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# microRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in Arabidopsis

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## SUMMARY

microRNA398 (miR398) is a conserved miRNA of plants that targets two of the three copper/zinc superoxide dismutases (SOD) of Arabidopsis (CSD1 and CSD2) by triggering cleavage or inhibiting translation of their mRNAs. We analysed the transcriptomes of mutants impaired in miR398 production, and found that the mRNAs encoding the copper chaperone for superoxide dismutase (CCS1), which delivers copper to CSD1 and CSD2 apoproteins in different cellular compartments, are undiscovered targets of miR398. We identified the cleavage site in *CCS1* mRNAs by 5'-RACE PCR. We further show that both *CCS1* protein and mRNA levels are tightly linked to the quantities of miR398, which are themselves dependent on the copper content in the medium. We generated transgenic plants carrying a *CCS1* mRNA version resistant to cleavage by miR398, and demonstrated that both *CCS1* mRNAs and proteins accumulate in these plants when miR398 is abundant and copper limiting. Moreover, we show that one of the ten ARGONAUTE proteins of Arabidopsis (AGO10) is involved in miR398-directed translational inhibition of *CCS1* mRNAs, as *CCS1* protein, but not *CCS1* mRNAs accumulates in *ago10* (*zll*) mutants. Thus, miR398 mediates the cleavage and translational inhibition of mRNAs encoding *CCS1*, the chaperone protein that is essential for generating the mature copper/zinc SODs of Arabidopsis. Our results also imply that new targets that have not been identified by computing analyses have yet to be discovered, even for an extensively studied miRNA such as miR398.

**Keywords:** copper chaperone for superoxide dismutase (CCS1), copper/zinc superoxide dismutase, miR398, ARGONAUTE, Arabidopsis.

## INTRODUCTION

microRNAs (miRNAs) are a class of small RNA regulators of 21–24 nucleotides that play key roles in developmental processes of both plants and animals by directing cleavage or translational inhibition of target mRNAs (Mallory and Bouché, 2008; Voinnet, 2009). miRNAs are processed from imperfectly paired hairpin precursors derived from transcription of *MIR* genes by RNA polymerase II. miRNAs direct a variety of developmental events in plants, and there is increasing evidence that points to potential roles in diverse physiological processes. For instance, miR395 and miR399 are involved in sulfate and phosphate deficiencies, respectively (Chiou, 2007), and miR398 appears to be involved in responses to environmental stresses (Sunkar *et al.*, 2006; Jia *et al.*, 2009; Trindade *et al.*, 2010).

miR398 is a conserved miRNA that was identified in Arabidopsis by both computational analyses (Bonnet *et al.*, 2004; Jones-Rhoades and Bartel, 2004) and sequencing of small RNA libraries from seedlings exposed to abiotic stresses (Sunkar and Zhu, 2004). Based on sequence homologies, miR398 was predicted to target mRNAs of both cytosolic CSD1 (copper/zinc superoxide dismutase 1) and chloroplastic CSD2, as well as a subunit of the mitochondrial cytochrome *c* oxidase, COX5b-1 (Bonnet *et al.*, 2004; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004). Cleavage of the target mRNAs was experimentally verified (Jones-Rhoades and Bartel, 2004) using a modified version of 5'-RACE PCR (Llave *et al.*, 2002). When plants are exposed to abiotic stresses such as high levels of Cu<sup>2+</sup>, Fe<sup>3+</sup>, ozone or

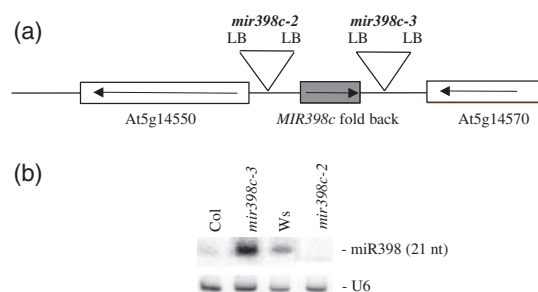
salt, or to biotic stresses such as a bacterial infection by *Pseudomonas*, the levels of miR398 decrease (Sunkar *et al.*, 2006; Jagadeeswaran *et al.*, 2009). For instance, at least two of the three *MIR* genes encoding miR398 (i.e. *MIR398b* and *MIR398c*) are transcribed only when copper is limiting (Sunkar *et al.*, 2006; Dugas and Bartel, 2008), in contrast to the *CSD2* gene, which is transcribed at the same rate under low- and high-copper conditions (Yamasaki *et al.*, 2007). However, *CSD2* mRNA is down regulated when copper is low, implying a post-transcriptional control that depends on copper availability. miR398 plays an essential role in this process, as demonstrated firstly by generating transgenic plants expressing modified versions of *CSD1* or *CSD2* mRNAs rendered resistant to cleavage by miR398, thus artificially increasing target mRNA levels in these plants (Dugas and Bartel, 2008), and secondly, by over-expressing *MIR398* precursors leading to the opposite phenomenon (Yamasaki *et al.*, 2007; Dugas and Bartel, 2008). In addition to transcript cleavage, miR398 also acts through translational repression of the target mRNAs when copper is limiting (Brodersen *et al.*, 2008). The exact molecular mechanism underlying this process remains to be elucidated, but probably involves two of the ten ARGONAUTE (AGO) proteins of Arabidopsis, AGO1 and AGO10 (Brodersen *et al.*, 2008). Indeed, *ago10* mutations restore leaf development and siRNA and miRNA pathways in *ago1* hypomorphic mutants and lead to increased AGO1 protein levels, thus implicating AGO10 as a negative regulator of AGO1 (Mallory *et al.*, 2009). Moreover, analyses of chimeric gene constructs indicate that the PAZ domain, which is thought to mediate small RNA binding, is exchangeable between both proteins, whereas the MID-PIWI and N-terminal domains appear to contribute to their specific functions (Mallory *et al.*, 2009). Very recently, Lanet *et al.* (2009) provided biochemical evidence for translational repression by showing that active miRNAs associate with polysomes via their target mRNAs.

In Arabidopsis, the copper cofactor is delivered to Cu/Zn superoxide dismutases (SODs) by a copper chaperone for superoxide dismutase (CCS1) (Abdel-Ghany *et al.*, 2005; Chu *et al.*, 2005). The Arabidopsis genome contains a unique *CCS1* gene encoding both a cytosolic and a chloroplastic form of CCS1, which deliver copper to SODs localized in different cellular compartments. Here, we show that miR398 directs the post-transcriptional regulation of *CCS1* mRNAs by cleavage and AGO10-mediated translational repression.

## RESULTS

### Isolation and molecular characterization of mutants with altered levels of miR398

In Arabidopsis, the miR398 family is represented by two members that differ by one nucleotide at the 3' end and are encoded by three genes, namely *MIR398a*, *MIR398b* and *MIR398c* (Bonnet *et al.*, 2004; Jones-Rhoades and Bartel,



**Figure 1.** Levels of miR398 in *mir398c* mutants.

(a) Schematic presentation of *MIR398c* and positions of the T-DNA inserts in *mir398c-2* (FLAG\_072C02) and *mir398c-3* (SALK\_017704) mutants, represented by triangles. LB, sequenced left borders of the T-DNA inserts. (b) RNA gel blot analysis of 10 µg total RNA prepared from rosette leaves of Col and Ws wild-type plants and *mir398c-2* and *mir398c-3* mutants. U6 RNA was used as a loading control.

2004; Sunkar and Zhu, 2004). Two mutations in the Arabidopsis *MIR398* loci have been described previously (Dugas and Bartel, 2008): *mir398a-1* (SALK\_043101) and *mir398c-1* (SALK\_039698). By comparing the *MIR398* sequences with T-DNA flanking genomic sequences deposited in the databases, we isolated additional T-DNA knockouts corresponding to putative *mir398* mutants. Their genomic DNAs were characterized by PCR, and the junctions between the T-DNAs and the *MIR398* genes were sequenced. Plants homozygous for the mutations were isolated, and the levels of miR398 were determined by Northern blot analysis. Plants were grown for 30 days on soil, and total RNAs from rosette leaves of five plants per homozygous line were extracted in bulk, blotted on a membrane and hybridized with an miR398-specific probe. We found two mutant alleles with T-DNAs inserted in *MIR398c* (Figure 1a) that contained lower and higher levels of miR398 compared to their respective wild-type (Figure 1b). Similar results were obtained with total RNAs extracted from flowers of the same plants (data not shown), and this experiment was repeated three times under the same conditions. In *mir398c-2* (FLAG\_072C02), the global production of miR398 is impaired by an unknown mechanism, as *MIR398b* and/or *c* precursors appear to be normally produced in these plants (Figure S1). In contrast, both miR398 (Figure 1b) and *MIR398b* and/or *c* precursors (Figure S1) accumulate in *mir398c-3* (SALK\_017704), probably because of enhancer sequences carried by the T-DNAs, such as CaMV 35S enhancers.

### Transcriptome analyses of *mir398c* mutants reveal a new target of miR398

*mir398c* mutants altered in the production of miR398 were then used to identify target mRNAs of miR398 by analysing their transcriptomes. We hypothesized that target transcripts would be up-regulated in *mir398c-2* and down-regulated in *mir398c-3*. Ten to fifteen *mir398c-2*, *mir398c-3*

**Table 1** List of genes up-regulated in *mir398c-2* and down-regulated in *mir398c-3*

AGI gene accession	Function	log <sub>2</sub> ( <i>mir398c-2</i> /Ws)	log <sub>2</sub> ( <i>mir398c-3</i> /Col)
AT1G08830	CSD1	+0.79	-1.7
AT1G12520	CCS1	+0.96	-1.41
AT1G78370	Glutathione transferase	+0.82	-0.71
AT2G28190	CSD2	+1.08	-1.23
AT5G08640	FLS1 (flavonol synthase 1)	+0.75	-1.36
AT5G13930	Chalcone synthase	+1.14	-1.45

Transcriptome analyses were performed using the CATMA microarray on RNAs extracted from rosette leaves of *mir398c-2*, *mir398c-3* and wild-type plants. The results with a Bonferroni *P* value < 0.05 confirmed in two independent biological repeats are presented as log<sub>2</sub> ratios. AGI, Arabidopsis Genome Initiative.

and wild-type plants were grown in culture chambers under standard growth conditions for 17 days. Rosette leaves from each plant were pooled, and total RNA was extracted in bulk for comparison by microarray analysis. We found only six mRNAs that were up-regulated in *mir398c-2* compared to Ws and also down-regulated in *mir398c-3* compared to Col (Table 1). Two of those were targets of miR398 characterized previously, namely the *CSD1* and *CSD2* mRNAs (Sunkar *et al.*, 2006). We compared the coding sequences of the four remaining putative targets to the sequence of miR398a using the RNAhybrid program, which identifies the energetically most favorable hybridization sites of a small RNA in a large RNA (Rehmsmeier *et al.*, 2004; Kruger and Rehmsmeier, 2006). Only one putative mRNA target, corresponding to *CCS1* (At1g12520), formed duplexes with miR398 that were predicted to be as stable as the ones formed by *CSD1* or *CSD2* mRNAs with miR398 (Table 2 and Figure S2). We therefore concluded that *CCS1* mRNAs are probably targeted by miR398. This was further confirmed using a modified 5'-RACE PCR technique that enables precise determination of the cleavage sites of miRNAs on their mRNA targets (Llave *et al.*, 2002). As already observed for many other targets of miRNAs in Arabidopsis (Mallory and

Bouché, 2008), we found that *CCS1* mRNAs were cleaved within the coding sequence at the predicted miR398 target site, between nucleotides 10 and 11 of the miRNA (Figure 2a). As *CCS1* is transcribed in various spliced forms (Chu *et al.*, 2005), and as all of them contain the last exon of *CCS1*, it is likely that miR398 targets all mRNA forms of *CCS1* in Arabidopsis. We aligned the *CCS1* genomic sequences retrieved from databases for various plant species, and found that the miR398-complementary site was conserved in all sequences (Figure 2b).

#### CCS1 mRNAs and protein abundance are determined by the level of miR398

When copper is limiting, *MIR398b* and *MIR398c* genes are expressed, as shown by *MIR398* promoter::GUS transcriptional fusion analyses (Figure 3a) (Sunkar *et al.*, 2006; Dugas and Bartel, 2008). Under these conditions, miR398 levels remain high, and, as a consequence, both *CSD1* and *CSD2* transcripts are cleaved, resulting in low amounts of proteins (Dugas and Bartel, 2008). To determine whether the abundance of *CCS1* mRNAs is post-transcriptionally regulated by copper availability, we grew *mir398c-2* and corresponding wild-type sibling plants on plates supplemented with various amounts of copper (0, 0.1, 0.2 and 0.3 μM of CuSO<sub>4</sub> added to the medium). After 15 days of growth, total RNAs and proteins were extracted in bulk from 10 to 15 plants. Levels of miR398 in the wild-type were determined by Northern blot analyses, and the results showed that the miRNA quantity decreases when copper is added to the medium and becomes undetectable at 0.3 μM of CuSO<sub>4</sub> (Figure 3b). Compared to the wild-type siblings, *mir398c* plants contain lower amounts of miR398 at 0, 0.1 and 0.2 μM of CuSO<sub>4</sub> (Figure 3b). These results confirmed that *mir398c-2* plants are impaired in miR398 production, and that levels of miR398 depend on copper availability in the medium. *CCS1* mRNA levels, determined by quantitative RT-PCR and compared to actin mRNA levels, were tightly linked to the quantities of miR398 in both mutant and wild-type plants (Figure 3c). This was also the case when sucrose was added to the medium (Figure S3). Similarly, *CCS1* protein quantities detected by Western blotting and a *CCS1*-specific

**Table 2** Characteristics of the duplexes formed by miR398 and target mRNAs validated by 5'-RACE PCR

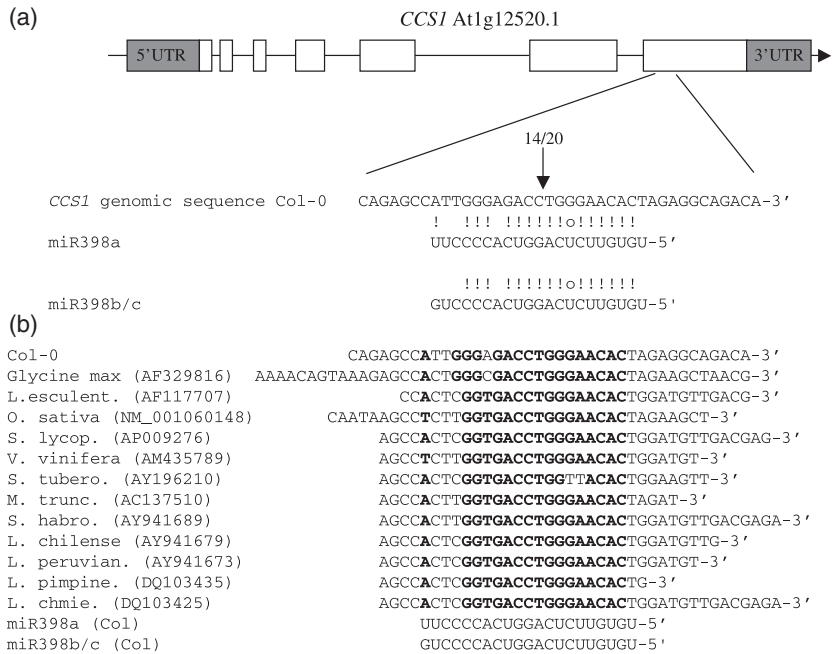
mRNA target	Duplex MFE (kcal/mol) <sup>a</sup>	Number of mismatches		Number of GU wobbles	
		miR398a	miR398b/c	In the seed region <sup>b</sup>	Outside the seed region <sup>b</sup>
<i>CSD1</i>	-34.7	3	4	0	0
<i>CSD2</i>	-34.7	3	2	1	2
<i>CCS1</i>	-33.3	4	5	1	0
<i>COX5b-1</i>	-36	2	3	0	0

<sup>a</sup>Values for MFE (maximum free energy) were obtained using the RNAhybrid program (Rehmsmeier *et al.*, 2004; Kruger and Rehmsmeier, 2006) with miR398a. The MFE of a perfectly match hybrid of miR398a with itself is 45.7 kcal/mol and the MFE of a perfectly matched hybrid of miR398b/c with itself is 46.9 kcal/mol.

<sup>b</sup>The seed region corresponds to nucleotides 2–6 starting from the 5' end of the miRNA.

**Figure 2.** miR398 targets *CCS1* mRNA. (a) Schematic presentation of the *CCS1* gene (drawn to scale). The location of the miR398 target site in *CCS1* is shown. Exons are represented by white boxes and UTRs by grey boxes. The sequence (Col-0) of the miR398 recognition site in the *CCS1* mRNAs is aligned with sequences of miR398a and miR398b/c. Cleavage was experimentally validated using a modified version of 5'-RACE PCR (Llave *et al.*, 2002). The arrow indicates the 5' terminus cleavage product for the number of clones indicated.

(b) Sequences of the *CCS1* miR398-complementary site in various plant species. The sequence accession numbers are given in parentheses. Nucleotides matching either miR398a or miR398b/c are shown in bold.



antibody follow the same pattern (Figure 3d). From this analysis, we concluded that the levels of *CCS1* transcripts and protein are linked to miR398 quantities, as already described for both *CSD1* and *CSD2* (Sunkar *et al.*, 2006).

**An miR398-resistant form of *CCS1* transcripts accumulates when miR398 is plentiful**

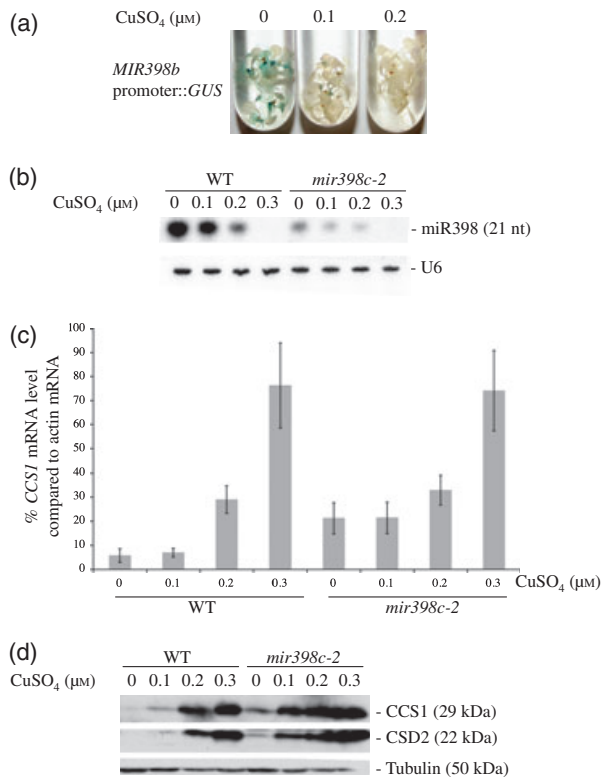
If *CCS1* mRNAs are post-transcriptionally regulated by miR398, we hypothesized that expression of an miR398-resistant form of these transcripts would result in their accumulation when copper is limiting (i.e. when miR398 levels are high). We therefore generated transgenic plants carrying an miR398-resistant form of the *CCS1* cDNA driven by the *CCS1* promoter and designated *proCCS1::3mCCS1*. We introduced three silent mutations into the miR398-complementary site (Figure 4a). As a control, we also generated transgenic plants carrying the same construct with an unmodified version of the *CCS1* coding sequence, designated *proCCS1::CCS1*. T<sub>1</sub> plants were selected, and, after two generations, T<sub>3</sub> plants containing at least one transgene in the homozygous state were grown for 12 days on medium supplemented or not with copper. *CCS1* mRNA levels were determined by quantitative RT-PCR. Consistent with our previous results (Figure 3c), we observed that *CCS1* transcripts are 11 times more abundant in wild-type plants grown on medium containing copper, compared with medium containing limiting amounts of copper (Figure 4b). This was also observed for four independent transgenic lines carrying the *proCCS1::CCS1* construct, but not in plants carrying *proCCS1::3mCCS1* (Figure 4b). Similarly, we detected high quantities of *CCS1* protein (Figure 4c) in *proCCS1::3mCCS1* plants grown without copper compared

to the control lines (i.e. *proCCS1::CCS1* and wild-type). Both *CSD1* and *CSD2* proteins were undetectable under low copper conditions (Figure 4c), probably because miR398 is still mediating cleavage of the corresponding mRNAs in these plants, including *proCCS1::3mCCS1* plants. As control, we verified that the levels of *CCS1* proteins were similar in all plants grown with copper added to the medium (Figure 4c). Therefore, we concluded from this experiment and the 5'-RACE PCR mapping experiment that miR398 mediates the cleavage of *CCS1* mRNA when copper is limiting.

**Mutations in *AGO10* affect the miR398-directed translational repression of *CCS1* mRNAs**

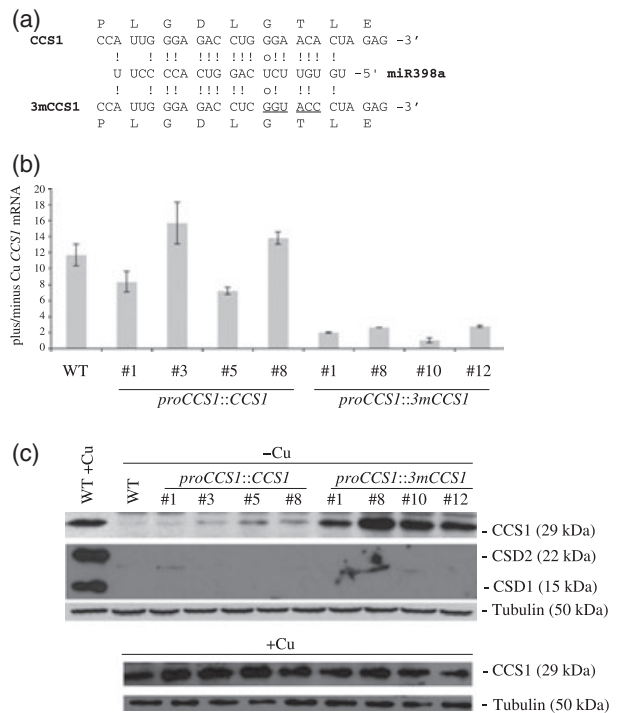
Although miRNAs of plants have mainly been described as acting by triggering cleavage of their targets, Brodersen *et al.* (2008) demonstrated that some of these miRNAs can additionally act by translational repression, through the action of *AGO1* and/or *AGO10* (also known as *ZWILLE/PINHEAD*; *ZLL*). For instance, the *CSD2* protein accumulates in *ago10* mutants but levels of the corresponding mRNA remain almost unchanged, suggesting that *AGO10* is probably involved in miR398-mediated translational inhibition of *CSD2* mRNA (Brodersen *et al.*, 2008). We therefore decided to quantify *CCS1* mRNAs and protein in *ago10* mutants. We used one of the *ago10* mutants, *zll-3* (Moussian *et al.*, 1998) in which we quantified miR398. Ten to fifteen *zll-3* and wild-type plants were grown for 12 days on plates containing various amounts of copper, and total RNA was extracted in bulk. The levels of miR398 decreased when the concentrations of copper increased in the medium for both wild-type and *zll-3* plants (Figure 5a). By contrast, *CCS1* transcripts, quantified by quantitative RT-PCR, accumulate





**Figure 3.** *CCS1* is post-transcriptionally regulated by miR398. (a) Transgenic plants expressing an *MIR398b* promoter::GUS transcriptional fusion. The three tubes each contain five seedlings grown on medium supplemented with various amounts of copper as indicated. GUS activity was revealed after 12 days of growth. A total of 11 independent transgenic lines were generated, showing the same pattern of expression. One representative line is shown. (b) RNA gel blot analysis of 1 µg total RNAs prepared from 12-day-old seedlings of *mir398c-2* and the corresponding wild-type sibling plants grown on various amounts of copper as indicated. U6 RNA was used as a loading control. (c) Quantitative RT-PCR of *CCS1* mRNAs in the plants described in (b). Mean values for three technical PCR repeats were compared to the level of actin mRNA, arbitrarily fixed at 100%. Error bars indicate SD ( $n = 5$ ). (d) Immunodetection of *CCS1* and *CSD2* proteins in the plants described in (b) using anti-*CCS1* or anti-*CSD2* polyclonal antibodies after SDS-PAGE of 5 µg of total proteins. Detection of tubulin served as a control for equal loading.

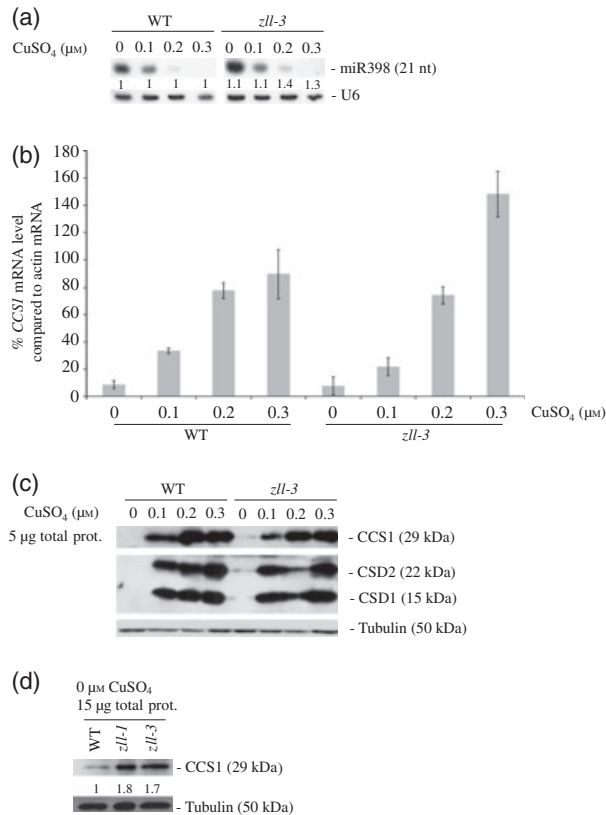
in similar ways in wild-type and *zll-3* when copper concentrations increase (Figure 5b). The same pattern was observed for *CCS1* protein, as for both *CSD1* and *CSD2* (Figure 5c). When 5 µg of total proteins were separated by SDS-PAGE, *CCS1*, *CSD1* and *CSD2* protein levels were found to be slightly elevated in *zll-3* mutants when copper was omitted from the medium (0 µM  $\text{CuSO}_4$ ) (Figure 5c). This was further confirmed for *CCS1* in both *zll-1* and *zll-3* alleles, when three times more proteins were loaded on the gel (Figure 5d). Thus, we concluded that the *CCS1* protein, like *CSD2* (Brodersen *et al.*, 2008), is more abundant in *ago10* mutants compared to wild-type plants when copper is limiting, while *CCS1* mRNA and miR398 levels stay unchanged.



**Figure 4.** miR398-resistant form of *CCS1* transcripts. (a) Pairing of miR398a with the corresponding complementary sites of both *CCS1* and a miR398-resistant form of *CCS1* (*3mCCS1*). The *KpnI* restriction enzyme site introduced in the mutated construct is underlined. (b) Quantitative RT-PCR of *CCS1* mRNAs in transgenic plants ( $T_3$  generation) carrying either unmodified *CCS1* cDNA or *3mCCS1* driven by the *CCS1* promoter (*proCCS1*), and the wild-type (Col accession). Plants were grown for 12 days *in vitro*, with 0.5 µM of  $\text{CuSO}_4$  or without copper added to the medium, and total RNAs were extracted in bulk (10–15 plants) to prepare the corresponding cDNAs and perform quantitative RT-PCR. Results are presented for four independent transgenic lines carrying *proCCS1::CCS1* (lines 1, 3, 5 and 8) and four independent lines for *proCCS1::3mCCS1* (lines 1, 8, 10 and 12). Values are the differences in *CCS1* mRNA levels between plants grown with or without copper. Error bars indicate SD ( $n = 3$ ). (c) Immunodetection of *CCS1*, *CSD1* and *CSD2* proteins in 15 µg of total proteins extracted from the plants described in (b). Proteins were detected using anti-*CCS1* or anti-*CSD1/2* polyclonal antibodies after SDS-PAGE. Detection of tubulin served as a control for equal loading.

## DISCUSSION

In the present study, we analysed the transcriptomes of two *mir398* T-DNA mutants containing lower and higher contents of miR398 to find targets that had not previously been identified by bioinformatics predictions. In addition to *CSD1* and *CSD2* mRNAs, we identified *CCS1* mRNAs encoding the chaperone that delivers copper to the Cu/Zn SODs of Arabidopsis. To demonstrate that miR398 triggers the cleavage of *CCS1* mRNAs, we mapped the 5' end of the cleavage products in the miR398-complementary site of *CCS1*. Then we generated transgenic plants carrying a *CCS1* cDNA form that was resistant to cleavage by miR398, and found that *CCS1* protein accumulates in these plants when the miRNA is abundant. Thus, we concluded from these experiments



**Figure 5.** *ago10* mutants are impaired in miR398-directed post-transcriptional regulation of *CCS1*.

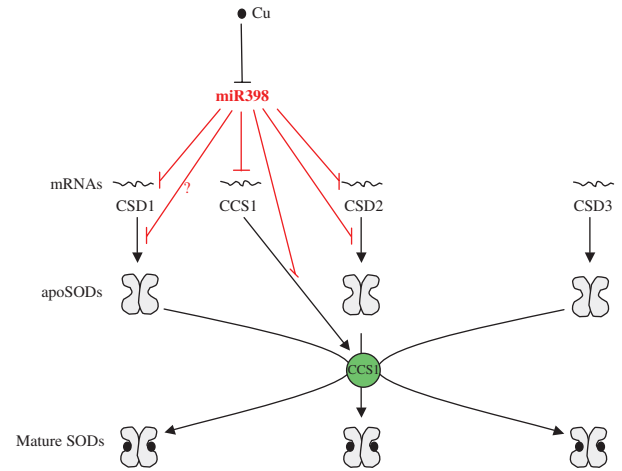
(a) RNA gel blot analysis of 1 μg total RNAs prepared from 12-day-old seedlings of *zll-3* mutants and the corresponding wild-type (*Ler*). Plants were grown on various amounts of copper as indicated. U6 RNA was used as a loading control, and values for miR398 normalized to those for U6 RNA are indicated. Values for wild-type controls were set at 1.

(b) Quantitative RT-PCR of *CCS1* mRNAs in the plants described in (a). Mean values for three technical PCR repeats were compared to the level of actin mRNA, arbitrarily fixed at 100%. Error bars indicate SD ( $n = 5$ ).

(c) Immunodetection of *CCS1*, *CSD1* and *CSD2* proteins in the plants described in (a) using anti-*CCS1* or anti-*CSD2/CSD1* polyclonal antibodies after SDS-PAGE of 5 μg of total proteins. Detection of tubulin served as a control for equal loading.

(d) Immunodetection of *CCS1* in 15 μg of total proteins extracted from 12-day-old seedlings of *zll-3* or *zll-1* mutants and the corresponding wild-type (*Ler*). Plants were grown without copper. *CCS1* was detected using an anti-*CCS1* polyclonal antibody after SDS-PAGE. Detection of tubulin served as a control for equal loading. Normalized values of *CCS1* protein to tubulin controls (with wild-type controls set at 1.0) are indicated.

that miR398 mediates the cleavage of *CCS1* mRNA when copper is limiting. In consequence, miR398 is controlling the level of both *CSD1* and *CSD2* post-transcriptionally by two means: directly by directing the slicing of their mRNAs, and indirectly by triggering cleavage of the mRNA encoding the copper chaperone necessary for their activation (Figure 6). We found no developmental phenotypes associated with the production of a *CCS1* miR398-resistant form when *CCS1* is normally down-regulated in copper limiting conditions. Similarly, null *ccs1* mutants, in which Cu/Zn SOD activities



**Figure 6.** miR398 controls the production of Cu/Zn SODs.

*CSD1*, *CSD2* and *CSD3* are the three Cu/Zn SODs of Arabidopsis, and are localized in the cytoplasm, chloroplast and peroxisome, respectively. miR398 controls the production of both *CSD1* and *CSD2* mRNAs by mediating their cleavage and production of the corresponding apo-proteins by translational repression, at least for *CSD2*. miR398 also controls the production of *CCS1*, the chaperone delivering copper to all Cu/Zn SODs, at both the mRNA and protein levels.

are almost undetectable (Chu *et al.*, 2005), present no particular phenotypes (N.B., data not shown). Therefore, it is probable that miR398 action on *CCS1*, *CSD1* and *CSD2*, when copper is absent, is crucial in stress situations that remained to be identified.

In previous studies, plants expressing miR398-resistant forms of *CSD1* or *CSD2* (five mutations introduced into the miR398-complementary sites) were shown to accumulate the corresponding mRNAs when miR398 is abundant, but, surprisingly, both *CSD1* and *CSD2* protein levels remained unchanged (Dugas and Bartel, 2008). The authors concluded that, despite the absence of cleavage, miR398 was able to repress translation of the mutated *CSD1* or *CSD2* mRNAs. All three Cu/Zn SOD isoforms of Arabidopsis, including both *CSD1* and *CSD2*, are nearly absent in a null *ccs1* knockout mutant (Chu *et al.*, 2005), with almost no detectable Cu/Zn SOD activities, and very low amounts of proteins detected by Western blotting (Chu *et al.*, 2005). This indicates that *CCS1* is necessary for the activation of both *CSD1* and *CSD2* apo-forms, as found previously for SODs of yeast cells (Culotta *et al.*, 1997) and *Drosophila* (Kirby *et al.*, 2008). In plants expressing mutated CSD mRNA forms, miR398 still triggers the cleavage of *CCS1* mRNAs, probably explaining why *CSD1* or *CSD2* mRNAs accumulate, but not the corresponding proteins. In contrast, in transgenic plants expressing a *CCS1* mRNA version resistant to cleavage by miR398, we observed that both *CCS1* mRNA and protein accumulate (Figure 4).

The regulation of miRNA targets differs in several ways between animals and plants (Brodersen and Voinnet, 2009).

In animals, miRNAs mainly cause translational repression of target mRNAs by pairing their 3' UTRs, often at multiple sites. The target site of most animal miRNAs is short (only 6–8 bases), with perfect pairing between the miRNA and its target. By contrast, plant miRNAs have a long target site, usually in mRNA coding regions, with extensive near-perfect pairing, and act mainly by triggering cleavage of the target mRNA. Thus, the requirements for sequence complementarity between miRNAs and mRNA target sites differ between plants and animals, explaining why the number of predicted plant miRNA targets is relatively low (Jones-Rhoades and Bartel, 2004). Likewise, the number of targets revealed by our transcriptome analyses (designed to identify cleaved targets) is also very limited. Indeed, we found only one mRNA target, namely *CCS1*, that was not previously identified by bioinformatics approaches, in addition to *CSD1* and *CSD2* mRNAs. In agreement with previous observations (Dugas and Bartel, 2008), we found no differences in the expression level of *COX5b-1*, a predicted target of miR398 confirmed by 5'-RACE PCR (Jones-Rhoades and Bartel, 2004). Nevertheless, we cannot exclude the possibility that *COX5b-1*, or other mRNAs, are stress-specific miR398 targets that were not revealed by our transcriptome analyses performed with plants grown under standard conditions. Alternatively, some targets might only be regulated by miR398-directed translational repression.

*CCS1* protein accumulates in *ago10* mutants (Figure 5), suggesting that AGO10 is involved in miR398-directed post-transcriptional regulation of *CCS1*. miR398 acts through translational repression and cleavage of at least two of its mRNA targets identified so far: *CSD2* and *CCS1* (Figure 6). Now that targets of plant miRNAs regulated by translational repression are being discovered, the picture becomes more complicated, as at least some miRNAs of plants are involved in triggering both cleavage and translational repression. Plant mRNA targets specifically regulated by translational repression remain to be identified by future studies, for instance by analysing the proteomes of mutants impaired in the production of miRNAs.

Finally, a crucial point about *CCS1* is to understand why *CCS1* mRNA escaped all bioinformatics predictions, and why the miR398 target site was not discovered previously. To identify real targets and minimize the number of false positives, the first prediction algorithms used were very restrictive, allowing only three mismatches between the miRNA and the putative mRNA target, with no bulges in the duplex, and treating GU wobbles as mismatches (Rhoades et al., 2002). Using a system of penalty scoring that takes into account GU wobbles and bulges, and restricting the results to target sequences conserved between species, more targets could be predicted and validated (Jones-Rhoades and Bartel, 2004). Finally, additional targets have been found (Wang et al., 2004; Allen et al., 2005; Schwab et al., 2005; Rajagopalan et al., 2006; Fahlgren et al., 2007; Alves-Junior

et al., 2009) using more sophisticated algorithms that take into consideration further parameters such as the minimum free energy of the duplex, expression profiles of the putative targets in miRNA biogenesis mutants, and the position of the mismatches, as the pairing in the 5' seed region of the miRNA and around the cleavage site (spanning nucleotides 10 and 11) appears to be the most important (Schwab et al., 2005; Mallory and Bouché, 2008). About 120 Arabidopsis miRNA/target mRNA duplexes have been validated by 5'-RACE PCR since the discovery of miRNAs in plants (Table S1), and we found only six duplexes that exceed four mismatches in this list (if we consider GU as a mismatch). Five of these, the duplexes formed by miR-JAW and TCP3, 4 or 10 (Palatnik et al., 2007), the one formed by miR159a and CSD3 (German et al., 2008), and finally the one between miR398 and *CCS1* mRNA, correspond to target genes identified experimentally. The targets of miR-JAW and miR398 were found by analysing the transcriptomes of *mir-jaw* (Palatnik et al., 2007) and *mir398* mutants (Table 1), respectively, and CSD3 was identified as a target of miR159 by analysing the degradome of Arabidopsis (German et al., 2008). The only exception is from a recent study (Alves-Junior et al., 2009), in which a target of miR161 containing six GU wobbles and two mismatches was predicted and validated by 5'-RACE PCR. In this study, GU wobbles were not considered as mismatches, but the predicted energy of the duplex was used as a filter. Such new algorithms, allowing more than four mismatches, and applying more flexible rules to the pairing in the seed region and the GU content, combined with transcriptomic analyses of mutants impaired in the production of miRNAs, are likely to result in discovery of new targets.

## EXPERIMENTAL PROCEDURES

### Plant materials and growth conditions

*Arabidopsis thaliana* ecotypes Columbia (Col), Landsberg *erecta* (Ler) and Wassilewskija (Ws) were used. Surface-sterilized seeds were plated on culture medium (Appendix S1) (Estelle and Somerville, 1987) containing 0.8% agar (plant cell culture tested, Sigma, <http://www.sigmaaldrich.com/>), incubated at 4°C for 48 h, and grown *in vitro* under the following conditions: day/night cycles of 16/8 h; light intensity 100–150  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ , temperature day/night 20/15°C; humidity 65%.

The Ws *mir398c-2* mutant (FLAG\_072C02) was isolated from the INRA (Institut National de la Recherche Agronomique, Versailles, France) collection of T-DNA mutants and genotyped using forward (5'-CCTAGTACCTCTTTGACGTTTC-3') and reverse (5'-GCGTTGATAAACATGGACGG-3') primers specific to the *MIR398c* gene, and the T-DNA primer TAG5 (5'-CTACAAATTGCCTTTCTTATCGAC-3'). The Col-0 *mir398c-3* mutant (SALK\_017104) was isolated from the Salk Institute collection of T-DNA mutants, and genotyped using forward (5'-CCTAGTACCTCTTTGACGTTTC-3') and reverse (5'-CACAAATCGTGTGATGAGCTC-3') primers specific to the *MIR398c* gene, and T-DNA primer LBa1 (5'-ATGGTTCACGTAGTGGGCCATC-3'). *zll* (*ago10*) EMS mutants have been described previously (Moussian et al., 1998).



## Generation of transgenic plants

To generate plants carrying an miR398-resistant form of CCS1, we cloned the *CCS1* promoter in front of the *CCS1* cDNA in pBIB-Hygro (Becker, 1990). The *CCS1* cDNA was amplified from clone pda15533 (Riken Institute; GenBank accession number AK221656) using forward primer 5'-ATTCTAGACTCGAATTCGTGAGTAAAT-3' (*Xba*I site underlined) and reverse primer 5'-GACTGCGGCCGCAAAAC-AAGTTCTCAATCAC-3' (*Not*I site underlined), and transferred to pBluescript KS+ (Stratagene, <http://www.stratagene.com>) at the corresponding sites. Clones were sequenced and then mutagenized using a QuikChange site-directed mutagenesis kit (Stratagene) and primers 5'-CCATTGGGAGACCTCGGTACCCTAGAGGCAGAC-3' (forward) and 5'-GTCTGCCTCTAGGGTACCGAGGTCTCCCAATGG-3' (reverse), which introduce a *Kpn*I site (underlined). In parallel, the promoter of *CCS1* (approximately 2 kb upstream of the first ATG) was amplified from Col genomic DNA and cloned into a pBIB-Hygro binary vector using primers 5'-GGAAGTTGTAGAGAAGCTTAAGG-3' (forward, native *Hind*III site underlined) and 5'-GTCATCTAGACTT-GCTTCTCAGTTTTTGGCGG-3' (reverse, *Xba*I site underlined). *CCS1* and *3mCCS1* cDNAs were then transferred from pBluescript to pBIB-Hygro by digestion with *Xba*I and *Sac*I to obtain the *proCCS1::CCS1* and *proCCS1::3mCCS1* constructs.

For *miR398b* promoter::GUS constructs, we amplified 2 kb upstream of the predicted fold-back structure from Col genomic DNA using forward primer 5'-ACGTAAGCTTTCTAAACCTAAAGAAACCTTAAG-3' (*Hind*III site underlined) and reverse primer 5'-ACGTTAGACAACCCTGTCGATCCACTACC-3' (*Xba*I site underlined). The PCR product was cloned into the corresponding sites of pBIB-Hygro, and the clones were sequenced.

## RNA analysis

Total RNA was extracted as described previously (Bouché *et al.*, 2006). Small RNAs were separated by electrophoresis on 15% polyacrylamide gels and blotted onto membranes. The miR398 <sup>32</sup>P-end-labelled oligonucleotide probe was 5'-CAGGGGTGACCTGAGAACACA-3'. RNA hybridization signals were quantified using a Fuji phosphorimager (Fuji, <http://www.fujifilm.com>).

For the analyses of mRNAs expression by quantitative RT-PCR, total RNA extraction was performed using an RNeasy plant kit (Qiagen, <http://www.qiagen.com/>), followed by a DNase treatment (Fermentas, <http://www.fermentas.com>). RT-PCR was performed on 500 ng of total RNAs using M-MLV reverse transcriptase (Fermentas), and cDNAs were diluted 10 times. A 5 µl aliquot was used for quantitative RT-PCR using a MasterCycler ep *realplex* (Eppendorf) with RealMasterMix SYBR ROX/SYBR solutions (5 PRIME, <http://www.5prime.com>) using the primers 5'-CCCATATGACAGTACCATCA-3' (forward) and 5'-CCATTTCAAGATCAAAGTGGCAC-3' (reverse) for *CCS1*, and 5'-GCACCCTGTTCTTCTTACCG-3' (forward) and 5'-AACCCCTCGTAGATTGGCACA-3' (reverse) for actin.

To identify the miR398 cleavage site in *CCS1* mRNA, we used a GeneRacer kit (Invitrogen, <http://www.invitrogen.com/>) as previously described (Llave *et al.*, 2002).

## Western blotting

Polyclonal antibodies against recombinant CSD2 or CCS1 proteins were obtained from Agrisera (<http://www.sera.com>) and used according to the manufacturer's instructions. Total proteins were extracted in a buffer containing 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 10% glycerol, separated by SDS-PAGE, and transferred to PVDF membranes. Blocking was performed in Tris Buffered Saline Tween-20 (T-BST) containing 5% milk. Antibodies against CSD2 were also used to detect CSD1 (dilution of 1/1000)

(Kliebenstein *et al.*, 1998, 1999). Tubulin was detected using monoclonal anti- $\alpha$ -tubulin (Sigma; reference number T5168) according to the manufacturer's instructions. Proteins were revealed using a Western Lightning kit (PerkinElmer Life Sciences, <http://www.perkinelmer.com>).

## Transcriptome studies and statistical analysis of microarray data

Microarray analysis was performed using the CATMA array (version 2.2) containing 24 576 gene-specific tags corresponding to 22 089 genes from Arabidopsis (Crowe *et al.*, 2003; Hilson *et al.*, 2004). For each genotype, two independent biological replicates were used. Total RNA extraction was performed using an RNeasy plant kit (Qiagen) as indicated by the manufacturer. RNA integrity, cDNA synthesis, hybridization and array scanning were performed as described previously (Lurin *et al.*, 2004).

Statistical analysis was based on two dye swaps (i.e. four arrays, each containing 24 576 gene-specific tags and 384 controls) as described previously (Gagnot *et al.*, 2008). To identify the genes that were differentially expressed, we performed a paired *t* test on the log ratios, assuming that the variance of the log ratios was the same for all genes. Spots displaying extreme variance (too small or too large) were excluded. The raw *P* values were adjusted by the Bonferroni method, which controls the family-wise error rate. We considered genes with a Bonferroni *P* value lower than 5% as being differentially expressed, as described previously (Gagnot *et al.*, 2008). We used the Bonferroni method (with a type I error equal to 5%) in order to maintain strong control of the false positives in a multiple-comparison context (Ge *et al.*, 2003). The microarray data are available at the following address; <http://urgv.evry.inra.fr/CATdb>.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Expression of *MIR398* precursors in *miR398* mutants.

**Figure S2.** Nucleotide pairing between miR398 and target mRNAs presented in Table 1.

**Figure S3.** *CCS1* mRNAs are post-transcriptionally regulated by miR398 when sucrose is added to the medium.

**Table S1.** miRNA/mRNAs duplexes validated by 5'-RACE-PCR.

**Appendix S1.** Supplemental details for the *in vitro* culture medium. Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

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