, botanically speaking an accessory fruit formed from an inferior ovary adhering to the receptacle, covered with spines (Fig. 1) and is considered a non-climacteric fruit (Campos-Guillén et al. 2012). An important physiological characteristic of the prickly pear cactus fruit is the variability in fruit ripening time. The time that elapses from the onset of the floral bud to a ripe fruit ranges from 45 to 154 days (Fig. 1) in different varieties, therefore, they have been classified into early, middle, and late ripening fruits (Campos-Guillén et al. 2012). Interestingly, precise transcriptional and post-transcriptional gene regulation during prickly pear cactus fruit development has been observed (Campos-Guillén et al. 2012), suggesting that miRNAs might participate. Altogether, this makes prickly pear cactus fruit an attractive species for miRNA analysis.

In the present study, bioinformatics and molecular biology approaches were combined to obtain miRNA expression profiles during prickly pear cactus fruit development. Furthermore, the spatio-temporal expression of miR164 during prickly pear cactus fruit development was analyzed in detail.

Materials and methods

Plant material

Prickly pear cactus fruits (*Opuntia ficus indica*) were collected at INIFAP (Mexican National Institute of Forestry, Agriculture, and Livestock Research) Campo Experimental Norte de Guanajuato, in San Luis de la Paz, Gto., Mexico. The tissues were grouped into different stages of prickly pear cactus fruit development: young floral bud (not fertilized yet), floral bud, flowering bud, young fruit, green fruit, and ripe fruit (Fig. 1). After harvesting, the tissues were washed, frozen in liquid nitrogen, and stored at −80 °C until further use. For RNA extraction, the samples were sliced and ground to a fine powder in a mortar with liquid nitrogen. For tissue print analysis, the samples were cut immediately and printed on nylon membranes.

Low molecular weight (LMW) RNA extraction and sRNAs analysis in polyacrylamide gel

Low molecular weight (LMW) RNA was extracted from different stages of prickly pear cactus fruit development according to our previously reported protocol (Rosas-Cárdenas et al. 2011). In summary, LiCl extraction buffer and phenol pH 8.0 was used for extraction, the samples were incubated for 5 min at 60 °C and centrifuged for 10 min at 13,400*g* at 4 °C. The supernatant was re-extracted with 600 µl of chloroform-isoamyl alcohol and centrifuged. For the separation of high molecular weight (HMW) RNA, the supernatant was incubated for 15 min at 65 °C and precipitated with 50 µl of 5 M NaCl and 63 µl of 40 % polyethylene glycol 8000 (w/v), followed by incubation on ice for at least 30 min and centrifuged. The

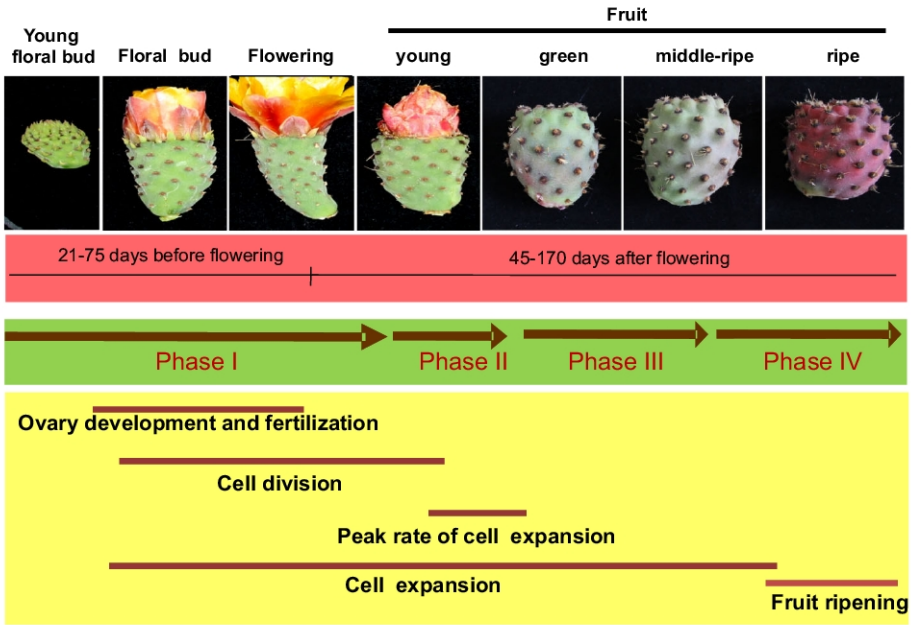

Fig. 1 Diagram of prickly pear cactus fruit development. **a** Prickly pear cactus fruit at different stages of development. The images illustrate major physiological events, their timing, and the sampling time points. **b** The phases represent: I, floral development and fruit-set; II, cell division during young fruit development; III, cell expansion; and IV, fruit ripening

supernatant that contained LMW RNA was extracted with 500 µl of phenol–chloroform-isoamyl alcohol and centrifuged. LMW RNA was precipitated with 50 µl of 3 M sodium acetate pH 5.2 and 1 ml of absolute ethanol, incubated overnight at −20 °C and recovered by centrifugation, dried and resuspended in RNAse-free water. LMW RNA was visualized in polyacrylamide gels. RNA concentration and quality was quantified in a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.), measuring absorbance to calculate the A260/A280 and A260/A230 ratios.

Northern blot miRNA analysis

A total of 2 µg of LMW RNAs of different developmental stages of prickly pear cactus fruit were separated according to their size in 12.5 % denaturing polyacrylamide gels using a mini-vertical electrophoresis gel system (GE Healthcare). A semidry trans-blot system (Biorad) was used to transfer the sRNAs from gel to Amersham Hybond-N membrane (GE Healthcare) in 0.5× TBE buffer for 1 h at 10 V. The membranes were dried at room temperature, fixed with 12 ml EDC cross-linking solution (0.16 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma-Aldrich) prepared in 0.13 M 1-methylimidazole at pH 8.0, incubated for 1.5 h at 65 °C, and rinsed twice with water. The membranes were dried at room temperature and stored at −20 °C till further use. The northern blot analysis was performed according to a previously reported protocol with some modifications (Pall and Hamilton 2008; Rosas-Cárdenas et al. 2011). The membranes were pre-hybridized with 15-ml hybridization solution (Rapid-hyb Buffer Amersham; GE Healthcare) for 1-h at 37 °C, followed by the addition of the labeled probe of interest, and then incubated for 24-h at 37 °C. Each probe used was labeled with [γ-^{32}P] ATP to detect the respective miRNA and control U6 (small nucleolar RNA; 5′-AGGGGCCATG CTAATCTTCTC-3′). The membranes were washed twice with wash solution (2× SSC/0.1 % SDS), first for 4 min, and then a second time for 2 min at room temperature, followed by exposure to a storage Phosphor Screen System (Amersham Biosciences) for approximately, 48 h.

Reverse northern blot hybridization

Fifty-three synthetic DNA oligonucleotides corresponding to the reverse complementary sequences of selected mature miRNAs were used to print the miRNA arrays or used as probe for northern blot hybridization (Suppl. Table S1). Each probe (5 µl of each oligonucleotide stock 100 µM) was manually spotted on a 2 × 2 cm Amersham Hybond-N nylon membrane (GE Healthcare), dried at room temperature, and fixed with 12 ml EDC cross-linking solution, as described previously. Printed membranes were wrapped in aluminum foil and stored at −20 °C until they were used for hybridization. miRNA fractions isolated from different tissues were used for miRNA array hybridization. A total of 16 µg of LMW RNAs of each tissue (divided over several lanes) were resolved in 12.5 % denaturing polyacrylamide gels using a mini-vertical electrophoresis gel system (GE Healthcare) and the sRNA fraction corresponding to 18–24 nt was excised from the polyacrylamide gel and stored at −20 °C until the purification.

The gel slices with the sRNA fractions were placed in two or three 1.5 ml tubes, crushed with a glass rod, and then 200 µl sterile nuclease-free water was added and continued to crush the gel into fine slurry. The sample was placed at 70 °C for 10 min. Following the manufacturer's recommendations, one column with a gel filtration matrix (DTR column; EDGE Biosystems, Gaithersburg, MD, USA) was prepared for each gel slice. The gel slice slurry was vortexed for 30 s and the entire volume transferred onto the DTR column and centrifuged at 850*g* for 3 min. Then 3 µl of 10 mg/ml glycogen, 25 µl of 3 M NaOAc (pH 5.2), and 900 µl of ice cold 100 % EtOH was added to the flow-through, mixed by inversion and placed at −80 °C for 30 min, subsequently the tubes were centrifuged at 13,400*g* for 10 min and the supernatant discarded. Finally, the RNA was air dried. The purified fractions with the sRNAs were resuspended in water (max. 20 µl), samples from same tissue were pooled, and stored at −20 °C until tested.

A total of 40 ng of each previously purified sRNA fraction corresponding to 18–24 nt was dephosphorylated with Antarctic Phosphatase (New England Biolabs) for 1 h and then radioactively labeled with T4 polynucleotide kinase (Invitrogen) and 1.5 µl [γ-^{32}P]-ATP 222 TBq/mmol (370 MBq/ml; AccesoLab, Mexico D.F., Mexico) for 1 h at 37 °C. The labeling reaction was stopped by incubation at 65 °C for 5 min and then directly incubated on ice for 3 min. The miRNA arrays were pre-hybridized in 15 ml of Rapid-hyb Buffer Amersham (GE Healthcare) for 1 h at 37 °C. The labeled miRNA samples were hybridized in Rapid-hyb Buffer Amersham (GE Healthcare) for 15 h at 37 °C. After hybridization, the miRNA arrays were washed with wash solution (2× SSC/0.1 % SDS) for 10 min at 37 °C. The membranes were then exposed using a storage Phosphor Screen System (Amersham Biosciences) for 2 days. The phosphor screen was scanned in a Storm 860 Gel and Blot Imaging System (Amersham Biosciences). Signals were quantified using the ImageQuant TL software (Amersham Biosciences).

Data analysis of reverse northern blot arrays

The signal intensity of each spot on the array was quantified using ImageQuant TL using the array module (Amersham Biosciences). Background correction was performed with the Spot Edge Average method to determine the average background intensity around each spot outline. Normalization of intensity data between arrays was performed using a normalization factor calculated based on the expression profile of miR166 of a traditional northern blot (Suppl. Fig. S1). First, the traditional northern blot of miR166 was normalized using the intensity values of U6 determined using ImageQuant TL (Suppl. Fig. S2). For each stage, this miR166 normalized expression value was compared to the miR166 value of the array, and the ratio between these two values was used to normalize the values of each miRNA on the respective array, allowing the comparison of miRNAs between arrays. An expression profile matrix was built representing the level of expression for each stage for each miRNA detected in at least one stage. The expression profile matrix was imported into the Multiple ArrayViewer program (MeV; Saeed et al. 2003) and hierarchical clustering was performed using the following options selected: 'gene tree', 'sample tree' 'optimize gene leaf order', 'Pearson correlation', and 'complete linkage clustering'.

miRNA detection by microarray hybridization

The expression profiles of miRNAs detected by reverse northern blot were verified by microarray hybridization. Briefly, total RNA of young fruit, green fruit, and ripe fruit was extracted with Trizol (Invitrogen). RNA of each condition was labeled with biotin and hybridized to separate arrays, GeneChip® miRNA 2.0 Array (Affymetrix) according to the manufacturer. In detail, first a hybridization was done with a pool of RNAs from each stage and second, each sample of each stage was hybridized in an independent way as second repetition (data not shown; readers can obtain details from the authors). The Affymetrix fluidic station 450 was used for the wash and stain operation of the arrays. Images were captured with the Scanner Unit model "3000 7G". These procedures were performed at the 'Unidad de Genotipificación y Análisis de Expresión' of the Instituto Nacional de Medicina Genómica (Inmegen, Mexico). The R software, available from the Bioconductor Project (http://www.bioconductor.org), was used to normalize data before comparison and to evaluate differential expression between pool samples. Data normalization was made using the Robust Multichip Average (RMA) method (Irizarry et al. 2003). It consists of three steps: a background adjustment, quantile normalization, and finally summarization. All differential expression values were selected from at least >2×-fold change using the Oligo (de Carvalho et al. 1992) and Limma (Smyth 2005) packages.

miRNA detection by stem-loop RT-PCR

In brief, total RNA was isolated using Trizol (Invitrogen). The stem-loop RT-PCR method used is described in Li et al. (2009). The oligonucleotide sequences used are as follows: miR164 RT loop, 5′-TCAACTGGTGTCGTGGAGTCCG GCAATTCAGTTGAGTGCACGTG-3′, miR164 forward, 5′-ACACTCCAGCTGGGTGGAGAAGCA-3′, and miR164 reverse, 5′-AACTGGTGTCGTGGAG-3′. The final PCR products were separated in a 4 % agarose gel.

miRNA detection by tissue printing and northern blot hybridization

Tissue printing provides a simple and rapid method to analyze the localization and expression of miRNAs at the tissue level. This is especially convenient for large tissues, such as prickly pear cactus fruit. For the spatio-temporal analysis miR164 was selected. The prickly pear cactus fruits were washed with water and dried at room temperature. The materials were cut in longitudinal and transverse sections. Transverse sections were made from basal, medial, and apical regions of prickly pear cactus fruits. After cutting the samples, they were immediately placed with its cut surface face down on Amersham Hybond-N membrane (GE Healthcare). The different sections were firmly pressed on the nylon membrane for 30 s. The membranes were treated in a similar way as described above, were dried at room temperature and stored at −20 °C till further use. To analyze the miRNA localization and expression, the miRNA detection was carried out as the northern blot analysis described above, at 37 °C. An oligonucleotide for U6 RNA (5′-GGGGCCATGCTAAT CTTCTC-3′) was used as positive control and the M13 forward oligonucleotide (5′-TGTAAAACGACGGC CAGT-3′) as negative control. Duplicate analyses assured reproducibility of the results.

Results

miRNA selection for expression analysis

Some miRNAs are highly conserved across the plant kingdom (Chavez-Montes et al. 2014), making it possible to detect them in many plant species using the available miRNA sequences. Based on this principle, we took the approach of screening for conserved miRNA expression profiles during prickly pear cactus fruit development. We generated miRNA arrays with a selection of miRNA sequences from different fruit bearing species (Suppl. Fig. S3). In summary, 53 miRNAs were selected based on in silico analysis, of which 49 are expressed in at least one fruit crop, 14 miRNAs are expressed in all fruit analyzed, and 4 are not expressed in the analyzed fruit crops (http://smallrna.udel.edu/; (Chavez-Montes et al. 2014) (Suppl. Fig. S3). To analyze which of the miRNAs are expressed in prickly pear cactus fruit, we selected four developmental stages: young floral buds, young cactus fruits, green cactus fruits, and ripe cactus fruits. These samples represent the most marked changes during fruit development, which is depicted in Fig. 1. The first sample, young floral bud, represents the phase I (ovary development). The young fruit sample represents the phase II (cell division and seed formation), the green fruit sample represents the phase III (cell expansion and embryo maturation), and the ripe fruit sample the phase IV (ripening fruit) (Fig. 1) (Gillaspy et al. 1993; Reyes-Agüero et al. 2006). The sRNA fractions of these different tissues were obtained and analyzed by spectrophotometric analysis and polyacrylamide gels, showing well-defined 5S and tRNA bands (Suppl. Fig. S2), suggesting a good quality of LMW RNA. Each sRNA sample was labeled and used for hybridization with the miRNA array.

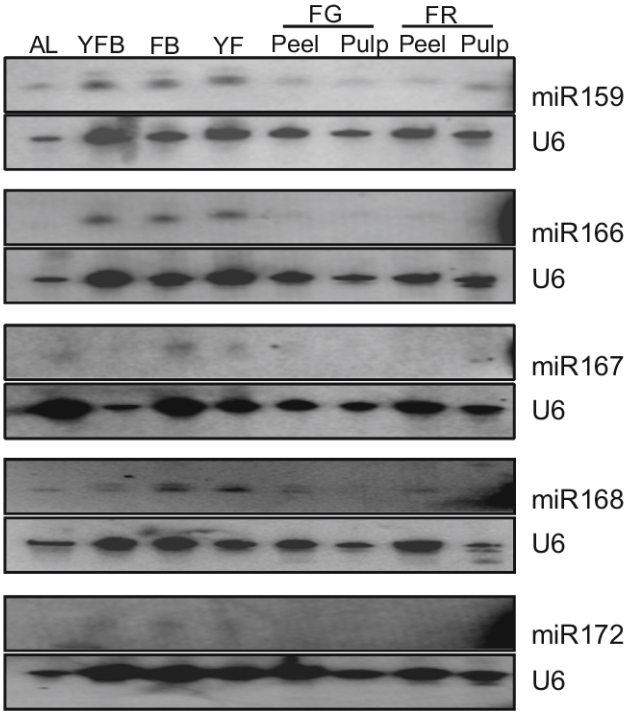

Fig. 2 Northern blot hybridization analysis of miRNA expression at different stages of prickly pear cactus fruit development. 2 µg per sample of LMW RNA were electrophoresed in 12 % polyacrylamide gels under denaturing conditions, blotted, and hybridized with ^{32}P-labeled oligonucleotide probes complementary to the indicated miRNAs. The U6 panel shows equivalent loading. *AL* Arabidopsis leaves (positive control), *YFB* young floral bud, *FB* floral bud, *YF* young cactus fruit, *FG* green cactus fruit, *FR* ripe cactus fruit

Detection of miRNAs at different stages of prickly pear cactus fruit development

To elucidate the potential roles of miRNAs in prickly pear cactus fruit development, global miRNA expression patterns at different fruit stages were studied by northern blot hybridization, reverse northern blot hybridization (arrays; Suppl. Fig. S4), and GeneChip® miRNA 2.0 microarrays (Suppl. Fig. S5). As a first approach to detect miRNA presence in prickly pear cactus we performed sRNA northern blot hybridizations with five miRNAs previously identified as highly conserved in plants (Chavez-Montes

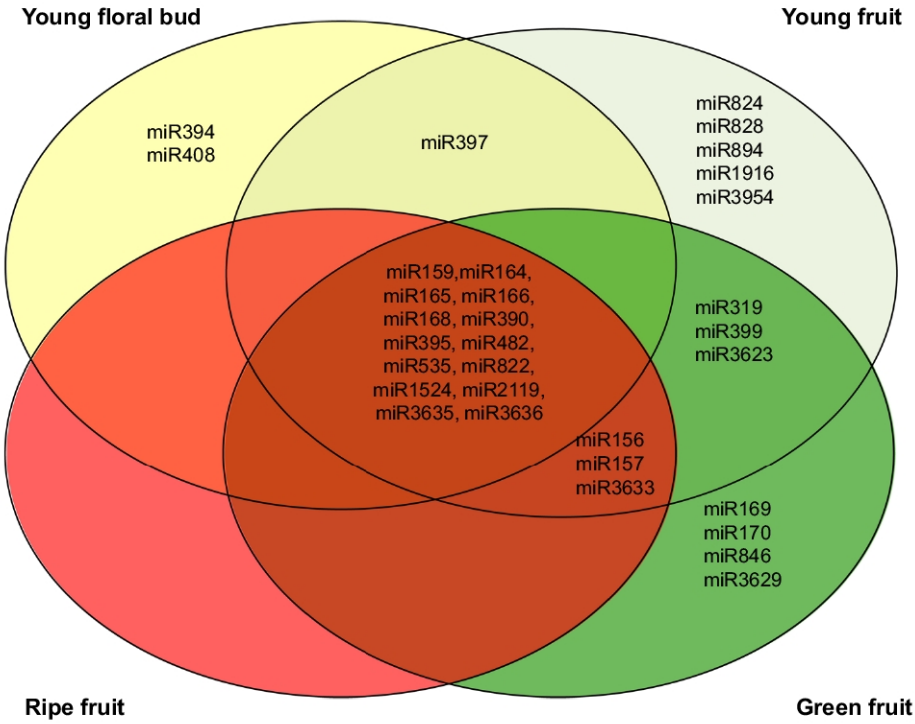

Fig. 3 Distribution of miRNAs detected at different stages of prickly pear cactus fruit development. The *Venn diagram* shows shared and specifically expressed miRNAs at different developmental stages of prickly pear cactus fruit. Note: miR393 and miR398 are not depicted in the figure because their expression overlap is only at the floral bud with the green fruit stage; for miR159 the northern blot result was also taken into account

et al. 2014). As expected, all five conserved miRNAs (miR159, miR166, miR167, miR168, and miR172) were detected during different developmental stages (Fig. 2). We observed that these miRNAs were highly expressed during early floral development and less expressed during fruit development.

The following experiment performed was a reverse northern blot analysis. For this, we prepared arrays with spotted oligonucleotides corresponding to sequences of 53 different miRNAs (see "Materials and methods" for details). sRNA fractions of the four developmental stages were individually hybridized to the array. Interestingly, a total of 34 different miRNAs were detected in prickly pear cactus: 22 miRNAs in floral buds, 26 in young fruit, 25 in green fruit, and 17 in ripe fruit (Figs. 3, 4; Suppl. Table S2). A good correlation was observed between the expression obtained by the miRNA reverse northern blot array experiment and the northern blot analysis for miR159, miR166, and miR168 (Fig. 2). In addition, miR167 and miR172, which were not detected by our array hybridizations, were detected by northern blot hybridization. However, they showed very low expression levels (Fig. 2), suggesting that different hybridization efficiencies may cause some lowly expressed miRNAs to be undetected by the array approach. On the other hand, several miRNAs previously reported as highly conserved and highly abundant in plants, as miR172, were expressed at very low levels in prickly pear cactus. This indicates that not all conserved miRNAs present a similar expression pattern in prickly pear cactus versus other species.

Finally, as third experiment, a preliminary microarray hybridization experiment was performed for floral bud, green fruit, and ripe fruit (see "Materials and methods" for details). Comparing this data with the expression profiles of the detected miRNAs by reverse northern blot hybridization (Fig. 4), 18 miRNAs show a similar profile (Suppl. Fig. S5). The rest of the miRNAs show some differences in their expression profile, most of them being miRNAs that are lowly expressed and therefore perhaps having more variation (Suppl. Fig. S5).

Expression profiles of miRNAs during prickly pear cactus fruit development

The reverse northern blot experiment revealed that 14 miRNAs (miR159, miR164, miR168, miR165, miR166, miR390, miR395, miR482, miR535, miR822, miR1524, miR2119, miR3635, and miR3636) could be detected in all developmental stages (Fig. 3). Other miRNAs were detected in two or three stages. miR397 was expressed both at the floral bud and the young fruit stage, while the expression of miR319, miR399, and miR3623 was detected at the young fruit and the green fruit stage. Moreover, miR156, miR157, and miR3633 were found to be expressed at the young fruit, green fruit, and ripe fruit stage. Furthermore, we also found miRNAs that were only

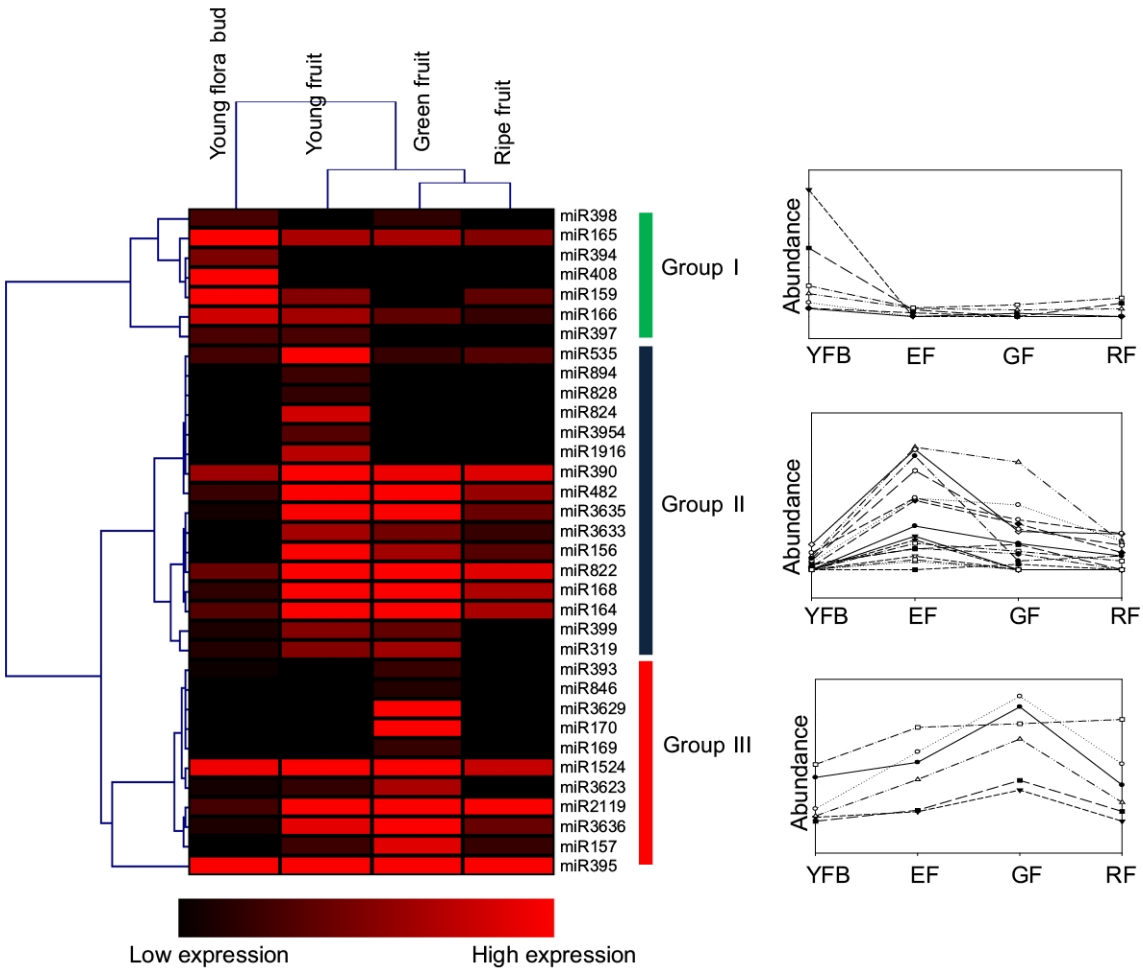

Fig. 4 Cluster analysis of miRNA expression at different stages of prickly pear cactus fruit. Heatmap of normalized miRNA expression levels during prickly pear cactus fruit development; gene-normalized data were used. Each *column* represents a stage and the intensity of the *red* color indicates the relative expression level at that stage. Hierarchical clustering resulted in three major groups marked with *color bars* on the *right vertical axis*. The *histograms* simulating the expression patterns of the corresponding clusters are presented on the *right*. Hierarchical clustering was performed using MeV with the following options selected: 'gene tree', 'sample tree' 'optimize gene leaf order', 'Pearson correlation', and 'complete linkage clustering'. *YFB* young floral bud, *YF* young cactus fruit, *GF* green cactus fruit, *RF* ripe cactus fruit

expressed at a single stage. For instance, miR408 and miR394 were expressed specifically in floral buds. At the young fruit stage, specific expression was detected for miR824, miR828, miR894, miR1916, and miR3954. miR169, miR170, miR846, and miR3629 were specific to the green fruit stage. Interestingly, we did not detect any of the selected miRNAs to be specifically expressed at the ripe fruit stage.

Although the miRNAs are present in one, some, or in all stages analyzed, the expression levels were different through different stages (Suppl. Table S2). miR159 and miR408 were the most abundant miRNAs in the floral bud sample; and miR164, miR390 and miR1524 were the most abundant miRNAs in the young fruit. A few miRNAs were expressed at high levels in ripe fruit, such as miR2119 and miR395. In general, the miRNA expression levels ranged from very low to high as fruit development progressed. However, stage specific miRNAs were expressed at relative low levels, with the exception of miR408.

Hierarchical cluster analysis of miRNA expression profiles

To identify trends among the detected miRNAs during development, we performed a hierarchical cluster analysis (Fig. 4). The hierarchical clustering resulted in three major groups of miRNA expression profiles, named as: Group I, II, and III (Fig. 4). miRNAs in the same group might be involved in a similar process during development. Group I contains 7 miRNAs: miR159, miR165,

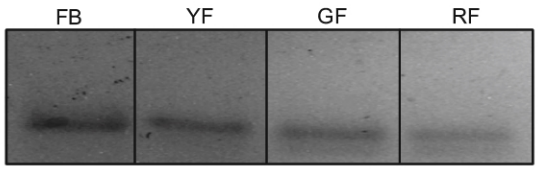

Fig. 5 Stem-loop RT-PCR of miR164 at different stages of prickly pear cactus fruit development. RT-PCR products were electrophoresed in 4 % agarose gel. *FB* floral bud, *YF* young cactus fruit, *GF* green cactus fruit, *RF* ripe cactus fruit

miR166, miR394, miR397, miR398, and miR408, which are highly expressed in floral buds and then decrease their expression during development, though miR159 and miR166 show a slight increase in ripe fruit. Group II is the largest group, containing 16 miRNAs, and can be divided into two subgroups. The first subgroup shows no or very low expression in young floral buds, a high expression in young fruit and a significant decrease in expression in green and ripe fruit (miR535, miR824, miR828, miR1916, and miR3954). The other subgroup contains miRNAs that maintain their expression relatively high, also after the young fruit stage: miR156, miR164, miR168, miR319, miR390, miR399, miR482, miR822, miR3633, and miR3635. Finally, Group III contains 11 miNAs that are mainly expressed at the green fruit stage, namely miR157, miR169, miR170, miR393, miR395, miR846, miR1524, miR2119, miR3623, miR3629, and miR3636.

Detailed expression analysis of miR164

Development is regulated by spatially and temporally coordinated regulatory networks that include regulation by miRNAs. For the prickly pear cactus fruit developmental process, we were interested in miRNAs of group II, which showed an increase in expression during fruit development and a decrease in ripe fruit. We confirmed the expression profile of one of the miRNAs in this group, miR164, in the four developmental stages of the prickly pear cactus fruit (Fig. 5) by stem-loop primer based RT-PCR, which is a highly sensitive and specific method for miRNA detection (Chen et al. 2005; Li et al. 2009). This temporal pattern was also previously reported for miR164 in tomato (Mohorianu et al. 2011). However, while this data provides information about the time of expression of this miRNA, it provides little information about its spatial pattern of expression, because tissues are homogenized to isolate RNA.

Until now, the spatio-temporal expression of miRNAs, which includes their localization in specific tissues has not been studied in fleshy fruits. The use of common in situ hybridization techniques provides information about the spatial pattern of expression, but it might be challenging for the tissues of large fruits. Therefore, we used tissue printing followed by miRNA hybridization to observe the spatio-temporal expression of miR164 during prickly pear cactus fruit development (Fig. 6; Suppl. Fig. S6). We found that miR164 was expressed in all analyzed developmental stages (Fig. 7). We also observed that, at early stages of floral development, miR164 was expressed in meristematic zones such as the floral meristem and the meristems producing the glochids (i.e., hair-like spines) (Fig. 6a). In open flowers, miR164 was highly expressed in fusion zones such as the carpel base, within the stamens and receptacle, and the compitum zone (Fig. 6b). High miR164 expression was observed in the ventral carpel vascular bundle (Fig. 6c). Moreover, in middle-ripe and ripe fruits, miR164 was mainly homogenously expressed in the complete fruit (Fig. 7). The observed hybridization patterns correlate well with the miRNA expression profile obtained with the reverse northern blot hybridization and the stem-loop RT-PCR experiment.

Discussion

In plants, miRNAs participate in diverse biological processes, including fruit development (Moxon et al. 2008; Karlova et al. 2013). High-throughput sequencing to identify plant miRNAs has greatly advanced our knowledge about their functions. However, without the availability of a genome or transcriptome, the identification of miRNAs in non-model plant species using high-throughput sequencing strategies is challenging. Fortunately, a high number of identified miRNAs are conserved among plant species, and even their target genes are also conserved (Axtell and Bartel 2005). Based on these observations, we performed an expression analysis of selected miRNAs throughout four stages of fruit development in the cactus species *Opuntia*, followed by a detailed analysis of the spatial pattern of expression of miR164 during fruit development.

Prickly pear cactus contains at least 34 miRNA families

miRNA expression analysis revealed that at least 34 (64 %) out of 53 studied miRNAs are expressed during prickly pear cactus fruit development. This indicates that the genome of the cactus species *Opuntia* also encodes for conserved miRNAs and has at least 34 miRNAs. Furthermore, it suggests that the detected miRNAs may have a role in prickly pear cactus fruit development. 39 % of the detected miRNAs were present in all four analyzed prickly pear cactus stages and 32 % of the detected miRNAs appeared to be stage-specific (Fig. 3), suggesting both

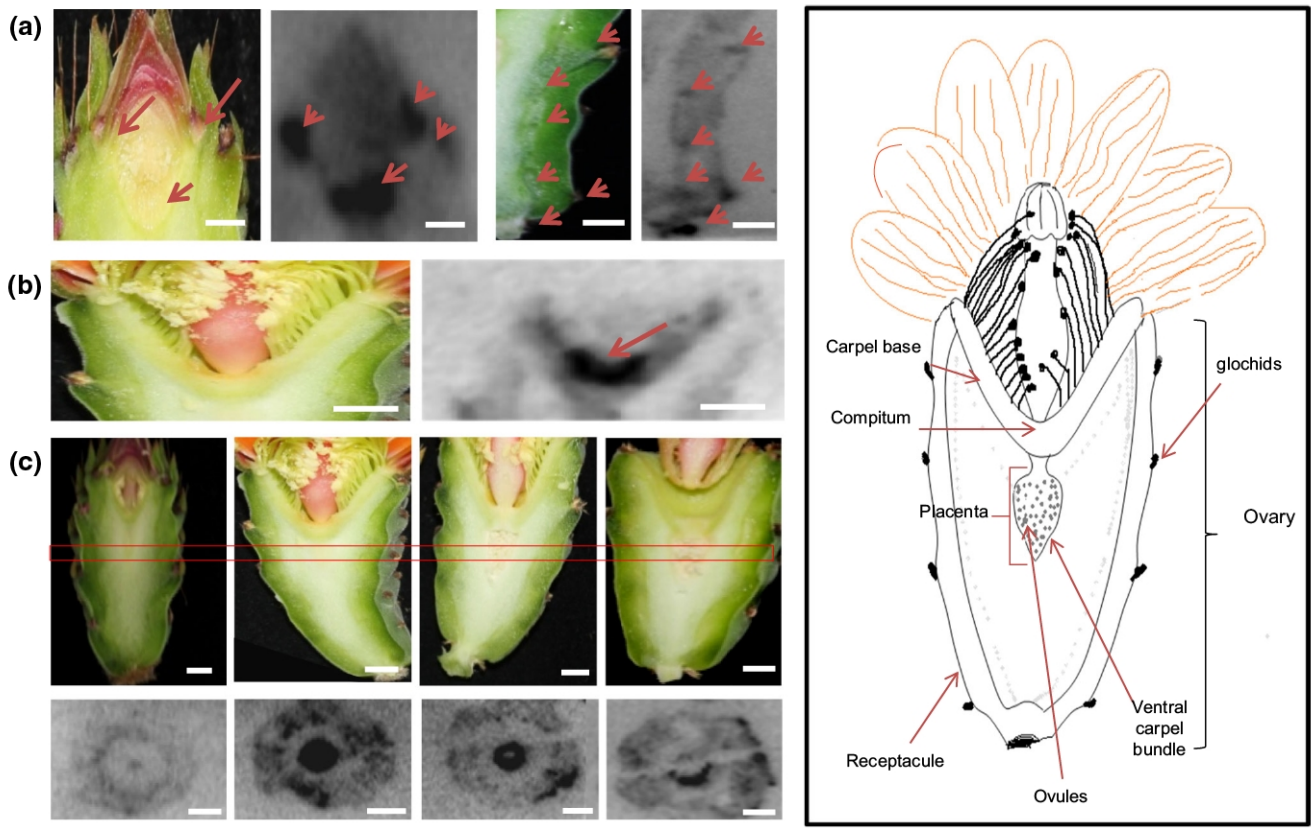

Fig. 6 miR164 spatio-temporal expression pattern during early stages of prickly pear cactus fruit development. Tissue print of prickly pear cactus followed by northern blot hybridization was performed. **a** miR164 expression in meristems. **b** miR164 expression at the compitum of prickly pear cactus. **c** miR164 expression in prickly pear cactus placenta. *Scale bars* indicate 0.5 cm

general and specific roles during fruit development, respectively. The lowest expression of miRNAs was detected in the ripe fruit, which could suggest that miRNAs are less important or that specific miRNAs, not included in this analysis, participate at this stage. Of the 34 detected miRNAs, 14 miRNAs are conserved with cucurbits (Jagadeeswaran et al. 2009), 18 miRNAs are conserved with tomato, 21 with chili (http://smallrna.udel.edu/; Chavez-Montes et al. 2014), and 24 miRNA families with grapevine (Pantaleo et al. 2010; Wang et al. 2012). Some miRNAs such as miR156, miR159, miR164, miR165, miR166, miR168, and miR390 are conserved between prickly pear cactus and all above-mentioned species, suggesting deeply conserved roles in these species. Based on phylogenetic analysis, grapevine is the closest related model species to prickly pear cactus (Tree of Life; http://www.tolweb.org), which is also reflected in the fact that prickly pear shares the highest miRNA family conservation with grapevine. Accordingly, it was previously reported that miR3636 and miR3635 were grapevine specific miRNAs (Pantaleo et al. 2010), but we detected them also in prickly pear cactus fruit (Fig. 3).

miRNA expression profiles during prickly pear cactus fruit development

Hierarchical cluster analysis of the detected miRNAs resulted in three groups: early developmental stage-associated miRNAs (Group I), middle late stage-associated (Group II), and late stage-associated miRNAs (Group III) (Fig. 4). This result supports the idea that the observed miRNA expression changes are not random, but that the miRNAs are highly regulated during fruit development, leading to specific target gene regulation that contributes to proper fruit development.

Group I contains seven miRNAs, which in general decrease their expression during fruit development. One of them is miR159, targeting GAMYB-related transcription factors involved in floral transition and anther development (Achard et al. 2004). A similar expression profile was reported in tomato for this miRNA that targets the *ACS* gene, which is involved in ethylene biosynthesis, a hormone important for fruit ripening (Zuo et al. 2012). Other members of this group are miR165/166 that regulate diverse aspects of plant development, including shoot