Identification and Profiling of Conserved MicroRNAs in Different Developmental Stages of Crown Imperial (*Fritillaria Imperialis* L.) using High-throughput Sequencing

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Abstract

Novel strategies for improvement of plants' ornamental and other properties relay on miRNA control of differential plant gene expression modulation. Still, in response to the same abiotic stresses, some conserved miRNA families show different expression patterns in different plant species. In parallel, the use of deep sequencing technologies reveals new levels of complexity of regulatory networks in plants through identification of new miRNAs. These are two major reasons why more studies are needed before envisioned new strategies may take their course in practical application domain. This research revealed 21 conserved miRNAs, matching 15 miRNA families, in *Fritilaria imperialis*. Among identified conserved miRNA families in crown imperial, miR166, miR169 and miR396 families were the most abundant ones. The expression of seven conserved miRNAs (Fim-miR156b, Fim-miR159, Fim-miR166a-5p, Fim-miR169d-5p, Fim-miR171c, Fim-miR393 and Fim-miR396e-3p) was further investigated in different tissues and three developmental stages, suggesting different roles these miRNAs have in growth and development of crown imperial. Gained knowledge from this research can open the door to find efficient ways to secure crown imperial survival, preservation and utilization and if proven useful may be applied in other plant species as well.

Introduction

Plants' microRNAs (miRNAs), as miRNAs in other living organisms, are represented by a group of small non-coding RNAs with approximately 18–25 nucleotides in length [1, 2]. They are conserved in eukaryotes with a main role in post-transcriptional gene expression regulation and based on the mature miRNAs' similarities they can be grouped into families [1, 3, 4]. In all analyzed eukaryotic species so far key biological processes like development, differentiation, proliferation, cell metabolism and programmed cell death are influenced by miRNAs [3–5]. In plants, in addition to abiotic stress, miRNAs were shown to be involved in developmental and morphogenesis processes [6]. Up to day a significant number of miRNAs' families was found to be conserved in different plant species, from mosses and gymnosperms to flowering plants [7]. For example, miRNAs' families like miR156, 159, 166, 169, 171, 393, and 396 were found to be highly conserved in many plant species [8–11]. Among them miR156, miR159, miR166, and miR169 were shown to be mainly involved in flower development [12–15]. On the other hand miR171 was found to be mainly involved in root development [16], miR393 in processes like flag leaf inclination and root growth [17], while miR396 was found mainly to control cell proliferation during leaf and root development [18]. Nevertheless, unraveling the exact roles of these and other miRNAs in plants' growth and development is still the subject of active research.

The miRNAs' discovery was enabled through different approaches like direct cloning, sequencing and/or bioinformatics prediction followed by experimental validation, but with the development of a high throughput sequencing technology the later became a method of preference due to providing a significant reduction of time and costs in miRNAs' research [19]. This technology has been successfully applied in plants' miRNAs investigation in peanut [20], trifoliate orange [21], *Brassica napus* [22], radish [23] and *Prunus mume* [24]. *Fritillaria imperialis* L. (crown imperial), a member of the *Liliaceae* family of

monocotyledonous plants is recognized as medicinal and ornamental plant [25, 26]. It is confirmed that this plant is rich in secondary metabolites with bioactive properties that have a high bioengineering potential, which make this species a valuable resource for the development of various pharmaceuticals and biocatalysts [27, 28]. The origin and the center of the genetic diversity of crown imperial is Iran [29], where fourteen native species (out of 100 from the *Fritillaria* genus) are located. However, in recent years, the wild populations of this species in Iran have been facing the extinction, partly due to the changes in the environmental conditions and partly due to the improper grazing, pasture degradation and pest attacks [29, 30]. Since plant growth and development are under the influence of multiple environmental and genetic factors, and miRNAs are one of the main communicators in the tuning of gene expression to meet the internal and external demands of growth, development and survival, we focused this research on the identification of members of this particular RNA class in different tissues during crown imperial development by combining RNAseq and qRT-PCR approach. The gained knowledge from this research can open the door to find efficient ways to secure crown imperial survival, preservation and utilization and if proven useful designated approach may be applied in other plant species as well.

Materials And Methods

Plant material

Fritillaria imperialis plants were collected from their natural habitats in Koohrang, Chaharmahalva Bakhtiari, Iran. Samples were collected during 2015–2016 on two occasions: (1) closed flowers were collected at the end of March 2015 and (2) seven tissue samples (stamen, pistil, petal, sepal, leaf, stem, bulb and fruit) were collected from the end of March 2015 until June 2016. These tissue samples were collected during three developmental stages: stem elongation stage (in which leaves formed a rosette and the maximum length of plant was up to 60 mm), flower development stage (in which plant formed a closed red flower with pedicle's maximum length of 32.5 mm) and seed head stage (in which plant formed a visible capsule fruit with the maximum width of 28.5 mm). All the samples were harvested, immediately placed in liquid nitrogen and deposited at -80°C until further processing.

Rna Extraction And Sequencing

The isolation of total RNA was performed from50-100 mg powdered samples by using TRIzol reagent (Invitrogen, USA) according to [31](Chomczynski 1993) RNA quality was determined by 1% agarose gel electrophoresis and its concentration was quantified using Bio photometer spectrophotometer (Eppendorf, Germany). Total RNAs isolated from sepal, stem, leaf, pistil and stamen samples were pooled together in an equal fraction ratio. The pair-end reads of 101 bp length were generated by TruSeq™ RNA Sample Preparation Kit (Illumina) and sequenced on HiSeq 2000 platform (Illumina) by MacrogenCompany (Korea). RNA-Seq data were obtained from the company's server in FASTQ format. The raw transcriptome data have been submitted to the NCBI's sequence reads archive (SRA) under the accession ID: SRR6242026.

Identification Of Conserved Mirnas In Fritillaria

Quality control of raw sequence data was performed in FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Reads were trimmed to remove adaptor sequences using the Trimmomatic (version 0.32) tool (http://www.usadellab.org/cms/?page =trimmomatic). All short reads were assembled *de novo* using Trinity (version r20140717) (http://trinityrnaseq.sourceforge.net) and obtained contigs were compared to all previously known mature plants miRNAs available in miRBase (version 21) (http://www.miRbase.org). Finally, contigs with three or fewer mismatches to their homologous miRNA were chosen for the secondary structure prediction using Mfold web server (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form). The following parameters were used to identify potential miRNAs: 1) the mature miRNA length should be between 18 and 24 nucleotides, 2) mismatches between miRNA and sequence pattern should be between 0–3, 3) miRNA sequences should not contain any loop or large fractures, 4) miRNA must be located in the stem-loop arms, 5) between miRNA and opposite sequence (miRNA*) should be less than six mismatches [5], 6) the MFEI index (Minimal Folding Free Energy Index) and MFE (Minimal Free Energy) for miRNA precursors (pre-miRNAs) should be higher than 0.67 [32].

Quantitative Rt-pcr Analysis Of Mirna Expression

Seven miRNAs which showed the perfect match to homologous miRNA were selected for quantitative RT-PCR analysis. Total RNA samples were extracted from seven tissues (stamen, pistil, petal, sepal, leaf, stem, bulb and fruit) collected during three developmental stages (stem elongation, flower development, and seed head stage). MiRNA primer designer software was used for primers' design (Table 1). The first strand cDNA synthesis was conducted according to Varkonye-Gasic et al [33]. 2μg of total RNAs was reversely transcribed to cDNA using Thermo Scientific RevertAid Reverse Transcriptase and stem-loop RT-PCR miRNA primers (Table S1). The expression level of miRNAs was monitored and quantified by real-time PCR in which each reaction included 2 μL of cDNA, 6 μL SYBR Premix Ex Taq™ II kit (Takara, Japan), 1 μL miRNA-specific forward primer, 1 μL reverse universal primer and 2 μL deionized water. The PCR amplification conditions were as in Mousavi et al [34]. The qPCR expression levels were analyzed according to the mean value of two biological and three technical replicates. GADPH (Glyceraldehyde 3-phosphate dehydrogenase) gene was used as an internal control for data normalization. Relative gene expression level was performed using the Comparative Quantitation method [35].

Table 1
The list of miRNA specific primers for confirmation by qPCR.

Primer Name	miRNA Primer sequences (5' to 3')	Tm (°C)
Fim-mir156b	GCGGCGGTGTGCTCACTCTCTTC	70
Fim-mir159	GCGGCGGTTTGGATTGAAGGGAG	68.2
Fim-mir166a-5p	GCGGCGGGGAATGTTGTCTGGC	69.6
Fim-mir169d-5p	GCGGCGGCCGGCAAGTCATCCTTG	73.8
Fim-mir171c	GCGGCGGTGATTGAGCCGCGCC	73.3
Fim-mir393	GCGGCGGTCCAAAGGGATCGC	69.2
Fim-mir396e	GCGGCGGGTTCAATAAAGCTGTG	66.4
Universal reverse	ATCCAGTGCAGGGTCCGAGG	64.6

Prediction Of Mirna Target Genes

To predict putative target genes of *F. imperialis* miRNAs, a small plant RNA target prediction server psRNATarget (http://plantgrn.noble.org/psRNATarget/) was used for querying mature miRNA sequences against RNA-seq reads/contings. Reads/contigs matching mature miRNA sequences were used in BLASTX search against non-redundant database with cut-off set to $1e^{-5}$ [36]. The hits obtained by BLASTX analysis were mapped to the GO database to reveal biological processes, cellular components, and molecular functions of the target genes. Additionally, the enzyme commission (EC) numbers were associated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to reveal biological pathways targeted by the respective miRNAs.

Results

Out of 57,150,222 high quality reads *de novo* assembly resulted in construction of 99,621 contigs ranging from 201 – 15,627 nucleotides in length, with N50 of 1132. From these contigs 1,004 (ca. 1%) showed high homology to conserved plant miRNAs and upon their secondary structures prediction we have successfully identified 21 conserved miRNAs sequences belonging to 16 miRNA families (Table S2, Fig. 1) among which miR166 and miR169 families were the most abundant ones (Fig. 2). While the length of pre-miRNAs ranged from 50 to 203 nucleotides, with an average length of 92 nucleotides (Fig. 3), the length of mature miRNAs ranged from 18 to 23 nucleotides, with 21-nucleotide mature miRNAs represented as the most abundant form (Fig. 4). Sequences analysis showed that miR166a-5p and miR166c-3p shared 66.6% similarity, while the reverse complement of miR166d-3p sequence showed 66.6% and 95.2% similarity with these two sequences, respectively. Similarly, sequences miR169d and miR169d-5p shared 80.95% similarity, while the reverse complement of miR169h-3p sequence displayed 85.7% and 80.95% similarity with these two sequences, respectively.

The results of the expression analysis of seven conserved miRNAs in seven crown imperial tissues during three developmental stages are given in the Fig. 5. Expression of miR156b and miR169d-5p was up-regulated in all seven analyzed tissues during all three analyzed developmental stages, displaying an increase ranging from 1.3-fold to 4.5-fold and from 1.5-fold to 21-fold, respectively. For miR156b up-regulation was the highest in the sepal during the stem elongation phase as well as in the pistil during the flower developmental phase and in the fruit during the seed head stage. For miR169d-5p up-regulation was the highest in the stamen during the stem elongation phase as well as in pistil and petal during the flower development and in fruit during the seed head stage. On the other hand, expression profile of miR159, miR166a-5p, miR171c and miR396e-3p varied much more across different developmental stages and tissues. During the stem elongation phase miR159 was up-regulated in the pistil, petal and sepal (with the highest expression in petals), down-regulated in stamen and expressed at its regular physiological level in leaf and bulb. During the flower developmental phase, it was up-regulated in pistil and bulb (with the highest expression in bulb) and down-regulated in stamen, petal, and sepal (with the minimal expression in stamen) and during the seed head stage it was equally up-regulated in fruit and bulb. During the stem elongation phase miR166a-5p was up-regulated in all analyzed tissues except bulb (with the highest expression in leaf and sepal). During the flower development it was up-regulated in pistil, petal, sepal and bulb (with the highest expression in bulb and pistil), while it was expressed at regular physiological level in stamen and leaf. During the seed head stage, it was up-regulated in fruit, while it was expressed at its regular physiological level in bulb. For miR171c was shown that it was up-regulated in bulb during all three stages as well as in fruit during the seed head stage (only it was ~ 12-fold higher in bulb than in fruit), but that it was down-regulated in pistil and stamen and expressed at its regular physiological level in petal, sepal and leaf during the steam elongation and flower development stages. During the stem elongation phase miR393 was up-regulated in petal, sepal, leaf and bulb (with the highest expression in bulb), while it was down-regulated in pistil and stamen. During the flower development phase, it was up-regulated in sepal, and bulb (with the highest expression in bulb) and down-regulated in pistil and stamen, while during the seed head stage it was up-regulated in bulb and fruit (only in bulb it was ~ 4-fold higher than in fruit). For miR396e-3p was shown that during the stem elongation phase it was up-regulated in petal, sepal and leaf (with the highest expression in leaf), down-regulated in stamen and bulb (with the minimal expression in bulb) and expressed at its regular physiological level in pistil. During the flower development it was up-regulated in all analyzed tissues except stamen and bulb (with the highest expression in petal), while during the seed head stage it was up-regulated in fruit and down-regulated in bulb.

The results of tissue-specific miRNAs expression profiles are given in the Fig. 6. In the pistil tissue during the stem elongation and flower development phase miR169d-5p showed the highest up-regulation (~ 5-fold increase), while miR171c showed the greatest down-regulation during both phases (~ 0.2-fold decrease). In the stamen tissue during the stem elongation phase miR169d-5p showed the highest up-regulation (~ 21-fold increase) and miR159 showed the greatest down-regulation (almost 100%), while during the flower development phase miR156b and miR169d-5p showed the highest up-regulation (~ 2-fold) and miR159 and miR393 showed the greatest down-regulation (almost 100%). In the petal tissue

during stem elongation phase miR393 showed the highest up-regulation (~ 18-fold increase), while during flower development miR169d-5p and miR396e-3p showed the highest up-regulation (~ 4-fold increase) and only miR159 showed down-regulation (~ 0.5-fold). In the sepal tissue during the stem elongation phase miR166a-5p showed the highest up-regulation (~ 11-fold increase), while during the flower development phase miR393 showed the highest up-regulation (~ 6-fold increase) and only miR159 showed down-regulation (~ 0.4-fold). In leaf tissue during the stem elongation phase miR166a-5p showed the highest up-regulation (~ 11-fold), while during the flower development phase miR156b, miR169d-5p and miR171c showed the highest up-regulation (~ 1.4-fold) and only miR159 showed down-regulation (~ 0.7-fold). In bulb during the stem elongation phase miR393 showed the highest up-regulation (~ 22-fold increase) and after that miR171 showed higher up-regulation (~ 13-fold increase) than another miRNAs and miR166a-5p and miR396e-3p showed the greatest down-regulation (almost 100%), during the flower development phase miR393 and miR171 showed up-regulation (~ 23-fold and ~ 19-fold increase respectively) and miR396e-3p and miR156b showed down-regulation (~ 1.3-fold) during the seed head stage again miR171 and miR393 showed up-regulation, miR171 showed the highest up-regulation (~ 44-fold increase) and miR396e-3p showed down-regulation (~ 0.5-fold). In fruit tissue during the seed head stage miR169d-5p showed the highest up-regulation (~ 5-fold increase).

The prediction of the genes targeted by these seven conserved miRNAs in *Fritillaria imperialis* has given the results presented in the Table 2.

Table 2
Predicted targets of seven conserved miRNAs in *F.imperialis*

miRNAs Name	Target Protein Family	Target Gene Names (Number Of Mismatch)
Fim-miR156b	squamosa promoter binding protein-like 9 (SPL9)	AT2G42200 (1)
	protein kinase 1B (PK1B)	AT2G28930 (2)
Fim-miR159	myb domain protein 33 (MYB33)	AT5G06100 (1)
Fim-miR166a-5p	cellulose synthase-like D3 (csld3)	AT3G03050 (2)
	AT2G45990	AT2G45990 (2)
	Disease resistance protein (TIR-NBS-LRR class) family (RPS4)	AT5G22540 (3)
	GTP binding protein (ARC3)	AT1G75010 (3)
Fim-miR169d-5p	Transducin/WD40 repeat-like superfamily protein (AT5G03450)	AT5G03450 (4)
Fim-miR171c	GRAS family transcription factor (HAM3)	AT4G00150 (0)
Fim-miR393	F-box/RNI-like superfamily protein (TIR1)	RCOM_0556140 (3)
	AT1G53710	AT1G53710 (4)
Fim-miR396e-3p	Transducin/WD40 repeat-like superfamily protein (LST8-1)	AT3G18140 (4)
	Nuclear transport factor 2 (NTF2) family protein with RNA binding (RRM-RBD-RNP motifs) domain-containing protein	AT5G60980 (5)
	fibrillin family protein	AT2G35490 (5)

Discussion

In respect to the size of identified miRNAs in *F. imperialis*, mature miRNAs of 21-nucleotides in lenght were the most abundant form, similarly to the ones detected in maize, chickpea and *Brassica rapa* [37–39]. Among 1,004 constructed contigs 21 conserved miRNAs were computationally detected, representing members of 15 known miRNA families (miR156, miR159, miR160, miR162, miR166, miR169, miR171, miR172, miR393, miR396, miR2390, miR2861, miR3960, miR5179, miR7399 and miR8112). Interestingly, miRNAs identified in crown imperial were highly similar to those identified in radish (miR156, miR166, miR172, and miR396) [23], *Prunus mume* (miR156, miR159, miR160, miR162, miR166, miR169, miR171 and miR393) [24], peanut (miR156, miR159, miR166, miR169, miR171, miR172, miR393 and miR396) [20], and larch (miR156, miR159, miR160, miR162, miR166, miR169, miR171, miR172, and miR396) [11].

Having in mind that crown imperial is a monocot angiosperm, while radish, *P. mume* and peanut belong to eudicot angiosperms of the Rosids monophyletic clade, and that larch is a gymnosperm, such finding confirms joint ancient origin and high evolutionary preservation of above-mentioned miRNAs families, at least among the vascular plants.

Among identified conserved miRNA families in crown imperial, miR166 and miR169 were the most abundant ones, followed by miR396 family. For miR166/165s in Arabidopsis is known to regulate developmental processes like shoot apical meristem maintenance, leaf polarity and flower and root development by negatively regulating class III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIP III) genes. Moreover, in Arabidopsis was found that seven *MIR166* genes encode same mature miR166, but that each of them shows specific and precise spatio-temporal expression [40]. Results obtained in our research detected at least three members of miR166 family in crown imperial, which sequences shared similarities in the range from 66–95%. Also, results suggested that crown imperial miR166a-5 is involved both in vegetative and reproductive tissues development (especially leaf, flower and fruit development), but its' up-regulation was more prominent in vegetative tissues. *In silico* analysis predicted that miR166a-5p developmental role in crown imperial involves targeting mRNAs coding for cellulose synthase-like protein D3 (CSLD3) (required for the synthesis of a cell wall polysaccharide during root hair elongation), ribosomal RNA small subunit methyltransferase G, AT5G22540 (involved in plant response to disease, i.e. bacterial infection) and ARC3 (responsible for chloroplast expansion and division). Predicted targets in crown imperial differ from the reports in Arabidopsis and maize, in which HD-ZIP genes were targeted by miR166, while cellulose synthase in maize was predicted to be targeted by miR397 [41]. However, in soybean, both evolutionary conservation and diversification of miR166 family members was reported [42], while in tomato a novel miRNA PC-102-5p targeting cellulose synthase was detected [43], thus miR166a-5p may indeed be a member of miR166 family in crown imperial that evolved to target different genes, which remains to be experimentally tested.

For miR169 is known to control flowering time in response to various stresses and are mostly up-regulated in response to abiotic stress. The main target of miR169 in plants is the NUCLEAR FACTORY, SUBUNIT A (NF-YA) TFs family associated with transcriptional regulation of many genes, like the ones involved in flower development in Antirrhinum and Petunia [44], drought response in soybean [45] and early flowering induced by abiotic stresses in Aabidopsis [46]. Results of our research suggest that it is involved in the flower and fruit development in crown imperial, predominantly up-regulated in the reproductive tissues (especially in stamen during the stem elongation phase), which is concordant with previous records. *In silico* prediction of miR169d-5p target involved AT5G03450, a RING-type domain-containing protein of transducin/WD40 repeat-like superfamily, which is involved in flowering stage as well as in plant embryo development and is expressed in leaf, flower, plant sperm cells, stem, root, apex, which is in accordance with observed miR169d-5p expression profile in crown imperial. Records for miR396 show high expression in root, leaf, and flower of radish and peanut [20, 23] as well as in petals of sweet potato [47] and leaves of *Arabidopsis* [48]. While it has been shown to be highly expressed in *Vitis vinifera* green berries, its expression decreased upon ripening [49]. Such findings revealed that miR396 is mostly involved in vegetative growth rather than reproductive transition stages and abundantly present in

vegetative tissues. This is concordant with our finding as well, only in crown imperial miR396 was also up-regulated in all analyzed tissues during reproductive phase, which potentially broadens its role in *F. imperialis*. Further, our study showed that miR396 was complementary to LST8-1 subunit of TORC1 (Target of Rapamycin Complex 1), which is consistent with previous results in Arabidopsis [50]. Two other predicted targets for miR396 in crown imperial involved Nuclear transport factor 2 (NTF2) family protein and AT2G35490 (fibrillin 2 – FBN2, role in photoprotection of photosystem II). While for interaction with NTF2 our finding differs from previous records that reported on NTF2 being targeted by miR397and MAV8 (predicted candidate miRNAs) in Arabidopsis [51, 52], there were no records on miRNA FBN2 regulation so far. However, there are also records on miR396 family exhibiting both conservativeness and diversification in Poaceae, especially in wheat [53], which may be the case in crown imperial as well, but it remains to be experimentally tested.

Considering expression profile of miR156b, it was found to be up-regulated in all analyzed tissues and developmental stages, indicating the general role of this miRNA in diverse aspects of plant development, as previously reported in *Prunus mume* [24] and *Arabidopsis* [15]. Predicted targets of miR156b in crown imperial involved *SPL9* and *PK1B* genes, encoding for a putative transcriptional regulator involved in the vegetative to reproductive phase transition and protein kinase, respectively. Previous studies have identified SPL family as target gene for miR156 in rice, Arabidopsis and potato, with role in controlling developmental processes in leaf, flower and apical dominance [54–56], while no records were found for PK1B regulation. On the contrary, expression profile of miR159 varied across tissues and stages, displaying almost total down-regulation in stamen during first stem elongation and flower development stages and up-regulation in bulb and fruit during seed head stage. Also, during transition from stem elongation phase to flower development phase miR159 showed change from predominantly up-regulation to predominantly down-regulation in all tissues, except pistil and bulb, in which it remained up-regulated. This finding is in agreement with report on significant down-expression of miR159 in transition from juvenile to adult phase in the *Trifoliate Orange* [6]. Also, in transgenic gloxinia plants, suppression of miR159 led to early flowering [57]. Additionally, miR159 displayed significant change in expression during the fruit formation in tomato [58] and strawberry [59]. So far all collected reports support the possible role of miR159 in the reproductive tissues, especially in flower and fruit development, which is concordant with our finding. Predicted target gene for miR159 in crown imperial was identified as MYB33, transcription factor involved in anther and pollen sperm cell development, cell differentiation, negative growth regulation, response to cytokinin, ethylene and gibberellin, etc. This prediction is concurrent with report by Achard et al. [60] that in Arabidopsis miR159 is involved in floral and anther development by targeting the expression of MYB33 and another target mRNA coding for GAMYB like transcription factor, which is involved in gibberellic acid mediated signaling underlying anther development and flowering time.

In contrast to other miRNAs in crown imperial, miR171c was almost completely down-regulated in all analyzed tissues and stages, except in bulb, in which it displayed continuous up-regulation through all three stages, and in fruit. So far it was reported to represses differentiation of axillary meristems, regulating shoot branch production in Arabidopsis, whereas its over-expression changes transition from

vegetative to reproductive phase in barley, preserving its roles shown in Arabidopsis, but displaying additional functions like activation of the miR156 pathway as well [61]. Similarly, in rice miR171c was reported to control the floral transition and maintenance of shoot apical meristem, while its over-expression led to decreased tolerance to salt stress in rice [62, 63]. Also, in strawberry miR171 was reported to be up-regulated in red fruit [64] as well as in tomato fruit after ethylene treatment, while miR171b was predicted to be involved in fruit ripening in banana [65], thus, miR171 family may have additional role in fruit ripening. In crown imperial, miR171c may have a role in regulating bulb and fruit developmental process. In our analysis, Scarecrow-like protein 6 (SCL6) was identified as miR171c target in crown imperial, which is in accordance with the results obtained in Arabidopsis, *Pinus densata* and maize [10, 66–68]. Similarly, miR393 showed complex expression profile in crown imperial that supports possible multiple roles, at least the ones involved in flower, bulb and fruit development. In barley miR393 showed multiple roles including regulation of seedling growth, stomatal density and drought stress tolerance [69]. In rice, expression of miR393a and miR393b exhibited different trends, with miR393a detected in roots and miR393b detected mostly in aerial organs, including young leaves and inflorescence tissues [17]. Additionally, in pear miR393 showed a gradually down-regulation during the fruit development [70]. Predicted gene targets of crown imperial miR393 included F-box/RNI-like superfamily protein TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and calcineurin-like metallo-phosphoesterase domain-containing protein. This target prediction is supported, at least partially, by earlier reports in Arabidopsis, in which miR393 was found to target TIR1 and AFB2 auxin receptors [71–73].

Even though the results of the analysis of majority of conserved Fim-miRNAs were concordant with previous reports, several potentially novel data, regarding some of Fim-miRNAs roles and target genes were revealed, requiring further investigation of these predictions by experimental testing. Due to the differences in plants' genetic backgrounds and different diversification of conserved miRNA families in different plant species, it would be recommendable to study sequences and functions of each of miRNAs families of interest in individual plant species, which was a guiding idea behind our research. In this way customized biotechnological approaches, based on miRNAs, could be developed in due time for treating individual plant species and enabling optimal outcome for intended improvement and/or preservation of various plant species.

Conclusion

In this study, using high-throughput sequencing method, 21 conserved miRNAs matching 15 miRNA families were identified in *F. imperialis*. Among identified conserved miRNA families in crown imperial, miR166 and miR169 were the most abundant ones, followed by miR396 family. The expression of seven conserved miRNAs was further investigated in different tissues and three developmental stages revealing the differential roles these miRNAs have in growth and development of crown imperial. The obtained results suggest that Fim-miR156b and Fim-miR169d-5p have wide developmental roles since they are abundantly present in all analyzed tissues during three analyzed developmental stages. On the other hand, Fim-miR169d-5p may be mostly involved in reproductive tissues development, Fim-miR393 and Fim-miR171c could be predominantly involved in the development of bulb, Fim-miR166a-5p could have a

role in the development of petal, sepal, and leaf, while Fim-miR396e-3p seems to be involved in both vegetative and reproductive tissues development. These findings have potential to significantly improve understanding of the molecular mechanisms underlying the growth and development of crown imperial, leading eventually to devising novel molecular strategies for preserving this endangered plant species.

Declarations

Author contributions

FAT, BS and SMF performed the experimental work. FAT, HF and SMF carried out the data analysis and interpretation. BS, BBĐ and HF designed and supervised the study. All authors have read and agreed to the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Research involving human participants and/or animals

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

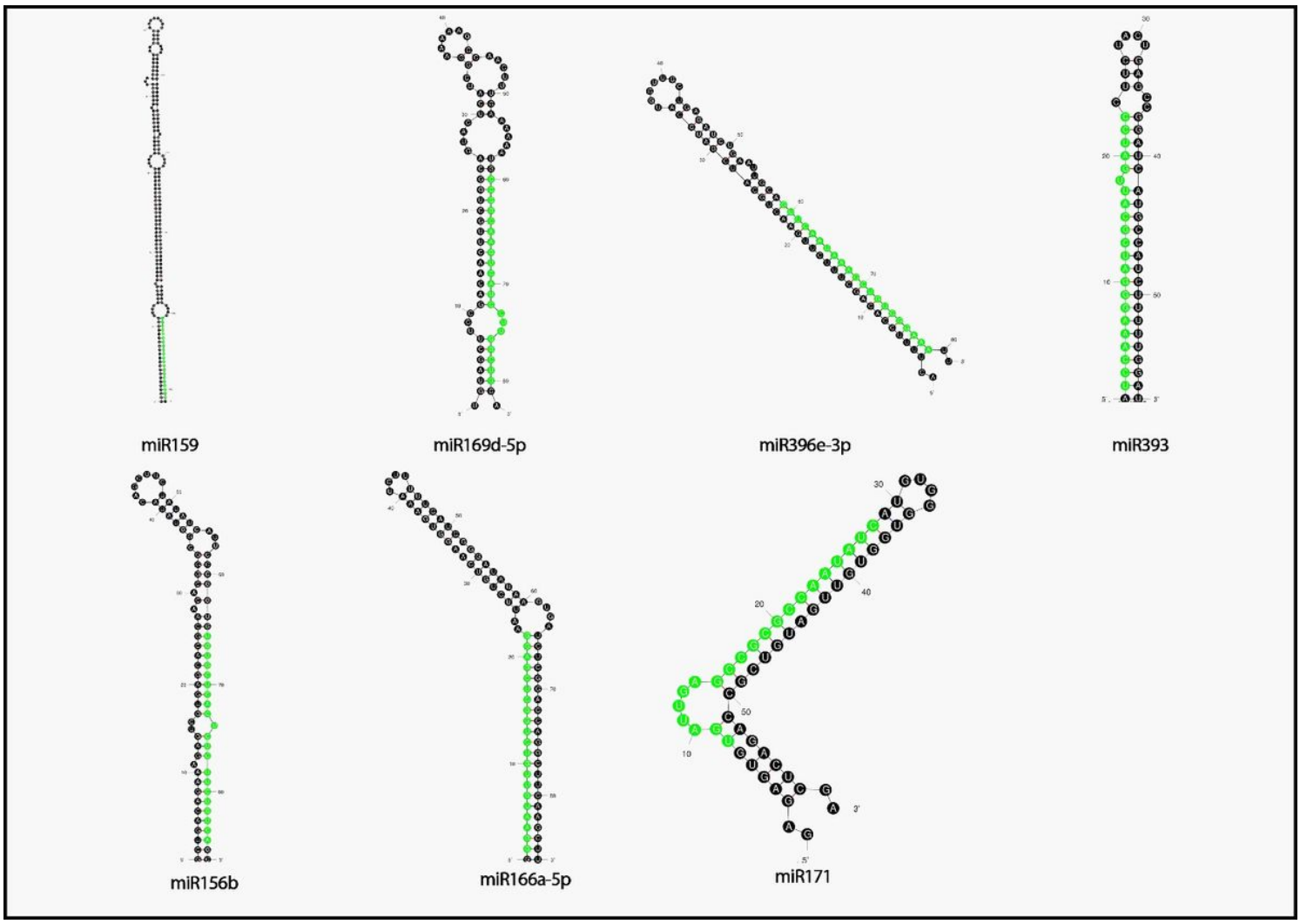

Figure 1

The structure of the 7 conserved miRNAs precursor in Fritillaria imperialis.

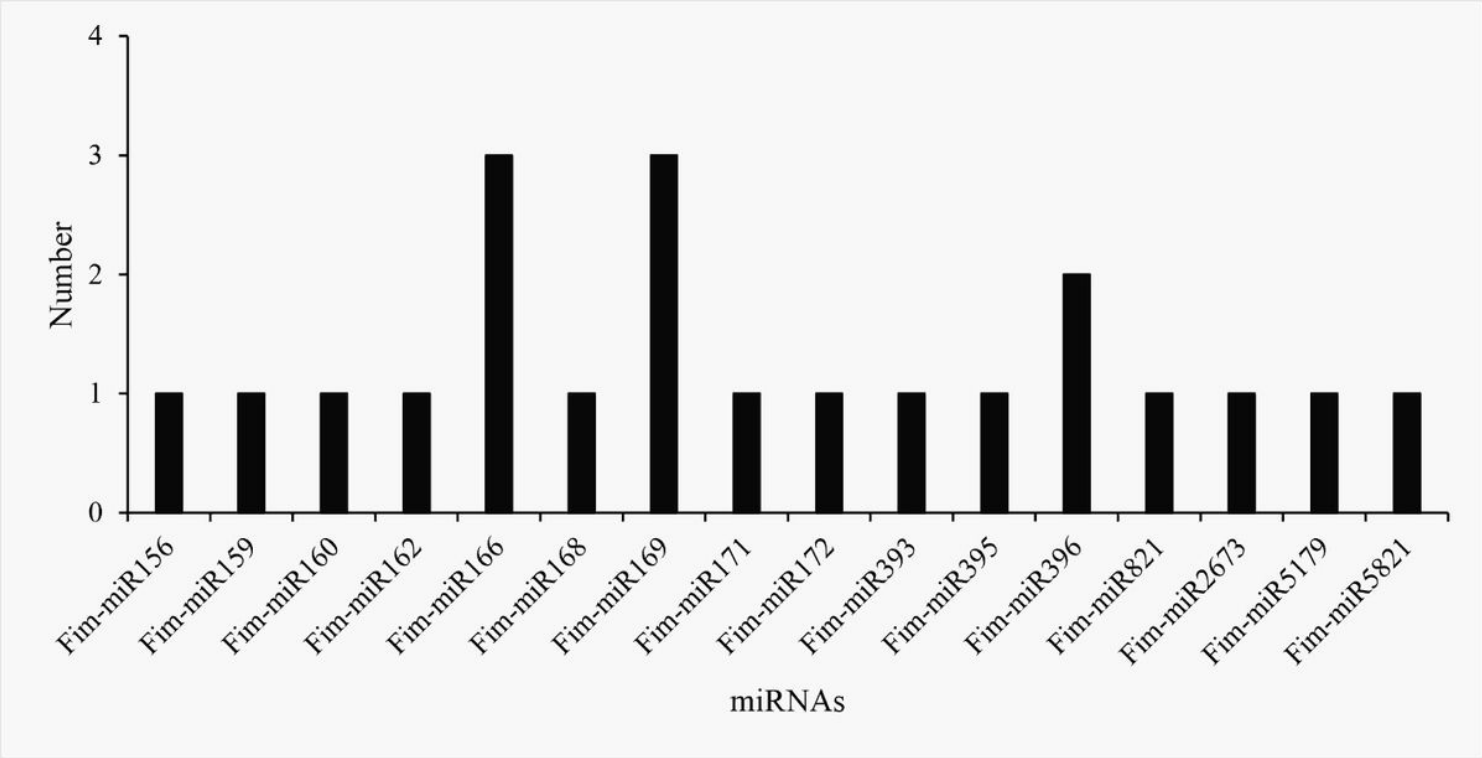

Figure 2

Conserved miRNA families and their members identified in F. imperialis.

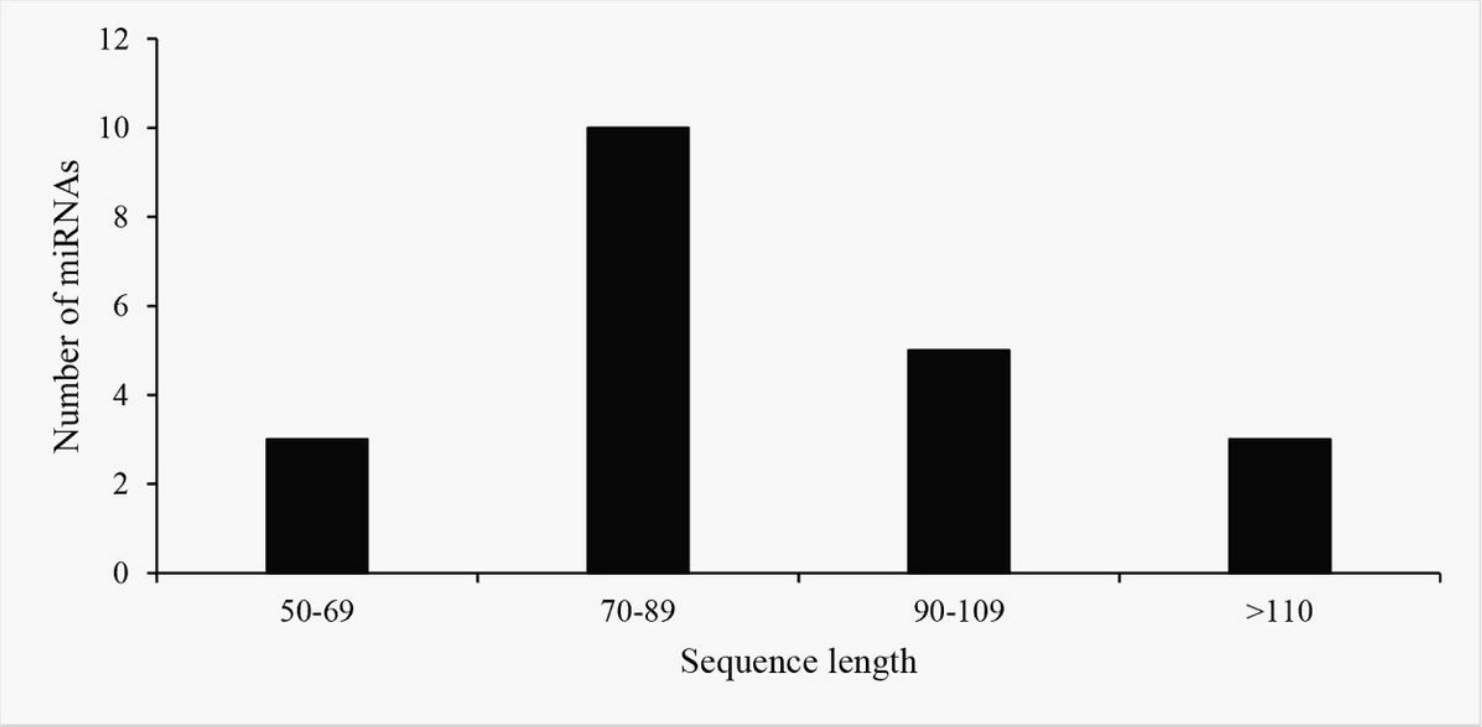

Figure 3

Size distribution of pre-miRNAs in F. imperialis.

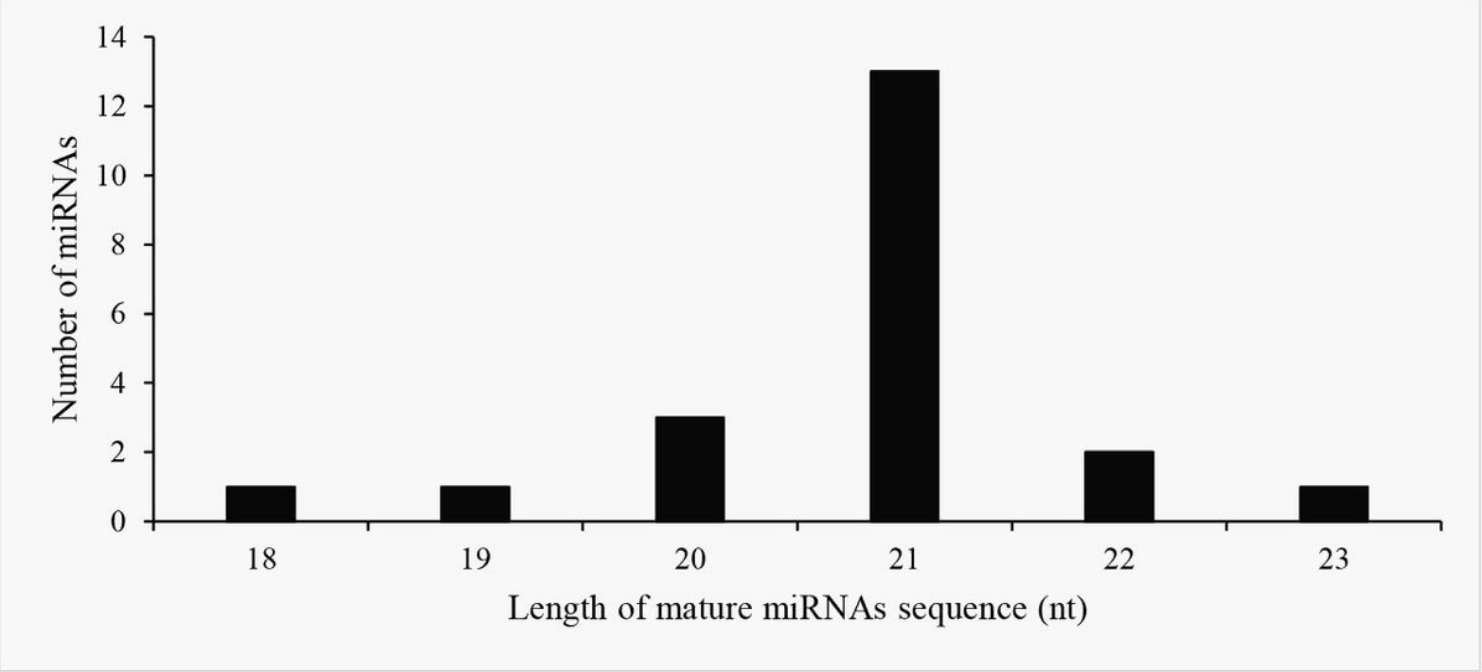

Figure 4

Size distribution of mature miRNAs in F. imperialis.

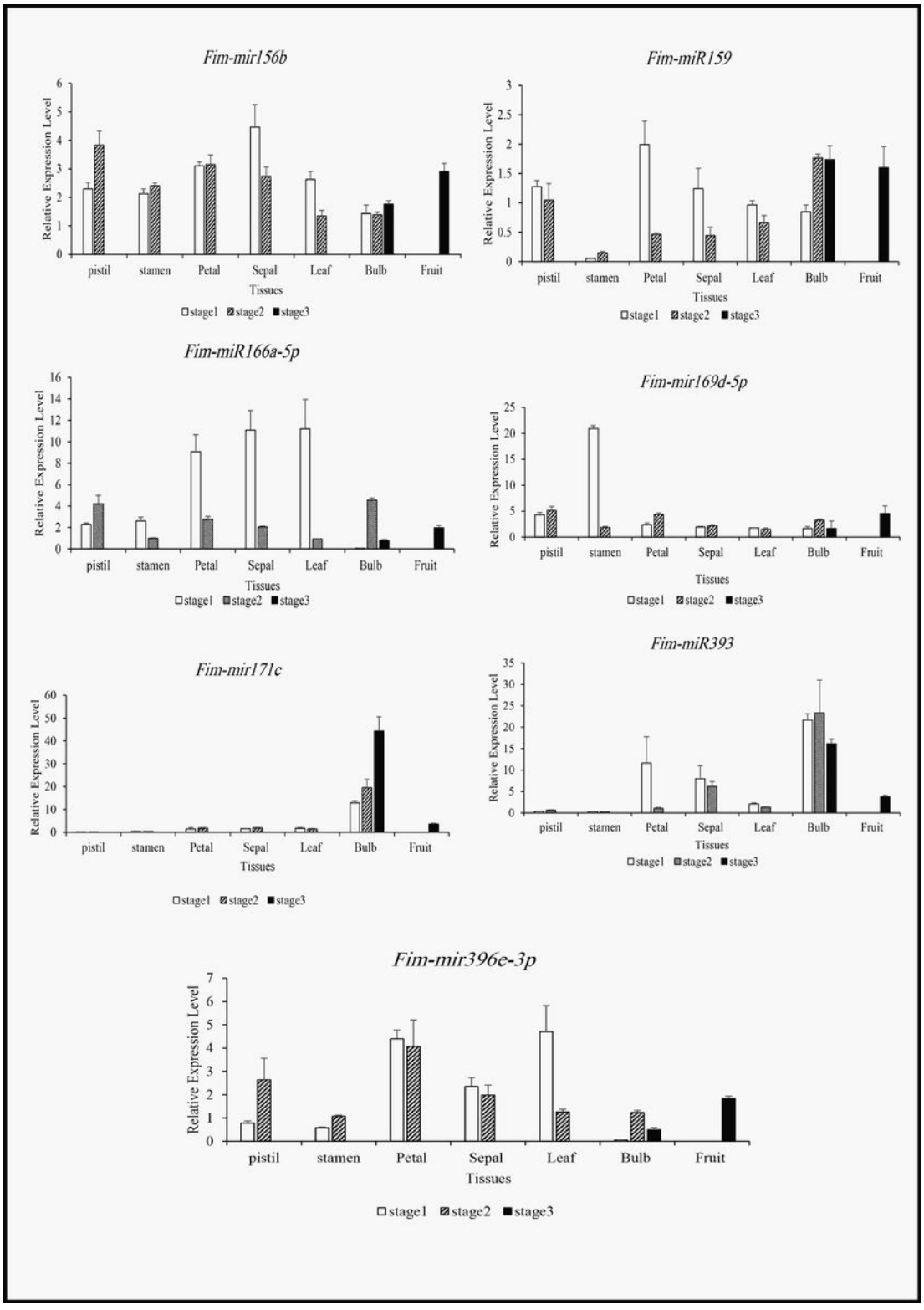

Figure 5

qRT-PCR validation and expression analysis of miRNAs in different tissues and developmental stages of F. imperialis.

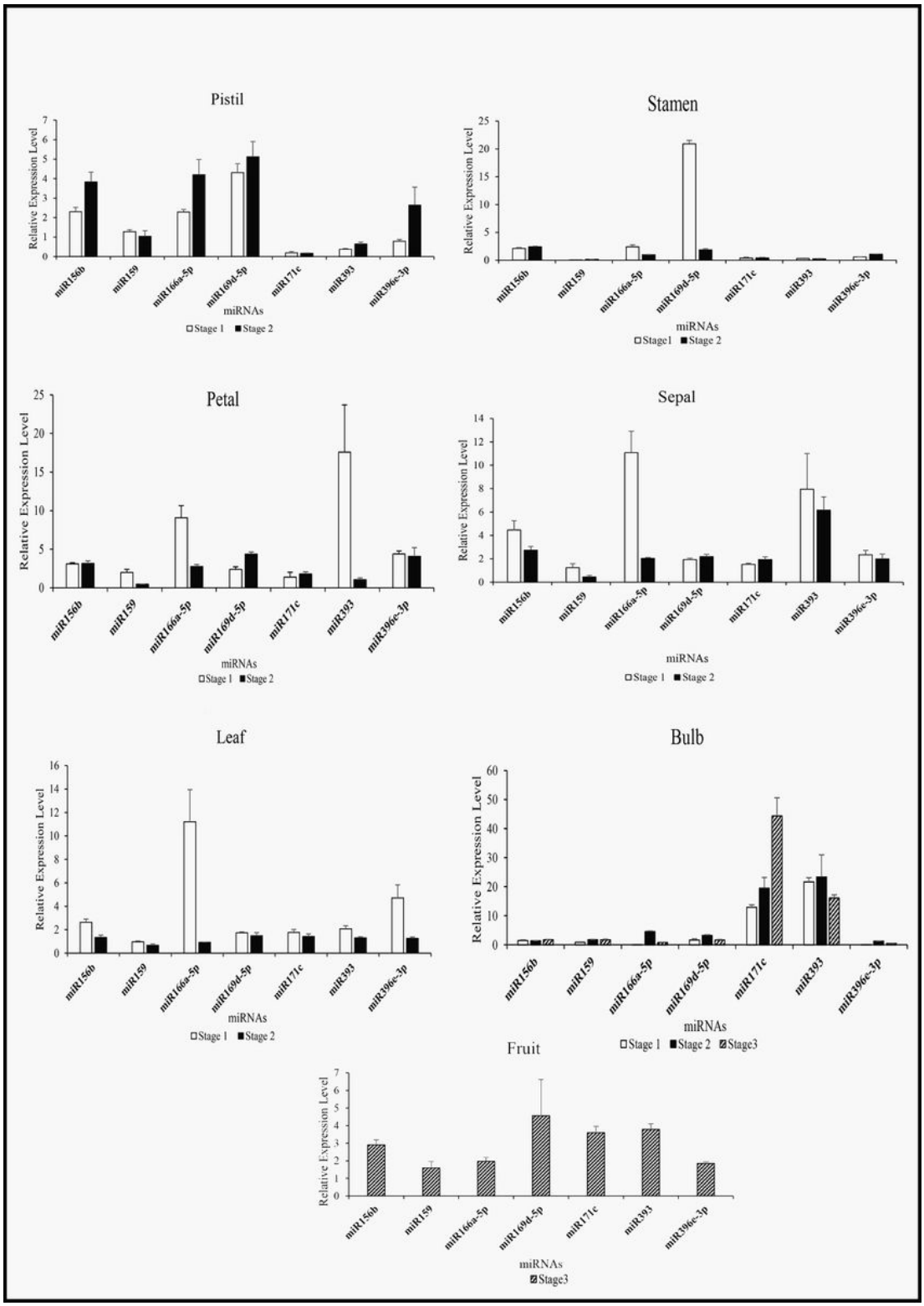

Figure 6

The pattern of expression of seven miRNAs in several stages at seven tissues of F. imperialis.

Supplementary Files

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- TableS1.xlsx
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