Scientia Horticulturae 122 (2009) 666–669

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Short communication

Differentially expressed microRNAs during solid endosperm development in coconut (*Cocos nucifera* L.)

Dongdong Li [1,*], Yusheng Zheng [1], Li Wan, Xiaoming Zhu, Zhekui Wang

Key Laboratory of Tropic Biological Resources of Ministry of Education, Hainan University, Renming Road 58, Haikou, Hainan 570228PR China

ARTICLE INFO

Article history:
Received 23 April 2009
Received in revised form 28 June 2009
Accepted 6 July 2009

Keywords:
Coconut
Endosperm
Expression
Development
miRNA

ABSTRACT

MicroRNAs (miRNAs) are a class of 20–24 nt, endogenously expressed, non-coding RNAs that play important regulatory roles in plants and animals. To identify miRNAs potentially involved in tissue development and compound anabolism, we studied miRNA expression profiles in endosperm of coconut at different developmental stages. Based on the annotation in miRBase (release 10.1), we measured a total of 179 miRNAs in immature (95 expressed miRNAs) and mature tissues (176 expressed miRNAs) using microarrays, respectively. The comparative analyses on miRNA expression profiles between these two groups of tissues showed that 23 miRNAs were up-regulated and nine miRNAs were down-regulated in matured endosperm. We further confirmed the increased expression of four miRNAs and decreased expression of a miRNA in immature endosperm using real-time PCR. Moreover, we computationally predicted the target genes of 32 miRNAs with differential expression ($p < 0.01$), and identified the lowest-score targets of six miRNAs. Finally, we discussed the potential functional relevance of several differentially expressed miRNAs.

1. Introduction

As one of the key plantation crops in tropics, the coconut palm (*Cocos nucifera* L.) is a member of the monocotyledonous family Aracaceae (*Palmaceae*) (Daniel et al., 2005). The coconut is one of very few seeds that store a major portion of its energy reserves as the medium chain length fatty acids (lauric acids) in endosperm. About 50% of coconut oil consists of fatty acids of medium chain length (C6–C12), among of which the majority are lauric acids (C12). Notably, coconut oil has the largest proportion of C6, C8 and C10 fatty acids among palm oils (López-Villalobos et al., 2001).

The endosperm is the most valuable component of the fruit, whose entire development process has an impact on fiber content and composition, lipid metabolism, and the levels of vitamins and various carbohydrates (Narayan et al., 2000). Therefore, it is important to understand key regulatory factors in the global control of development or in specific metabolism processes, such as lipid, vitamin, amylose and flavor volatiles, which would allow us to manipulate nutrition and quality characteristics associated with fruit development (Lee et al., 2007). However, few studies have focused on the endosperm development of coconut. Through comparative analyses of expressed tags from developing endosperm of coconut, we recently identified genes related to sugar storage, lipid anabolism, water flow and cell proliferation and differentiation (Li and Fan, 2008). The analysis on protein homology revealed many genes involved in endosperm development and compound biosynthesis. However, little has been known about the molecular mechanisms associated with development and metabolic regulation in the endosperm of coconut at present.

Recently, small RNAs have been recognized as a new class of growth regulators involved in plant development. So far, more than 800 miRNAs have been identified from flowering plant species, including both eudicots and monocots (Hewezi et al., 2008). By targeting mRNAs for cleavage or translational repression, miRNAs are involved in many functional processes in plants, such as leaf development (Ori et al., 2007), shoot and root development (Guo et al., 2005), floral development (Allen et al., 2005) and stress responses (Sunkar et al., 2006). In addition, plant miRNAs have been considered to regulate the biogenesis and function of miRNAs and siRNAs themselves (Bartel, 2004).

In this study, we examined the expression profiles of miRNAs at two different developmental stages of coconut endosperm, and identified a set of significantly differentially expressed miRNAs associated with endosperm development and substance anabolism. We further predicted the targets genes of these miRNAs, and we then discussed the potential function of several differentially expressed miRNAs.

Abbreviations: RT, reverse transcription; RQ, relative quantity; qRT-PCR, quantitative real-time PCR; RACE, rapid amplification of cDNA ends.

* Corresponding author. Fax: +86 0898 66279227.
E-mail address: liddfym@hotmail.com (D. Li).

[1] Dongdong Li and Yusheng Zheng contributed equally to the work.

0304-4238/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.scienta.2009.07.002

2. Materials and methods

2.1. Plant material and total RNA isolation

Two endosperms were analyzed including immature (8-month-old) and mature (12-month-old) endosperm of coconut, which were obtained from the Grand View Garden of Wenchang City, Hainan Province, China. Endosperms were isolated respectively, frozen in liquid nitrogen, and then kept at −70 °C. Total RNA was extracted from frozen endosperm with Trizol (TIANGEN, Catalog number, DP407-02) according to the manufacturer's instructions. Briefly, the frozen tissue was homogenized by Trizol, and the total RNA was precipitated with ethanol and sodium citrate. Then, the RNA pellet was suspended with DEPC-treated ddH_2O. Finally, RNA quality and quantity were determined by UV spectrum measurement of OD_{260} nm and agarose gel electrophoresis.

2.2. miRNA microarray

Total RNA samples were sent to LC Sciences (Houston, TX, USA) for miRNA microarray assays. The custom μparaflo™ microfluidic chip contained 653 unique plant miRNAs of release version 10.1, representing 877 miRNAs from 17 plant species (http://microrna.sanger. ac.uk/). The 877 miRNAs comprised 154 from *Arabidopsis thaliana*, 115 from *Oryza sativa*, 187 from *Physcomitrella patens*, 100 from *Populus trichocarpa*, 43 from *Zea mays* and 278 from 12 other plant species. Immature tissue RNA samples were labeled with Cy3 dye (in green color) and mature tissue RNA samples were labeled with Cy5 dye (in red color), followed by hybridization with the probe-containing chip. The signals were extracted after background subtraction, normalization and detection evaluation. The cut-off of statistic significance was set as p-value < 0.01. Mature miRNAs were sorted separately according to the differential ratio. The ratio values are shown in $\log_2$ scale in order to quickly and easily assess the differential direction as well as the magnitude. A positive $\log_2$ value indicates an up-regulation, while a negative $\log_2$ value indicates a down-regulation.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

The expression profiles of five mature miRNAs were also assayed by stem–loop reverse transcription-PCR (RT-PCR) (Chen et al., 2005). Total RNA from coconut pulp during two different developmental stages (8- and 12-month-old) was isolated as above. The stem–loop reverse transcription primers were designed following the method described by Chen et al. (2005). To generate cDNAs, the total RNA was reversely transcribed in the reaction solution, which contained RT primers respectively (Sangon, Shanghai, China), 1 × RT buffer, each dNTP of 0.25 mM, 3.33 U/ml MultiScribe reverse transcriptase, and 0.25 U/ml RNase inhibitor (P/N: KR103-03, Tiangen, Beijing China). The 7.5 μl reaction solution were incubated in a 96- or 384-well plate within the 9700 Thermocycler (Applied Biosystems, USA), and the parameters for the reaction were as follows: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and then held at 4 °C. All reverse transcriptase reactions, including no-template controls and RT minus controls, were run in duplicate (Lee et al., 2007). The reverse transcription product is amplified using a miRNA-specific forward primer and reverse primer. Real-time PCR was carried out on the 7500 HT Sequence Detection System (Applied Biosystems, USA), and SYBR green PCR master mix (Tiangen, Beijing, China) was used to detect PCR products. The β-actin was chosen as a reference gene. The reaction was performed as previously described (Chen et al., 2005) with some minor modifications, and the resulting melting curves were visually inspected to ensure specificity of production detection. Quantification of miRNA expression was performed in terms of comparative threshold cycle (Ct) with the $2^{-\Delta\Delta Ct}$ method, and the results were expressed as $\log_2$ of the relative quantity (RQ) of the normalized target miRNA ($\log_2$ RQ) (Livak and Schmittgen, 2001). For each gene, the experiment was performed in quintuplicate, including no-template and no-reverse transcriptase controls. The results were shown as mean ± standard derivation (S.D.). The primers used in this part are shown in Table 1.

Table 1
Primers used for reverse transcription and real-time PCR.

Name	RT Primer (5′–3′)
athm168aRT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTCCCG
athm168aFW	GCCGTCGCTTGGTGCAGGTC
athm168aRE	TCCAGTGCAGGGTCCGAGGTATTC
athm171ART	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATATT
athm171aFW	CTGATTGAGCCGCGCCAATATC
athm171aRE	ATCCAGTGCAGGGTCCGAGGTATT
athm397aRT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCATCAA
athm397aFW	CTCATTGAGTGCAGCGTTGATGG
athm397aRE	TCCAGTGCAGGGTCCGAGGTA
athm398aRT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGGG
athm398aFW	CGCGGCTGTGTTCTCAGGTC
athm398aRE	CAGTGCAGGGTCCGAGGTATTC
athm166aRT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGGAA
athm166aRE	CCAGTGCAGGGTCCGAGGTATTC
athm166aFW	GGCTCGGACCAGGCTTCATTC
β-ActinRE	AATCTATGAAGGATATGCCCTACC
β-ActinFW	TTTACCATAGTCACTGCAAGCACC

Note: underlined sequences are hybridized to a miRNA molecule to initiate reverse transcription.

2.4. Prediction of miRNA targets

All the miRNAs that showed significantly differential expression ($p < 0.01$) between the two different mature stages of endosperm were submitted to the miRU web sever (http://bioinfo3.noble.org/miRNA/miRU.htm) with default parameters. Combined with previous references, the potential targets for each miRNA were predicted.

3. Results

We measured a total of 176 miRNAs in immature and mature tissues using microarray. Of these miRNAs, 92 were expressed in both immature and mature tissues; three miRNAs (ath-miR398a, ath-miR398b and bna-miR397a) were expressed only in immature endosperm; and 81 only in mature tissues. The comparative analyses on the miRNA expression profiles from these two groups of tissues showed that 23 miRNAs were up-regulated and nine miRNAs were down-regulated in matured endosperm ($\log_2$-RQ > 1.0) (Table 2). The contrasting expression patterns between the two developmental stages suggested that miRNAs play a role in tissue development and compound metabolism of coconut endosperm (Fig. 1 and Table 2). Among differentially expressed miRNAs, miR171 showed the largest variation (8 times); miR396, miR397, miR398, miR395, miR474, miR162 and miR168 showed moderate variations (2–3 times), and the rest ones showed only variations of one time ($\log_2$ RQ > 1.0).

Real-time PCR (RT-PCR) is one of the standards or methods for quantifying gene expression. To verify the microarray data, we used RT-PCR to examine miRNA expression in the two groups of tissues. We confirmed the increased expression of ath-miR168a, ath-miR166a, ath-miR397a and ath-miR398a in immature endosperm as well as the decreased expression of ath-miR171a in

Table 2
Differentially expressed miRNAs in endosperm of coconut ($\log_2$ RQ > 1.0) and predicted function of miRNAs.

miRNA	Mean fold change	Predicted function of miRNAs	Score
vvi-miR156e	2.02	No hit found	
osa-miR156l	1.18	Post-transcriptional repression of SPL3	1.5
osa-miR162b	1.35	Modulating defence responses related to DEAD/DEAH box	3.0
zma-miR162	1.05	No hit found	
ath-miR166a	−1.46	spatially defined adaxial expression	1.5
ath-miR168a	−1.24	Regulation of chromatin and gene expression (SUVR4)	2.5
osa-miR168a	−1.16	Regulation of chromatin and gene expression (SUVR4)	2.5
ath-miR171a	1.06	Control asymmetric cell division	0
zma-miR171a	1.89	No hit found	
ppt-miR171b	2.66	Control asymmetric cell division	1.0
ptc-miR171c	1.23	Control asymmetric cell division	0
ptc-miR171e	1.62	Control asymmetric cell division	0.5
ptc-miR171k	1.41	Control asymmetric cell division	0
ptc-miR171j	2.14	Control asymmetric cell division	1.5
bna-miR171	1.42	Control asymmetric cell division	0
ath-miR395b	2.05	Regulating sulfate homeostasis	1.0
ppt-miR395	2.65	No hit found	
osa-miR396d	3.85	Transcription activator GRL3, post-transcriptional regulation	1.0
vvi-miR396b	1.80	No hit found	
ptc-miR396f	2.28	Stress-inducible protein, post-transcriptional regulation	2.0
ptc-miR396g	1.93	Post-transcriptional regulation based on cDNA AP2 domain	2.0
ath-miR397a	−4.55	Maintaining the cell in a meristematic state	0.5
ptc-miR397b	−3.83	Maintaining the cell in a meristematic state	1.5
ath-miR398a	−3.62	Regulate reactive oxygen species	3.0
ath-miR398b	−3.32	Regulate reactive oxygen species	2.5
ptc-miR474a	1.53	Post-transcriptional processes	2.0
ptc-miR474b	1.78	F-box-related post-transcriptional processes	2.5
ptc-miR474c	1.82	COV1-like protein, regulation of vascular patterning	3.0
ppt-miR477a	3.48	Involved in DNA repair and RNA processing	2.5
osa-miR528	−3.84	Ubiquitin-like specific protease 1	2.5
ath-miR854a	1.54	Similar to receptor lectin kinase 3	0.5
ppt-miR894	−1.37	No hit found	

mature endosperm. As shown in Fig. 2, the expression of these five miRNAs showed the same trends as microarray data, although the dosage changes measured by RT-PCR were smaller than those calculated by microarray. The smaller expression fold might be due to the unspecific binding of SYBR Green used in RT-PCR.

To unravel the role of these differentially expressed miRNAs in regulating the development of endosperm in coconuts, we predicted their potential targets using the miRU web server (http://bioinfo3.noble.org/miRU.htm), which is based on the penalty-scoring system for bulges and mismatches at a target site, combined with its possible conserved targets in another genome. All miRNAs with a significantly differential expression ratio ($p < 0.01$) were submitted to miRU with default parameters. For all 32 miRNAs, we identified the target genes with the lowest scores (Table 2). Among them, six miRNAs (ppt-miR395, vvi-miR156e, zma-miR171a, ppt-miR894, zma-miR162 and vvi-miR396b) had no predicted targets. Four genes were predicted to be targets for more than one miRNAs, such as scarecrow transcription factor (SCAR), which was predicted to be targets of six miRNAs (ppt-miR171b, ptc-miR171j, ptc-miR171e, bna-miR171, ptc-miR171k, ptc-miR171c and ath-miR171a). Meanwhile, four transcription factors, including transcription activator

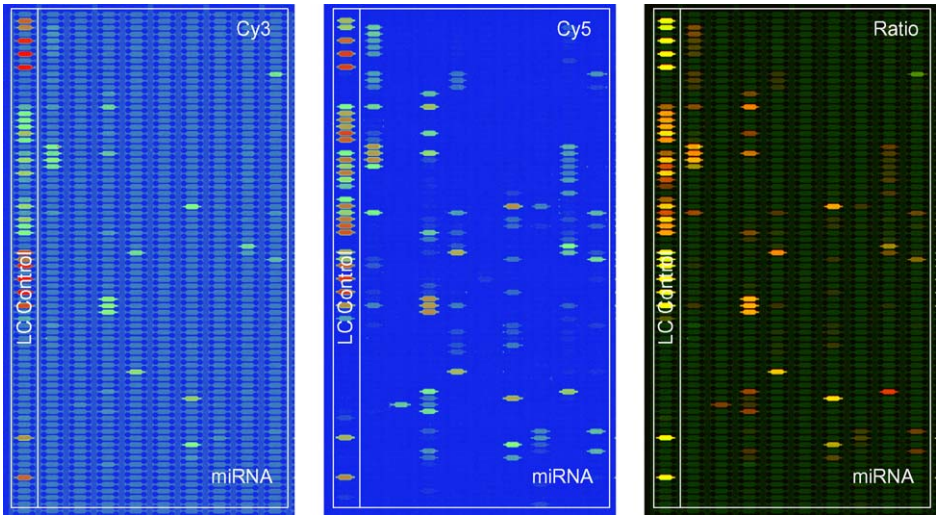

Fig. 1. Differential expression analysis of miRNAs between the immature (left) and mature (middle) endosperm of coconut (*Cocos nucifera* L.). Left: miRNA expression in immature tissue (labeling dye: Cy3); middle: miRNA expression in mature tissue (labeling dye: Cy5); and right: ratio image.

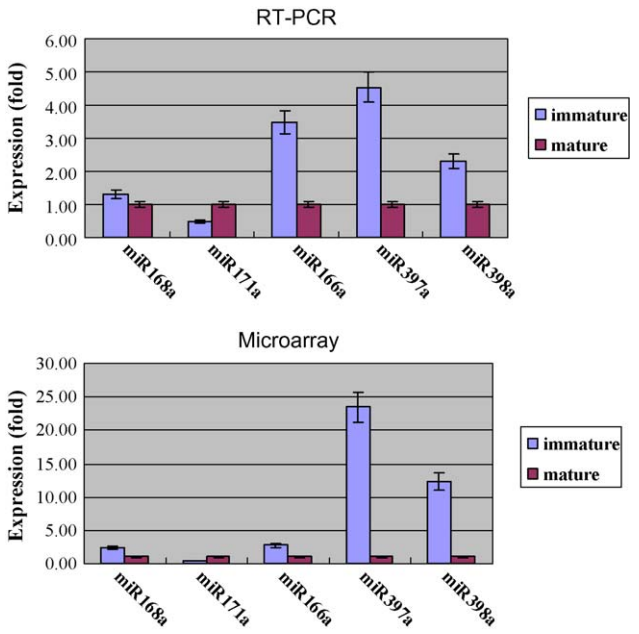

Fig. 2. Comparison of five miRNA expression in immature and mature endosperm of coconut (*Cocos nucifera* L.) between real-time PCR and microarray.

GRL3, homeobox-leucine zipper family protein (HB8), DEAD/DEAH box and S locus F-box-related domain, were also predicted to be miRNA targets (Table 2).

4. Discussion

The endosperm is an essential part of the seed that sustains embryo development and reserve storage in many cereal species (Berger, 2003). Although many recent studies report that miRNAs control the key components of signaling pathways and thereby regulate related development processes in plants (Kim et al., 2009), the relationship between miRNA expression and cellular development/compound anabolism in coconut endosperm remains largely unexplored. In this study, we compared the expression profiles of miRNAs between two different mature stages of endosperm and identified 32 miRNAs with differential expression, suggesting that miRNAs are involved in endosperm development of coconut.

We found that some miRNAs, such as ath-miR398a, ath-miR398b and bna-miR397a, were uniquely expressed in immature endosperm. In plant, miR398 and miR397 have been suggested to function in maintaining the cell in a meristematic state (Luo et al., 2008). Previous studies show that the expression level of miR397 is very low in mature organs such as leaf, flower and stem (Abdel-Ghany and Pilon, 2008). Our results verified the expression of these two miRNAs, which is consistent with the previous studies. miR397 has been shown to target laccases, a group of polyphenol oxidase (Sunkar and Zhu, 2004). Thus, the high expression of this miRNA in endosperm of coconut can repress the expression of laccase genes, which may provide one explanation for endosperm to stay in a meristematic state.

Although we only examined a few known targets here, our results provide some crucial insights into molecular mechanisms of tissue development and proliferation in coconut endosperm. In addition, because the genomic sequence and genome-wide expression data are not available in coconut, we had to predict the target genes of miRNAs in this study. Using a simplistic five-mismatch rule to identify candidate targets of a miRNA undoubtedly gives numerous false positives (Gleave et al., 2008), and the number of mismatches alone is insufficient to accurately predict targets (Schwab et al., 2005). A more accurate prediction of miRNA target candidates requires more efforts in future.

Acknowledgement

This research was supported by the National Natural Science Foundation of PR China (NSFC) (Project No. 30560092).

References

Abdel-Ghany, S.E., Pilon, M., 2008. MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in Arabidopsis. J. Biol. Chem. 283, 15932–15945.
Allen, E., Xie, Z., Gustafson, A.M., Carrington, J.C., 2005. microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell 121, 207–221.
Bartel, D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.
Berger, F., 2003. Endosperm: the crossroad of seed development. Curr. Opin. Plant. Biol. 6, 42–50.
Chen, C., Ridzon, D.A., Broomer, A.J., Zhou, Z., Lee, D.H., Nguyen, J.T., Barbisin, M., Xu, N.L., Mahuvakar, V.R., Andersen, M.R., Lao, K.Q., Livak, K.J., Guegler, K.J., 2005. Real-time quantification of microRNAs by stem–loop RT-PCR. Nucleic Acids Res. 33, e179.
Daniel, Z.V., Miguel, F.B., Nelson, T.H., Patricia, C.G., 2005. Morphological variation of fruit in Mexican populations of *Cocos nucifera* L. (Arecaceae) under in situ and ex situ conditions. Genet. Resour. Crop Evol. 52, 421–434.
Gleave, A.P., Ampomah-Dwamena, C., Berthold, S., Dejnoprat, S., Karunairetnam, S., Nain, B., Wang, Y.Y., Crowhurst, R.N., MacDiarmid, R.M., 2008. Identification and characterisation of primary microRNAs from apple (*Malus domestica* cv. Royal Gala) expressed sequence tags. Tree Genet. Genomes 4, 343–358.
Guo, H.S., Xie, Q., Fei, J.F., Chua, N.H., 2005. MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for Arabidopsis lateral root development. Plant Cell 17, 1376–1386.
Hewezi, T., Howe, P., Maier, T.R., Baum, T.J., 2008. Arabidopsis small RNAs and their targets during cyst nematode parasitism. Mol. Plant. Microbe Interact. 21, 1622–1634.
Kim, J.H., Woo, H.R., Kim, J., Lim, P.O., Lee, I.C., Choi, S.H., Hwang, D., Nam, H.G., 2009. Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis. Science 323, 1053–1057.
Lee, Y.P., Yu, G.H., Seo, Y.S., Han, S.E., Choi, Y.O., Kim, D., Mok, I.G., Kim, W.T., Sung, S.K., 2007. Microarray analysis of apple gene expression engaged in early fruit development. Plant Cell Rep. 26, 917–926.
Li, D.D., Fan, Y.M., 2008. Construction and characterization of a cDNA library from the pulp of coconut (*Cocos nucifera* L.). Agric. Sci. China 7, 101–105.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−delta delta *C*(*T*)) method. Methods 25, 402–408.
López-Villalobos, A., Dodds, P.F., Hornung, R., 2001. Changes in fatty acid composition during development of tissues of coconut (*Cocos nucifera* L.) embryos in the intact nut and in vitro. J. Exp. Bot. 52, 933–942.
Luo, Y.C., Zhou, H., Li, Y., Chen, J.Y., Yang, J.H., Chen, Y.Q., Qu, L.H., 2008. Rice embryogenic calli express a unique set of microRNAs, suggesting regulatory roles of microRNAs in plant post-embryogenic development. FEBS Lett. 580, 5111–5116.
Narayan, K.K., Deo, J.V., Abani, M., 2000. Natural tritium levels in tender and ripe coconut fruit (*Cocos nucifera* L.): a preliminary examination. Sci. Total. Environ. 256, 233–237.
Ori, N., Cohen, A.R., Etzioni, A., Brand, A., Yanai, O., Shleizer, S., Menda, N., Amsellem, Z., Efroni, I., Pekker, I., Alvarez, J.P., Blum, E., Zamir, D., Eshed, Y., 2007. Regulation of LANCEOLATE by miR319 is required for compound-leaf development in tomato. Nat. Genet. 39, 787–791.
Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M., Weigel, D., 2005. Specific effects of microRNAs on the plant transcriptome. Dev. Cell 8, 517–527.
Sunkar, R., Zhu, J.K., 2004. Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. Plant Cell 16, 2001–2019.
Sunkar, R., Kapoor, A., Zhu, J.K., 2006. Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell 18, 2051–2065.