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BRIEF COMMUNICATION

Tissue Culture Responsive MicroRNAs in Strawberry

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Abstract MicroRNAs (miRNAs) are 20–24 nucleotide (nt) non-coding regulatory RNAs which play critical roles in plant growth and development. miRNA-encoding genes, which are transcribed by RNA polymerase II, are involved in a variety of processes, including developmental and morphogenesis systems, and hormone and stress responses. To investigate miRNA responses to tissue culture conditions, quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was used to detect differences in miRNA expression between in vitro micropropagated strawberry plants and transplanted micropropagated strawberry plants. Four miRNAs were differentially expressed between them, including one up-regulated gene (miR156) and three down-regulated genes (miR164, miR172 and miR390). The ratios of miRNA expression levels in in vitro micropropagated strawberry plants to micropropagated plants transplanted into soil in greenhouse for 4 months for miR156, miR164, miR172 and miR390 were 6.757, 0.046, 0.035 and 0.050, respectively. The ratio of miR156 expression levels in micropropagated plants transplanted into soil for 5 months to levels in the conventionally propagating runner plants was 3.785. miR156 was expressed highly and was strikingly inversely proportional to the expressions of its target gene *SPL9* and miR172 in in vitro micropropagated strawberry plants. We speculate that high expression of miR156 is the main reason for rejuvenation in micropropagated plants.

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Abbreviations

qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
IBA	Indole-3-butyric acid
SPL	Squamosa promoter binding protein like
dNTP	Deoxynucleotide triphosphate
RT	Reverse transcription
PCR	Polymerase chain reaction

Introduction

MicroRNAs (miRNAs) are 20–24 nucleotide (nt), non-coding regulatory RNAs which are generated from endogenous loci that produce transcripts with internal stem-loop structures (Rhoades et al. 2002; Yu et al. 2010). miRNAs down-regulate gene expression by guiding targeted mRNA cleavage or translational inhibition (Bartel 2004). miRNAs in plants arise from defined genetic loci known as MIR genes. Most characterized eukaryotic MIR genes are transcribed by RNA polymerase II (Lee et al. 2004), suggesting that miRNA transcription may be regulated by a similar mechanism as that established for protein-coding genes (Megraw et al. 2006).

Multiple lines of evidence suggest that miRNAs are important regulators of many aspects of plant growth and development (Chen 2004; Lauter et al. 2005). Mutations in individual miRNAs or disruptions in their ability to properly regulate their targets have been shown to cause abnormal developmental phenotypes, e.g., loss of organ polarity and altered vascular development, floral and leaf-

patterning defects, defective organ separation and aberrant number of floral organs, floral development and timing defects, aberrant phyllotaxis and abortion of the shoot apical meristem, and symmetry defects in cotyledon and leaf shape (Dugas and Bartel 2004; Axtell and Bartel 2005; Tsaftaris et al. 2011). Increasing evidence indicates that some miRNAs respond to biotic and abiotic stresses (Sunkar et al. 2007). miRNA expression were altered in plants infected with viruses (Bazzini et al. 2007), fungi (Lu et al. 2007) or bacteria (Navarro et al. 2008). Under abiotic stresses, such as cold, drought, salinity, UV-B radiation, phosphate or sulfate starvation, oxidative stress, or mechanical strain, the expression of specific plant miRNAs was also altered (Lu et al. 2008). For example, in *Arabidopsis* plants subjected to oxidative stress inducers, such as high light and heavy metal, Ath-miR398 is down-regulated (Sunkar et al. 2006).

Plant tissue culture is now a proven technology for the in vitro production of large numbers of genetically identical plants. One drawback to this method is the appearance of epigenetic variations in plants. Each plant tissue culture is a microsystem in which cell division, cell differentiation and developmental processes take place on successively renewed, identical or different culture media, in different types of semi-hermetic containers. From the beginning of culture to the end of the cycle, the explants are submitted to unusual culture and environmental conditions compared to the greenhouse or field, such as high osmoticity, abnormal mineral nutrition, unusual hormonal treatment (high cytokinin and/or auxin application), high relative humidity in the flask atmosphere, and possible accumulation of different gases (ethylene notably) in the contained atmosphere (Gaspar et al. 2002). Under these conditions, epigenetic variations will appear (Joyce et al. 2003; Fiuk et al. 2010; Swapna et al. 2011), but the molecular basis of epigenetic variation induced by tissue culture is not precisely known.

The strawberry plant (*Fragaria* spp.) is a herbaceous perennial plant that propagates vegetatively, and is also one of the most widely consumed fruits throughout the world. Micropropagation using tissue culture technology has been used commercially in Europe since the 1970s (Boxus 1974) to multiply elite cultivars, and its use is now growing in China. Despite the widespread cultivation of micropropagated strawberry plants, reports have shown that micropropagation affects the growth characteristics of strawberry plants. For instance, micropropagated strawberry plants exhibit epigenetic variation in leaf and leaflet area, hairiness of leaf stalks, runner number, and tissue culture-induced rejuvenation (Mohamed et al. 1991; Karhu 2001; Szczygiel et al. 2002; Zhang et al. 2006). Although there are no reports on changes in miRNAs expression induced by tissue culturing of strawberry plants, in an earlier study we found that miR390 expression was lower in micropropagated strawberry plants that were transplanted into pots and grown in a greenhouse for 110 days compared to miR390 expression in conventionally propagated plants (Li et al. 2009). In order to determine whether the changes in miRNAs expression in transplanted micropropagated plants are affected during the tissue-culture process and to elucidate the relationship between the epigenetic variation in tissue-cultured plants and the expression of miRNAs, we compared the expression of some conserved miRNAs in in vitro strawberry plants to that in transplanted plants using quantitative reverse transcription–polymerase chain reaction (qRT-PCR).

Materials and Methods

Plant Material

The strawberry plant (*Fragaria*× *ananassa*) cultivar 'Allstar' was maintained in a greenhouse at Shenyang Agricultural University and conventionally propagated by runner plants. Tissue culture propagation was done as follows: runners were collected from actively growing plants and disinfected with 0.1% w/v $HgCl_2$ as described by Dai et al. (2007). Shoot tips of ~0.3 mm in length were excised from each runner and placed on MS medium (Murashige and Skoog 1962) supplemented with 0.2 mg/l 6-benzylaminopurine, 0.1 mg/l gibberellic acid and 0.01 mg/l indole-3-butyric acid (IBA) to establish in vitro shoot proliferation. In vitro plants were sub-cultured on the same medium at 5-week intervals. Unrooted plantlets were transferred to half-strength MS medium supplemented with 0.01 mg/l IBA for rooting. The explants were cultured in 100-ml bottles under white fluorescent light (60 μmol s^{-1} m^{-2}) in a 14-h light photoperiod. The temperature of the culture room was maintained at 23±2°C.

In the spring the rooted plants were transferred to plug trays containing peat moss, vermiculite and soil (2:1:1 v/v) in the greenhouse, and then the surviving micropropagated plants were replanted in 12-cm plastic pots. After 1 month, the plants were replanted in the field to propagate the first runner generation of micropropagated plants. During autumn, the runner plants were planted in the greenhouse, and they began flowering and fruiting the next spring.

To analyze the changes in miRNA expression in response to the conditions of tissue culture, five types of plants were used: (1) in vitro micropropagated plants (M0), (2) micropropagated plants transplanted into soil in the greenhouse for 4 months and which were beginning to produce runners (M0-4), (3) the first runner generation of micropropagated plants at the flowering stage (M1-F), (4) the first runner generation of micropropagated plants at the beginning of berry harvest stage (M1-B), (5) the first runner generation of micropropagated plants at the end of berry harvest and the

beginning of runner development stage (M1-R). In order to detect whether the changes in miR156 expression in transplanted micropropagated plants are affected during the tissue-culture process, two types of plants similar in plant size, vigor and developmental stage were used: (1) micropropagated plants transplanted into soil for 5 months (M0-5) and (2) conventionally propagating runner plants (C0). In order to validate whether the expression of miR156 is inversely related to the expression of its target gene *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (SPL) as well as miR172, micropropagated strawberry plants at six different stages were used: in vitro plants (M0) and micropropagated plants transplanted into soil for 1 month (M0-1), 2 months (M0-2), 3 months (M0-3), 4 months (M0-4) and 5 months (M0-5). Young leaves were collected from all above-mentioned plants, flash frozen in liquid nitrogen and stored at −70°C.

Total RNA Extraction

Total RNA was isolated from young leaves as described by Chang et al. (2007). Before precipitation, the RNA was treated with 15 U RNase-free DNase I (TaKaRa Biotechnology Co., Dalian, China) at 37°C for more than 4 h. RNA integrity was assessed on a 1.0% agarose gel, and concentration was estimated using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

qRT-PCR

qRT-PCR using a stem-loop primer was performed using a standard *TaqMan* PCR protocol (Chen et al. 2005) on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The RT primers (Table 1) were based on those described by Chen et al. (2005). Their 3′ end was complementary to both the ~8 nt at the miRNA 3′ end and the ~7 nt at the primer's 5′ end, to ensure the formation of a stem-loop structure. Subsequent PCRs used a 5′ primer matching the ~10 nt at the 5′ end of the target miRNA and a universal reverse 3′ primer (Table 1).

The 20-μl RT reactions contained 2 μg total RNA, 1 μl 5 μM stem-loop primer, 1 μl 10 mM dNTPs, 1 μl 5 U/μl AMV reverse transcriptase (TaKaRa Biotechnology Co, Dalian, China), 4 μl 5×AMV buffer and 1 μl 40 U/μl RNase inhibitor. The template, primer and dNTP mixture was heated to 65°C for 5 min, and quenched on ice for at least 5 min. Then the remaining reagents were added, and the complete reaction incubated at 16°C for 30 min, followed by 60 cycles of 20°C/30 s, 42°C/30 s and 50°C/1 s. The AMV reverse transcriptase was inactivated by heating the reactions to 85°C for 10 min, and then cooling and holding at 4°C. The RT reactions for 18S ribosomal RNA (rRNA) and *SPL9* were only different in RT-primer and cycling conditions which were completed by using 0.5 μl 50 μM oligo d(T)18 and 0.5 μl 50 μM random primer (9-mer) and incubating at 37°C for 2.5 h and 70°C for 15 min.

The 20-μl PCR reactions included 1 μl RT product, 8 μl 2.5× realMaster Mix (TianGen, Beijing, China), 1 μl 20× probe enhancer solution, 0.5 μl 10 μM TaqMan probe, 1 μl 10 μM forward primer and 1 μl 10 μM reverse primer. The reactions were incubated in a 96-well plate (Applied Biosystems) at 95°C for 10 min, followed by 40 cycles of 95°C/15 s and 60°C/60 s.

Relative fold changes in miRNA expression were calculated using the comparative C_t ($2^{-\Delta\Delta Ct}$) method with 18S rRNA as the endogenous control. Two biological replicates were performed, and in each biological replicate the genes and no template control were carried out in triplicate. The threshold cycle (C_t) was defined as the cycle number at which the fluorescence signal exceeded the fixed threshold.

Results and Discussion

In order to quantify the changes in miRNAs expression in strawberry plants in response to the tissue culture conditions, five types of plants were used (M0, M0-4, M1-F, M1-B and M1-R). RT-PCR using stem-loop primers is a method with high sensitivity and specificity for detecting miRNAs in animals and plants (Chen et al. 2005; Tang et al. 2006; Varkonyi-Gasic et al. 2007). In this study, qRT-PCR with the stem-loop primer was performed using a standard *TaqMan* PCR protocol to detect the presence of miRNAs in young strawberry leaves. According to our previous study, 74 miRNAs in strawberry were detected by microarray, and 14 of them were verified by RT-PCR (Li et al. 2009). Considering their biological functions in other plants, six highly conserved miRNAs (miR156, 159, 164, 165, 167 and 172) were selected for further analysis of their expression difference in the five types of plants. The results showed that all six miRNAs were detectable in all five types of strawberry plants.

A criterion of fold change >2 was used to examine the differentially expressed miRNAs by qRT-PCR. The in vitro micropropagated plants (M0) are of the same genotype as the transplanted micropropagated plants including M0-4, M1-F, M1-B and M1-R. The expression levels of six miRNAs were analyzed between the in vitro micropropagated plants and the transplanted micropropagated plants (Fig. 1). When the relative quantification of six miRNAs in M0 was set as 1, the results revealed that the highest expression of miR156 was in M0 (1), much higher than the quantification of M0-4 (0.148), M1-F (0.036), M1-B (0.067) and M1-R (0.074). The ratios for M0/M0-4, M0/M1-F, M0/M1-B and M0/M1-R were 6.757, 27.778, 14.925 and 13.512, respectively. The expression trend among the five types of plants for miR164 and miR172 was the same. The expression of miR164 and miR172 was lowest in M0. The ratios of miR164 and miR172 levels for M0/M0-4

Table 1 Primers used to perform qRT-PCR on miRNAs and *SPL9*

Name		Sequence (5′ to 3′)
miR156	RT primer	ctcaactggtgtcgtggagtccggcaattcagttgaggtgctcac
	PCR primer	Forward: acactccagctgggtgacagaaga
		Reverse: aactggtgtcgtggag
	Probe	FAM-ttcagttgaggtgctcac-TAMRA
miR159	RT primer	ctcaactggtgtcgtggagtccggcaattcagttgagtagagctc
	PCR primer	Forward: acactccagctgggtttggattga
		Reverse: aactggtgtcgtggag
	Probe	FAM-ttcagttgagtagagctc-TAMRA
miR164	RT primer	ctcaactggtgtcgtggagtccggcaattcagttgagtgcacgtg
	PCR primer	Forward: acactccagctgggtggagaagca
		Reverse: aactggtgtcgtggag
	Probe	FAM-ttcagttgagtgcacgt-TAMRA
miR165	RT primer	ctcaactggtgtcgtggagtccggcaattcagttgaggggggatg
	PCR primer	Forward: acactccagctgggtcggaccagg
		Reverse: aactggtgtcgtggag
	Probe	FAM-ttcagttgaggggggatg-TAMRA
miR167	RT primer	ctcaactggtgtcgtggagtccggcaattcagttgagtagatcat
	PCR primer	Forward: acactccagctgggtgaagctgcc
		Reverse: aactggtgtcgtggag
	Probe	FAM-ttcagttgagtagatcat-TAMRA
miR172	RT primer	ctcaactggtgtcgtggagtccggcaattcagttgagatgcagca
	PCR primer	Forward: acactccagctgggagaatcttga
		Reverse: aactggtgtcgtggag
	Probe	FAM-ttcagttgagatgcagca-TAMRA
miR390	RT primer	ctcaactggtgtcgtggagtccggcaattcagttgagggcgctat
	PCR primer	Forward: acactccagctgggaagctcagga
		Reverse: aactggtgtcgtggag
	Probe	FAM-ttcagttgagggcgctat-TAMRA
SPL	RT primer	oligo d(T)18; random primer (9 mer)
	PCR primer	Forward: ggagaatgcgtcacaggagtcac
		Reverse: gcctcatttcccttaaaacccca
	Probe	FAM-tctcttctgtcaagtcaaccatgggcc-TAMRA
18S rRNA	RT primer	Oligo d(T)18; random primer (9 mer)
	PCR primer	Forward: gtagtcatatgcttgtct
		Reverse: gaatgatgcgtcgccagcacaaagg
	Probe	FAM-cagaagtcgggatttgttgc-TAMRA

FAM 6-carboxy-fluorescein, reporter fluorophore, *TAMRA* 6-carboxy-tetramethylrhodamine, quencher fluorophore

were 0.046 and 0.035, respectively. The expression of miR159 and miR167 in M0 were also lower than those in M0-4, but higher than those in M1-F. There was no obvious trend for miR159 and miR167 expression levels in in vitro micropropagated plants compared to transplanted micropropagated plants. Moreover, the fold changes of the expression levels of miR159 and miR167 were obviously lower than those of miR164 and miR172 among the five types of plants. This similar phenomenon was also found with miR165. In our previous study, both the microarray and qRT-PCR data showed that the expression of miR390 was down-regulated in the transplanted micropropagated strawberry plants compared to the conventionally-propagated plants, and the ratios of transplanted micropropagated plant/conventionally propagated plant were 0.441 (microarray) and 0.262 (qRT-PCR), respectively (Li et al. 2009). To investigate whether the down-regulated expression of miR390 in transplanted micropropagated plants was affected by tissue culture conditions, we compared the

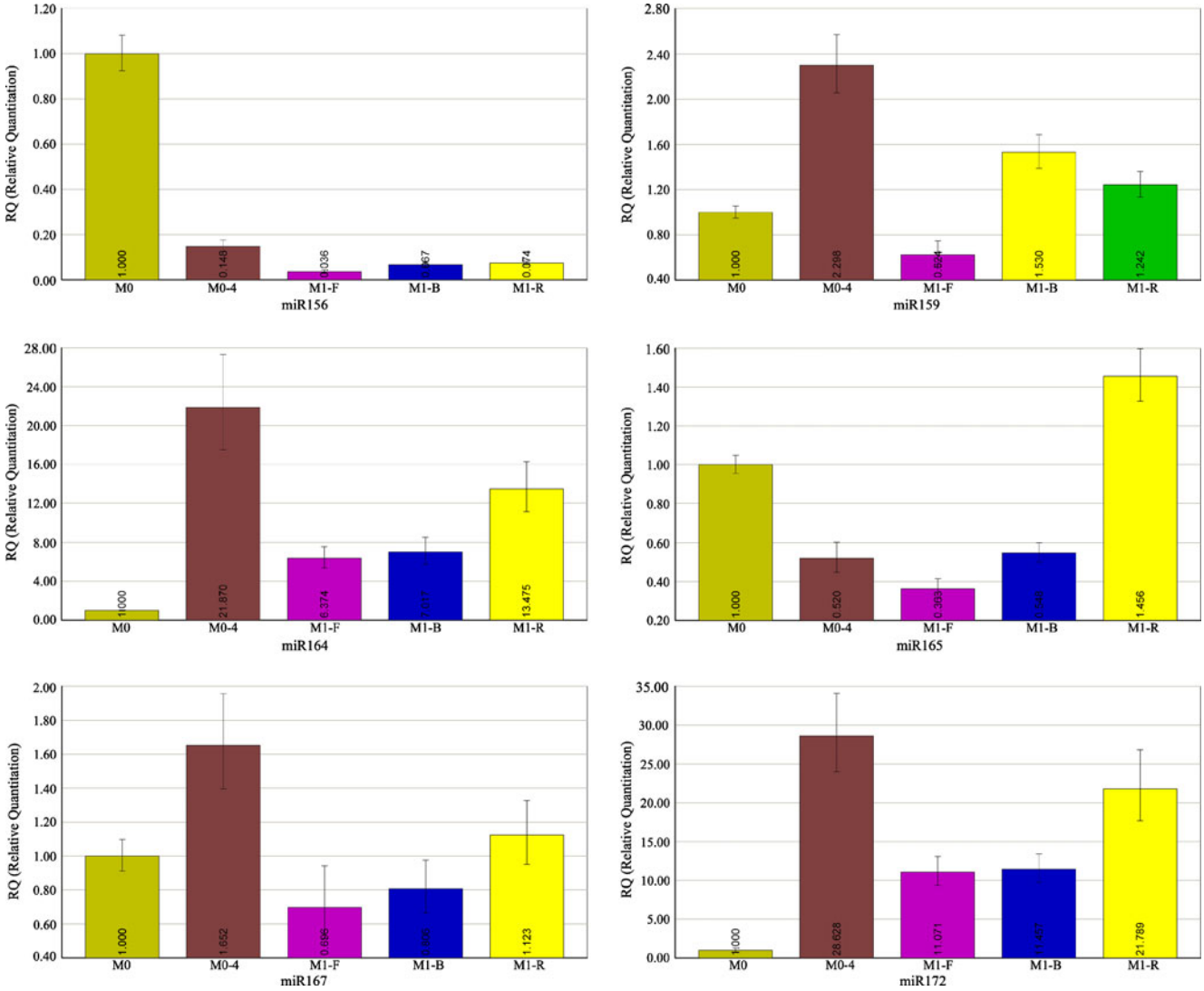

Fig. 1 miRNAs were expressed differentially among M0, M0-4, M1-F, M1-B and M1-R. *M0* in vitro micropropagated plants, *M0-4* micropropagated plants transplanted into soil in greenhouse for 4 months, *M1-F* the first runner generation of micropropagated plants at flowering stage, *M1-B* the first runner generation of micropropagated plants at the beginning of berry harvest stage, *M1-R* the first runner generation of micropropagated plants at the end of berry harvest and the beginning of runner development stage

expression of miR390 between the in vitro micropropagated plants (M0) and the transplanted micropropagated strawberry plants (M0-4). The ratio of M0/M0-4 (ratio=1:20.08) was 0.050. So, the above results showed that the expressions of miR156, miR164, miR172 and miR390 were obviously affected by tissue culture conditions. Among them, miR156 was up-regulated and miR164, miR172 and miR390 were down-regulated in in vitro micropropagated plants.

In order to determine whether the changes of miRNA expression in tissue-cultured plants would recover to normal after the in vitro plants were transplanted into soil, miR156, the up-regulated gene was selected and analyzed in two types of plants—M0-5 and C0—of similar size, vigor and developmental stage. The ratio of M0-5/C0 (ratio=1:0.2642) was 3.785. Thus, we speculated that the expression difference in miR156 was transmitted from in vitro micropropagated plants to transplanted plants.

In both maize and *Arabidopsis*, miR156 and miR172 expression levels are inversely related (Chuck et al. 2007; Wu et al. 2009). The above results (Fig. 1) also showed the same pattern. Additionally, a growing body of evidence suggests that the *SPL* gene family has been validated as the target of miR156. Ten out of the 16 *SPL* genes in *Arabidopsis*—including *SPL2*, *SPL3*, *SPL4*, *SPL5*, *SPL6*, *SPL9*, *SPL10*, *SPL11*, *SPL13* and *SPL15*—have a miR156 target site (Wu et al. 2009). The mechanistic relationship between miR156 and miR172 in *Arabidopsis* is that *SPL9* and *SPL10* activate the gene encoding a miR172 precursor and that *SPL9* binds directly to the promoter of this precursor gene (Fornara and Coupland

2009; Wu et al. 2009). We have isolated the *SPL9* gene (accession no. JN979457) from cultivated strawberry (*F.* × *ananassa*) (Zhao et al. 2011), which contains the miR156 (5′-UGACAGAAGAGAGUGAGCAC-3′) recognition sites (5′-GTGCTCTCTCTCTTCTGTCA-3′). To validate whether the *SPL9* gene is repressed by miR156 and the expression of miR172 is inversely related to the expression of miR156 in strawberry, the expressions of miR156, *SPL9* and miR172 in strawberry leaves were compared in micropropagated plants at different stages. When the relative quantification of three genes in M0 was set as 1 (Fig. 2), the results showed that the highest expression of miR156 was also in M0 (1), higher than that in M0-1 (0.588), M0-2 (0.161), M0-3 (0.253), M0-4 (0.195) and M0-5 (0.130). However, the lowest expression of *SPL9* was in M0 (1), obviously lower than that in M0-1 (4.196), M0-2 (5.923), M0-3 (4.374), M0-4 (3.395) and M0-5 (2.907). So we consider that the expression levels of *SPL9* gene showed some trade-off correlation with those of miR156 (Fig. 2a, b), especially in M0, M0-1 and M0-2. Additionally, the lowest expression of miR172 (Fig. 2c) was also in M0 (1), obviously lower than that in M0-1 (9.063), M0-2 (22.668), M0-3 (26.759), M0-4 (22.289) and M0-5 (29.922). Among them, the qRT-PCR result showed that the expression of miR172 (Fig. 2c) was inversely related with the expression of miR156 (Fig. 2a), and it was on the whole consistent with the expression of *SPL9* gene from M0 to M0-2 (Fig. 2b).

Epigenetic changes occurring in in vitro culture can result in 'rejuvenation' affecting woody and herbaceous plants (Cassells and Curry 2001). The term rejuvenation implies a reversal of the maturation process. There are numerous reports that in vitro multiplication of shoots from mature shoot tips or axillary buds through several subcultures results in restoration of some juvenile characteristics, including increased vigor (Jones and Hadlow 1989), high rooting competency (Welander 1985), juvenile leaf morphology (Joyce and Cassells 2002) and delayed flowering (Hammatt 1999). However, the molecular mechanism of rejuvenation is still unknown. The transition from the juvenile to the adult phase of shoot development in plants is accompanied by changes in vegetative morphology and an increase in reproductive potential. miR156 is necessary and sufficient for the expression of the juvenile phase, and regulates the timing of the juvenile-to-adult transition by repressing the expression of *SPLs* transcription. miR172 acts downstream of miR156 to promote adult epidermal identity (Wu et al. 2009). miR156 and miR172 are positively regulated by the transcription factors they target, suggesting that negative feedback loops contribute to the stability of the juvenile and adult phases (Rubio-Somoza and Weigel 2011). In this study, we found that miR156 was expressed highly in in vitro strawberry plants (Figs. 1 and 2a). This may be the main reason why micropropagated plants exhibit rejuvenation. Further studies are required to investigate why the expression of miR156 responds to tissue culture conditions.

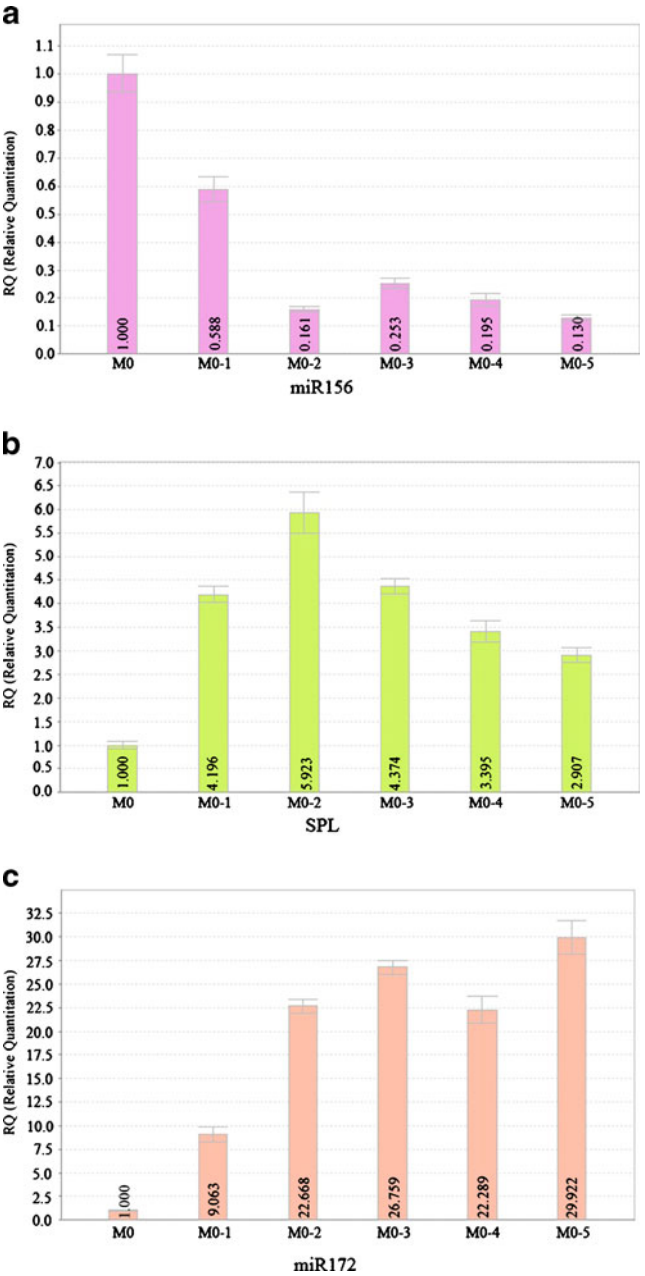

Fig. 2 Comparative **a** miR156, **b** *SPL* and **c** miR172 expression in six different types of plants: in vitro plants (*M0*) and micropropagated plants transplanted into soil for 1 month (*M0-1*), 2 months (*M0-2*), 3 months (*M0-3*), 4 months (*M0-4*) and 5 months (*M0-5*)

miRNAs have emerged as an essential regulatory component in plants. Many of the known miRNAs are evolutionarily conserved across diverse plant species and function in the regulatory control of fundamentally important biological processes such as developmental timing, patterning and response to environmental

changes (Xie et al. 2010). A growing body of evidence suggests that functional diversity of plant miRNA has been found. For example, several NAC-domain genes, including *CUC1*, *CUC2* and *NAC1* in *Arabidopsis*, are subject to negative control by miR164 (Yang et al. 2007; Larue et al. 2010). Analysis of miR164 mutations and overexpression has further revealed the importance of these genes for proper plant development (Mallory et al. 2004; Sieber et al. 2007; Raman et al. 2008). Expression of a miR164-resistant form of CUC1 in wild type plants resulted in reduced sepal number, increased petal number, and broadened leaves (Mallory et al. 2004). ORE1, which is also a NAC transcription factor, positively regulates aging-induced cell death in *Arabidopsis* leaves. *ORE1* expression is up-regulated concurrently with leaf aging by *EIN2* but is negatively regulated by miR164 (Kim et al. 2009). A *NAP* sequence of *Crocus* contains a possible target site for miR164, and *CsatNAP* showed increased expression in senescence leaves compared to the green ones (Kalivas et al. 2010). In our study, the expression of miR164 is obviously affected by tissue culture conditions. Further experiments are needed to find out why it is suppressed in in vitro micropropagated plants.

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