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Full Length Article

The miRNAomes in *Rehmannia glutinosa* Roots Exposed to Different Levels of Replanting Disease Pressure

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Abstract

Root development in the medicinal plant *Rehmannia glutinosa* is severely disordered by replanting disease, which occurs when it is grown in the same soils over consecutive seasons. Following the demonstration of changes to the miRNAome of the *R. glutinosa* plants in the second year of cultivation, a fuller experiment was organized to track the root miRNAomes over four successive plantings. Four small RNA libraries were generated from the roots of one year's planted (free of replanting disease) and 2–4 years' continuous cultivation (replanting disease) *R. glutinosa* plants, respectively. Sequencing of these libraries revealed the presence of 18 novel and 344 conserved miRNA families. The abundance of many of the miRNAs varied between the four libraries, consistent with the notion that replanting disease reflects the outcome differential miRNA transcription. A combined bioinformatic and degradome sequencing approach allowed the identification of 69 differentially abundant (DA) miRNA targets, leading to the recognition that particular miRNA/target modules may be important for the expression of the disease. The expression profiles of 28 key miRNAs and their targets implied that the longer years of continuous cultivation was extended, the more severe tendency of these profiles' changes would be. The indication was that the miRNAs-responsive continuous cultivation pressure reprogrammed gene expression patterns, which disordered hormone signaling, repressed core mechanisms, weakened stress tolerance and resulted in forming the disease in *R. glutinosa*. In conclusion, the key miRNAs involved in important roles of replanting disease and were a crucial basis for exploration in the disease forming repertoire.

Keywords: Continuous cultivation; Differentially abundant; miRNA/target module; Function analysis; Expression profile

Introduction

An estimated 70% of medicinal plants cultivated for their roots suffers from "replanting disease", which is manifested when a species is grown as a continuous cultivation (Zhang and Lin, 2009; Zhang *et al.*, 2011). The syndrome is manifested by a severe reduction in plant growth, economic yield and end-use quality. One of these medicinal species is the perennial herbaceous plant *Rehmannia glutinosa* (*Scrophulariaceae*), the tuberous roots of which are an important ingredient in traditional Chinese medicine. The severity of replanting disease is such that, after one season of *R. glutinosa* cultivation, the land must be used for growing other crops for the next 8–10 years (Wen *et al.*, 2002). The disease damages the economic viability of the *R. glutinosa* crop because the plants shift from developing tuberous roots to fibrous ones (Zhang *et al.*, 2012; Yang *et al.*; 2015; Yang and Li, 2016).

The sustainable and profitable cultivation of *R. glutinosa* will require a sufficient understanding of the mechanistic basis of the disease. It has been suggested that replanting disease is induced by autotoxins exuded by the roots, and some research effort has been dedicated to identifying the nature of these autotoxins (Wu *et al.*, 2011; Li *et al.*, 2012; Gu *et al.*, 2013). As yet, however, there has been little focus on the molecular regulation of the disease.

microRNAs (miRNAs) have been shown to represent an important class of transcription regulators in the eukaryotes (He *et al.*, 2014; Asha *et al.*, 2016; Niu *et al.*, 2016). They inhibit transcription for the most part by interacting with their complementary mRNA, and in so doing target them for degradation (Tang *et al.*, 2012; Lu *et al.*, 2014). They are intimately involved in the adaptive response to both abiotic and biotic stress, and are regulated by complex gene networks (Kumar, 2014; Feng *et al.*, 2015; Niu *et al.*, 2016). A number of miRNAs have been associated already with the expression of replanting disease in a second-year crop of *R. glutinosa* whole plants (Yang *et al.*, 2011; Li *et al.*, 2013). Although this finding has been taken to imply that they play a regulatory role in the onset of the disease, preliminarily screening some miRNAs-responsive replanting disease, it is still unclear about which miRNAs play a vital role in response to the disease. *R. glutinosa* roots, medicinal parts, is the most sensitive

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tissue-responsive replanting disease (Zhang and Lin, 2009), and little is known about the miRNA components and changes of the species' roots under different years of continuous cultivation.

To capture key miRNAs-responsive replanting disease, here, the effect of continuously cultivating 2–4 years' *R. glutinosa* on the miRNA content of its root*s* was explored in order to gain a handle on the diversity of miRNAs in plants exposed to different levels of replanting disease pressure. Some key miRNAs will accurately be locked to elaborate molecular regulation mechanisms of the disease. The research forms part of an attempt to establish a proper scientific basis for controlling replanting disease, so tha*t R. glutinosa* roots can become a viable crop.

Materials and Methods

Plant Materials and RNA Extraction

R. glutinosa cultivar "Wen 85-5" plants were grown at the Wen Agricultural Institute, Jiaozuo City, Henan Province, China. The materials of four isolated plots were planted on April 22 and harvested on November 30 of each of the years 2012 through 2015. Plot R1 (free of replanting disease) was planted in the soil which had not been grown with *R. glutinosa* for more than ten years; R2 was first planted in 2014, then replanted in 2015; R3 was first planted in 2013 and replanted in both 2014 and 2015; R4 was first planted in 2012, and replanted in 2013, 2014 and 2015. R1 was considered as a control and the other three plots as treatments.

For construction of sequencing libraries, the roots of five independent R1, R2, R3 and R4 plants, respectively were collected at the tuberous root expansion stage (July 22, 2015). Five sequencing libraries were established in present study, i.e. four sRNA libraries, R1 (roots of R1), R2 (roots of R2), R3 (roots of R3) and R4 (roots of R4), one degradome library from a root mixture of R1, R2, R3 and R4.

Total RNA from each sample was isolated using a TRIZOL® Reagent (Invitrogen). The RNA quality (including its concentration and integrity) was measured using a NanoDrop 2000/2000C spectrophotometer (Thermo Scientific) and agarose gel electrophoresis.

SRNA Sequencing and miRNA Identification

For construction of sRNA libraries, 16–30 nt small RNAs (sRNAs) of total RNA from each sample were separated by denaturing a polyacrylamide gel (PAGE) PAGE electrophoresis. The small RNAs were ligated sequentially to 5' and 3' chimeric oligonucleotide adaptors and then the products were purified by a denaturing PAGE PAGE electrophoresis, and reversely transcribed cDNAs. The obtained cDNAs were sequenced by the Illumina HiSeq™ 2000 system of Next generation sequencing (NGS). The adaptors and the low quality reads were removed from sequenced raw reads, clean reads were remained and analyzed as follows. For identify conserved miRNAs, clean reads were subjected to search against Rfam database (xfam.org), RepBase database (www.girinst.org/server/RepBase) and miRBase database (www.mirbase.org). For the identification of novel miRNAs, sRNA sequences were first an alignment with the de novo assembled *R. glutinosa* transcriptome sequences from our previous report (www.ncbi.nlm.nih.gov.sra) (Li *et al.*, 2013) by MIREAP tool (sourceforge.net/projects/mireap), and then the secondary structures of the candidate miRNA precursors were predicted by mFold program (Zuker *et al.*, 2003; Jones-Rhoades *et al.*, 2006; Meyers *et al.*, 2008).

DA miRNA Screening

Differentially abundant (DA) miRNAs in comparing R1 (Control) with the three (R2, R3 and R4) treatment libraries were analyzed using the Poisson distribution algorithms (Audic and Claverie, 1997). Fewer than 10 sequencing frequencies of the miRNAs were first removed in both sRNA libraries and then the frequencies of remained miRNAs were normalized. Moreover, DA miRNAs were screened according to the thresholds of P-value (<0.01) and FDR (<0.001) and the significance of the DA miRNAs was assessed by calculating the absolute value of $\log_2^{ratio} \geq 1$ (Benjamini *et al.*, 2001).

miRNA RT-PCR and qPCR Analysis

For miRNA reverse transcription reaction, sRNAs (size range 16–30 nt) of total RNA from each sample were isolated by denaturing a PAGE electrophoresis, the 3' ends of the sRNAs were polyadenylated by poly(A) polymerase reaction (Exiqon, Denmark), and the polyadenylated sRNAs were added Universal Adaptor Primer (containing Oligo dT Adaptor) and reversely transcribed using PrimeScript® RTase (Exiqon, Denmark). Synthesized cDNAs were used for miRNA RT-PCR and qPCR.

RT-PCR was processed using an ExiLENT SYBR® Green master mix kit (Exiqon, Denmark). A 5 μL aliquot of each reaction product was tested by agarose gel electrophoresis. qPCR of each sample (3 technical replicates) was performed using ExiLENT SYBR® Green master mix kit (Exiqon, Denmark) on a BIO-RAD iQ5 real-time PCR detection system (Bio-Rad laboratories, Inc.). The relative expression level of the miRNAs was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), and a part fragment (21 bp) of an 18S ribosome RNA gene (Genbank ID: EU787015.1) was used to as a house-keeping gene normalized the $2^{-\Delta\Delta CT}$ data.

Identification and Functional Analysis of DA miRNA Targets

To identity DA miRNA targets, mRNAs from the roots of

R1, R2 R3 and R4 plants were pooled and then the mRNA mixture was prepared for constructing the root degradome library. The constructed method of degardome sRNA library was described previously in the published reports (Addo-Quaye *et al.*, 2008; German *et al.*, 2009). The sRNA library was sequenced by the Illumina HiSeq™ 2000 system. After obtained raw reads were removed the adaptors and the low quality reads, clear reads were extracted. And then filtering out the clean reads mapped to house-keeping RNAs, i.e. rRNA, tRNAs, snoRNAs and snRNAs (xfam.org), the remained reads with the length of 20–21 nt were then used to validate potentially cleaved targets by the CleaveLand tool (Addo-Quaye *et al.*, 2009; Zhou *et al.*, 2012). The degradome reads were mapped to the *R. glutinosa* transcriptome sequences (Li *et al.*, 2013). All the identified targets were subjected to BlastX analysis (www.ncbi.nlm.nih.gov/BLAST) to search for similarity.

mRNA qPCR Analysis

For mRNA reverse transcription reaction, 5 μg total RNA of each sample was performed with MMLV Reverse Transcriptase (Clontech, USA) according to the kit's manual. The qPCR of each gene from each sample (3 technical replicates) were processed by SYBR® Green kit (Takara, Japan) to detect transcript abundance. The relative expression levels of each gene were assessed by calculating it's $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). A part fragment (205 bp) of the 18S ribosome RNA gene (Genbank ID: EU787015.1) was used as a house-keeping gene normalized the $2^{-\Delta\Delta CT}$ data.

Results

The Construction of sRNA Libraries

Four sRNA libraries were constructed using total RNA isolated from R1, R2, R3 and R4 roots, respectively. NGS yielded 17,621,287 (R1), 15,624,681 (R2), 20,186,972 (R3), and 16,365,785 (R4) raw reads. After removal of adaptor and poor quality sequences, those in the range 16–30 nt were extracted. As a result, the number of relevant unique reads was 6,822,331 (R1), 5,429,466 (R2), 8,477,451 (R3) and 6,677,971 (R4). Sequences in the size range 21–24 nt represented ~90% of these unique reads (Table 1).

Identification of Conserved miRNAs

The sRNAs obtained were subjected to a BlastN search against known non-coding RNAs deposited in the Rfam 11.0 and NCBI Genbank databases in order to enable the removal of tRNA, rRNA, snRNA, snoRNAs and scRNA sequences: these accounted for 5.32% of the reads in R1, 8.20% in R2, 4.66% in R3 and 5.64% in R4 (Table 1). Repetitive sequences were identified by reference to the RepBase 19.06 database these represented 0.14% (R1), 0.16% (R2), 0.13% (R3) and 0.12% (R4) of the reads. After their removal, the remaining sequences were compared with the miRNAs documented in the miRBase 21.0 database. In all, 779 (772 in R1, 758 in R2, 764 in R3 and 757 in R4) of the *R. glutinosa* miRNAs were classified as belonging to 344 miRNA families (339 in R1, 341 in R2, 342 in R3 and 337 in R4) (Table 2).

Identification of Novel miRNAs

The filtered sRNA sequences were pooled and mapped onto the *R. glutinosa* transcriptome to identify potentially novel miRNAs. This resulted in the identification of 31 miRNA candidates (size range 18–24 nt), of which 23 were present in R1, 23 in R2, 24 in R3 and 22 in R4 (Table 3). The predicted hairpin structures corresponding to these ranged in length from 64 to 99 nt, and their minimum free energy energies from -18 to -36.2 kcal mol^{-1}. In all four libraries, most of these miRNA candidates (the exception were miRn1 and miRn15) were in the rare class, with a read count of less than 20. The transcription of 18 of these miRNAs was validated using RT-PCR based on a cDNA template prepared from "Wen 85-5" roots. These 18 miRNAs, which have not been identified in any other plant species, are considered to be novel or specific to *R. glutinosa*. They have been assigned the names rgl-miRn1 to 18 (Table 3).

DA miRNA Profiles from *R. glutinosa* Roots

After removing the 14 novel and 47 conserved miRNA families in which the read count was <10, the read counts in the remaining four novel and 296 conserved families were normalized. Comparisons were made between the abundance in R1 and that in each of R2, R3 and R4. 69 miRNA families were identified as being significantly differentially expressed (Table 4); the R2 *vs* R1 contrast produced 18 up- and 17 down-regulated families, the R3 *vs* R1 one 24 up- and 22 down regulated and the R4 *vs* R1 one 34 up- and 31 down-regulated miRNA families. Of these, 17 were up-regulated in all of R2, R3 and R4, and 14 were down-regulated. Seven families were more abundant in only R3 and R4, while eight were less abundant in these two treatments. Besides, there were significantly 19 DA miRNA families in only R4. Interestingly, the fold-changes of the DA miRNA expression levels mostly had the same trend of which R4 had the highest, and followed by R3 (Table 5). For example, the fold changes with respect to the down-regulated miR533 family were 1.95 in R2 *vs* R1, 2.65 in R3 *vs* R1 and 3.87 in R4 *vs* R1. The implication was that continuous cultivation induced changes in the miRNA profiles and individual miRNA abundances, and these changes became more apparent as the number of consecutive cultivation years was increased.

Table 1: Abundance of different sRNAs from the four sRNA libraries

Class	Unique sRNA number (percentage)			
	R1	R2	R3	R4
Total unique sRNAs	6,822,331(100.00)	5,429,466(100.00)	8,477,451(100.00)	6,677,971(100.00)
21-24nt unique sRNAs	6,196,723(90.83)	4,965,247(91.45)	7,715,328(91.01)	6,098,323(91.23)
Non-coding RNA	362,757(5.32)	445,451(8.20)	395,379(4.66)	376,513(5.64)
rRNA	326,124(4.78)	406,111(7.48)	354,458(4.18)	337,376(5.05)
scRNA	6(0.00)	1(0.00)	4(0.00)	4(0.00)
snRNA	1,125(0.02)	1,229(0.02)	1,270(0.01)	1,094(0.02)
snoRNA	679(0.01)	801(0.01)	736(0.01)	639(0.01)
tRNA	34,823(0.51)	37,309(0.69)	38,911(0.46)	37,400(0.56)
Repeat	9,380(0.14)	8,892(0.16)	10,780(0.13)	7,687(0.12)
Known miRNA	19,901(0.29)	15,975(0.29)	21,239(0.25)	16,663(0.25)
Unmapped reads	6,430,293(94.26)	4,959,148(91.34)	8,050,053(94.96)	6,277,108(94.00)

Table 2: Summary of conserved miRNAs in the four sRNA libraries

Class	miRNA famlies (members)				
	R1	R2	R3	R4	Total
Group I	4(32)	4(32)	4(32)	4(32)	4(32)
Group II	10(56)	10(52)	10(53)	10(54)	10(56)
Group III	325(684)	327(674)	327(678)	322(671)	329(691)
Total	339(772)	341(758)	341(763)	336(756)	343(779)

Table 3: Candidates of novel miRNAs from *R. glutinosa* roots

miRNA ID	Count of reads				Sequences	Energy (kcal mol^{-1})	RT-PCR
	R1	R2	R3	R4			
rgl-miRn1	56	15	21	23	AUUUCCGAUACUCGUAGGCGG	-36.2	Yes
rgl-miRn2	2	5	5	4	UUUCUUUUGAACCUUGCACGU	-27.7	Yes
rgl-miRn3	1	2	3	9	UCAAUCUUUGAUGGGAUAUC	-18.4	Yes
m0025-5p	0	1	2	2	ACGGUAAGAUCUCCGCGAGUGAGU	-28.9	No
m0033-3p	1	0	2	6	AGGAAAGUAGCGAAAAACCCU	-21.8	No
rgl-miRn4	0	9	0	0	AUUUACUACAUUCUGACAGU	20.9	Yes
rgl-miRn5	0	5	10	12	CUUUUUGGAUCUAUUGCAGA	-24	Yes
m0053-3p	0	1	0	0	UUUAGGAGUCGAGGAGCCUUCA	-19	No
rgl-miRn6	1	8	1	5	ACUUACUGUAGCAUAUUUGA	-22.2	Yes
m0060-5p	3	4	10	0	CAUAGGUCACUAGUCGAGGAGG	-20	No
m0063-5p	11	8	8	5	UUGGAUUGGCUGUGAGUUCUG	-19.7	Yes
m0066-5p	9	3	9	5	AAGGUCACUGGAACUGGUUGAUUC	-20.5	No
rgl-miRn7	2	1	4	7	UCGUUCGAUUGUAAAACUCGCU	-20.1	Yes
rgl-miRn8	1	7	9	0	AGGUGACAUCGGCAGUUGUUC	22.3	Yes
rgl-miRn9	1	0	8	0	AGGAGGUGGGACACUUGACU	-20.52	Yes
rgl-miRn10	1	1	9	0	UUAUCGAUCUGAGCCGGAAU	-21.9	Yes
m0083-5p	3	0	0	2	UUCUUGCAGGAAGAUUGGCA	-20.1	No
m0087-3p	1	0	0	2	CCAUGAGUUGGAAAAGCUUCU	21.22	No
rgl-miRn11	6	4	9	6	ACAUUUCUAGACAGUCAAUCCA	-23.1	Yes
m0094-5p	2	1	3	1	CAUUUUUUUUAAUAAGUUGGGAGU	-19.2	No
rgl-miRn12	0	2	16	13	AACAGUUUUCAUUUCGCUACGUUCUU	-19.54	Yes
rgl-miRn13	4	5	9	9	AGUGUGUUUGAAGAGGUGAAGA	-23.7	Yes
rgl-miRn14	3	7	2	0	CAGGUUGCGAGAUAAAGAAG	-27	Yes
rgl-miRn15	0	0	0	152	CAAUGUGCUAAUUUCAGGGUU	-18.9	Yes
rgl-miRn16	5	2	4	8	UGAUAAUUCUGUAUAUUUCGCC	-19.7	Yes
rgl-miRn17	3	5	3	9	ACUCUUGCUGUAGUGGCACU	-29	Yes
m0117-5p	1	2	0	0	GCAUGGUCAAAGGCACUGAGGGUG	-19.2	No
m0121-5p	0	0	0	1	AUUUAAGCAGUCAGCUGGGGAUC	-20	No
rgl-miRn18	2	0	2	7	AUUUCUUUACUUGGAGGGAUCUU	-18.9	Yes
m0131-5p	2	1	0	1	GAAGAACUUUCCAGAGAUGGU	-18	No
m0132-5p	0	0	5	0	UUGCUGAAAGCUCCAAAAGUU	-24.8	No

Identification and Function Analysis of miRNA Targets

To further identify the putative miRNA targets, a degradome library was therefore constructed using total RNA isolated from a root mixture from R1, R2, R3 and R4. This captured ~20 million raw reads of 3' cleavage fragments and ~19 million clean reads. After filtering to remove rRNA, tRNA, snRNA and snoRNA sequences, the data were analysed using the CleaveLand 3.0 pipeline. A set of 73 targets matching 33 of the DA miRNA families was identified. A BlastX search of the Nr (non-redundant proteins) and Swissport databases showed that the predicted translation

Table 4: Summary of different expression profiles from the four sRNA libraries

Class	Significance DA miRNA number		
	Up-regulated	Down-regulated	Total
R2/R1	18	17	35
R3/R1	24	22	46
R4/R1	34	31	65
All of R2/R1, R3/R1 and R4/R1	17	14	31
Both of R2/R1 and R3/R1	0	0	0
Both of R2/R1 and R4/R1	0	0	0
Both of R3/R1 and R4/R1	7	8	15
only R2/R1	1	2	3
only R3/R1	0	0	0
only R4/R1	10	9	19
Total	35	34	69

Table 5: Identified key DA miRNA profiles by NGS and their targets by the *R. glutinosa* root degradome library

miRNA familiy	Fold-change(log_2ratio)^{signficiance}			miRNA ID	Target annotation	Target abbreviation
	R2/R1	R3/R1	R4/R1			
miR1161	1.02**	1.35**	1.79**	miR1161a	uridine 5'-monophosphate synthase	UMP
miR1508	1.08**	1.54**	1.41**	miR1508b	nitrate transporter NRT1-2	NRT1-2
miR1513	1.34**	1.48**	2.09**	miR1513a	global transcription factor group	GPFG
miR1919	1.25**	2.29**	2.20**	miR1919a	ethylene-insensitive 3f	EIN3F
miR2088	0.98	1.80**	2.21**	miR2088a	disease resistance protein RGA2	RGA2
miR2592	1.03**	1.82**	1.70**	miR2592f	translational elongation factor 1	TEF1
miR3630	1.08**	1.47**	1.58**	miR3630a	MYB transcription factor 34	MYB34
miR399	1.15**	1.57**	1.68**	miR399d	MYB family transcription factor-like protein	MYB-L
				miR399i	potassium channel	PC
miR4356	1.07**	1.54**	1.90**	miR4356b	potassium ion transmembrane transporter	PITT
miR5160	0.66	1.76**	1.78**	miR5160a	DNA primase	DPR
miR531	1.25**	1.29**	1.45**	miR531a	60S acidic ribosomal protein	ARP60S
miR533	1.95**	2.65**	3.87**	miR533a	enhanced disease susceptibility 5	EDS5
				miR533e	DNA mismatch repair protein	DMR
miR822	1.98**	2.39**	2.45**	miR822a	DNA repair protein Rad4 family	RAD4
rgl-miRn15	0.00	0.00	10.25**	rgl-miRn15	histone acetyltransferase	HAT
miR1024	-0.11	-0.21	-1.46**	miR1024a	dehydration-responsive protein-related	DRP
miR164	-1.80**	-2.24**	-3.24**	miR164f	stomatal cytokinesis-defective 1	SCD1
miR167	-1.03**	-1.71**	-2.37**	miR167a	Auxin response factor 6	ARF6
				miR167a	Auxin response factor 8	ARF8
miR168	-1.24**	-1.42**	-2.66**	miR168b	ethylene-responsive transcription factor ERF14	ERF14
miR171	-1.56**	-2.41**	-3.32**	miR171a	scarecrow-like protein 6	SCL6
				miR171b	Laccase-11	LAC11
miR172	-1.34**	-1.85**	-2.76**	miR172a	floral homeotic protein APETALA 2	AP2
miR2600	-0.63	-1.22**	-3.34**	miR2600a	1-aminocyclopropane-1-carboxylate oxidase 1	ACCS1
miR405	-1.04**	-1.58**	-3.72**	miR405a	Ethylene receptor	ETR
miR408	-1.19**	-2.04**	-1.88**	miR408a	LOB domain-containing protein 37	LOB37
miR5240	-0.17	-0.44	-1.55**	miR5240b	ethylene-responsive transcription factor	ERTF
miR946	-0.25	-1.34**	-2.29**	miR946a	cytokinin oxidase	CKX1

product of all of the putative and identified miRNA targets shared homology with other plant proteins, resulting in that key 29 targets of the 28 DA miRNAs were screened (Table 5). Two auxin response factor (*ARF6* and *ARF8*) genes were targeted by miR167, an ethylene response factor (*ERF14*) gene by miR168, an enhanced disease susceptibility (*EDS5*) by miR533. Of the novel miRNAs, rgl-miRn15 targeted a gene encoding a histone acetyltransferase (*HAT*). The targets of the miRNA families which were highly abundant in R2-R4 roots included genes encoding a DNA mismatch repair protein (DMR), a nucleotide excision repair protein (RAD4), a potassium channel (PC) and three MYB family transcription factors. Among the targets of the miRNA families, which were less abundant R2-R4 than in R1 were *DRP* (encoding a dehydration-responsive protein), *CKX1* (cytokinin oxidase) and *ERTF* (an ethylene-responsive transcription factor).

Expression Profiles of miRNA/target Modules

Based on the Nr annotation and Swissport analysis, a set of 28 of the DA miRNAs and 29 of their targets (Table 5) was estimated transcript abundances via QPCR. Among the 16 up-regulated miRNAs, most of the targets proved to be present at low abundance, while most of the 12 down-regulated ones were associated with abundant target transcript (Fig. 1). This behavior is consistent with the miRNAs repressing the transcription of their target mRNAs.

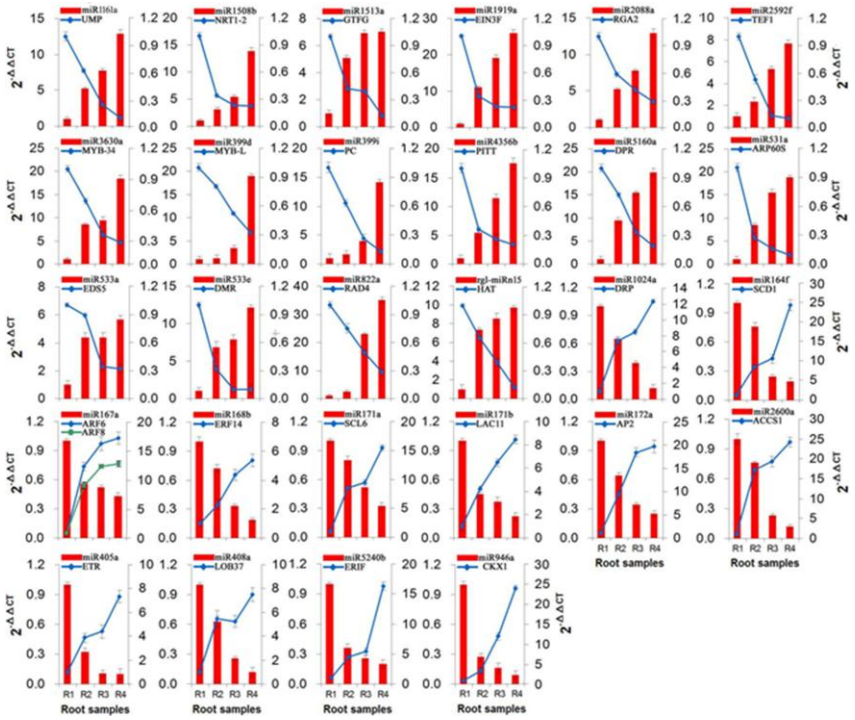

Fig. 1: Expression profiles of the key DA miRNA/target modules from four root samples

Discussion

Many aspects of plant development and their response to stress are regulated by miRNAs (Ma *et al.*, 2015; Niu *et al.*, 2016), reflected by the growing volume of entries deposited in the miRBase 21.0 database. While some hundreds of miRNA sequences have been identified in *Arabidopsis thalian*a and rice (Reinhart *et al.*, 2002; Gielen *et al.*, 2016), only 37 *R. glutinosa* ones have been lodged in the database so far (Yang *et al.*, 2011; Li *et al.*, 2013). In the study, the application of NGS has identified 18 novel miRNA families of *R. glutinosa* roots, which enriched the species' sRNA information.

An earlier comparison of miRNAs and their mRNA targets between plants growing in fresh soil and second-year plants suggested that miRNAs were likely involved in replanting disease (Yang *et al.*, 2011; Li *et al.*, 2013). Here, the survey has been extended to plants grown for a third and fourth year. It is a reasonable assumption that some key miRNAs in plants suffering from different levels of replanting disease pressure may be involved in and perhaps causative of the plant's response to the disease. As a result, 69 miRNA families were varied in abundance in the roots caused by continuous cultivation. The expression profiles of the key miRNA/target modules indicated that the longer the period of continuous cultivation was extended, the more severe the tendency of the expression profiles' changes would be. The details were showed as follows.

Ethylene is a ubiquitous signaling molecule used by plants during episodes of stress and during senescence (Jourda *et al.*, 2014; Arraes *et al.*, 2015). Four miRNAs with targets involved in ethylene signal transduction were identified as being significantly different expression in all the continuously cultivated *R. glutinosa* roots. For example, miR2600a was further down-regulated in R3 and R4; its target is *ACCS1,* a gene encoding ACC oxidase, which is responsible for the final stage of ethylene synthesis (Sallmann *et al.*, 2015; Somyong *et al.*, 2016), an accumulation of *ACCS1* would be expected to promote the ethylene synthesis. Furthermore, the down-regulation of miR5240b targeted the gene encoding ERTF, which underlie the positive regulation of ethylene-based signal transduction (Ohta *et al.*, 2000; Shahin *et al.*, 2016). With the replanting-year prolongation, the miR5240b expression levels from the *R. glutinosa* roots got lower and lower (i.e., R2>R3>R4), which could more seriously accumulate ERTF abundance, enhancing ethylene signaling in continuously cultivated *R. glutinosa*. Thereby, this implied that ethylene signaling response was more sensitive in R3 and R4 than in R2, one effect of which could be to accelerate senescence, manifested by the suppression of tuberous root growth. In the roots of R3 and R4 plants, the down-regulated miR946a signified a high expression of targeted *CKX1*, the product of which is a potent degrader of cytokinin (Gao *et al.*, 2014; Niemann *et al.*, 2015). As a result, the expectation is that cytokinesis metabolism would be disrupted, thereby inhibiting cell division and accelerating the aging of the *R. glutinosa* roots.

Specially, in R4 plants, there was a substantial increase in the abundance of rgl-miRn15, up-regulated rgl-miRn15

targeted the *HAT* product, which is linked to transcriptional activation (Marmorstein and Roth, 2001). *TEF1* (encoding a translation elongation factor) is targeted by miR2592f, its product is involved in protein synthesis (Saito *et al.*, 2010). The miR2592f were all up-regulated in the roots of R2, R3 and R4 plants, implying the repression of *TEF1* and hence a restriction in protein synthesis. The resulting increase in the stress would likely inhibit much of the plant's core metabolism.

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One of the miRNAs up-regulated by continuous cultivation was miR533a; its miR533a target is the gene encoding EDS5, which are likely involved in the positive regulation of stress tolerance (Ishihara *et al.*, 2008; Liu *et al.*, 2016). Our present study suggested that, in the roots of continuous cultivation *R. glutinosa* plants, miR533a expression levels of R4 is the highest, the second is R3. This may have served to limit the accumulation of EDS5, and thereby the plant's tolerance of the replanting disease with increase of replanting years could compromise and become weaker and weaker.

A target of miR408a is the gene *LOB37*, the product of which is a LOB domain protein; such proteins are known to be required for adventitious or fibrous root formation (Liu *et al.*, 2005; Xu *et al.*, 2016). This miRNA was down-regulated in the roots of R2-R4 plants, implying a greater accumulation of *LOB37*, and hence favoring the growth of fibrous roots at the expense of tuberous ones. The product of the miR171a target *SCL6* is involved in the control of flowering and the regulation of apical meristem development (Curaba *et al.*, 2013; Ma *et al.*, 2016). The gene is likely to have been more active in R2-R4 plants, since miR171a abundance was low in these roots. As a result, the plants may have been induced to develop more fibrous roots and/or reach flowering earlier. *AP2*, a target of the down-regulated miRNA miR172a, is a further genetic component of floral initiation (Wollmann and Weigel, 2010); again, its up-regulation in plants suffering from replanting disease may have had the consequence of favoring the growth of the flower over that of the tuberous roots.

Conclusion

A clear effect of the continuous cultivation years of *R. glutinosa* was the alteration in the miRNA content of the roots. The up-regulation of some miRNAs and the down-regulation of others imply considerable changes to the transcriptome, since each miRNAs targets a specific transcript(s). This led to the disorder of the *R. glutinosa* plant's hormone signaling and the repression of its core metabolisms, which consequently declined its stress tolerance and resulted in forming of the disease. The analysis has provided a number of informative leads regarding the molecular basis of replanting disease. Although it is not yet clear which (if any) of the reprogrammed gene(s) is/are causal for the disease, the differential abundance of certain of the miRNAs could certainly serve as a diagnostic for the presence of the disease.

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