DOI: 10.1007/s10535-014-0426-5 BIOLOGIA PLANTARUM 58 (3): 491-498, 2014

Copper stress induces the differential expression of microRNAs in non-heading Chinese cabbage

J. REN, J.J. ZHOU, W.K. DUAN, X.M. SONG, T.K. LIU, X.L. HOU, and Y. LI*

Horticultural Department, Nanjing Agricultural University, State Key Laboratory of Crop Genetics & Germplasm Enhancement, and Key Laboratory of Southern Vegetable Crop Genetic Improvement, Ministry of Agriculture, Nanjing 210095, P.R. China

Abstract

To gain a deep understanding of the regulatory mechanism of Cu-responsive microRNAs (miRNAs) in non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino), the transcription of 10 annotated stress-inducible miRNAs and their target genes were investigated in two cultivars Suzhouqing and Wutacai exposed to excess of copper. Results show that these miRNAs were negatively correlated with their target genes under the Cu stress and showed different transcriptions in different tissues and cultivars. The transcriptions of bra-miR1530a and bra-miR1533v were highest in petioles and lowest in roots. Bra-miR1533ah, bra-miR1533m, bra-miR1533t, bra-miR414a, and bra-miR398b had the highest and lowest transcriptions in leaves and roots, respectively. In contrast, the transcription of bra-miR172f was highest in roots and lowest in leaves. Bra-miR1533aj and bra-miR1533d had similar transcriptions in petioles and leaves. The promoter analysis further revealed that seven miRNAs contained the Cu-response element (CuRE). In addition, miRNAs with more CuREs in the 5'-flanking sequences showed a lower expression following the Cu treatment. It imply that CuREs likely played a role in increasing the response to Cu in non-heading Chinese cabbage.

Additional key words: *Brassica campestris*, *cis*-responsive element; Cu-response element.

Introduction

MicroRNAs are non-coding RNAs that are approximately 19 - 25 nt in length (Yin *et al*. 2012). They are well known to negatively regulate the post-transcription of genes *via* an interaction with 3' untranslated regions or coding regions of their targets (Bartel *et al.* 2004). According to *miRBase* (http://www.mirbase.org/, V 20, June 2013), thousands of miRNAs have been isolated from plants, animals, and viruses (Kozomara and Griffiths-Jones 2014). These miRNAs are crucial for plant development (Guo *et al.* 2005, Lauter *et al.* 2005, Millar and Gubler 2005, Chuck *et al*. 2007, Alonso-Peral *et al.* 2010, De Lima *et al*. 2012). In addition, miRNAs have also important functions in the regulation of stress response in plants (Zhou *et al.* 2007, 2008, 2010, Sunkar *et al.* 2008, Zeng *et al.* 2010, Waters *et al.* 2012).

Copper is a micronutrient essential for plant growth, nevertheless, it can be highly toxic at supra-optimal amounts not only to plants but also to animals and humans (Ku *et al.* 2012, Min *et al.* 2013, Song *et al.* 2013b). It has been previously reported that plants develop an important regulatory network to adapt to the frequently changing availability of Cu (Lu *et al.* 2011). Due to wastewater irrigation, over-use of fungicides and pesticides containing Cu, and unconscionable Cu mining, Cu stress has become a more serious issue. Thus, researchers have turned their attention to studying the mechanism of Cu tolerance in plants. Lu *et al.* (2011) demonstrated an important involvement of a set of miRNAs in this network. To date, studies on miRNAs transcription in response to Cu stress have mainly focused on model plants such as *Arabidopsis thaliana* (Yamasaki *et al*. 2007) and *Populus tremula* (Lu *et al*. 2011) with very little attention paid to a popular vegetable, the non-heading Chinese cabbage. Cu-tolerance varies

Submitted 20 July 2013, *last revision* 4 March 2014, *accepted* 24 March 2014.
Abbreviations: CuRE - copper-response element; miRNA - microRNA.
Acknowledgements: The research was supported by the National Basic Research Program of China (973 Program, 2009CB119001), the National Science Foundation of China (31272173), the Fundamental Research Funds for the Central Universities of China (KYZ201111), and the Project Funded by the Priority Academic Program Development of the Jiangsu Higher Education Institutions.
* Corresponding author; fax: (+86) 25 84395262, e-mail: yingli@njau.edu.cn

widely in different cultivars of this species and a previous study (Li *et al.* 2009) has shown a higher Cu-tolerance in cv. Wutacai than in cv. Suzhouqing.

Recently, Wang *et al*. (2011) analyzed 168 miRNAs in non-heading Chinese cabbage using bioinformatics. To investigate whether miRNA-associated Cu-regulatory networks exist in non-heading Chinese cabbage cvs. Wutacai and Suzhouqing, we selected 10 miRNAs (Table 1) that are stress-responsive. The target gene of these 10 miRNAs and the function of these target genes had been analyzed by Wang *et al.* (2011). The transcriptions of these miRNAs and their target genes were investigated in different tissues of both the cultivars exposed to excess of Cu.

Materials and methods

Two cultivars (Wutacai and Suzhouqing) of non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino) were grown in a greenhouse in the Nanjing Agricultural University (32° 02′ N, 118° 50′ E) in September 2012. For acclimation, seedlings that had 4 to 5 leaves were cultured in plastic containers (24 seedlings in each 18 dm^3 container) with a half-strength Hoagland's nutrient solution (Guo *et al.* 2003). After 3 d, the plants were sub-divided and cultured according to one of the following three treatments: *1*) control, *2*) 100 μM Cu, and *3*) 1 mM Cu. Roots, petioles, and leaves of the two cultivars treated with 100 μM Cu were harvested at 0, 2, and 4 h, and the leaves of two cultivars were also harvested after treatment with 100 μM or 1 mM Cu for 3 d. The samples of the untreated controls were harvested at the corresponding time points. The samples were imme-diately frozen in liquid nitrogen for storage at -80 °C until further analyses. Three replicates were prepared at each variant and time point.

Total RNA was extracted using an RNA kit (*Tiangen*, Beijing, China) according to the manufacturer's instructions. Reverse transcription (RT) reactions of miRNA and a target gene were independently analyzed using a *One Step PrimeScript*® miRNA cDNA synthesis agent kit and reverse transcriptase M (*TaKaRa*, Tokyo, Japan). All real-time quantitative PCR analyses were performed using a *iQ5* multicolor real-time PCR detection system (*Bio-Rad*, Hercules, USA) with a *SYBR Premix Ex TaqTM II* agent kit (*TaKaRa*). The RT-qPCR analysis was biologically repeated three times, and each sample was repeated as three technical replicates. The PCR conditions were as follows: 94 °C for 30 s, followed by 40 cycles of 94 °C for 10 s, 60 °C for 30 s, and 72 °C for 20 s, and then a melting curve (61 cycles at 65 °C for 10 s) was generated to confirm the specificity of the amplification. Data were collected at 72 °C in each cycle, and the expression of the genes (normalized Ct values), which were calculated using the *iQ5* optical system software *v. 2.0*, were used to determine the gene expression variations in the samples analyzed according to the $2^{-\Delta\Delta CT}$ method (Livak *et al.* 2001). miRNA forward primers and target gene primer sequences are listed in Table 1 Suppl. *Actin* (accession No. AF111812) was used as internal reference gene because the expression of this gene has been shown to be stable in non-heading Chinese cabbage (Xiao *et al*. 2012).

The 1 500 bp upstream promoter regions of ten miRNAs were analyzed to search for known stress-responsive *cis*-elements using the *PlantCARE* database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot *et al.* 2002). The position and number of the GTAC core sequence of CuRE were searched within the miRNA promoter region (600 bp) of 10 miRNAs, including bra-miR1530a, bra-miR1533ah, bra-miR1533aj, bra-miR1533d, bra-miR1533m, bra-miR1533t, bra-miR1533v, bra-miR172f, bra-miR414a, and bra-miR398b, from the *Brassica* database (http://brassicadb.org/brad/).

Our study includes three biological replicates of each experiment. A variance analysis was performed using the *SAS* software ($\alpha = 0.05$).

Results

To investigate whether miRNA-associated Cu-regulatory networks exist in non-heading Chinese cabbage, the expressions of 10 miRNAs were investigated in two cultivars (Fig 1). Cu modified the expressions of these miRNAs. In Suzhouqing, the amount of transcripts of bra-miR1530a, bra-miR1533ah, bra-miR1533aj, bra-miR414a, and bra-miR398b significantly decreased when treated with 100 μM Cu^{2+}. In addition, 1 mM Cu further decreased the transcriptions of these five miRNAs. The transcription of bra-miR1533v was significantly decreased under 100 μM Cu^{2+}, but not under 1 mM Cu. The transcriptions of bra-miR1533m and bra-miR1533t were not decreased when treated with 100 μM Cu^{2+}, but were significantly decreased under the 1 mM Cu treatment. The expressions of bra-miR1533d and bra-miR172f remained unchanged. However, in Wutacai, the transcriptions of bra-miR1533m, bra-miR1533t, bra-miR1533d, and bra-miR172f were significantly decreased under both the 100 μM and 1 mM Cu treatments.

We further analyzed the expressions of 10 miRNA-targeted genes in leaves of two non-heading Chinese

cabbage cultivars under the Cu stress (Fig 2). The expressions of these 10 genes were up-regulated as the Cu^{2+} concentrations increased, and down-regulated as the available Cu^{2+} concentrations decreased in the media. In addition, except contig4123, the expressions of other nine target genes in Wutacai were higher compared to those in Suzhouqing.

The transcriptions of these miRNAs in different tissues were examined in Suzhouqing (Fig. 3). Except bra-miR1530a, the transcriptions of the other nine miRNAs showed similar expression patterns in which the highest and lowest expressions were observed in leaves and roots, respectively. For bra-miR1530a, the highest expression was observed in petioles.

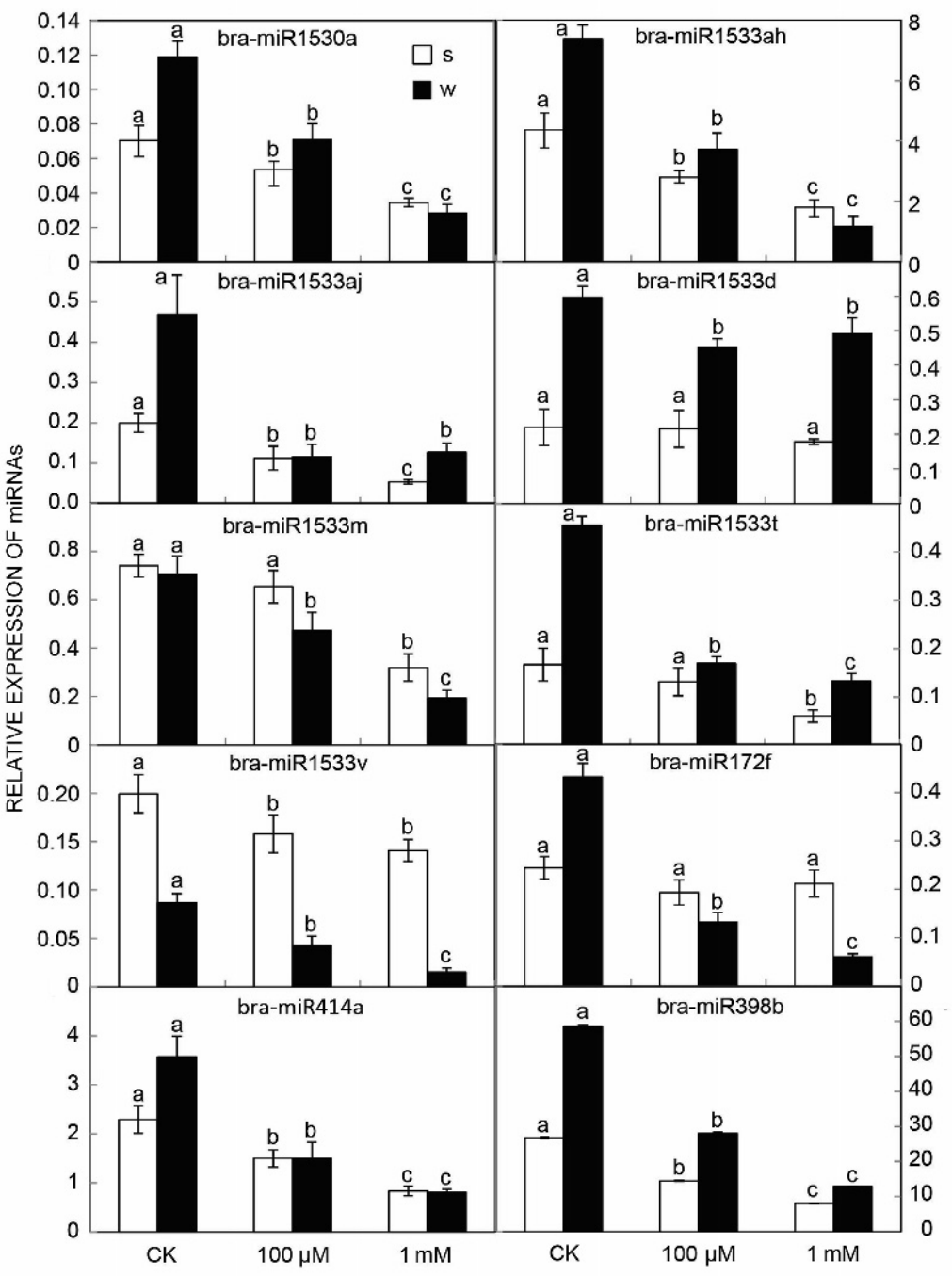

Fig. 1. The transcriptions of miRNAs in leaves of Wutacai (W) and Suzhouqing (S) cultivars of non-heading Chinese cabbage. Plants were treated with 0 (CK), 100 μM, or 1 mM Cu^{2+} for 3 d. The transcriptions were normalized according to those of *actin* (control). *Error bars* represent SE from three independent replicates. Different letters indicate significant differences ($P < 0.05$) between the control and Cu-treated plants.

Discussion

Many studies have indicated that miRNAs are involved in the stress responses (Lu *et al.* 2005, 2008, 2011, Sunkar *et al.* 2006, 2008). Previous studies have shown that excess of Cu may induce oxidative stress (Ku *et al.* 2012, Min *et al.* 2013, Song *et al.* 2013a). To investigate the Cu-responsive miRNAs in non-heading Chinese cabbage, we selected 10 miRNAs that may play important roles in plant stress responses. The miR398 is conserved in various plant species (Jones-Rhoades and Bartel 2004, Lu *et al*. 2011). Thus, we randomly selected one miR398 (bra-miR398b) for analysis in this study. Lu *et al.* (2011) reported the miR398s cleaves transcripts encoding cytosolic (AtCSD1) and plastidic (AtCSD2) Cu-Zn superoxide dismutases and a zinc-binding subunit 5b of cytochrome *c* oxidase (AtCOX5b-1) in *Arabidopsis thaliana* and *Populus trichocarpa*. Thus, zinc finger family proteins may be involved in the miRNA-associated Cu-regulatory network. Further, we randomly selected three miRNAs (bra-miR1530a, bra-miR1533v, and bra-miR414a), whose target gene is a zinc finger family protein, to investigate the miRNA-associated Cu-regulatory network in non-heading Chinese cabbage. AP2 proteins, one of the most important families of transcriptional regulators, play a crucial role in response to biotic and abiotic stressors (Song *et al*. 2013b). The bra-miR1533 is the largest miRNA family in non-heading Chinese cabbage. We randomly selected two miRNAs (bra-miR1533d and bra-miR1533t) in this family and one in another family (bra-miR172f) in which the target genes are AP2 proteins. Next, we randomly selected three miRNAs (bra-miR1533ah, bra-miR1533aj, and bra-miR1533m) that are involved in plant stress responses in the bra-miR1533 family.

Examining the 5′-flanking sequences of these 10 miRNAs show that *cis*-responsive elements were present in these miRNAs promoters (Table 2). Each miRNA had more than one *cis*-responsive element that is involved in regulation of stress. These *cis*-reponsive elements include ABRE (ABA-responsive element), MBS (MYB binding site), TCA (salicylic acid-responsive element), TGA (auxin-responsive element)，HSE (heat shock-responsive element), LTR (low temperature-responsive element), GC-motif (enhancer-like element involved in anoxic-specific inducibility)，ARE (anaerobic-response element), and GARE (gibberellin-responsive element). In *Arabidopsis*, the ABREs in the gene promoter region respond to many types of biotic stresses, and the AREs respond to hypoxia, low temperature, and drought (Dieterich *et al.* 2005). Our in-*silico* analysis revealed that the analyzed miRNAs exhibited on average seven *cis*-elements, many of which respond to different abiotic and biotic stresses including Cu stress (Ding *et al.* 2009, Zeng *et al.* 2010).

Table 1. The position and number of GTAC in the 5-flanking region (600 bp) of the mature miRNAs.

miRNA name	GTAC positon	GTAC number
bra-miR1530a	-43	1
bra-miR1533ah	-46	1
bra-miR1533aj	-338	1
bra-miR1533d		0
bra-miR1533m	-325	1
bra-miR1533t	-241	1
bra-miR1533v	-250/ -331	2
bra-miR172f		0
bra-miR414a		0
bra-miR398b	-115/-146/ -410/-540	4

Table 2. Stress-responsive *cis*-elements in miRNA promoters. The *cis*-responsive elements were searched using the *PlantCARE* (Lescot *et al.* 2002) database for the promoter regions (1 500 bp) of 10 miRNAs. ABRE - ABA-responsive element, ARE - anaerobic-responsive element, GARE - gibberellin-responsive element, GC - GC-motif (enhancer-like element involved in anoxic-specific inducibility), HSE - heat shock element, LTR - element involved in low-temperature response, MBS - MBY binding site, TCA - salicylic acid-responsive element, TGA - auxin-responsive element.

miRNA name	ABRE	ARE	GARE	GC	HSE	LTR	MBS	TCA	TGA	Total
bra-miR1530a		4		1	2			1	1	9
bra-miR1533ah					1				2	3
bra-miR1533aj						1	2			3
bra-miR1533d	3	2						1		6
bra-miR1533m	1	2			1	1	5		1	11
bra-miR1533t		2	1		1	1	1			6
bra-miR1533v	2	2					5			9
bra-miR172f	1	5	3		1	1	3	2	1	17
bra-miR414a	1	2	2				1	1		7
bra-miR398b		2				1	3	1	1	8
Total	8	21	6	1	6	5	20	6	6	79

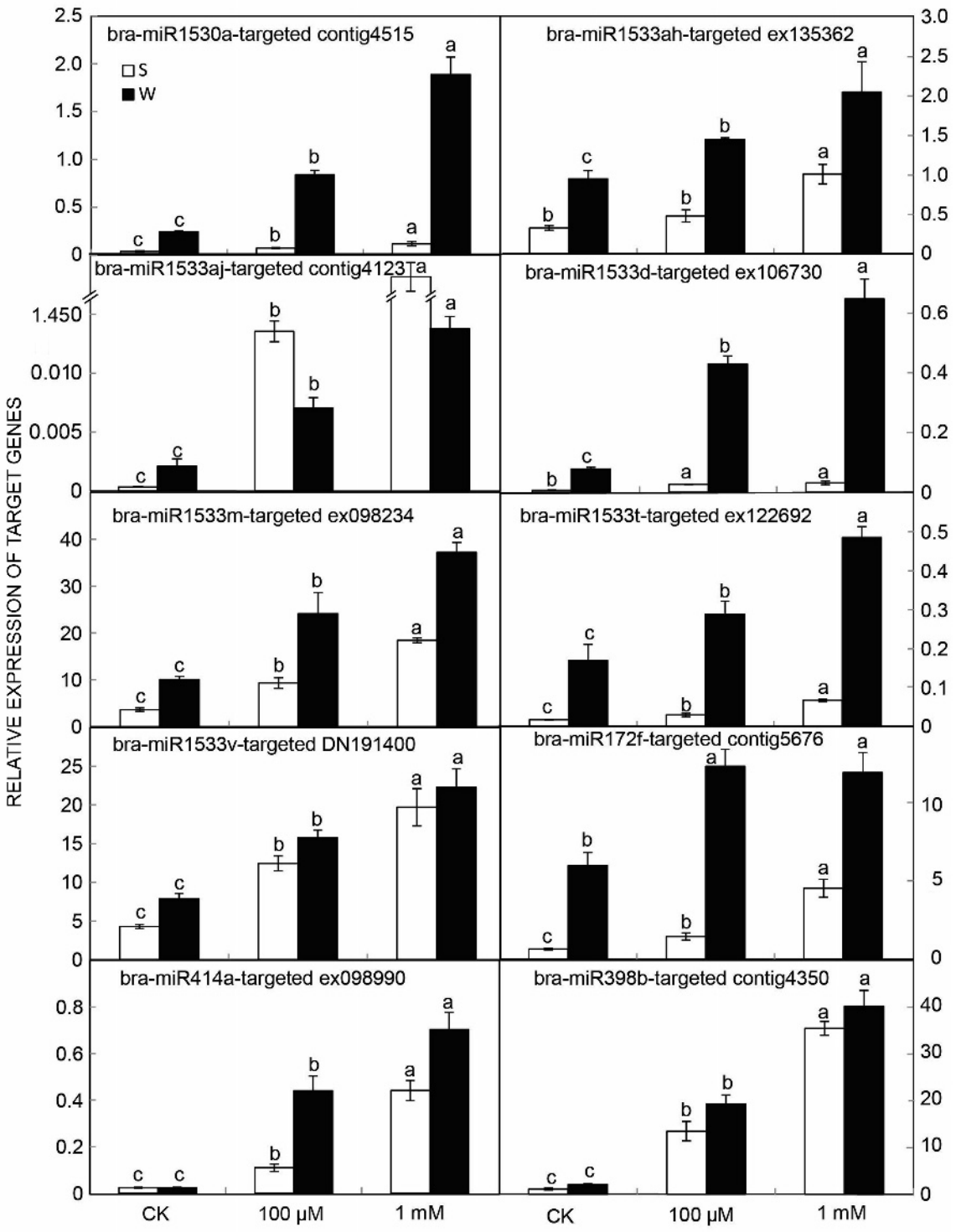

Fig. 2. The transcriptions of target genes in different cultivars of non-heading Chinese cabbage. The targets include bra-miR1530a-targeted contig4515, bra-miR1533ah-targeted ex135362, bra-miR1533aj-targeted contig4123, bra-miR1533d-targeted ex106730, bra-miR1533m-targeted ex098234, bra-miR1533t-targeted ex122692, bra-miR1533v-targeted dn191400, bra-miR172f-targeted contig5676, bra-miR414a-targeted ex098990, and bra-miR398b-targeted contig4350. Plants were treated with 0, 100 μM, or 1 mM Cu^{2+} for 3 d. The transcriptions were normalized according to those of *actin*. Values represent means ± SE of three replicates. Different letters indicate significant differences ($P < 0.05$) between the control and Cu-treated plants.

To resist an environmental stress, a plant may regulate its stress-related gene expression *via* the induction or restriction of corresponding miRNA expression. For example, Pant *et al.* (2009) performed the RT-qPCR and

found that a low nitrogen content restricts the expression of miR169 and miR398. Sunkar *et al.* (2007) demonstrated that miRNAs that have an increased expression under stress may target to some negative regulatory factor. However, miRNAs with decreasing expression levels may target to some positive regulatory factor. In this study, 10 miRNAs were remarkably down-regulated (Figs. 1 and 3) and the expressions of target genes of those miRNAs were increased (Fig. 2) when the Cu concentration increased suggesting that these miRNAs might respond to positive regulatory factors and that these target genes might be the positive regulatory factors that played important roles in the Cu stress. These results demonstrate that the Cu-associated miRNA

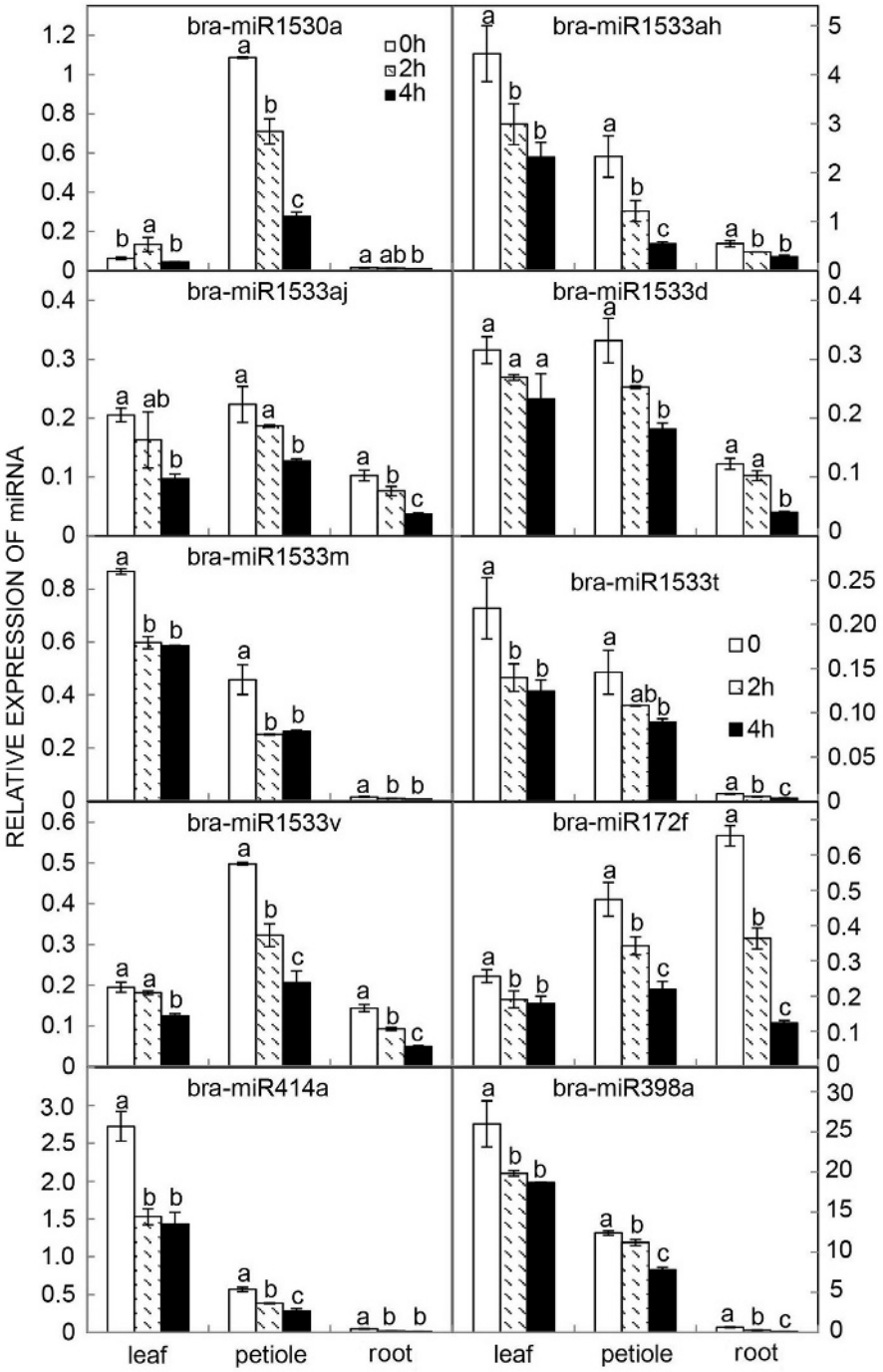

Fig. 3. The transcriptions of miRNAs in leaves, petioles, and roots of cv. Suzhouqing grown at 100 μM Cu^{2+} for 0, 2, and 4 h. Values represent means ± SE of three replicates. Different letters indicate significant differences ($P < 0.05$). between the control (0 h) and Cu-treated plants.

suppression occurred in non-heading Chinese cabbage which is consistent with results observed in *A. thaliana* and *P. trichocarpa* (Lu *et al.* 2011).

The promoters of the conserved Cu-associated miRNAs carry the Cu response element (CuRE) (Quinn *et al*. 1995, 2000, Sunkar *et al*. 2006, Nagae *et al.* 2008,Lu *et al*. 2011). Nagae *et al.* (2008) demonstrated the CuRE contains the GTAC core sequence. This study found that except for the promoters of bra-miR 1533d，bra-miR 172f, and bra-miR 414a, the other seven miRNAs exhibited the GTAC core sequence (Table 1) and those miRNAs were conserved Cu-associated miRNAs. Lu *et al.* (2011) proposed CuRE as negative *cis*-responsive element, and miRNAs with more GTAC core sequences show a lower expression in Cu-treated than in the untreated *P. trichocarpa*. The GTAC core sequence likely plays a role in increasing the response to Cu. In this study, bra-miR398 which has the most copies of the GTAC core sequence among the 10 miRNAs determined**,** had the most dramatic decrease of transcription under the Cu stress. These results further confirmed the previous findings.

Interestingly, the transcriptions of most 10 miRNAs were higher in Wutacai than in Suzhouqing (Fig. 1). Although the transcriptions of these miRNAs in Wutacai demonstrated the same changing trends as in Suzhouqing, the decrease in the former was obviously larger than in the latter under the same Cu concentration. Ding *et al.* (2009) found that miRNAs show different expression profiles in the salt-tolerant maize inbred line NC286 and the salt-sensitive line Huangzao4, and this miRNA genotype-specific expression model can explain the distinct salt sensitivities between maize lines. The transcriptions of 10 miRNAs were different in Wutacai and Suzhouqing. Li *et al.* (2009) showed that the Cu-tolerance of Wutacai is higher than that of Suzhouqing. Thus, the different transcriptions of miRNAs between Wutacai and Suzhouqing might contribute to and partially explain the distinct Cu sensitivities between the two non-heading Chinese cabbage cultivars.

In addition to the differences among genotypes, the miRNAs were also spatially distributed in one genotype. For example, in each cultivar, the transcriptions of bra-miR1533ah, bra-miR1533m, and bra-miR398b in leaves were higher than in petioles and roots. This result is consistent with previous studies which showed that several miRNAs are tissue-specific (Chapman and Carrington 2007, Robertus *et al.* 2009). Zhao *et al.* (2007) showed that this phenomenon is due to the special functions of miRNAs in organs and cells.

In conclusion, Cu-responsive miRNAs in non-heading Chinese cabbage included not only conserved miR398 but also bra-miR1533 which suggests that the conservation and diversity of Cu-associated suppression of miRNAs also exists in non-heading Chinese cabbage. These results further confirm the previous findings that the 5'-flanking sequences of Cu-responsive miRNA genes containing multiple copies of CuREs likely play a role in increasing the response to Cu, and that there is a stronger down-regulation following a Cu treatment. However, the miRNA-associated Cu-regulatory network is a complex system, and further research is required to elucidate the complexity of this network in plants.

References

Alonso-Peral, M.M., Li, J., Li, Y., Allen, R.S., Schnippenkoetter, W., Ohms, S., White, R.G., Millar, A.A.: The microRNA159-regulated GAMYB-like genes inhibit growth and promote programmed cell death in *Arabidopsis*. - Plant Physiol. **154:** 757-771, 2010.

Bartel, D.P.: MicroRNAs: genomics, biogenesis, mechanism and function. - Cell **116**: 281-297, 2004.

Chapman, E.J., Carrington, J.C.: Specialization and evolution of endogenous small RNA pathways. - Nature Rev. Genet. **8**: 884-896, 2007.

Chuck, G., Cigan, A.M., Saeteurn, K., Hake, S.: The heterochronic maize mutant *Corngrass1* results from overexpression of a tandem microRNA. - Nat. Genet. **39**: 544-549, 2007.

De Lima, J.C., Loss-Morais, G., Margis, R.: MicroRNAs play critical roles during plant development and in response to abiotic stresses. - Genet. mol. Biol. **35**(4 Suppl): 1069-1077, 2012.

Dieterich, C., Grossmann, S., Tanzer, A., Röpcke, S., Arndt, P.F., Stadler, P.F., Vingron, M.: Comparative promoter region analysis powered by CORG. - BMC Genomics **6**: 24, 2005.

Ding, D., Zhang, L.F., Wang, H., Liu, Z., Zhang, Z.X., Zheng, Y.L.: Differential expression of miRNAs in response to salt stress in maize roots. - Ann. Bot. **103**: 29-38, 2009.

Guo, H.S., Xie, Q., Fei, J.F., Chua, N.H.: MicroRNA directs mRNA cleavage of the transcription factor *NAC1* to downregulate auxin signals for *Arabidopsis* lateral root development. - Plant Cell **17**: 1376-1386, 2005.

Guo, S.R.: Nutrient solution. - In: Guo, S.R. (ed.): Soilless Cultivation Science. Pp. 111-115. China Agricultural Press, Beijing 2003.

Jones-Rhoades, M.W., Bartel, D.P.: Computational identification of plant microRNAs and their targets, including a stress induced miRNA. - Mol. Cell **14**: 787-799, 2004.

Kozomara, A., Griffiths-Jones, S.: MiRBase: annotating high confidence microRNAs using deep sequencing data. - Nucl. Acids Res. **42**: D68-D73, 2014.

Ku, H.M., Tan, C.W., Su, Y.S., Chiu, C.Y., Chen, C.T., Jan, F.J.: The effect of water deficit and excess copper on proline metabolism in *Nicotiana benthamiana*. - Biol. Plant. **56**: 337-343, 2012.

Lauter, N.，Kampani, A., Carlson, S., Goebel, M., Moose, S.P.: *MicroRNA172* down-regulates *glossy15* to promote vegetative phase change in maize. - Proc. nat. Acad. Sci. USA. **102**: 9412-9417, 2005.

Lescot, M., Dehais, P., Thijs, G., Marchal, K., Moreau, Y.,

Van de Peer, Y., Rouzé, P., Rombauts, S.: PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for in *silico* analysis of promoter sequences. - Nucl. Acids Res. **30**: 325-327, 2002.

Li, Y., Song, Y.P., Shi, G.J., Wang, J.J., Hou, X.L.: Response of antioxidant activity to excess copper in two cultivars of *Brassica campestris* ssp. *chinensis* Makino. - Acta Physiol. Plant **31**: 155-162, 2009.

Livak, K.J., Schmittgen, T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, - Methods **25**: 402-408, 2001.

Lu, S.F., Sun, Y.H., Chiang, V.L.: Stress-responsive microRNA in *Populus*. - Plant J. **55**: 131-151, 2008.

Lu, S.F., Sun, Y.H., Shi, R., Clark, C., Li, L., Chiang, V.L.: Novel and mechanical stress-responsive microRNA in *Populus trichocarpa* that are absent from *Arabidopsis*. - Plant Cell **17**: 2186-2203, 2005.

Lu, S.F., Yang, C.M., Chiang, V.L.: Conservation and diversity of microRNA-associated copper-regulatory networks in *Populus trichocarpa*. - J. Integr. Plant Biol. **53:** 879-891, 2011.

Millar, A.A., Gubler, F.: The *Arabidopsis GAMYB-like* genes, *MYB33* and *MYB65*, are microRNA-regulate genes that redundantly facilitate anther development. - Plant Cell **17**: 705-721, 2005.

Min, H.L., Cai, S.J., Rui, Z., Sha, S., Xie, K.B., Xu, Q.S.: Calcium-mediated enhancement of copper tolerance in *Elodea canadensis*. - Biol. Plant. **57**: 365-369, 2013.

Nagae, M., Nakata, M., Takahashi, Y.: Identification of negative *cis*-acting elements in response to copper in the chloroplastic iron superoxide dismutase gene of the moss *Barbula unguiculata*. - Plant Physiol. **146**: 1687-1696, 2008.

Pant, B.D., Musialak-Lange, M., Nuc, P., May, P., Buhtz, A., Kehr, J., Walther, D., Scheible, W.R.: Identification of nutrient-responsive Arabidopsis and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing. - Plant Physiol. **150**: 1541-1555, 2009.

Quinn, J.M., Barraco, P., Eriksson, M., Merchant, S.: Coordinate copper- and oxygen-responsive *Cyc6* and *Cpx1* expression in *Chlamydomonas* is mediated by the same element. - J. biol. Chem. **275**: 6080-6089, 2000.

Quinn, J.M., Merchant, S.: Two copper-responsive elements associated with the *Chlamydomonas Cyc6* gene function as targets for transcriptional activators. - Plant Cell **7**: 623-628, 1995.

Robertus, J.L., Harms, G., Blokzijl, T., Booman, M., Jong, D.D., Imhoff, G.V., Rosati, S., Schuuring, E., Kluin, P., Berg, A.V.D.: Specific expression of miR-17-5p and miR-127 in testicular and central nervous system diffuse large B-cell lymphoma. - Mod. Pathol. **22**: 547-555, 2009.

Song, J., Yang, Y.Q., Zhu, S.H., Chen, G.C., Yuan, X.F., Liu, T.T., Yu, X.H., Shi, J.Y.: Spatial distribution and speciation of copper in root tips of cucumber revealed by μ-XRF and of copper in root tips of cucumber revealed by μ-XRF and μ-XANES. - Biol. Plant. **57**: 581-586, 2013a.

Song, X.M., Li, Y., Hou, X.L.: Genome-wide analysis of the AP2/ERF transcription factor superfamily in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*). - BMC Genomics **14**: 573, 2013b.

Sunkar, R., Chinnusamy, V., Zhu, J., Zhu, J.K.: Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. - Trends Plan Sci. **12**: 301-309, 2007.

Sunkar, R., Kapoor, A., Zhu, J.K.: 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, 2006.

Sunkar, R.，Zhou, X.，Zheng, Y.，Zhang, W.X., Zhu, J.K.: Identification of novel and candidate miRNAs in rice by high throughput sequencing. - BMC Plant Biol. **8**: 1-10, 2008.

Wang, J.Y., Hou, X.L., Yang, X.D.: Identification of conserved microRNAs and their targets in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*). - Genome **54**: 1029-1040, 2011.

Waters, B.M., Mclnturf, S.A., Stein, R.J.: Rosette iron deficiency transcript and microRNA profiling reveals links between copper and iron homeostasis in *Arobidopsis thaliana*. - J. exp. Bot. **63**: 5903-5918, 2012.

Xiao, D., Zhang, N.W., Zhao, J.J., Guusje, B., Hou, X.L.: Validation of reference genes for quantitative PCR normalization in non-heading Chinese cabbage. - Funct. Plant Biol. **39**: 342-350, 2012.

Yamasaki, H., Abdel-Ghany S.E., Cohu, C.M., Kobayashi, Y., Shikanai, T., Pilon, M.: Regulation of copper homeostasis by micro-RNA in *Arabidopsis*. - J. biol. Chem. **282**: 16369-16378, 2007.

Yin, Z.J., Li, Y., Han X.L., Shen, F.F.: Genome-wide profiling of miRNAs and other small non-coding RNAs in the *Verticillium dahliae* -inoculated cotton roots. - PloS ONE **7(4)**: e35756, 2012.

Zeng, H.Q., Zhu, Y.Y., Huang, S.Q., Yang, Z.M.: Analysis of phosphorus-deficient responsive miRNAs and *cis*-elements from soybean (*Glycine max* L.). - J. Plant Physiol. **167**: 1289-1297, 2010.

Zhao, B., Liang, R., Ge, L., Li, W., Xiao, H., Lin, H., Ruan, K., Jin, Y.: Identification of drought-induced microRNAs in rice. - Biochem. biophys. Res. Commun. **354**: 585-590, 2007.

Zhou, L., Liu, Y., Liu, Z.C., Kong, D., Duan, M., Luo, L.J.: Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa*. - J. Exp. Bot. **61**: 4157-4168, 2010.

Zhou, X.F., Wang, G.D., Sutoh, K., Zhu, J.K., Zhang, W.X.: Identification of cold-inducible microRNAs in plants by transcriptome analysis. - BBA **1779**: 780-788, 2008.

Zhou, X.F., Wang, G.D., Zhang, W.X.: UV-B responsive microRNA genes in *Arabidopsis thaliana*. - Mol. syst. Biol. **3**: 103-115, 2007.