Involvement of MicroRNA in Copper Deficiency-Induced Repression of Chloroplastic CuZn-Superoxide Dismutase Genes in the Moss Physcomitrella patens

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Superoxide dismutases (SODs) are metallo-enzymes that catalyze the dismutation of superoxide radicals. In Arabidopsis thaliana, the expression of CuZn-SOD in both the chloroplast and cytosol was reported to be down-regulated by microRNA398 (miR398) during growth on low copper. The moss Physcomitrella patens contains chloroplastic and cytosolic CuZn-SOD genes, but lacks miR398. From analysis of P. patens microRNA, miR1073 was predicted to target CuZn-SOD mRNAs. We noticed that two chloroplastic CuZn-SOD genes contain the miR1073 target sequence in the 3’ untranslated region; however, the cytosolic isozyme genes lack this sequence. In this study, we investigated the involvement of miR1073 in the expression of CuZn-SOD genes in P. patens. When protonemata of P. patens were cultured on a copper-depleted medium, SOD activity and mRNA levels of chloroplastic CuZn-SODs were decreased markedly. In contrast, cytosolic CuZn-SODs showed little or no change in mRNA levels or SOD activity. The precursor transcript and the mature form of miR1073 were induced by copper deficiency. The chloroplastic CuZn-SOD (PpCSD1) mRNA was cleaved at the miR1073 target site under copper deficiency. These results suggest that miR1073 is involved in the down-regulation of PpCSD1 expression. In addition to PpCSD1 mRNA, antisense RNAs of PpCSD1 were also detected under normal conditions; however, under copper deficiency, they were cleaved within the open reading frame (ORF) region. The cleavage of sense PpCSD1 mRNA was also detected within the ORF region. Although only miR1073 exists in the database, it is presumed that RNA cleavage, other than that mediated by miR1073, is involved in the regulation of PpCSD1 expression.

Keywords: Copper deficiency • MicroRNA • Physcomitrella patens • Superoxide dismutase.

Abbreviations: CCS, copper chaperone for CuZn-SOD; CuSO₄, copper sulfate; CuZn-SOD, copper/zinc-superoxide dismutase; DDC, diethyldithiocarbamate; EST, expressed sequence tag; Fe-SOD, iron-superoxide dismutase; H₂O₂, hydrogen peroxide; KCN, potassium cyanide; LAC, laccase; miRNA, microRNA; Mn-SOD, manganese-superoxide dismutase; ORF, open reading frame; PC, plastocyanin; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR; SOD, superoxide dismutase; UTR, untranslated region.

Introduction

MicroRNAs (miRNAs) are endogenously transcribed single-stranded non-coding RNA species that are ~21 nucleotides long. They are present in all eukaryotes and exhibit evolutionary conservation. Hundreds of miRNAs have been reported in animals and plants; however, there is generally no sequence conservation among them. There are two regulatory modes of the target mRNA; translational repression and endonucleolytic mRNA cleavage (Hutvágner and Zamore 2002, Song et al. 2004). The degree of miRNA–mRNA complementarity is a key determinant of the mechanism used, such that perfect complementarity enables cleavage, and central mismatch promotes translational repression. It is well known that animal miRNAs bind the 3’ untranslated region (UTR) of target mRNAs and mediate translational repression. In contrast, most plant miRNAs target the open reading frame (ORF) of mRNA, which leads to mRNA cleavage, although translation repression was recently recognized as an alternative mode in plants (Jones-Rhoades et al. 2006, Brodersen et al. 2008, Voinnet 2009).

Plant miRNAs are involved in responses to various abiotic and biotic stresses, such as oxidative stress, copper and phosphorus deficiency, UV stress, salt stress, etc. (Sunkar and Zhu 2004, Axtell and Bartel 2005, Sunkar et al. 2012). Copper is an essential micronutrient for normal growth and development of plants, while excess copper is toxic because it can generate toxic hydroxyl radicals. Therefore, plants have developed an important regulatory network to maintain the appropriate intracellular copper ion concentration (Clemens 2001, Grotz and Guerinet 2006, Puig et al. 2007). In Arabidopsis thaliana, miR397, miR398, miR408 and miR857 target the transcripts of
genes encoding copper proteins, including cytosolic CuZn-SOD (CSD1), chloroplastic CuZn-SOD (CSD2), a subunit of the mitochondrial Cyt c oxidase (COXsb1), a copper chaperone for CuZn-SOD (CCS), laccase (LAC) and phytofycin (plastocyanin) (Jones-Rhoades and Bartel 2004, Sunkar and Zhu 2004, Abdel-Ghany and Pilon 2008, Mendoza-Soto et al. 2012). miR397 and miR857 only regulate LAC genes, and miR408 regulates LAC and phytofycin genes. miR398 regulates the remaining four genes, including CuZn-SOD genes. Since CuZn-SODs are abundant copper proteins and play a crucial role in the response to oxidative stress and copper homeostasis, of the four copper miRNAs, miR398 has been investigated in the greatest detail (Zhu et al. 2011).

Seven SOD genes have been identified in A. thaliana: three CuZn-SOD genes (AtCSD1, AtCSD2 and AtCSD3), three Fe-SOD genes (AtFSD1, AtFSD2 and AtFSD3) and one Mn-SOD gene (AtMSD) (Kliebenstein et al. 1998). AtCSD2, AtFSD2 and AtFSD3 are localized in the plastids, AtCSD1 and AtFSD1 are localized in the cytosol, and AtMSD and AtCSD3 are localized in the mitochondria and peroxisome, respectively (Kliebenstein et al. 1998, Myouga et al. 2008). The expression of chloroplastic CuZn-SOD (AtCSD2) and cytosolic CuZn-SOD (AtCSD1) genes was down-regulated under copper-deficient conditions (Sunkar et al. 2006, Dugas and Bartel 2008). In this process, miR398 expression is induced by copper deficiency and then targets the mRNAs of AtCSD2 and AtCSD1 (Yamasaki et al. 2007). miR398 also represses the expression of genes for several copper proteins; for example, CCS, which is essential for CuZn-SOD activation (Abdel-Ghany and Pilon 2008, Cohu et al. 2009).

In A. thaliana, it was proposed that the mechanism underlying copper protein down-regulation under copper-limited conditions allows preferential delivery of copper to plastocyanin (PC), which is essential for photosynthetic electron transport and survival (Weigel at al. 2003, Burklehead et al. 2009).

From recent reviews on the phylogenetic distribution of plant miRNAs (Axtell and Bowman 2008, Jones-Rhoades 2012), several species of monocots and eudicots, in addition to A. thaliana, possess miR398 and its target sites in CuZn-SOD mRNAs. Gymnosperms also have a miR398-like gene. In contrast, pteridophytes and the moss Physcomitrella patens have no miR398 gene. Therefore, regulation of CuZn-SODs by miR398 is likely to be conserved among seed plants (Zhu et al. 2011). Copper deficiency-induced repression of CuZn-SOD was also reported in the moss, Barbula unguiculata (Shiono et al. 2003). Under normal growth conditions, cytosolic and chloroplastic CuZn-SODs were expressed but Fe-SOD was repressed completely. Under copper-deficient conditions, CuZn-SODs were repressed and Fe-SOD was induced. However, it was unclear whether miRNA was involved in the down-regulation of CuZn-SOD in moss under copper-deficient conditions because no miRNA-related sequence was identified in the Barbula CuZn-SOD mRNA.

The moss, P. patens, is used as a model plant due to its high frequency of homologous recombination (Schafer and Zryd 1997), and the availability of its entire genome sequence, which has been published (Rensing et al. 2008). Global analysis of miRNA in P. patens revealed that this moss lacks miR398 (Axtell et al. 2007, Fattash et al. 2007). miR1073 was first identified in P. patens by Axtell et al. (2007). Then, Addo-Quaye et al. (2009) predicted that two CuZn-SOD mRNAs in P. patens have a dedicated target site for miR1073. From the P. patens database, we identified two unique features in miR1073. Of the four CuZn-SOD genes in P. patens, the two chloroplastic CuZn-SOD genes possess the miR1073 target sequence while the two cytosolic CuZn-SOD genes lack it. This is distinct from other data suggesting that Arabidopsis miR398 targets both cytosolic and chloroplastic CuZn-SOD mRNAs. The second feature is the presence of the miR1073 target sequences in the 3’ UTR of the CuZn-SOD mRNAs. This is also distinct from Arabidopsis miR398, because miR398 targets sequences located within either the 5’ UTR or the ORF. Thus, miR1073 may regulate CuZn-SOD expression through modes different from those of other plant miRNAs.

To date, no report concerning the expression of SOD isozyme genes in P. patens has been published. In this study, we characterized SOD isozymes in terms of their composition and response to copper deficiency. We show that miR1073 is involved in the repression of chloroplastic CuZn-SOD gene expression during copper deficiency by cleavage of target mRNAs.

**Results**

**Identification of P. patens SOD genes**

To identify SOD isozyme genes in P. patens, BLAST and keyword searches were performed in the JGI (Rensing et al. 2008) and expressed sequence tag (EST) (Nishiyama et al. 2003) databases. The search revealed SOD isozyme genes for four CuZn-SODs (PpCSD1, PpCSD2, PpCSD3 and PpCSD4), three Fe-SODs (PpFSD1, PpFSD2 and PpFSD3) and one Mn-SOD (PpMSD). TargetP (Emanuelsson et al. 2000) and ChloroP (Emanuelsson et al. 1999) programs predicted PpCSD1 and PpCSD2 to localize to chloroplasts and PpCSD3 and PpCSD4 to localize to the cytosol (Supplementary Fig. S1). The alignment showed that the four P. patens CSDs possessed residues related to copper and zinc binding, and cysteine residues forming internal S–S bonds. The alignment also showed that chloroplastic and cytosolic CuZn-SODs in P. patens have characteristic residues conserved in each group. A phylogenetic tree was constructed using amino acid sequences of plant CuZn-SODs (Supplementary Fig. S2). PpCSD1 and PpCSD2 were grouped into a clade of chloroplastic CSDs, while PpCSD3 and PpCSD4 were in a clade of cytosolic CSDs. These findings suggest that P. patens has typical CSDs.

Next, we predicted that PpMSD would localize to the mitochondrial and that PpFSD1 and PpFSD2 would be found in the apoplast and chloroplast, respectively (Supplementary Fig. S3). PpFSD3 is likely to be a pseudogene, because its 3’ half is truncated in the genome. Since PpMSD and two Fe-SODs (PpFSD1 and PpFSD2) possessed all the residues necessary for
expression of SOD activities in P. patens. PP, protoplast; CP, chloroplast. 

Six bands were detected after SOD activity staining in the gels (Fig. 1A). SOD isozymes are generally classified according to their sensitivity to inhibitors, KCN and H2O2 (Hernández et al. 1999): Fe-SOD was resistant to KCN but sensitive to H2O2, Mn-SOD was resistant to KCN but sensitive to H2O2, Mn-SOD was KCN and H2O2 resistant, but CuZn-SOD was sensitive to both inhibitors. Based on their responses to inhibitors, the lower three activity bands were identified as CuZn-SOD and the upper three bands as Mn-SOD. No Fe-SOD activity was detected. This was unexpected because Fe-SOD activity has been reported in other mosses: B. unguiculata (Nagae et al. 2008), Mnium undulatum (Rzepeka et al. 2005) and Plagiomnium cuspidatum (Wu et al. 2009). Physcomitrella patens has two complete Fe-SOD genes (Supplementary Fig. S3A), the transcripts of which were detected (Fig. 3). Thus, why Fe-SOD activity was not detected remains unclear at present, given the presence of Fe-SOD genes.

CuZn-SOD bands were clearly discriminated from other bands using a copper chelator, diethyldithiocarbamate (DDC). In the isolated chloroplasts, only one CuZn-SOD activity band was detected (Fig. 1B). Therefore, we defined the lowest activity band as chloroplastic CuZn-SOD and the remaining two CuZn-SOD bands as cytosolic isozymes. Considering the high sequence identity (94% in the mature proteins) of the two chloroplastic CuZn-SODs (Supplementary Fig. S1), the lowest CuZn-SOD band was expected to include the two chloroplastic CuZn-SODs. The molecular weight and pi of mature CuZn-SOD proteins were calculated from the amino acid sequences: PpCSD1 (mol. wt = 15.7 kDa, pi = 5.1); PpCSD2 (mol. wt = 15.7 kDa, pi = 5.3); PpCSD3 (mol. wt = 15.9 kDa, pi = 5.5); and PpCSD4 (mol. wt = 15.6 kDa, pi = 5.9). Since lower pi proteins of identical molecular weight have higher mobility in native-PAGE, two features of PpCSD1 and PpCSD2 support the conclusion that the lowest CuZn-SOD band included two chloroplastic isozymes. We defined the upper CuZn-SOD band as PpCSD4 and the middle band as PpCSD3.

Expression pattern of P. patens SOD genes under copper-deficient conditions

To examine the effect of copper deficiency on CuZn-SOD activity, protonemata maintained on medium containing 0.2 μM CuSO4 were transferred to media containing 0 or 0.2 μM CuSO4 and subcultured on the same medium. SOD activity was assayed after incubation for 10, 20 or 30 d (Fig. 2). The activity of chloroplastic CuZn-SOD was repressed by copper deficiency, while that of the dominant cytosolic CuZn-SOD was unaffected.

To examine the effect of copper deficiency on expression of CuZn-SOD genes, reverse transcription–PCR (RT–PCR) was performed after culture for 30 d (Fig. 3). Copper deficiency resulted in the repression of two chloroplastic CuZn-SOD genes (PpCSD1 and PpCSD2), but did not affect the expression of two cytosolic CuZn-SOD genes (PpCSD3 and PpCSD4). Expression of the extracellular and chloroplastic Fe-SOD genes (PpFSD1 and PpFSD2) and the mitochondrial Mn-SOD gene (PpMSD) was unaffected by copper deficiency. Because the cytosolic PpFSD3 showed a degenerated gene structure, it seemed likely to be a pseudogene. However, its expression was strongly induced by copper deficiency. This observation will be discussed, along with the copper responsive cis-element, in the Discussion.

Cleavage of PpCSD1 mRNA and expression of miR1073 during copper deficiency

Addo-Quaye et al. (2009) predicted that the two CuZn-SOD mRNAs in P. patens have a miR1073 target site. Of the four CuZn-SOD genes in P. patens, the two chloroplastic CuZn-SOD genes (PpCSD1 and PpCSD2) were predicted to have the miR1073 target site in their 3’ UTR (Fig. 4A). To identify the cleavage site in the PpCSD1 mRNA, we performed 5’ rapid amplification of cDNA ends (RACE) using total RNA extracted from protonemata cultured under copper-deficient conditions; three bands were observed (Fig. 4B). The uppermost band suggests cleavage site 1, which is close to the 5’ end of the 5’ UTR. Considering the product size, 1.1 kb, it is possible that the product was derived from incomplete cDNA synthesis of full-length PpCSD1 mRNA. However, the other two bands were
smaller and were considered to be derived from cleaved PpCSD1 mRNA. One cleavage site (site 3) is within the miR1073 target sequence in the 3' UTR, while the other (site 2) is unrelated to miR1073 and is located within the ORF.

To investigate whether miR1073 responds to copper deficiency, expression levels of the MIR1073 precursor transcript and mature miR1073 were examined (Fig. 5). RT–PCR showed that the MIR1073 precursor transcript and mature miR1073 were induced by copper deficiency. Conversely, PpCSD1 and PpCSD2 mRNA levels were decreased by copper deficiency. These results suggest that miR1073 is involved in the down-regulation of the expression of chloroplastic CuZn-SOD in P. patens under copper-deficient conditions.

Occurrence of antisense RNAs of PpCSD1 mRNA

Another cleavage site (site 2) was located within the ORF of PpCSD1 mRNA (Fig. 4). No homologous miRNA with the sequence around cleavage site 2 was present in the miRNA database. In the experiments to identify the RNA targeting cleavage site 2, we found antisense RNAs of PpCSD1 mRNA (Fig. 6).

In the genome, the PpCSD1 gene contains seven introns (Fig. 6A). The nucleotide sequences of 3' RACE-PCR products revealed that antisense RNAs of PpCSD1 were divided into two groups. One group lacked introns (Fig. 6B; PCR products #2, #4 and #6), whereas another contained introns (Supplementary Fig. S5). It seems probable that antisense RNAs lacking the intron region were derived from mature PpCSD1 mRNA and involved in regulation of sense PpCSD1 mRNA. Furthermore, they had the following unusual structural feature: 3' RACE-PCR products #2 and #6 contained the sequences of the 3' UTR in sense PpCSD1 (red box) at their 3' ends. Product #4 contained a longer sense strand of the 3' UTR (red and blue boxes) at its 3' end. The nucleotide sequences of inserted regions are shown in Supplementary Fig. S6.

3' RACE-PCR products #2 and #4 differed in their 3' end structures. The 5' end of these antisense RNAs was determined by 5' RACE-PCR (Fig. 6C). Under copper-replete conditions, several antisense RNAs with differing 5' ends were detected. Among them, the largest antisense RNAs contained the flanking region of the miR1073 target site at the 5' end while the others lacked the flanking region. Under copper-deficient conditions, antisense RNAs became more fragmented. These findings suggest that antisense RNAs covering cleavage site 2 are produced under normal conditions, and that their cleavage is regulated by copper.

Other targets of miR1073 and their response to copper deficiency

In addition to CuZn-SOD genes, three copper protein genes, ascorbate oxidase (PpAAO2 and PpAAO4) and glyoxal oxidase (PpGLX11) genes, were predicted to have miR1073 target sequences (Addo-Quaye et al. 2009). We found that mRNAs of these genes contained an miR1073 target sequence in the 3'
From a miRBase search (http://www.mirbase.org/) of other copper protein genes, we found that PpLAC2, PpGLX12 and PpGLX19 contain miR1073 target sequences. The target sites were located in the 5′ UTR of the PpLAC2 mRNA and the 3′ UTR of the PpGLX12 and PpGLX19 mRNAs. Complementarity in the target sequences of these six genes was comparable with that in chloroplastic CuZn-SOD genes (Fig. 7B). Therefore, these six mRNAs for copper proteins are also likely to be degraded by copper deficiency, as are mRNAs for chloroplastic CuZn-SOD. We examined the effect of copper deficiency on mRNA levels of miR1073 target genes other than CuZn-SOD genes (Fig. 7C). Accumulation of PpAAO2 and PpAAO4 mRNAs decreased markedly, while PpLAC2, PpGLX11 and PpGLX12 did not show any change in expression under copper deficiency. PpGLX19 expression was increased slightly by copper deficiency. These results suggest that the PpAAO2 and PpAAO4 genes may be down-regulated via miR1073 under copper-deficient conditions.

In A. thaliana, the down-regulation of CuZn-SOD is considered to be important for supplying copper to PC, the mRNA level of which does not decrease under copper-deficient conditions (Burkehead et al. 2009). Thus, we examined the mRNA level of PC in response to copper deficiency in P. patens. Three PC genes were found in the P. patens database (PpPC1, PpPC2 and PpPC3). The mRNA levels of the three PC genes did not change under copper deficiency (Fig. 7C). In A. thaliana, the expression of CCS genes was also down-regulated by cleavage of its mRNA via miR398 (Cohu et al. 2009). Two CCS genes (PpCCS1 and PpCCS2) were identified in the P. patens database, the mRNA levels of which were unaffected by copper deficiency (Fig. 7C).

**Discussion**

Physcomitrella mir1073 resembles Arabidopsis miR398 in two respects. First, mir1073 targets mRNAs of copper protein genes, including chloroplastic CuZn-SOD genes (Figs. 4, 7). Secondly, mir1073 expression can be induced by copper deficiency (Fig. 5). Considering the close correlation of the expression of mir1073 and chloroplastic CuZn-SOD genes in response to copper deficiency, mir1073 in P. patens is expected to play...
an important role in the oxidative stress response and copper homeostasis, as does Arabidopsis miR398.

However, miR1073 has several unique properties distinct from Arabidopsis miR398, as listed below.

**The chloroplastic CuZn-SOD gene is preferentially repressed by miR1073**

Both miR1073 and miR398 target multiple mRNAs encoding copper proteins. Among them, mRNAs for chloroplastic CuZn-SOD are targeted by both miRNAs ([Supplementary Table S1](#)). Further, they differ in the mode of regulation of cytosolic CuZn-SODs. In *A. thaliana*, both cytosolic and chloroplastic CuZn-SOD mRNAs are targeted by miR398, and are greatly repressed by copper deficiency. In contrast, in *P. patens*, the two chloroplastic CuZn-SOD mRNAs targeted by miR1073 were repressed under copper deficiency, but the two cytosolic CuZn-SOD mRNAs were not repressed by miR1073 and were less affected by copper deficiency ([Figs. 2, 3](#)). How is this specificity of miR1073 conserved in the course of evolution?

To address this question, we surveyed the distribution of miR398 target sequences in cytosolic and chloroplastic CuZn-SOD genes in various plants ([Supplementary Fig. S4](#)). Unlike *P. patens*, most seed plants with miR398 target sequences in the chloroplastic CuZn-SOD gene also have such a sequence in at least one cytosolic CuZn-SOD gene. Therefore, the preferential repression of chloroplast CuZn-SOD in *P. patens* is an alternative mode of CuZn-SOD expression under copper-deficient conditions.

The response of cytosolic CuZn-SOD activity to copper deficiency in *P. patens* was distinct from that of the moss *B. unguiculata* (Shiono et al. 2003), in which both chloroplastic and cytosolic CuZn-SOD activities were repressed by copper deficiency. The full-length sequence, including the 5′ UTR and 3′ UTR, was reported for chloroplastic CuZn-SOD mRNA from *B. unguiculata*, although the cytosolic CuZn-SOD mRNA sequence was partial. We did not find an miRNA target sequence in the *B. unguiculata* chloroplastic CuZn-SOD mRNA sequence. Recently, Kanematsu et al. (2011) reported three CuZn-SOD
genes from the moss *Pogonatum inflexum*: Psod-1, Psod-2 and Psod-3. We found the miR1073 target sequence in the 3′ UTR of chloroplastic Psod-1 and Psod-2, but not in cytosolic Psod-3 (Supplementary Fig. S4). Therefore, the preferential repression of chloroplastic CuZn-SOD by miR1073 may be conserved among moss species.

A copper-economy model has been hypothesized, in which the miRNA-mediated mechanism saves copper for essential copper proteins, such as Cyt c oxidase in mitochondria and PC in chloroplasts, during copper deficiency (Burkhead et al. 2009). In the present study, the growth and morphology of *P. patens* protonemata showed no change after repeated subculture in copper-deficient medium (data not shown). Therefore, the external supply of copper contained in the culture media and intracellular trafficking of copper might maintain levels of essential copper proteins and support normal growth. Since CuZn-SOD is a major copper protein in the stroma and cytosol (Pilon 2011), copper availability is usually discussed in relation to CuZn-SOD expression. miRNA-directed repression of two chloroplastic CuZn-SODs during copper deficiency is consistent with the general view that copper is relocalized from CuZn-SOD to PC under copper-deficient conditions. In contrast, persistence of cytosolic CuZn-SODs under copper-deficient conditions is unusual, and indicates that copper proteins other than CuZn-SODs may contribute to the supply of copper to Cyt c oxidase. The miR1073 target genes, other than CuZn-SODs, encode apoplastic proteins: ascorbate oxidase, laccase and glyoxal oxidase (Fig. 7; Supplementary Table S1). Although the precise physiological role of these proteins remains unclear, LAC is expected to play an important role in copper homeostasis because the LAC mRNA is targeted by multiple miRNAs in both *A. thaliana* and *P. patens* (Supplementary Table S1). Further, ascorbate oxidase expression was down-regulated by copper availability (Fig. 7C), suggesting that ascorbate oxidase plays an important role in copper homeostasis in the cytosol.

CCS is also important for copper homeostasis. In *A. thaliana*, miR398 targets the CCS mRNA, which can produce two forms; one is targeted to the cytosol and the other to the chloroplast. Repression of CCS by copper depletion assists in the efficient relocation of copper into copper proteins other than CuZn-SOD. In *P. patens*, the two CCS mRNAs have no predicted miRNA target site, and the level of the CCS mRNAs did not decrease under copper-deficient conditions (Fig. 7C). Like the Arabidopsis CCS gene, *Physcomitrella* CCS genes are predicted to produce separate forms for the cytosol and chloroplast.

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**Fig. 7** Effects of copper deficiency on transcription of miR1073 target genes. (A) Positions of miR1073 target sites in target mRNAs. The gray and white boxes represent the ORF and UTR, respectively. The black lines represent predicted target sites of miR1073. The 5′ UTR sequence of *PpGLX12* mRNA is unknown. (B) The alignments of target mRNAs and miR1073 are shown. Watson–Crick pairings and G–U wobble pairs are indicated as colons and periods, respectively. (C) Effect of copper deficiency on mRNA accumulation of miR1073 target genes and other copper protein genes. After repeated subculture for 30 d in media lacking or containing 0.2 μM CuSO₄, total RNA was extracted and subjected to RT–PCR.
Therefore, both the unchanged CCS and cytosolic CuZn-SOD expression under copper deficiency facilitate maintenance of the activity of cytosolic CuZn-SOD under copper-deficient conditions.

### Cleavage mechanism of chloroplastic CuZn-SOD transcripts

5’ RACE-PCR predicted two cleavage sites in *PpCSD1* mRNA (Fig. 4). One is the miR1073 target site in the 3’ UTR (site 3), and the other site is within the ORF region unrelated to miR1073 (site 2). Occurrence of the miRNA target site in the 3’ UTR of the mRNA is rare in plants. In *P. patens*, miR408 targets the transcripts of eight phycocyanin genes and one LAC gene (Supplementary Table S2). We found that all of the phycocyanin genes contain miR408 target sequences within the ORF, while in the LAC gene it is in the 5’ UTR. miR398 target sites in *CuZn-SOD* mRNAs of other plant species (Supplementary Fig. S4) are located outside of the 3’ UTR. In contrast, in the moss *P. inflexum*, the miR1073 target site is in the 3’ UTR of two chloroplastic CuZn-SOD mRNAs. Therefore, preferential targeting to the 3’ UTR is also a prominent feature of miR1073; this property may be conserved among bryophytes. In *A. thaliana*, miR156 is an exceptional example of an miRNA with a target site in the 3’ UTR, which targets the mRNAs of three SBP box genes, SPL3, SPL4 and SPL5 (Wu and Poethig 2006). These findings suggest that mRNA cleavage at the 3’ UTR via miRNA may be rare but is present among seed plants and mosses.

The secondary structure of the target site located in the UTR is important for target accessibility and miRNA target recognition (Ameres et al. 2007, Kertesz et al. 2007). Of the eight miR1073 target mRNAs, the complementary sequences for miR1073 were present within the 3’ UTR, but in the 5’ UTR in the *PpLAC2* mRNA (Figs. 4, 7). The target sites within the 3’ UTR of *PpCSD1*, *PpCSD2* and *PpAAO2* mRNAs were predicted to form a large loop structure (Supplementary Fig. S5). Thus, the RNA-induced silencing complex (RISC) containing miR1073 might easily access the target sites of these mRNAs. Indeed, mRNA levels of *PpCSD1*, *PpCSD2* and *PpAAO2* decreased during copper deficiency (Figs. 3, 7C). In contrast, mRNA levels of *PpLAC2*, *PpGLX11*, *PpGLX12* and *PpGLX19* did not decrease during copper deficiency, although the target sites of these mRNAs showed high complementarity to the miR1073 sequence (Fig. 7B, C). The target sites of these mRNAs were predicted to form a stem structure (Supplementary Fig. S7). These findings indicate that the secondary structure is important for cleavage of mRNAs via UTR-targeting miR1073. The miR156 target sites of Arabidopsis SPL3, SPL4 and SPL5 mRNAs were also predicted to form loop structures (data not shown), supporting this assumption.

Of the eight miR1073 target genes, levels of the transcripts of *PpLAC2*, *PpGLX11*, *PpGLX12* and *PpGLX19* did not decrease in abundance during copper depletion. SPL3 mRNA was reported to be targeted by miR156 in the 3’ UTR (Wu and Poethig 2006). However, translational repression of SPL3 was reported to be an additional mode of action of miR156 (Gandikota et al. 2007). Further research is required to determine whether miR1073 is involved in translational control of *PpLAC2*, *PpGLX11*, *PpGLX12* and *PpGLX19*.

### Regulation of miR1073 and miR398 by the copper-responsive cis-element

In addition to CuZn-SOD, Fe-SOD showed a different mode of expression in *P. patens* and *B. unguiculata*. In *B. unguiculata*, transcript accumulation and activity of Fe-SOD were completely repressed under normal (copper-sufficient) conditions but induced under copper-deficient conditions (Shiono et al. 2003). Nagae et al. (2008) identified a copper-responsive cis-element containing a GTAC core sequence in the promoter of the *Barbula* Fe-SOD gene. This element is present in the promoter of Fe-SOD gene in *A. thaliana* (Supplementary Fig. S8). Interestingly, this element is also present in the *PpFSD3* gene, which was predicted to be a pseudogene. Indeed, *PpFSD3* gene expression was induced markedly by copper deficiency. We suggest that the *PpFSD3* gene did function as a copper-responsive gene in ancient times and that it subsequently became a pseudogene, having a degenerated ORF but maintaining the integrity of its promoter region. Further, it is expected that GTAC regulation would be conserved among mosses and seed plants. In addition to Fe-SOD, miR398 is also regulated by GTAC elements. Of three *MIR398* family genes in *A. thaliana*, *MIR398b* and *MIR398c* contain GTAC elements in their promoter regions (Supplementary Fig. S8). Although three miR398 family members target CuZn-SOD mRNA, only miR398b and miR398c regulate the copper-induced response (Yamasaki et al. 2009). As shown in Supplementary Fig. S8, we found GTAC elements in the promoter region of the *MIR1073* and *MIR408b* genes in *P. patens*. These findings suggest that miR408 is also involved in copper homeostasis under copper-deficient conditions. Further work is required to determine the regulatory network associated with miR1073 and miR408.

### Occurrence of antisense RNAs of *PpCSD1* mRNA

From 3’ and 5’ RACE-PCRs, various forms of antisense RNAs of *PpCSD1* were identified under copper-replete conditions (Fig. 6). Among them, the largest antisense RNAs (Fig. 6C, 5’ RACE products #8 and #12) possessed the flanking region of the miR1073 target site at the 5’ end. 3’ RACE products #8 and #12 were amplified using the primers specific for 3’ RACE products #2 and #4, respectively. Based on the several unique structural properties of these antisense RNAs, we suggest a mechanism for the synthesis of the two antisense RNAs (Supplementary Fig. S9). The process involves four steps. First, antisense RNA synthesis starts at the downstream region of the miR1073 target site in sense *PpCSD1* mRNA and stops at the palindromic sequences, which are also present in the flanking region of the miR1073 target site. Then, the antisense RNA forms a secondary structure. Elongation of the 3’ end follows addition of the poly(A) tail. Finally, the 5’-terminal region, including the
miR1073 target site, is cleaved. Palindromic sequences seem to play a key role in advancing these reactions. Next, we predicted the secondary structure of the two antisense RNAs using the mfold software. A double strand of the two palindromic sequences was maintained in the secondary structure of antisense RNAs, consistent with our suggestion (data not shown).

Synthesis of antisense RNA of SOD mRNA was reported in the A. thaliana cytosolic CuZn-SOD (AtCSD1) gene (Ronemus et al. 2006). AtCSD1 mRNA has an miR398 target site within the 5’ UTR (Supplementary Fig. S4). In the wild type, antisense RNA of AtCSD1 was detected under normal growth conditions. However, in mutants of argonaute 1 and RNA-dependent RNA polymerase, no antisense RNA of AtCSD1 was detected. They proposed that antisense RNAs are synthesized using sense mRNA cleaved via miRNA (Ronemus et al. 2006). In our proposed explanation of antisense RNA synthesis, the antisense RNA is synthesized from downstream of the miR1073 target site in the sense PpCSD1 mRNA. 3’ RACE products of antisense RNAs lacking intron sequences (Figs. 2, 4, and 6 in Fig. 6B) have complete miR1073 target sequences. This indicates that miR1073 is not involved in the cleavage of PpCSD1 mRNA. However, the origin of antisense RNA synthesis is close to the miR1073 target site. Further research is required to determine whether miR1073 is involved in antisense RNA synthesis.

In addition to the largest antisense RNAs of PpCSD1, smaller antisense RNAs were also generated under copper-replete conditions. Because smaller antisense RNAs lacked the 5’-terminal sequences present in the largest (Fig. 6C), it seems likely that the smaller antisense RNAs were formed by cleavage of primary antisense RNAs of PpCSD1. Under copper-deficient conditions, the largest antisense RNAs were not detected, but smaller ones were present. This suggests that the cleavage of antisense RNA of PpCSD1 is also regulated by copper.

Several mechanisms are proposed for the antisense-mediated regulation of sense mRNA, such as mechanisms related to transcription, RNA–DNA interactions, RNA–RNA interactions in the nucleus and RNA–RNA interactions in the cytosol (Lapidot and Pilpel 2006, Faghihi and Wahlestedt 2009). We speculate that the antisense RNAs of PpCSD1 mRNA can be synthesized and function in the cytosol because they lack introns. Thus, it is possible that the sense–antisense RNA duplex in the cytosol can be involved in mRNA degradation or mRNA stability. Further research is required to reveal the degradation mechanism of sense PpCSD1 mRNA via antisense RNAs.

Materials and Methods

Plant materials and Methods

Protonemata of P. patens (Gransden Wood strain) were cultured at 25°C under continuous light in a BCDAT agar medium (Nishiyama et al. 2000). For vegetative propagation, the plant was grown with a homogenizer ULTRA-TURRAX (IKA Labortecnik) in sterile water and soaked in the BCDAT agar medium overlaid with a layer of cellophane.

Characterization of P. patens SOD genes

The P. patens JG1 genome database (Phytozome, http://www.phytozome.net/) (Rensing et al. 2008) and EST database (PHYSCOBase, http://moss.nibb.ac.jp/) (Nishiyama et al. 2003) were searched using keywords, and tBlastN with the amino acid sequences of CSD1, CSD2, CSD3, FSD1, FSD2, FSD3 and MSD from A. thaliana. Analysis of nucleotide and amino acid sequences was performed using the Genetyx software (Genetyx Cooperation).

SOD activity assay

Crude extracts were prepared from protonemata (0.2 g FW) by homogenization in a buffer containing 1 mM EDTA, 10 mM dithiothreitol and 40 mM Tris–HCl (pH 7.8) in the presence of 20 mg of polyvinylpyrrolidone. Chloroplasts were isolated as described by Hattori et al. (2007) and broken by adding buffer. Protein was quantified spectrophotometrically as described by Bradford (1976) with bovine serum albumin as the standard. Native-PAGE was performed at 4°C on a 12% polyacrylamide gel using Laemmli’s system (Laemmli 1970) in the absence of SDS. The gel was then stained for SOD activity using the riboflavin/nitro blue tetrazolium method (Beyer and Fridovich 1987).

RT–PCR analysis

Total RNA was isolated with the Sepasol RNA I Super kit (Nacalai Tesque). For RT–PCR, cDNA was synthesized from 1 μg of total RNA at 42°C for 30 min with 1 μl of oligo(dT) primer from the RT–PCR kit (TAKARA BIO INC.). The primer pairs used for PCR are listed in Supplementary Table S3. Amplification conditions used were initial denaturation at 94°C for 5 min followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min.

Detection of RNA cleavage product

Total RNA isolated from P. patens protonemata cultured on medium lacking CuSO4 for 6 d was used for 5’ RACE using the SMARTer RACE cDNA Amplification Kit (Clontech). PCR was performed using the UPM Primer Mix in combination with gene-specific primers (reported in Supplementary Table S3). Cleavage products were excised from the gel, cloned and sequenced.

Detection of small RNA by RT–PCR

The RT–PCR analysis of miR1073-5p was carried out as described previously (Varkonyi-Gasic et al. 2007) using TaqMan MicroRNA Assays (Applied Biosystems).

Detection of PpCSD1 antisense-RNA by 3’ RACE-PCR

Total RNA was isolated with the Sepasol RNA I Super kit (Nacalai Tesque). 3’ RACE-PCR was performed using the RNA PCR Kit (AMV) Ver.3.0 (TAKARA BIO INC.). The primers used for PCR are described in Supplementary Table S3. PCR products were excised from the gel and sequenced.
Disclosures

The authors have no conflicts of interest to declare.

References


