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SHORT REPORTS

Identification and characterization of microRNAs and their targets in high-altitude stress-adaptive plant maca (*Lepidium meyenii* Walp)

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Abstract MicroRNAs (miRNAs) are endogenous, short (~21-nucleotide), non-coding RNA molecules that play pivotal roles in plant growth, development, and stress response signaling. In this study using recently published draft genome sequence of a high-altitude plant maca (*Lepidium meyenii* Walp) and applying genome-wide computational-based approaches, a total of 62 potentially conserved miRNAs belonging to 28 families were identified and four (lme-miR160a, lme-miR164c, lme-miR 166a, and lme-miR 319a) of them further validated by RT-PCR. Deploying psRNATarget tool a total of 99 potential miRNA target transcripts were also identified in maca. Targets include a number of transcription factors like Squamosa promoter-binding, NAC, MYB, auxin response factor, APETALA, WRKY, and F-box protein. To the best of my knowledge, this is the first genome-based miRNA profiling of a high-altitude plant.

Keywords Maca (*Lepidium meyenii* Walp) · High-altitude plant · Stress · miRNA · MFEI · miRNA targets

Abbreviations

miRNA	MicroRNA
MFE	Minimum folding free energy
MFEI	Minimum folding free energy index

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Introduction

Maca (*Lepidium meyenii* Walp), belonging to the Brassicaceae family, is an economically important plant cultivated in the central Andean region at 4000–4450 m above sea level. Its tremendous health benefits, particularly to reproduction and fertility, have drawn great investments from pharmacological research in recent years (Piacente et al. 2002; Shin et al. 2010). Maca roots contain several secondary metabolites of interest including glucosinolates, fatty acid esters, phytosterols, alkaloids, and alkamides (macamides) (Piacente et al. 2002). Due to its restricted cultivation area at high altitude, maca manifests strong endurance to extreme environmental conditions such as low temperatures in combination with high irradiance, strong winds, and oxidizing air pollutants.

MicroRNAs are endogenous, non-coding, small RNAs ranging in length from 20 to 24 nucleotides (Huang et al. 2016). Post-transcriptional gene regulation mediated by endogenous miRNAs play a crucial role in various aspects of plant development as well as adaptation to biotic and abiotic stresses (Naya et al. 2014; Khraiwesh et al. 2012; Paul et al. 2011; Kundu et al. 2017). In plants, mature miRNAs are generated from longer stem-loop RNA precursors (Pre-miRNA) with the aid of ribonuclease III-like Dicer (DCL1) enzyme (Fukudome and Fukuhara 2017). Despite the fact that miRNAs have a great role in stress responses, till date no scientific initiative has been taken to study maca miRNAs. Moreover, very few reports are available on medicinal plants for miRNA regulation of their bioactive compounds or secondary metabolites. With the recent draft genome sequence available (http://www.herbal-genome.cn/) (Zhang et al. 2016), it is important to exploit this information for better understanding the physiological processes in maca.

Materials and methods

Computational prediction of maca miRNAs

A set of total of 811 mature *Arabidopsis* miRNAs (downloaded from miRbase 21) were BLASTn search against maca genome and sequences with exact match were chosen manually. It is well documented that during in silico miRNA prediction least number of allowed mismatches between putative miRNAs and known miRNAs produce more accurate results and that is why maca miRNAs which showed 0 mismatches with known *Arabidopsis* miRNAs were chosen in this study. The possible precursor (pre-miRNA) sequences of approximately 400-nt (200 nt upstream and 200 nt downstream to the BLAST hit region) were extracted and sequences coded for proteins were removed. Stable secondary structures of the remaining precursor sequences were predicted using mfold web server (http://unafold.rna.albany.edu/?q=mfold/mfold-references) following previously described filtering criteria (Zhang et al. 2008) as follows: (1) the secondary structure of the precursor sequences should have the stem-loop structure that contains a mature miRNA sequence within one arm and no loop or break in the mature miRNA sequences; (2) the potential miRNA sequence should not be located on the terminal loop of the hairpin structure; (3) mature miRNAs should have fewer than nine mismatches with the opposite miRNA* sequence (Yang et al. 2010); and (4) the predicted stem-loop candidates should have higher MFEIs and negative minimum folding free energies. The formula for calculating MFEI is as follows:

$$\text{MFEI} = \frac{(\text{MFE}/\text{length of RNA sequence}) \times 100}{\%\text{GC content}}$$

Analysis of miRNA expression

For the experimental validation of some predicted maca miRNAs such as lme-miR160a, lme-miR164c, lme-miR 166a, and lme-miR 319a by RT-PCR (reverse transcription), small RNA was first isolated from maca leaves using mir Premier microRNA Isolation Kit (Sigma-Aldrich). 1 µg of aforesaid maca small RNA was polyadenylated (using modified oligo dT primer) and reverse transcribed at 37 °C for 1 h in 10 µl reaction mixture using Mir-X miRNA First-Strand Synthesis kit (Clontech). The obtained cDNA was then amplified by GeneAmp PCR system 2400 (Perkin Elmer) using entire predicted miRNA sequence as sense primer and adapter-specific mRQ 3′ primer provided with Mir-X miRNA qRT-PCR SYBR kit (Clontech) as antisense primer. 100 ng cDNA was used as template for the PCR. The PCR was programed as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 25 s, and a final elongation step at 72 °C for 7 min. The resulting PCR products (~70 bp) were checked in 2% agarose gel with EtBr staining.

Prediction of miRNA targets and their functional annotation

The Plant Small RNA Target Analysis Server (psRNATarget) was used in this study to predict maca miRNA targets (http://plantgrn.noble.org/psRNATarget/). Due to non-availability of maca protein database in psRNATarget server target transcript search was performed against protein database of *Arabidopsis thaliana*. The following parameters were employed in prediction of miRNA targets in maca: (a) maximum exception of 3.0, length of complementarity score: 20. (b) Target accessibility—allowed maximum energy to unpair the target site (UPE): 25. (c) Flanking length around the target accessibility analysis: 17 bp upstream and 13 bp downstream. (d) Range of central mismatch leading to translation inhibition: 9–11 nt.

Gene ontology analysis of the identified target transcript was executed by AmiGo (http://amigo.geneontology.org/amigo) and three important components such as biological process, cellular component, and molecular function associated with each GO term were inferred.

Results and discussion

Characterization of maca miRNAs

With high stringent filtering approach, a total of 62 potential conserved miRNAs belonging to 28 families were identified in maca (Table 1). Among them, 28 miRNAs (~45%) were located in 5′ arm of the precursor while 34 (~55%) located in 3′ arm suggesting that maca miRNAs are located in both the arms of the precursor void any preference. Precursors of maca miRNAs also showed great variability in their size ranging from 76 to 227 with an average of 117 ± 33 (Table 1) which represent good agreement with those reported for other plant species such as soybean, cotton, and maize (Zhang et al. 2008; Wang et al. 2011, 2012). lme-miR 2111b-3p showed the shortest precursor length of 76 nt while vra lme-miR 169a showed the longest one of 227 nt. The MFEI is a useful criterion for distinguishing miRNAs from other types of coding or non-coding RNAs. In this study, the identified precursors have higher MFEI values (0.73–1.43) with an average of 1.00 ± 14.0 which is much higher than that of tRNAs (0.64), rRNAs (0.59), or mRNAs (0.62–0.66), respectively (Zhang et al. 2006). The secondary structure of the

Table 1 Potential conserved miRNAs in maca

Identified miRNAs	LM (nt)	miRNA sequences	Input sequence	Strand	Location	LP (nt)	MFEs (ΔG)	MFEI
lme-miR156a	20	UGACAGAAGAGAGUGAGCAC	scaffold675	+/+	5′	91	−44.30	1.05
lme-miR156a-3p	22	GCUCACUGCUCUUUCUGUCAGA	scaffold568	+/+	3′	128	−47.90	0.87
lme-miR156b-3p	23	UGCUCACCUCUCUUUCUGUCAGU	scaffold236	+/+	3′	101	−45.50	0.97
lme-miR156c-3p	22	GCUCACUGCUCUAUCUGUCAGA	scaffold675	+/+	3′	86	−43.80	1.07
lme-miR157a	21	UUGACAGAAGAUAGAGAGCAC	scaffold344	+/+	5′	131	−49.40	1.01
lme-miR157a-3p	21	GCUCUCUAGCCUUCUGUCAUC	scaffold78	+/+	3′	86	−44.40	1.35
lme-miR157c-3p	21	GCUCUCUAUACUUCUGUCACC	scaffold344	+/+	3′	151	−54.90	0.96
lme-miR159c	21	UUUGGAUUGAAGGGAGCUCCU	scaffold613	+/−	3′	201	−66.60	0.89
lme-miR160a	21	UGCCUGGCUCCCUGUAUGCCA	scaffold249	+/+	5′	81	−45.70	1.04
lme-miR160a-3p	21	GCGUAUGAGGAGCCAUGCAUA	scaffold249	+/+	3′	96	−48.70	0.99
lme-miR160c-3p	21	CGUACAAGGAGUCAAGCAUGA	scaffold468	+/−	3′	90	−40.30	1.03
lme-miR161.2	21	UCAAUGCAUUGAAAGUGACUA	scaffold216	+/+	5′	102	−40.20	0.91
lme-miR162a	22	UGGAGGCAGCGGUUCAUCGAUC	scaffold1363	+/+	5′	116	−37.20	0.77
lme-miR162a-3p	21	UCGAUAAACCUCUGCAUCCAG	scaffold1363	+/+	3′	131	−44.30	0.76
lme-miR164a	21	UGGAGAAGCAGGGCACGUGCA	scaffold668	+/−	5′	126	−45.20	0.92
lme-miR164c	21	UGGAGAAGCAGGGCACGUGCG	scaffold369	+/+	5′	83	−38.90	0.97
lme-miR165a	22	UGGAGGCAGCGGUUCAUCGAUC	scaffold1363	+/+	5′	117	−41.90	0.86
lme-miR165a-3p	21	UCGGACCAGGCUUCAUCCCCC	scaffold830	+/+	3′	125	−45.40	1.05
lme-miR166a	21	GGACUGUUGUCUGGCUCGAGG	scaffold316	+/+	5′	151	−64.30	1.07
lme-miR166a-3p	21	UCGGACCAGGCUUCAUUCCCC	scaffold774	+/+	3′	92	−32.30	0.73
lme-miR167a	21	UGAAGCUGCCAGCAUGAUCUA	scaffold785	+/+	5′	97	−50.70	1.24
lme-miR167c	21	UAAGCUGCCAGCAUGAUCUUG	scaffold695	+/−	5′	146	−68.00	1.26
lme-miR 168a	21	UCGCUUGGUGCAGGUCGGGAA	scaffold468	+/−	5′	101	−46.60	0.91
lme-miR 168a-3p	21	CCCGCCUUGCAUCAACUGAAU	scaffold369	+/+	3′	101	−47.40	0.89
lme-miR 169a	21	CAGCCAAGGAUGACUUGCCGA	scaffold191	+/+	5′	227	−67.80	1.09
lme-miR 169b	21	CAGCCAAGGAUGACUUGCCGG	scaffold872	+/+	5′	216	−59.50	0.94
lme-miR 169d	21	UGAGCCAAGGAUGACUUGCCG	scaffold792	+/+	5′	121	−45.90	0.90
lme-miR 169 h	21	UAGCCAAGGAUGACUUGCCUG	scaffold1032	+/+	5′	131	−43.30	0.92
lme-miR 169b-3p	22	GGCAAGUUGUCCUUCGGCUACA	scaffold467	+/+	3′	112	−58.60	1.43
lme-miR miR170	21	UAUUGGCCUGGUUCACUCAGA	scaffold1132	+/+	5′	83	−33.60	0.96
lme-miR 170-3p	21	UGAUUGAGCCGUGUCAAUAUC	scaffold1132	+/−	3′	81	−27.40	0.78
lme-miR 171b	21	AGAUAUUAGUGCGGUUCAAUC	scaffold490	+/−	5′	91	−36.80	0.99
lme-miR 171a-3p	21	UGAUUGAGCCGCGCCAAUAUC	scaffold299	+/+	3′	85	−33.60	0.93
lme-miR 171b-3p	21	UUGAGCCGUGCCAAUAUCACG	scaffold433	+/+	3′	96	−40.30	1.01
lme-miR 172a	21	AGAAUCUUGAUGAUGCUGCAU	scaffold981	+/+	3′	105	−35.90	1.12
lme-miR 172c	21	AGAAUCUUGAUGAUGCUGCAG	scaffold997	+/+	3′	106	−40.70	1.13
lme-miR 319a	21	UUGGACUGAAGGGAGCUCCCU	scaffold1047	+/+	3′	181	−76.20	1.14
lme-miR 319c	21	UUGGACUGAAGGGAGCUCCUU	scaffold488	+/−	3′	173	−69.60	0.98
lme-miR 390a	21	AAGCUCAGGAGGGAUAGCGCC	scaffold237	+/+	5′	91	−43.40	1.11
lme-miR 391	21	UUCGCAGGAGAGAUAGCGCCA	scaffold432	+/+	5′	82	−34.80	0.87
lme-miR 391-3p	21	ACGGUAUCUCUCCUACGUAGC	scaffold432	+/+	3′	87	−36.30	0.84
lme-miR 393a	22	UCCAAAGGGAUCGCAUUGAUCC	scaffold997	+/+	5′	156	−53.90	1.00
lme-miR 393b-3p	21	AUCAUGCGAUCUCUUUGGAUU	scaffold997	+/+	3′	141	−50.90	1.04
lme-miR 394a	20	UUGGCAUUCUGUCCACCUCC	scaffold420	+/+	5′	126	−47.00	0.85
lme-miR 394b-3p	21	AGGUGGGCAUACUGCCAAUAG	scaffold356	+/+	3′	111	−48.70	1.08
lme-miR 395a	21	CUGAAGUGUUUGGGGGAACUC	scaffold904	+/−	3′	92	−44.30	1.10
lme-miR 395b	21	CUGAAGUGUUUGGGGGGACUC	scaffold904	+/+	3′	106	−35.90	1.00
lme-miR 396a	21	UUCCACAGCUUUCUUGAACUG	scaffold339	+/−	5′	106	−34.50	0.88

Table 1 continued

Identified miRNAs	LM (nt)	miRNA sequences	Input sequence	Strand	Location	LP (nt)	MFEs (ΔG)	MFEI
lme-miR 396b	21	UUCCACAGCUUUCUUGAACUU	scaffold127	+/+	5′	132	−43.70	1.15
lme-miR 396a-3p	21	GUUCAAUAAAGCUGUGGGAAG	scaffold306	+/+	3′	117	−41.30	0.96
lme-miR 396b-3p	21	GCUCAAGAAAGCUGUGGGAAA	scaffold127	+/+	3′	156	−55.50	1.13
lme-miR 397a	21	UCAUUGAGUGCAGCGUUGAUG	scaffold299	+/−	5′	96	−26.80	0.96
lme-miR 398a-3p	21	UGUGUUCUCAGGUCACCCCUU	scaffold302	+/+	3′	101	−42.90	1.13
lme-miR 398b-3p	21	UGUGUUCUCAGGUCACCCCUG	scaffold534	+/−	3′	115	−42.80	0.82
lme-miR 399a	21	UGCCAAAGGAGAUUUGCCCUG	scaffold904	+/−	3′	126	−41.60	0.97
lme-miR 399b	21	UGCCAAAGGAGAGUUGCCCUG	scaffold971	+/−	3′	146	−51.80	1.01
lme-miR 399c-3p	21	UGCCAAAGGAGAGUUGCCCUG	scaffold971	+/−	3′	141	−51.20	1.04
lme-miR 408	21	ACAGGGAACAAGCAGAGCAUG	scaffold613	+/+	5′	96	−39.50	0.90
lme-miR 408-3p	21	AUGCACUGCCUCUUCCCUGGC	scaffold613	+/−	3′	101	−39.60	0.84
lme-miR 828	22	UCUUGCUUAAAUGAGUAUUCCA	scaffold236	+/+	5′	101	−35.80	1.02
lme-miR 2111a	21	UAAUCUGCAUCCUGAGGUUUA	scaffold668	+/+	5′	101	−38.30	1.18
lme-miR 2111b-3p	21	AUCCUCGGGAUACAGUUUACC	scaffold602	+/+	3′	76	−29.90	1.11

LM length of mature miRNAs, *LP* length of precursor

precursors with higher MFEI values is presented in Fig. 1 (Top 20). Previous reports suggested that uracil at the first position of the sequence of miRNA play an important role in miRNA-mediated regulations in plant (Zhang et al. 2008). In this study, uracil was observed to be predominant at the first position of ∼60% mature miRNA sequences.

Experimental validation of putative maca miRNAs

The efficiency of the computational strategy was further verified by RT-PCR based experimental procedure. The randomly selected four miRNAs lme-miR160a, lme-miR164c, lme-miR 166a, and lme-miR 319a from maca

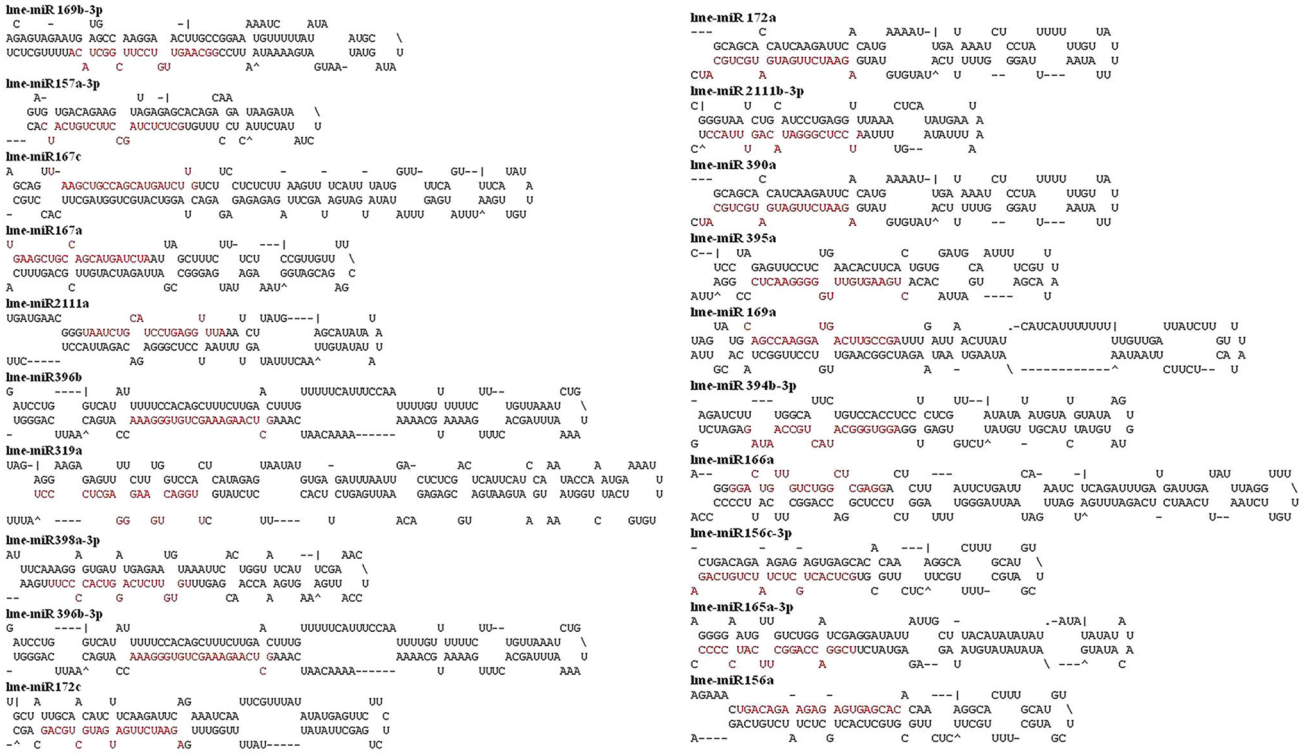

Fig. 1 Secondary structure (stem-loop) of the maca miRNA precursors with higher MFEI values (*Top 20*). Respective miRNAs are represented with *red font*

were subjected to validation studies. All these maca miRNAs showed confirmation through experimental validation (Fig. 2).

Potential targets of putative maca miRNAs and their function

A total of 99 potential targets were identified and most of them were functionally categorized as transcription factors. Important transcription factors targeted by maca miRNAs include Squamosa promoter-binding protein/SPB (miR156/157), Auxin-responsive factor (miR160), Cytochrome P-450 (miR162), NAM protein (miR164), Class III HD-Zip (miR165/166), MYB (miR172/319), F-box protein (miR394/399) (Table 2). These transcription factors are known to play a role in metabolic processes and stress response signaling in plants. Moreover, to improve the efficient understanding of miRNA regulation in maca, gene ontology analysis of the identified target transcript was executed by AmiGo (http://amigo.geneontology.org/amigo), and high involvement of the target transcripts in the biological, molecular, and cellular process was observed (Fig. 3).

Surrounding environment is the key factor for proper growth and development of plants. Stress-sensitive plants often show limited growth during environmental stresses while stress-tolerant plants employ several complex defense mechanisms including miRNA-mediated post-transcriptional gene silencing (Sunkar et al. 2007). Although few discrete studies have been performed to check the alterations of miRNAs during cold and irradiance stresses in tolerant plants, the exact molecular mechanism is still unclear. Zhang et al. (2014) reported that during cold stress 31 miRNAs were up-regulated and 43 were down-regulated in cold tolerant tea variety 'Yingshuang' while 46 miRNAs were up-regulated and 45 down-regulated in sensitive variety 'Baiye 1'. Casadevall et al. (2013) showed that up-regulation of miR396 enhances survival of *Arabidopsis thaliana* under UV-B radiation. Nevertheless, regulation of plant miRNAs at high-altitude environment (combined effect of extreme cold, strong wind, and oxidizing air pollutants) has been poorly studied and hence maca could provide new insights into the understanding of stress-responsive miRNAs at higher altitude. On the other hand, few workers also reported that miRNAs can influence the production of bioactive compounds/secondary metabolites in the medicinal plants (Robert-Seilaniantz et al. 2011; Singh et al. 2016). Nonetheless, identification of miRNAs and their targets is the key step to initiate a miRNA-related study in a plant. This study can be of immensely helpful for future research on miRNA-mediated

Table 2 Potential targets of identified maca miRNAs

miRNA	Targeted proteins (number)
156/157	Squamosa promoter-binding-like protein (24)
159	MYB (4)
	Pectin acetylesterase (1)
	Pyruvate orthophosphate dikinase (5)
160	Auxin response factor (4)
162	Cytochrome P450-like protein (1)
164	CUP-SHAPED COTYLEDON/CUC (3)
	NAM protein-like (6)
165	HD-Zip protein (2)
166	HD-Zip protein (2)
168	Glutathione transferase (1)
171	Scarecrow-like protein (2)
	Protein kinase (2)
172	APETALA 2 (8)
	MYB (2)
319	MYB (3)
390	Protein kinase (1)
	Glutamate dehydrogenase (1)
391	Protein kinase (2)
393	WRKY transcription factor (1)
394	F-box only protein (1)
	Lipase-like protein (1)
	Loricrin-like protein (1)
395	ATP sulfurylase (9)
	Transcription factor bZIP (2)
397	Laccase precursor (3)
399	F-box protein (1)
	Cytochrome P450-like protein (1)
408	Ethylene-responsive transcription factor (1)
828	Kinesin 2 (2)
2111	Flavanone 3-hydroxylase-like protein (2)

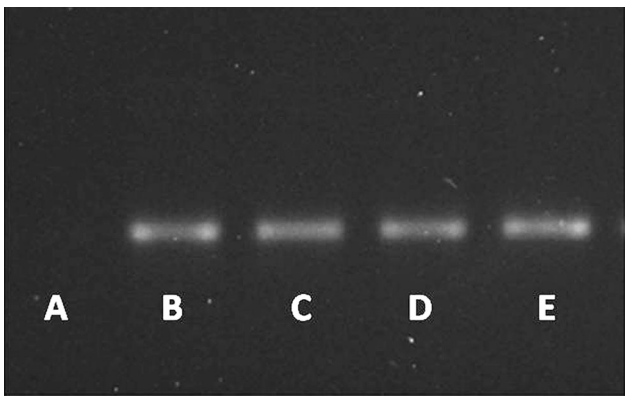

Fig. 2 Validation of some maca miRNAs by RT-PCR. The resulting PCR products are checked in 2% agarose gel with EtBr staining. **a** Negative control, **b** lme-miR160a, **c** lme-miR164c, **d** lme-miR 166a and **e** lme-miR 319a

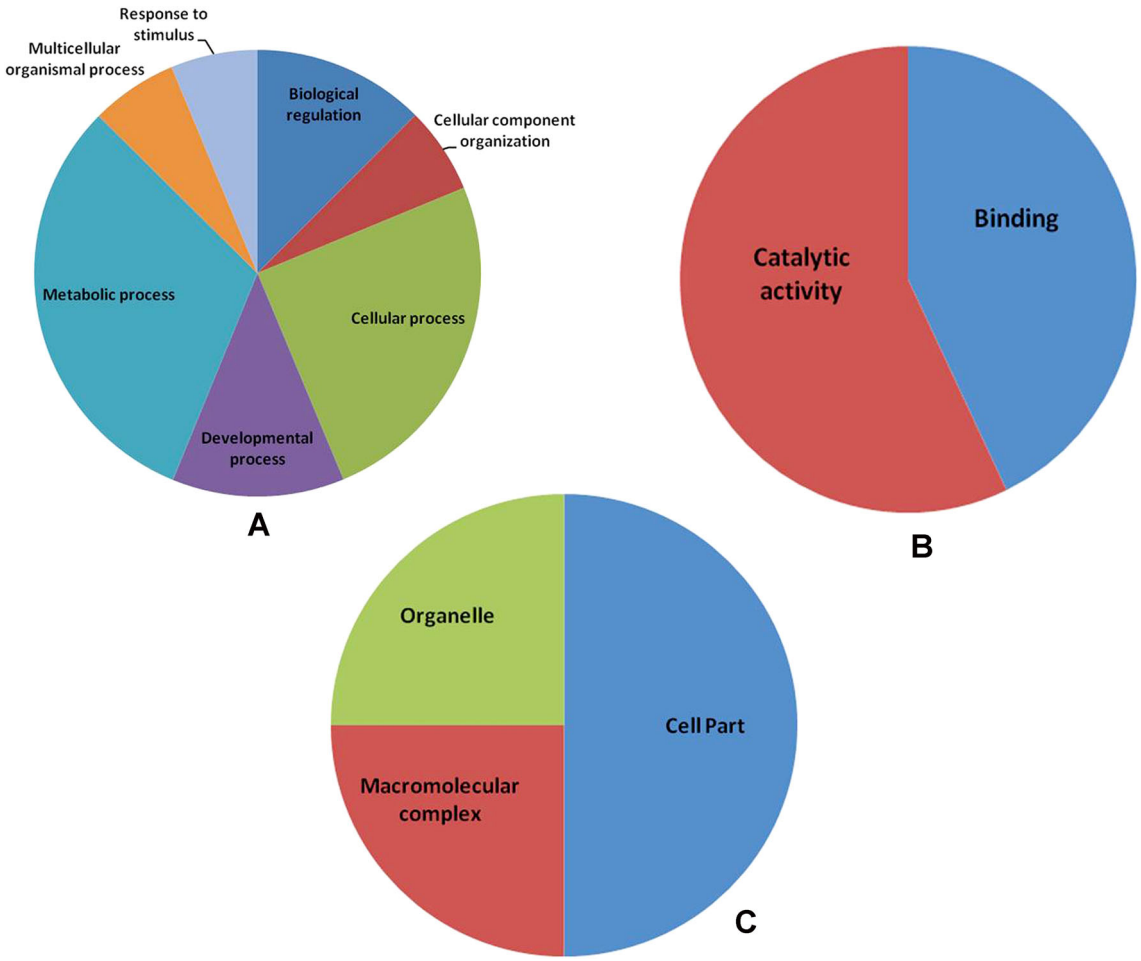

Fig. 3 GO analysis of target transcripts regulated by identified miRNAs: **a** biological process, **b** molecular function and **c** cellular component

stress response signaling as well as the production of bioactive compounds/secondary metabolites in medicinal plants.

Conclusion

In this study a total of 62 conserved miRNAs belonging to 28 families were first time identified in a high-altitude plant such as maca. To validate the expression of potential miRNAs in maca, a RT-PCR approach was performed and 4 miRNA families were detected. Moreover, a total of 63 potential targets were predicted and they were found to be involved in development, metabolism and stress responses.

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Compliance with ethical standards

Conflict of interest The author declares that he has no conflict of interest.

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