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Binding of miR398 to mRNA of Chaperone and Superoxide Dismutase Genes in Plants

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***Abstract*—**Among all microRNAs (miRNAs) in 12 plant species investigated in this study, only miR398 targeted the copper chaperone for superoxide dismutase (CCS). The nucleotide sequences of miRNA binding sites were located in the mRNA protein-coding sequence (CDS) and were highly homologous. These binding sites in *CCS* mRNA encoded a conservative GDLGTL hexapeptide. The binding sites for miR398 in the CDS of superoxide dismutase 1 mRNA encoded GDLGN pentapeptide. The conservative miR398 binding site located in the CDS of superoxide dismutase 2 mRNA encoded the GDLGNI hexapeptide. The miR398 binding site in the CDS of superoxide dismutase 3 mRNA encoded the GDLGNI or GDLGNV hexapeptide. Gene expression of the entire superoxide dismutase family in the studied plant species was regulated only by miR398. All members of the miR398 family, i.e. miR398a,b,c were connected to one site for each CuZnSOD and chaperone mRNA.

***Keywords*—**MicroRNA, mRNA, plant, superoxide dismutase.

I. Introduction

COPPER/ZINC superoxide dismutase (CuZnSOD) has been identified in cells of archaea, eukaryotes, protists, fungi, plants, and animals [1]. In plants, the CuZnSOD family consists of CuZnSOD1, CuZnSOD2 and CuZnSOD3. CuZnSOD1 is a homodimer, while CuZnSOD2 and CuZnSOD3 consist of four subunits each. The copper chaperone for superoxide dismutase (*CCS*) is involved in ensuring CuZnSOD activity [2]. The *CCS* gene is expressed in roots and shoots and is upregulated in response to copper and senescence. The CCS activates all three CuZnSODs which are located in three different subcellular compartments [3], [4]. CuZnSOD family proteins protect cells against superoxide anions, catalyzing their transformation into molecular oxygen and hydrogen peroxide [5], [6]. In plants, superoxide dismutases are involved in many processes and are expressed during 14 stages of growth, thereby having a large functional load. Changes in the activity of CuZnSODs occur under abiotic and biotic stresses, indicating their important role in plant productivity [7]. Recently, microRNAs (miRNAs) were identified as key players in many plant responses to stress [2], [8]-[10]. In response to drought, the plant *Triticum turgidum ssp. Dicoccoides* shows changes in the expression of miR1867, miR896, miR398, miR528, miR474, miR1450, miR396, miR1881, miR894, miR156, miR1432, miR166 and miR171 [11]. Significant changes in the concentrations of many miRNAs in the leaves and roots of *Prunus persica L.* were also identified in response to drought [12]. miR398 is involved in the plant responses to oxidative stress, water deficit, salt stress, UV stress, temperature stress, abscisic acid, copper and phosphate deficiency, the concentration of sucrose and bacterial infections [5], [6], [9], [13]-[18]. miR398 is also involved in plant response to cold stress and supports the habitus of plants [19]. miR398 has been shown to affect the activity of *Arabidopsis thaliana* CuZnSOD1 and CuZnSOD2 by promoting degradation of their mRNAs or, by inhibiting translation [2]. Additionally, miR398 has been shown to regulate the expression of the major chloroplast copper-dependent proteins, i.e. plastotsiana, CuZnSOD and Csd2. Suppression of CCS expression leads to inactivation of CuZnSODs in *A. thaliana* cells [20]. Therefore, it is necessary to elucidate the involvement of miRNAs in the regulation of CuZnSOD and CCS expression.

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II. Materials and Methods

We studied CuZnSOD genes and amino acid sequences of following plants: *Arabidopsis lyrata* (Aly), *Arabidopsis thaliana* (Ath), *Brachypodium distachyon* (Bdi), *Glycine max* (Gma), *Lycopersicon esculentum* (Les), *Medicago truncatula* (Mtr), *Oryza sativa* (Osa), *Physcomitrella patens* (Ppa), *Populus trichocarpa* (Ptr), *Ricinus communis* (Rco), *Selaginella moellendorffii* (Smo), *Sorghum bicolor* (Sbi), *Triticum aestivum* (Tae), *Vitis vinifera* (Vvi), *Zea mays* (Zma). Gene nucleotide sequences and protein amino acid sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov). Accession of CCS: Ath-GI:145335438, Aly-GI:297849624, Bdi-GI:357165368, Gma-GI:351722110, Lec-GI:5759319, Mtr-GI:357476979, Osa-GI:115460026, Ptr–GI:224074743, Rco-GI:255537177, Tae-TC450218, Vvi-GI:225426700, Zma-GI:226496707. Accession of CuZnSOD1: Ath–GI:15223944, Aly-GI:297843670, Bdi-GI:357121554, Gma-GI:351721628, Mtr-GI:357512147, Osa-Os07t0665200-01, Ppa-GI: 168016534, Ptr-GI:224130836, Rco-GI:255542449, Sbi-GI:242081805, Smo-GI:302798056, Vvi-GI:225451122, Zma-GI:162463248. Accession of CuZnSOD2: Ath-GI:145360415, Aly-GI:297826125, Bdi-GI:357148946, Gma-GI:356542677, Mtr-GI:357472086, Ptr-GI:224118332, Osa-Os08t0561700, Rco-GI:255565475, Sbi-GI:242081805, Vvi-GI:225436450, Zma-GI:225436450. Accession of CuZnSOD3: Ath-GI:15238738,

Aly-GI:297807835, Bdi-GI:357113374, Gma-GI:363814340, Mtr-GI:357497317, Osa-GI:115473931, Ppa-GI:168005768, Ptr-GI:224123758, Rco-GI:255568894, Sbi-GI:242036479, Smo-GI:30280781513, Vvi-GI:225441597, Zma-GI:162462124. The nucleotide sequences of 338 miRNAs of *A. thaliana* were received from miRBase (http://www.mirbase.org). The free energy of hybridization (ΔG), position of potential binding sites and interaction schemes were calculated using the RNAHybrid program (bibiserv.techfak.uni-bielefeld.de/rnahybrid/). $\Delta G/\Delta G_m$ ratio, *p*-value, and localization of miRNA binding sites on mRNA were calculated by E-RNAhybrid script (http://sites.google.com/site/malaheenee/software).

The maximal interaction energy (ΔGm) of ath-miR398a was equal to the binding energy of the perfectly complementary sequence (-191kJ/mol). 2-dimensinal (2D) mRNA structure was built using the UNAFold program (http://mfold.rna.albany.edu/). Graphs of nucleotide and amino acid sequences variabilities were created using the WebLogo program (weblogo.berkeley.edu/).

III. Results

A. Mir398 Binding to mRNA of CCS Gene

The binding energies of miR398 to CCS mRNA in 12 plant species are shown in Table I. miR398 binding energy to CCS mRNA varied from -140 to -158kJ/mol. While calculating the energy of intramolecular interactions in miRNA binding sites in the 2D structure of mRNA, we found that the binding energy between mRNA sites was significantly lower than the energy of miRNA and mRNA interaction. Thus, the binding of miRNA to these sites was favoured over the intramolecular interactions of mRNA sites. In the 2D structure of mRNA, nearly all miRNA binding sites were not paired at the 5′ and 3′ ends located in the loops of single-stranded sections of mRNAs (Fig. 1). This facilitated the separation of mRNA strands by helicase, which is composed of the RNA-induced silencing complex (RISC) [21]. During the separation of mRNA strands, RISC can freely communicate with mRNA by miRNA in this complex. This mechanism of miRNA binding to mRNA is necessary even in the case of siRNA and mRNA with full complementarity. Without the helicase in RISC siRNA is less likely to bind to the mRNA [21].

Table I shows the nucleotide sequences of the binding sites for miR398 in plant CCS mRNA. The nucleotide sequences of miR398 binding sites were located in the CDS. These sites were homologous to CCS mRNAs in the studied plants and encoded the same GDLGTL hexapeptide. Fig. 1 shows a diagram indicating the conservation of nucleotide and amino acids sequences.

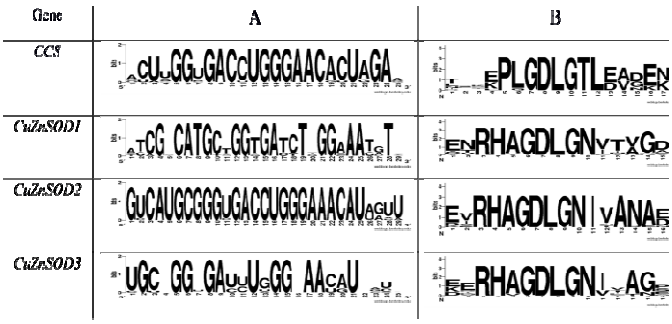

Fig. 1 The variability of nucleotides in the miR398 binding site in the CDSs of CCS and CuZnSOD mRNAs (A), and the variability of amino acids in the corresponding regions of their proteins (B)

The amino acids before and after the hexapeptide were variable, indicating the conservation of the binding site in the evolution of the studied plants for tens of millions of years. Accordingly, the system of regulation of CCS gene expression by miRNA arose at an early stage in plants evolution. Changes in the nucleotide sequences were usually present in the third position of codons. These changes did not affect the coding of amino acids, but varied the degree of miRNA:mRNA interactions. The binding energy of miRNA to mRNA was more than 70% of its maximum value and in cases of similar concentrations of these molecules, most of them will be bound.

TABLE I
Characteristics of miR398 Binding Sites in the CDS of *CCS* Orthologs

Object	Region of CDS in mRNA of *CCS* ortholog genes	Region of orthologous CCS	Position,nt	ΔG, kJ/mol
Ath	CCAUUG**GGAGACCUGGGAACACUAGA**GGC	TGTEPL**GDLGTL**EADKNG	698	-156
Aly	·····	·N·····	692	-156
Bdi	··CC·U··U·····G·····A··	ICEK·····GE··	702	-154
Gma	··CC·U··U·····A··	NSK·····NEK·	653	-158
Lec	···C·C··U·····G··U·U	LYSL·····DV·EK·	692	-148
Mtr	···C·U··U·····U·U	NTK·····DVNEK·	678	-147
Osa	··UC·U··U·····A··	RSNK·····GEK·	705	-147
Ptr	···C·U·····U····U·U	·EQ·····DV·EK·	711	-147
Rco	···CA···U···U·····U	·EK··Q·····EV·E··	731	-158
Tae	··CC·U··U·····A··	LSDK·····GE··	759	-153
Vvi	··UC·U··U·····G··U·U	·DE·····DV·E··	708	-147
Zma	···C·U··U·····A··	LSDK·····GE··	693	-140

Note: The dots are equal to nucleotides or amino acids in the data presented in Tables I–IV. The nucleotide sequences of miR398 binding sites are in bold face.

TABLE II
CHARACTERISTICS OF MIR398 BINDING SITES IN CDSS OF CUZNSOD1 ORTHOLOGS

Object	Region of CDS	Region of CuZnSOD1	Position, nt	ΔG, kJ/mol
Ath	AUCGC**CAUGCUGGUGAUCUAGGAAA**CAUC	ANRHA**GDLGN**ITVGD	237	-111
Aly	·····	·····	237	-111
Bdi	C·····U·····UG·G	ET·····V·A·V	237	-100
Gma	····U·····U··G··UG·U	V·····VN···	237	-111
Mtr	C···A·····U·····UG··	ET·····V····	237	-89
Osa	·C·····U·····U··A	EN·····A·A	240	-92
Ppa	U···U·····A··A··C··G·····UG·U	EV·····VIA··	246	-120
Ptr	····U·····G·····UG··	E·····V····	237	-113
Rco	·····G·····G·····UG··	E·····V····	237	-120
Sbi	UC··U·····G·····C··G·····U	EV·····VANA	396	-146
Smo	CCA·U····U·····C··U··C···C··	DT··V·····L·A··	234	-127
Vvi	····U·····U·····UG··	E·····VI··E	237	-89
Zma	·C·····C·····U·····UG·G	E·····V·A·A	237	-100

Note: The dots are equal to nucleotides or amino acids in the data presented in Tables I–IV. The nucleotide sequences of miR398 binding sites are in bold face.

B. Mir398 Binding to mRNA of Cuznsod1 Gene

The binding energy of miR398 to SOD1 mRNA was less than that of CCS mRNA and varied from -89 to -146kJ/mol (Table II). However, through the presence of the helicase in RISC and the location of the binding site ends in the 2D loop structure of mRNA (Fig. 1), the interaction between the miRNA and mRNA was possible. The nucleotide sequences of binding sites in CuZnSOD1 mRNA were homologous (Table II), but the variability of the nucleotides in the third codon position reduced the binding energy of miR398. The binding site oligonucleotides in CuZnSOD1 mRNA encoded the same GDLGN pentapeptide in all studied plants. It should be noted that this pentapeptide was also present in CuZnSOD1 protein in sponges, hydra, insects, fish, and mammals (unpublished data). The importance of this pentapeptide was confirmed by Fig. 1, which shows the high variability of amino acids before and after the pentapeptide.

C. Mir398 Binding to mRNA of Cuznsod2 Gene

The characteristics of the interaction between miR398 and CuZnSOD2 ortholog mRNAs are shown in Table III. The nucleotide sequence of CuZnSOD2 gene and amino acid sequences of the protein were conserved in 11 plant species (Table III). GGUGACCUGGGAAACAU oligonucleotide encoded the GDLGNI hexapeptide, located in a conserved region of CuZnSOD2 protein. The high homology of the binding sites was reflected in the low variability of miR398 and mRNA interaction energies. Its values ranged from -139 to -152kJ/mol.

D. Mir398 Binding to mRNA of Cuznsod3 Gene

CuZnSOD3 is localized in the extracellular region, but, is subjected to regulation by miR398 during synthesis. We found that the binding site for miR398 was located in the protein-coding region of the mRNA and that the nucleotide sequence had less homology than that of CuZnSOD1 and CuZnSOD2 mRNAs (Tables I and II). Differences were observed in the third nucleotide position in codons and two synonymous codons (UUG and CUG) were used to encode for leucine. However, such variability of oligonucleotided did not affect the amino acid sequence encoding the GDLGN heptapeptide (Table IV). The energy of miR398 binding to mRNA suggested a strong interaction between these molecules. miR398a, b, c were bound to one site in each orthologous mRNA.

TABLE III
CHARACTERISTICS OF MIR398 BINDING SITES IN CDSS OF CUZNSOD2 ORTHOLOGS

Object	Region of CDS in mRNA of *CCS* ortholog genes	Region of CuZnSOD2	Position,nt	ΔG, kJ/mol
Ath	GUCAUGCG**GGUGACCUGGGAAACAU**AAA	DECRHA**GDLGNI**NANADGV	425	-149
Aly	·····	·····T·····	437	-149
Bdi	·····UGU	··V·····V···E·I	389	-139
Gma	·····GU	··V·····V···E··	389	-149
Mtr	·····U	··I·····I·D·N··	383	-149
Osa	·····UGU	··V·····V···E··	410	-149
Ptr	·····GU	··I·····V·····	407	-149
Rco	GC·····C·····GU	·DI·····V·····	416	-152
Sbi	·····UGU	··V·····V···E··	393	-144
Vvi	·····GU	·DV·····V···E··	440	-149
Zma	·····UGU	··V·····V···E·I	395	-144

Note: The dots are equal to nucleotides or amino acids in the data presented in Tables I–IV. The nucleotide sequences of miR398 binding sites are in bold face.

TABLE IV
CHARACTERISTICS OF MIR398 BINDING SITES IN CDS OF CUZNSOD3 ORTHOLOG GENES

Object	Region of CDS in mRNA of *CCS* ortholog genes	Region of CuZnSOD3	Position,nt	ΔG, kJ/mol
Ath	UGCU**GGCGAUUUGGGUAAC**AUUCUU	EERHA**GDLGN**ILAGSNG	254	−107
Aly	···A·····	·····D·	254	−107
Bdi	··UG··U··CC····A·····A·AG	D···V·····Q·NND·	266	−149
Gma	·····U·····C·····GC·	DK·····A··PD·	254	−124
Mtr	···C··U·····G··	D·····V··PD·	257	−111
Osa	···C··U···C·U··A··U··AACA	·N·····T··AD·	239	−112
Ppa	···G··A··C·····G·CA·C	·V·····VI··ED·	245	−142
Ptr	·····A·····G·····CA··	K·····I···D·	248	−111
Rco	···A··G·····C·····G·C	·····VT··D·	252	−120
Sbi	···G··U··CC····A·····AG·A	K·····V·NED·	263	−149
Smo	C·····C·A··G··UG·GACC	KI·····VT··PD·	236	−136

Note: The dots are equal to nucleotides or amino acids in the data presented in Tables I–IV. The nucleotide sequences of miR398 binding sites are in bold face.

IV. CONCLUSION

Using the RNA hybrid program, we identified miR398 binding sites in the protein-coding region of the mRNA in orthologous plant CCS and CuZnSOD genes. The nucleotide sequences of miR398 binding sites were homologous and encoded highly conserved oligopeptides in chaperone and all superoxide dismutase proteins. Conserved miR398 binding sites in mRNAs of chaperone and superoxide dismutase orthologs were present in plants that diverged more than 100 million years ago. Consequently, the system of chaperone and superoxide dismutase gene expression control by miRNA was formed during the early stages of the evolution of these enzymes.

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