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Posttranscriptional Induction of Two Cu/Zn Superoxide Dismutase Genes in *Arabidopsis* Is Mediated by Downregulation of miR398 and Important for Oxidative Stress Tolerance [W]

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MicroRNAs (miRNAs) are a class of regulatory RNAs of ~21 nucleotides that posttranscriptionally regulate gene expression by directing mRNA cleavage or translational inhibition. Increasing evidence points to a potential role of miRNAs in diverse physiological processes. miR398 targets two closely related Cu/Zn superoxide dismutases (cytosolic CSD1 and chloroplastic CSD2) that can detoxify superoxide radicals. *CSD1* and *CSD2* transcripts are induced in response to oxidative stress, but the regulatory mechanism of the induction is unknown. Here, we show that miR398 expression is downregulated transcriptionally by oxidative stresses, and this downregulation is important for posttranscriptional *CSD1* and *CSD2* mRNA accumulation and oxidative stress tolerance. We also provide evidence for an important role of miR398 in specifying the spatial and temporal expression patterns of *CSD1* and *CSD2* mRNAs. Our results suggest that *CSD1* and *CSD2* expression is fine-tuned by miR398-directed mRNA cleavage. Additionally, we show that transgenic *Arabidopsis thaliana* plants overexpressing a miR398-resistant form of *CSD2* accumulate more *CSD2* mRNA than plants overexpressing a regular *CSD2* and are consequently much more tolerant to high light, heavy metals, and other oxidative stresses. Thus, relieving miR398-guided suppression of *CSD2* in transgenic plants is an effective new approach to improving plant productivity under oxidative stress conditions.

INTRODUCTION

Regulation of gene expression at the transcriptional level is known to determine the developmental progression and physiological status in plants and animals. With the discovery of microRNAs (miRNAs) and small interfering RNAs (siRNAs), the importance of posttranscriptional gene regulation is also widely recognized now. miRNAs are ~21-nucleotide noncoding RNAs and are processed from hairpin precursors by the Dicer family of enzymes (Carrington and Ambros, 2003; Bartel, 2004; Baulcombe, 2004; He and Hannon, 2004). They repress gene expression by guiding effector complexes (miRNA ribonucleoproteins or RNA-induced silencing complexes) to complementary sites on mRNAs (Bartel, 2004). Because of the extensive sequence complementarity between plant miRNAs and their target mRNAs, RNA-induced silencing complex recruitment in plants typically leads to target mRNA cleavage (Carrington and Ambros, 2003; Bartel, 2004; Schwab et al., 2005). Animal miRNAs are only partially complementary to their targets and thus repress expression by blocking translation initiation or elongation (Ambros, 2004; Bartel, 2004; Pillai et al., 2005; Petersen et al., 2006) and enhancing P-body sequestration (Liu et al., 2005; Pillai et al., 2005), although mRNA cleavage might also occur (Mansfield et al., 2004; Yekta et al., 2004; Bagga et al., 2005; Pillai et al., 2005).

The involvement of plant miRNAs in various developmental processes, such as phase transitions, flowering, and leaf and root development, has been demonstrated (Aukerman and Sakai, 2003; Palatnik et al., 2003; Chen, 2004; Mallory et al., 2004, 2005; Vaucheret et al., 2004; Baker et al., 2005; Guo et al., 2005). Increasing evidence also points to the potential role of miRNAs in various physiological processes. For example, miR395 and miR399 were recently shown to be induced by sulfate and phosphate deprivation, respectively, and the induction is important for the downregulation of certain genes under nutrient deficiency stress (Jones-Rhoades and Bartel, 2004; Fujii et al., 2005; Chiou et al., 2006). Environmental stresses induce the expression of many genes. The induction of some of the genes important for stress adaptation might be mediated by a downregulation of miRNAs. However, thus far no miRNAs have been reported to be downregulated by stress.

Accumulation of reactive oxygen species (ROS) as a result of various environmental stresses is a major cause of loss of crop productivity (Allen et al., 1997; Mittler, 2002; Apel and Hirt, 2004; Bartels and Sunkar, 2005; Foyer and Noctor, 2005). ROS affect many cellular functions by damaging nucleic acids, oxidizing proteins, and causing lipid peroxidation (Foyer et al., 1994). Stress-induced ROS accumulation is counteracted by intrinsic

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antioxidant systems in plants that include a variety of enzymatic scavengers, such as superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, glutathione *S*-transferase, and catalase. In addition, nonenzymatic low molecular mass molecules, such as ascorbate, tocopherol, carotenoids, and glutathione, may also be important (Mittler, 2002; Mittler et al., 2004). Plant stress tolerance may therefore be improved by the enhancement of in vivo levels of antioxidant enzymes (Mittler, 2002).

Superoxide dismutases (SODs) constitute the first line of defense against highly toxic superoxide radicals by rapidly converting superoxide to hydrogen peroxide (H_2O_2) and molecular oxygen (Fridovich, 1995). On the basis of the metal cofactor used, SODs are classified into three groups: iron SOD (FeSOD), manganese SOD (MnSOD), and copper-zinc SOD (Cu/Zn-SOD), which are localized in different cellular compartments (Mittler, 2002). Overexpression of a Cu/Zn-SOD (a cytosolic SOD from pea [*Pisum sativum*]) in transgenic tobacco (*Nicotiana tabacum*) plants increased ozone tolerance (Pitcher and Zilinskas, 1996); MnSOD-overproducing plants showed improved tolerance against freezing, water deficit, winter survival (McKersie et al., 1993, 1996, 1999), and methyl viologen–induced oxidative stress (Bowler et al., 1991; Slooten et al., 1995). Overexpression of FeSOD in transgenic plants also led to increased tolerance against methyl viologen (Van Camp et al., 1996; Van Breusegem et al., 1999) and winter survival (McKersie et al., 2000). Overexpression of a chloroplastic Cu/Zn-SOD from pea (homolog of *Arabidopsis thaliana CSD2*) in transgenic tobacco plants resulted in increased tolerance against high light and low temperature stresses (Sen Gupta et al., 1993a, 1993b).

The recent discovery that miR398 targets *CSD1* and *CSD2* genes (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004) has suggested a direct connection between the miRNA pathway and *CSD1* and *CSD2* regulation. miR398 and its target sites on *CSD1* and *CSD2* mRNA are conserved in dicotyledonous and monocotyledonous plants (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Lu et al., 2005; Sunkar et al., 2005), but the potential functional consequences of miR398-guided *CSD1* and *CSD2* regulation has not been investigated.

Here, we report that a miRNA is downregulated by stress: the expression of miR398 is downregulated by oxidative stress. This downregulation is important for the posttranscriptional induction of *CSD1* and *CSD2* expression under oxidative stress conditions. Furthermore, we show that relieving miRNA-directed suppression by overexpression of a miR398-resistant version of *CSD2* leads to great improvement of plant resistance to oxidative stress conditions such as high light, heavy metal, and methyl viologen. Thus, our findings provide evidence that suppressing the expression of a miRNA is important for plant adaptation to abiotic stresses.

RESULTS

miR398 Targets *CSD1* and *CSD2* mRNA for Cleavage

miR398 is one of the recently discovered miRNAs in *Arabidopsis* and rice (*Oryza sativa*), and it is also conserved in other flowering plants (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Axtell and Bartel, 2005; Sunkar et al., 2005). miR398 targets two closely related Cu/Zn-SODs: cytosolic *CSD1* (At1g08830) and plastidic *CSD2* (At2g28190) (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004). Furthermore, Jones-Rhoades and Bartel (2004) have shown that miR398 can direct the cleavage of the *CSD1* and *CSD2* transcripts. We probed the regulatory relationship between miR398 and its target genes, *CSD1* and *CSD2*, in a transient coexpression assay in *Nicotiana benthamiana* leaves. After 2 d of coexpression with miR398, *CSD1* and *CSD2* mRNA decreased substantially, which demonstrated that the miR398 can direct the degradation of *CSD1* and *CSD2* mRNAs in vivo (see Supplemental Figures 1A and 1B online).

The Spatial and Temporal Expression Pattern of *CSD1* and *CSD2* mRNAs Is Determined by miR398

Understanding the spatial and temporal dynamics of a miRNA is important for understanding the miRNA function. The differential expression of a miRNA is expected to have opposite effects on its target gene(s) expression. Negative correlations have been observed between plant miRNAs and their target mRNAs, as shown for miR166 and target mRNA *rolled leaf1* in maize (*Zea mays*) (Juarez et al., 2004). Both *CSD1* and *CSD2* transcripts and miR398 are readily detected on RNA gel blot analysis, which allowed us to examine their expression patterns. We compared the spatial and temporal expression of miR398, *CSD1*, and *CSD2* in the same RNA samples. A high level of miR398 expression in cauline leaves and almost no expression in inflorescence tissue were reported previously (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004). In this study, a broader range of tissues was examined. miR398 was expressed highly in cauline leaves and stem, moderately in root and inflorescence, and at low levels in rosette leaves of adult plants, and it was almost undetectable in floral tissues (Figure 1A). By contrast, the *CSD1* and *CSD2* transcripts showed a clear opposite expression pattern compared with miR398 (Figure 1A). Tissues with high levels of miR398 (cauline leaves, stem, and root) have low levels of the *CSD1* and *CSD2* mRNAs, whereas tissues with low expression of miR398 (old rosette leaves and inflorescence) have high levels of *CSD1* and *CSD2* transcripts. *CSD1* and *CSD2* mRNA levels are the most abundant in floral tissues from which miR398 expression cannot be detected on the RNA gel blots (Figure 1A). The miR398 level is also negatively correlated with the *CSD1* and *CSD2* mRNA levels in young, 2-week-old seedlings (Figure 2A). These results clearly show an inverse correlation between miR398 and *CSD1* and *CSD2* mRNA levels in *Arabidopsis*.

To corroborate the role of miR398 in the observed differential expression pattern of *CSD1* and *CSD2* mRNA, we monitored *CSD1* and *CSD2* expression levels in the miRNA biogenesis mutant *hen1-1*, which fails to accumulate miR398 (Figure 1B). In the Landsberg *erecta* (L*er*) wild type, as in Columbia, the *CSD1* and *CSD2* mRNA levels in cauline leaves were hardly detectable, since miR398 was abundantly expressed, while the opposite was true for flowers (Figure 1B). By contrast, the *CSD1* and *CSD2* mRNA levels in cauline leaves of *hen1* mutant plants were as abundant as in floral tissues (Figure 1B). These observations suggest that the spatial and temporal expression pattern and the

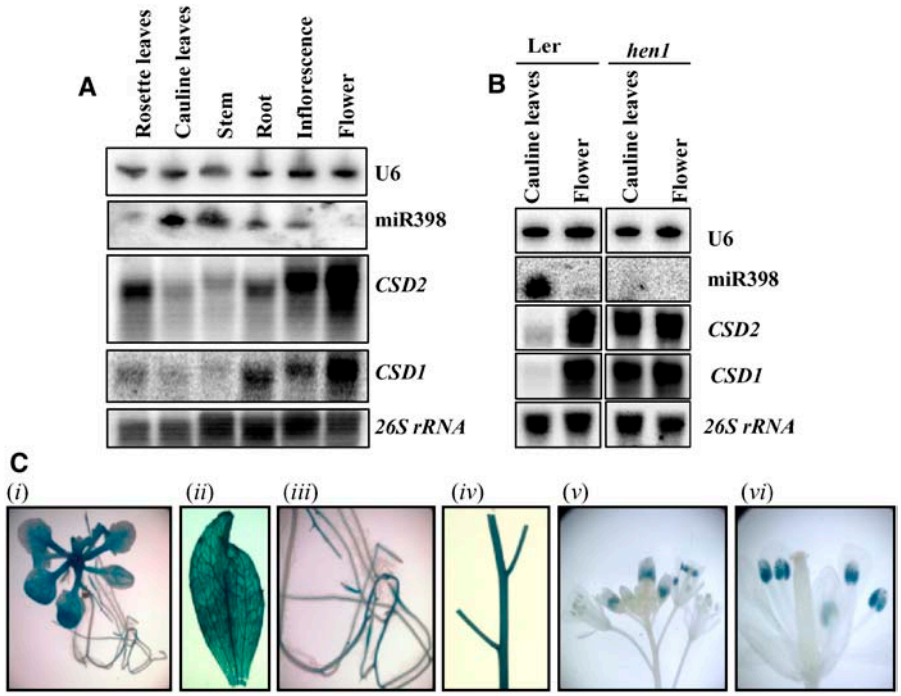

Figure 1. miR398, *CSD1*, and *CSD2* Expression Patterns in Plants.

(A) Tissue expression patterns. For small RNA gel blots, 10 μg of total RNA was loaded and the blot probed for miR398 or U6 RNA (as a loading control). For high molecular mass RNA, 20 μg of total RNA was loaded and the blot probed with full-length cDNA probes of *CSD1* and *CSD2*. The blot reprobed with 26S rRNA is shown as a loading control.

(B) miR398, *CSD1*, and *CSD2* expression in indicated tissues of the *hen1-1* mutant and wild-type L*er*. For small RNA gel blots, 10 μg of total RNA was loaded and the blot probed for miR398 or U6 RNA (as a loading control). For high molecular mass RNA, 20 μg of total RNA was loaded and the blot probed with full-length cDNAs of *CSD1* and *CSD2*. The blot reprobed with 26S rRNA is shown as a loading control.

(C) Transgenic *Arabidopsis* carrying a 2.0-kb *miR398b* promoter-GUS construct was processed for histochemical GUS staining. GUS activity is visualized in blue. The staining is mainly observed in the vascular tissue of leaves (i and ii) and root of GUS-stained *Arabidopsis* seedling (iii). The staining is visible in the primary and secondary roots. The staining is also seen in stem (iv). Inflorescence with anthers stained (v) and a closer view of a flower to visualize anther staining (vi) are shown.

abundance of *CSD1* and *CSD2* mRNAs are determined by miR398 in *Arabidopsis*.

To further analyze the spatial and temporal expression pattern of miR398, an ~2-kb putative promoter sequence upstream of the predicted fold-back structure of miR398b precursor was isolated and fused to the coding region of β-glucuronidase (GUS) to generate miR398b promoter-*GUS* transgenic plants. Analysis of the transgenic plants (Figure 1C) revealed strong GUS activity in the leaves of young seedlings, particularly in the vascular tissues. GUS expression was also detected in roots and hypocotyls. However, little or no GUS expression was observed in the stalk of inflorescence, petals, or sepals. GUS expression was observed in anthers (Figure 1C). The GUS staining pattern is consistent, in general, with the results from miR398 RNA gel blot analysis, except that RNA gel blot analysis was not able to detect any expression in flowers despite the clear GUS staining in anthers (Figure 1A). miR398 expression in anthers as indicated by the GUS staining is supported by a very low level of *CSD2* transcript in pollen documented in Genevestigator (an *Arabidopsis* microarray database).

Oxidative Stress Suppresses miR398 Expression

The *CSD1* and *CSD2* transcripts are known to be induced by oxidative stress (Perl-Treves and Galun, 1991; Tsang et al., 1991; Kliebenstein et al., 1998), although the mechanism of this induction is unknown. We investigated whether the level of miR398 that targets *CSD1* and *CSD2* mRNAs might be altered under oxidative stress conditions. Two-week-old wild-type seedlings grown under regular intensity light (100 μmol m^{-2} s^{-1}) were exposed to high light (800 μmol m^{-2} s^{-1}) for 8 or 24 h. The miR398 level was downregulated at 8 h, and the signal decreased further with longer treatment (Figure 2A). To further test miR398 regulation by oxidative stress, miR398 expression was studied in seedlings exposed to Cu^{2+}, Fe^{3+}, and methyl viologen (MV). Heavy metals, such as Cu^{2+} and Fe^{3+}, are involved in Fenton-type reactions and have a potential to generate hydroxyl radicals (Dietz et al., 1999; Estevez et al., 2001; Babu et al., 2003). MV binds to thylakoid membranes of the chloroplast and transfers the electrons to O_2 in a chain reaction causing continuous formation of superoxide radicals in the presence of light (Asada,

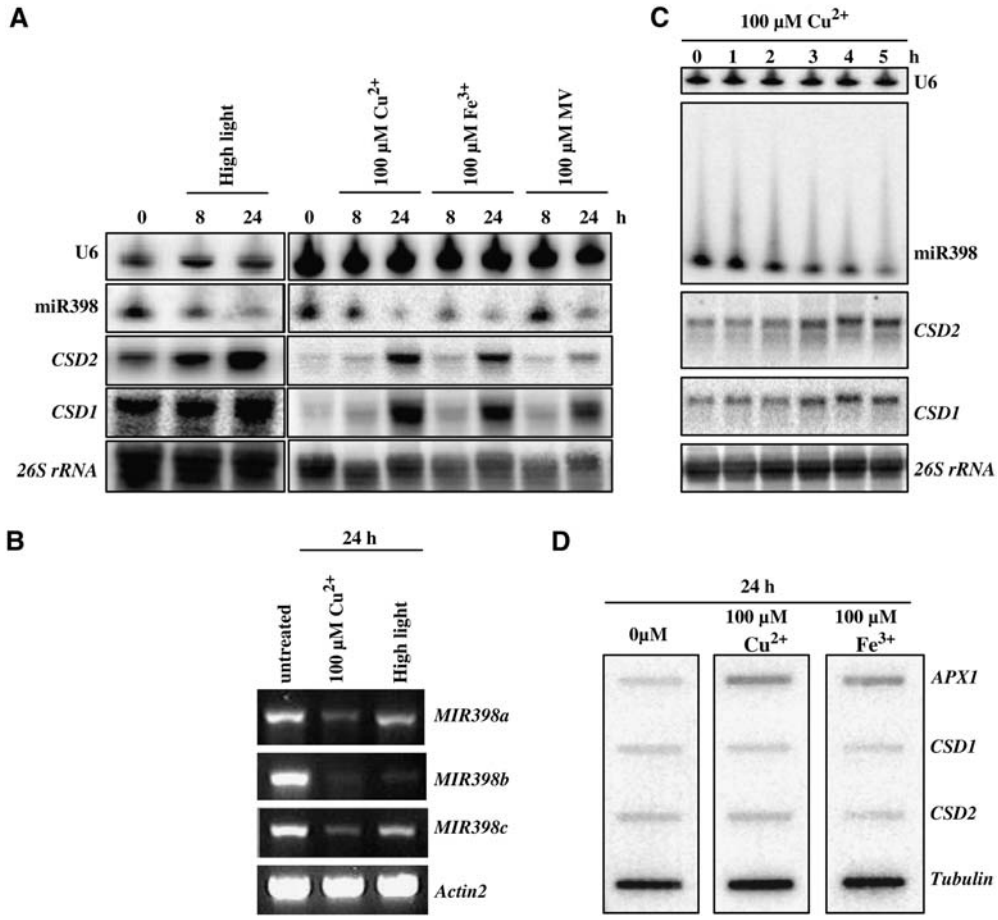

Figure 2. miR398, *CSD1*, and *CSD2* Expression in Response to High Light, Cu^{2+}, Fe^{3+}, and MV Treatments.

(A) miR398, *CSD1*, and *CSD2* in response to high light, Cu^{2+}, Fe^{3+}, and MV treatment. Each lane contained 10 μg (miR398 analysis or *CSD1* and *CSD2* analysis) of total RNA isolated from 15-d-old wild-type seedlings either transferred to high light (800 μmol m^{-2} s^{-1}) or sprayed with 100 μM Cu^{2+} or 100 μM Fe^{3+}, and seedlings were harvested after 8 and 24 h of treatment. RNA gel blot analysis was performed as indicated in Figure 1.

(B) RT-PCR analyses of precursor transcripts of *MIR398* family members in response to stress. Total RNA was isolated from 15-d-old wild-type seedlings either transferred to high light (800 μmol m^{-2} s^{-1}) or sprayed with 100 μM Cu^{2+}, and seedlings were harvested after 24 h of treatment. Actin served as a loading control.

(C) Time course of miR398, *CSD1*, and CSD2 expression pattern in response to 100 μM Cu^{2+} treatment. RNA gel blot analysis was performed as indicated in **(A)**.

(D) Three-week-old wild-type seedlings were assayed by nuclear run-on to determine the *CSD1* and *CSD2* transcriptional response to 100 μM Cu^{2+} or 100 μM Fe^{3+} treatment after 24 h.

1996). RNA gel blot analysis showed that miR398 expression was decreased after 8 h of the stress treatment, and the levels were greatly reduced after 24 h of treatment (Figure 2A).

The miR398 family is represented by two members with three loci (*MIR398a*, *MIR398b*, and *MIR398c*) in *Arabidopsis* (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004). miR398b and miR398c are identical in sequence, while miR398a differs from miR398b and miR398c only in its last (3′ most) nucleotide (2′ *O*-methyl and 3′-hydroxyl uracil in miR398a and a 2′ *O*-methyl and 3′-hydroxyl guanine in miR398b and miR398c). Using miR398b/miR398c or miR398a probes, we detected similar patterns of expression under stress conditions (data not shown). These results suggested that the miR398 family members cannot be differentiated in a small RNA gel blot analyses because of a potential cross-hybridization problem. To gain insights into which *MIR398* loci are responsive to oxidative stress conditions, we performed RT-PCR analysis using locus-specific primers designed to amplify precursor transcript, including the precursor fold-back sequence in *Arabidopsis*. In a recent study, Xie et al. (2005) provided evidence for the expression of *MIR398b* and *MIR389c* but not *MIR398a* in *Arabidopsis*. Here, we provide evidence for the expression of all three *MIR398* loci in 2-week-old *Arabidopsis* seedlings. By increasing the RNA quantity used for reverse transcription coupled with an increased number of PCR cycles, we were able to detect the expression of the primary *MIR398a* transcript, suggesting that *MIR398a* is

expressed at low abundance relative to *MIR398b* and *MIR398c*. The expression of *MIR398a*, *MIR398b*, and *MIR398c* loci was monitored in response to Cu^{2+} and high light stress. As shown in Figure 2B, the expression of *MIR398a*, *MIR398b*, and *MIR398c* precursor transcripts was downregulated under oxidative stress conditions (Figure 2B), suggesting that the downregulation occurs at all three loci.

CSD1 and *CSD2* expression was simultaneously monitored in the seedlings exposed to high light, Cu^{2+}, Fe^{3+}, or MV (Figure 2A). The same total RNA samples were used for both *CSD1* and *CSD2* mRNAs and miR398 expression analysis. The *CSD1* and *CSD2* mRNA levels were increased in response to high light, Cu^{2+}, Fe^{3+}, and MV treatments (Figure 2A). Increased levels of *CSD1* and *CSD2* were apparent after 8 h of exposure to the stress and continued to increase with prolonged (24 h) exposure (Figure 2A).

To further correlate the stress regulation of *CSD1*, *CSD2*, and miR398, we compared their expression levels at short intervals under Cu^{2+} stress. The miR398 level was decreased within 2 h of exposure to Cu^{2+} (Figure 2C). By contrast, *CSD1* and *CSD2* upregulation became apparent only at 3 h after exposure to the stress. Thus, the time course study shows that the downregulation of miR398 preceded that of *CSD1* and *CSD2* mRNA upregulation. Taken together, the above findings suggest that the lack of *CSD1* and *CSD2* expression in unstressed plants depends on miR398-mediated posttranscriptional regulation, and the stress induction of *CSD1* and *CSD2* mRNA is mediated by the downregulation of miR398.

To gain insight into the mechanism of miR398 regulation, miR398b promoter-GUS transgenic plants were subjected to the same oxidative stress conditions (high light, Cu^{2+}, and Fe^{3+}) and analyzed for GUS activity. Analysis of the seedlings revealed a decrease in the GUS intensity after 8 h of stress treatment, with a more pronounced decrease after 24 h of stress (Figure 3A). A quantitative analysis of GUS activity in high light, Cu^{2+}, and Fe^{3+} treatment substantiated the histochemical staining result (Figure 3B) and mirrored the RNA gel blot and RT-PCR results (Figures 2A to 2C). The results indicated that the downregulation of miR398 by stress is caused by stress-induced suppression of transcription of *MIR398* genes.

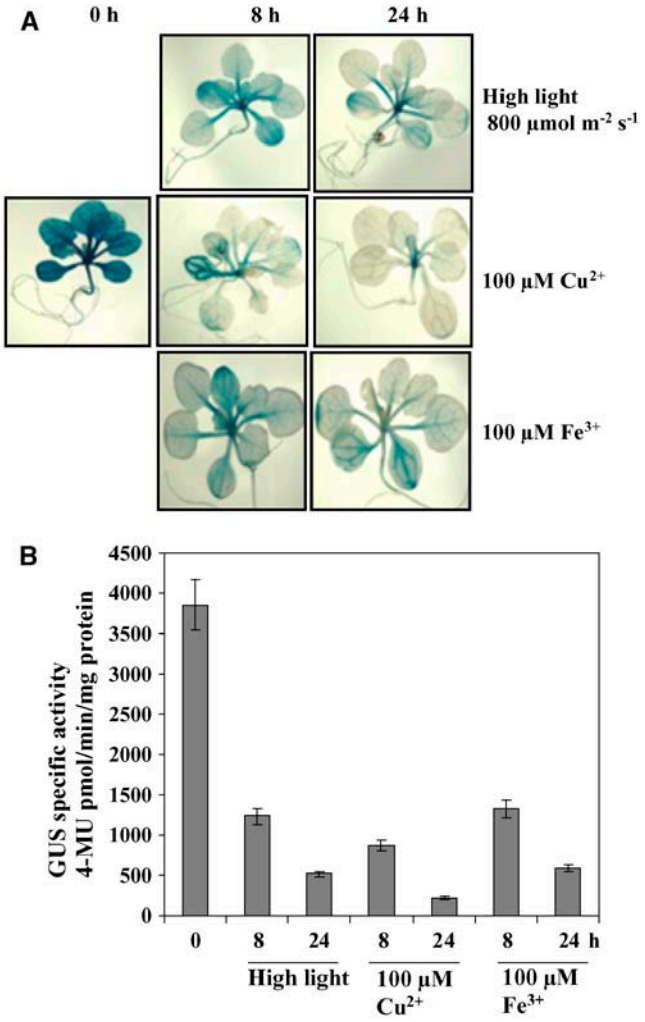

Figure 3. Response of miR398b Promoter-GUS to High Light, Cu^{2+}, or Fe^{3+} Treatments.

(A) miR398b promoter-GUS staining. Three-week-old transgenic seedlings on MS-agar medium were either transferred to high light (800 μmol m^{-2} s^{-1}) or sprayed with 100 μM Cu^{2+} or 100 μM Fe^{3+}. After 24 h of exposure, the seedlings were stained for GUS activity.

(B) Quantification of GUS activity in 3-week-old transgenic seedlings grown on MS-agar medium either transferred to high light (800 μmol m^{-2} s^{-1}) or sprayed with 100 μM Cu^{2+} or 100 μM Fe^{3+}, and the GUS activity was assayed after 24 h of treatment. The results are means ± SD of GUS activities from three independent experiments. Specific GUS activities are expressed as picomoles of 4-methylumbelliferone per milligram of total protein per minute.

Oxidative Stress–Induced *CSD1* and *CSD2* Expression Is Posttranscriptional

The results presented above clearly indicate that the stress-induced *CSD1* and *CSD2* mRNA is possibly caused by the suppression of miR398 expression and hence a decrease in miR398-guided *CSD1* and *CSD2* mRNA cleavage. However, we cannot exclude the possibility that the *CSD1* and *CSD2* mRNA levels may be transcriptionally upregulated during these stress treatments. To determine whether there is any transcriptional regulation of the *CSD1* and *CSD2* genes, we performed nuclear run-on assays with 2-week-old seedlings exposed to Cu^{2+} or Fe^{3+} for 24 h. The nuclear *CSD1* and *CSD2* RNA levels did not differ between control and Cu^{2+} or Fe^{3+} treatments (Figure 2D). By contrast, the At *APX1* (At1g07890) nuclear RNA level in the treated seedlings was substantially higher compared with the control and served as a positive control for the nuclear run-on assay (Figure 2D). At *APX1* has been shown to be induced transcriptionally under oxidative stress conditions (Fourcroy et al., 2004). These results indicate that *CSD1* and *CSD2* are being transcribed in vivo at all times, with no transcriptional induction by stress. Taken together, our results show that *CSD1* and *CSD2* mRNA accumulation in response to oxidative stresses is a result of decreased miR398-guided posttranscriptional regulation rather than increased transcription.

miR398 Cosuppression in Transgenic Plants

Ectopic expression has been successfully used to analyze the role of miRNAs because each miRNA is encoded by multiple loci and this approach obviates potential problems posed by functional redundancy. Overexpression of miRNA precursors in transgenic plants can lead to increased miRNA levels and decreased target mRNA level, and such transgenic plants often phenocopy mutants with deficiencies in the target mRNA. We used *MIR398b* precursor sequence for overexpression in transgenic plants. Despite repeated attempts, we were not able to obtain transgenic plants overexpressing miR398b and only recovered plants where cosuppression had occurred (Figure 4A). We examined whether cosuppression is due to silencing of one or more of the three *MIR398* loci using RT-PCR designed to amplify the locus-specific precursor transcripts. We detected very low primary transcript levels for both *MIR398b* and *MIR398c* in cosuppression lines compared with wild-type plants (Figure 4B), suggesting that these two loci were silenced. *MIR398c* primary transcript has extensive similarity with primary *MIR398b* transcript, and the similarity extends beyond the predicted fold-back structure both upstream and downstream (see Supplemental Figure 2A online). However, primary *MIR398a* transcript was not silenced in the cosuppression lines (Figure 4B), and this could be due to highly divergent *MIR398a* and *MIR398b* precursor transcript sequences outside the mature miRNA (see Supplemental Figure 2B online). Note that the level of *MIR398a* transcript is much lower compared with the *MIR398b* or *MIR398c* transcripts, and many more PCR cycles were required to detect *MIR398a* transcript. To determine whether the suppression in miR398 levels affects its target gene expression, we examined the levels of *CSD1* and *CSD2* transcript in two of these lines using RNA gel blot analysis. *CSD1* and *CSD2* mRNA levels were substantially increased in the cosuppression lines compared with the wild type (Figure 4A). After Cu^{2+} or Fe^{3+} treatment, the *CSD1* and *CSD2* transcript levels in the cosuppression lines were similar to those in the wild type (data not shown). The result confirms that miR398 is required for *CSD1* and *CSD2* expression in unstressed plants.

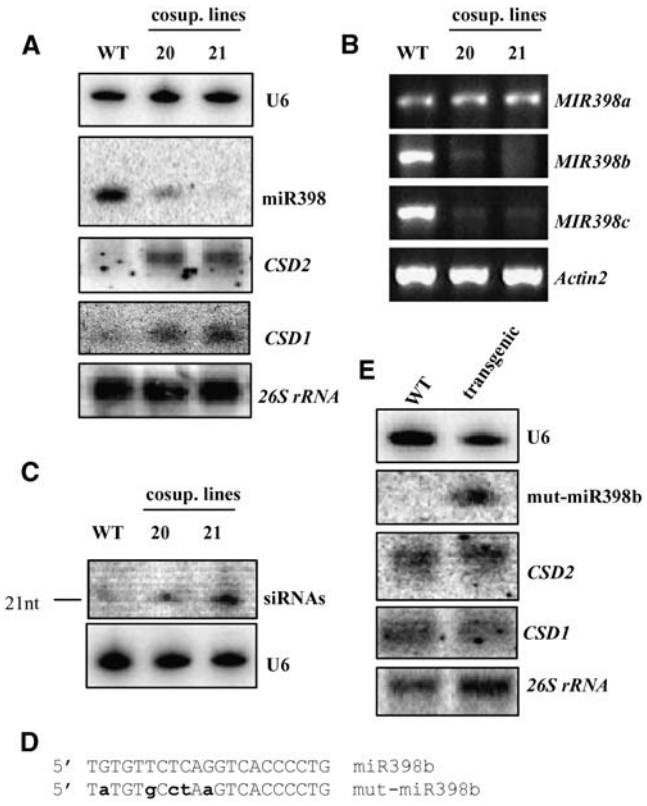

Figure 4. Cosuppression of miR398.

(A) RNA gel blot analysis of representative transgenic lines transformed with a miR398 overexpression construct. RNA gel blot analysis was performed as indicated in Figure 1.

(B) RT-PCR analyses of precursor transcripts of *MIR398* family members in cosuppression lines. Actin served as a loading control.

(C) Detection of siRNAs corresponding to miR398b precursor transcripts in cosuppression lines. Each lane contained 50 μg of total RNA isolated from 15-d-old seedlings. Small RNA gel blot analysis was performed as indicated in Figure 1. nt, nucleotides.

(D) The introduced mutations in mut-miR398b are shown in lowercase letters.

(E) Overexpression of mutated miR398b (mut-miR398b) in transgenic plants and RNA gel blot analysis of a representative transgenic line. RNA gel blot analysis was performed as indicated in Figure 1.

An important characteristic of cosuppression in plants is the production of siRNAs corresponding to the cosuppressed genes. To detect if there were siRNAs associated with the cosuppression, we used a 50-nucleotide oligonucleotide probe (5′-AGTAATCAACGGCTGTAATGACGCTACGTCATTGTTACAGCTCTCGTTTT-3′) spanning the region between the miR398b and miR398b* sequences in the miR398b hairpin precursor. As shown in Figure 4C, the probe detected 21-nucleotide siRNAs in the cosuppressed lines but not in the wild-type plants. The result further supports that there was cosuppression of the miR398b and miR398c precursor transcripts.

In contrast with our inability to overexpress wild-type miR398, a mutated miR398 (mut-miR398b; Figure 4D) that cannot target the wild-type *CSD1* and *CSD2* mRNAs could be overexpressed in transgenic plants (Figure 4D). mut-miR398b differed by five nucleotides compared with miR398b (Figure 4D). As expected, the *CSD1* and *CSD2* transcript levels were unaffected in these transgenic plants (Figure 4E).

Overexpression of a miR398-Resistant Form of *CSD2* Leads to More Dramatic Improvements in Stress Tolerance Than Overexpression of Wild-Type *CSD2*

Chloroplast is a particularly rich source of ROS, especially under stress conditions (Foyer et al., 1994; Asada, 1996). Efficient removal of ROS from chloroplasts is critical because very low concentrations of ROS can inhibit photosynthesis by oxidizing the thiol-modulated enzymes in the photosynthetic carbon reduction cycle (Kaiser, 1979). Analysis of the *Arabidopsis CSD2* knock-down mutant demonstrated an important role for CSD2 not only during high light stress but also in the absence of stress, particularly for the water–water cycle that is essential for protection of the chloroplasts under normal growth conditions (Rizhsky et al., 2003). These observations point to a critical role of CSD2 in ROS detoxification. Therefore, we focused on the functional analysis of *CSD2* in this report.

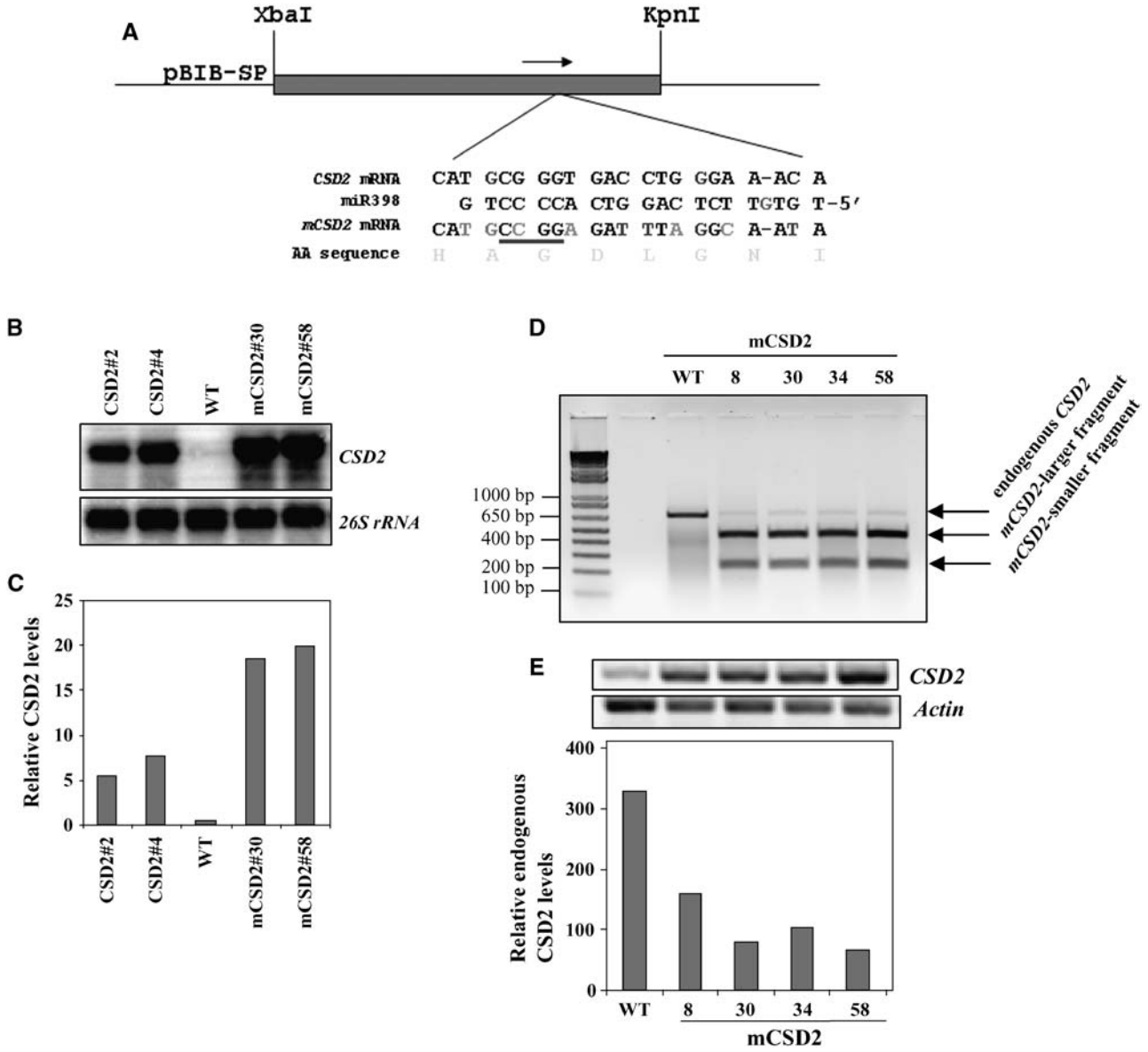

Figure 5. *CSD2* Expression Analysis in *CSD2* and *mCSD2* Transgenic Lines and Wild-Type Plants.

(A) CSD2 construct in pBIB binary vector. Blowup showing *CSD2* and *mCSD2* mRNA sequence corresponding to miR398 sequence. The introduced point mutations used to disrupt miR398 complementarity in *CSD2* are shown in bold red. Wobble base pairing is shown in pink. Introduced wobble base pairings are shown in blue. Amino acid sequence is shown in green. Introduced point mutation in the miR398 complementary sequence creates an *Msp*I site and is underlined.

(B) RNA gel blot analysis of *CSD2* expression with 10 μg of total RNA isolated from *CSD2* or *mCSD2* transgenic and wild-type plants.

(C) Expression levels were quantified by use of a phosphor imager and ImageQuant software.

(D) Determination of endogenous *CSD2* levels in mCSD2 transgenic lines and in the wild-type plants. Endogenous and miRNA-resistant (mCSD2) transcripts were amplified by RT-PCR and distinguished by digestion with the restriction enzyme *Msp*I, which cuts only the mutant form. Endogenous *CSD2* transcript is decreased substantially in *mCSD2* plants, which indicates a feedback regulation of *CSD2*. Agarose gel separation and ethidium bromide staining revealed the full-length PCR product (651 bp) and the *Msp*I digestion fragments (428 and 223 bp).

(E) *CSD2* expression levels in mCSD2 transgenic lines as determined by RT-PCR. As a control, *Actin2* fragment was amplified. Bottom panel shows the quantification of endogenous *CSD2* levels in *mCSD2* transgenic plants and wild-type plants with use of a phosphor imager and ImageQuant software.

Because *CSD2* is under posttranscriptional regulation of miR398, we hypothesized that ectopic expression of a miR398-resistant form of *CSD2* likely results in higher accumulation of *CSD2* transcript and more pronounced increase in oxidative stress tolerance. We generated a miR398-resistant version of *CSD2* construct (designated *mCSD2*) by introducing silent mutations into the miR398 recognition site in the CSD2 open reading frame (ORF) along with a wild-type *CSD2* construct for overexpression in transgenic *Arabidopsis*. When designing the miR398-resistant *mCSD2*, we did not alter the corresponding amino acid sequence (Figure 5A). Both the wild-type and *mCSD2* genes were overexpressed under control of the strong, constitutive super promoter (Li et al., 2001). RNA gel blot analysis of the resulting transgenic plants showed that overexpression of wild-type *CSD2* resulted in an ~8- to 10-fold increase in transcript levels, and overexpression of *mCSD2* brought about a further doubling of *CSD2* mRNA levels (Figures 5B and 5C).

To evaluate the effects of miR398-mediated *CSD2* regulation on plant stress tolerance, we exposed the wild-type and transgenic plants (normal *CSD2* and *mCSD2*) to high light. By visual observation, wild-type plants showed severe symptoms of loss of chlorophyll and drying of leaves, *CSD2* transgenic plants showed moderate symptoms, and the *mCSD2* plants showed only mild symptoms under high light stress conditions (Figure 6A). The physiological basis of high light stress tolerance was monitored by quantification of chlorophyll, anthocyanin, lipid peroxidation, and photosynthetic efficiency. The total chlorophyll content was decreased in the wild-type and transgenic lines exposed to high light stress, although the extent of decline was significantly lower in the transgenic plants. The decline in total chlorophyll content was the lowest in the *mCSD2* transgenic lines (Figure 6B). Another indicator of stress sensitivity is the accumulation of the purple flavonoid anthocyanin in leaves. Anthocyanin levels were determined in the wild-type and transgenic plants (*CSD2* and *mCSD2*) (Figure 6C) after 8 d of high light stress treatment. Anthocyanin levels were increased by ~20-, 10-, and 3-fold in wild-type, *CSD2*, and *mCSD2* transgenic plants, respectively (Figure 6C). To analyze the effect of high light stress on PSII activity, we measured chlorophyll fluorescence yield (Figure 6D). The maximum quantum yield of PSII photochemistry was similar in transgenic and wild-type plants under unstressed control conditions. The differences between the wild-type and transgenic plants after 1 d of high light stress were marginal (Figure 6D). However, after 2 d and later, the decrease in quantum yield of control plants was significantly greater than in the transgenic plants. Furthermore, the extent of decline in quantum yield was less in *mCSD2* than in *CSD2* transgenic lines (Figure 6D).

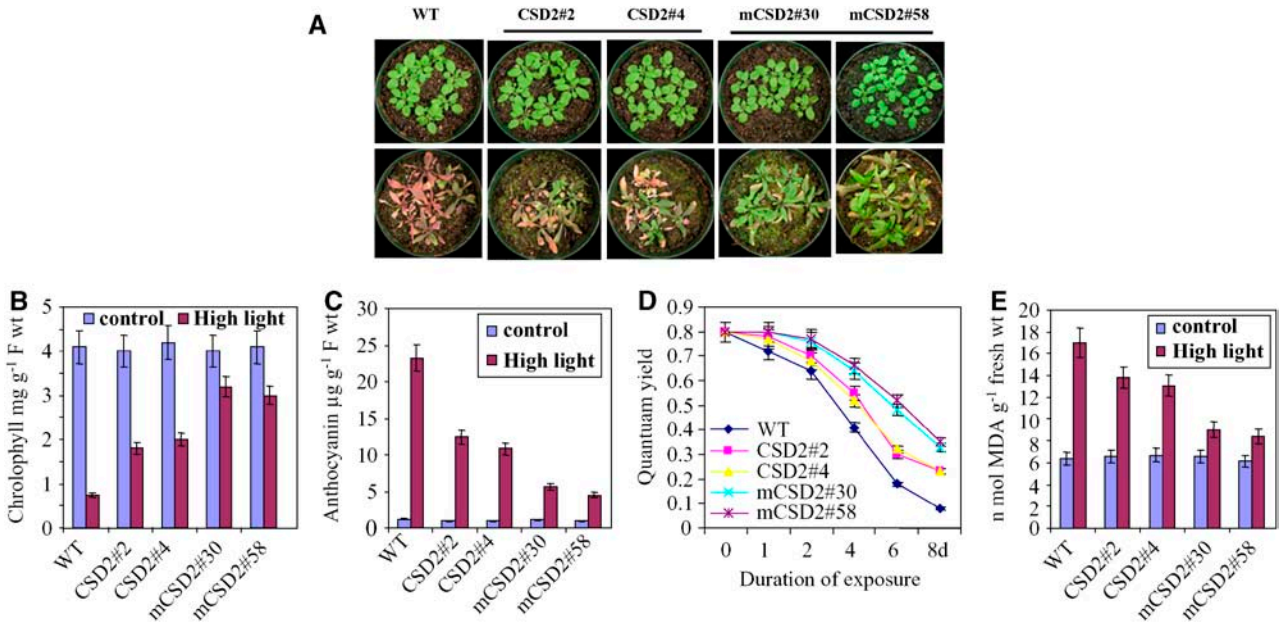

Figure 6. Response of Transgenic and Wild-Type Plants to High Light Treatment.

(A) Top panel shows plants grown under normal light intensity (100 μmol m^{-2} s^{-1}) throughout the experimentation. Bottom panel shows *CSD2* and *mCSD2* transgenic lines and the wild type exposed to continuous high light intensity (800 μmol m^{-2} s^{-1}). The photographs were taken after 8 d of exposure.

(B) and **(C)** Chlorophyll **(B)** and anthocyanin **(C)** content in leaves with or without high light for 8 d. Data are the mean ± SD of three independent experiments. F wt, fresh weight.

(D) Changes of quantum yield in *CSD2* and *mCSD2* transgenic lines and the wild type during high light stress. Data are the mean ± SD of three independent experiments.

(E) Lipid peroxidation expressed as MDA content in seedlings of *CSD2* and *mCSD2* transgenic lines and the wild type after 8 d of exposure to high light stress. Data are the mean ± SD of three independent experiments.

As an estimate of general lipid peroxidation, we determined the amount of malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids in wild-type and transgenic plants exposed to high light (Figure 6E). Mean MDA content did not differ substantially between wild-type and transgenic plants under control conditions, but the MDA levels were elevated in wild-type and transgenic plants exposed to high light. The lipid peroxidation was greatest in wild-type plants, lower in *CSD2* plants, and lowest in *mCSD2*-overexpressing plants (Figure 6E). Thus, *Arabidopsis* plants transformed with *mCSD2* showed better resistance to high light stress than those transformed with *CSD2* (Figure 6), as reflected by retention of more chlorophyll coupled with the higher PSII activity and lower levels of anthocyanin and lipid peroxidation.

To investigate whether *mCSD2* transgenic plants are more tolerant than *CSD2* transgenic plants in response to Cu^{2+} stress, wild-type and transgenic seeds were sown on Murashige and Skoog (MS)-agar plates containing different concentrations of Cu^{2+} (0, 75, 100, 150, and 175 μM), and seed germination and seedling development were monitored 18 d after imbibition (Figure 7). Seed germination was significantly better in the transgenic plants compared with the wild type at 150 μM Cu^{2+} (Figures 7A and 7B). Among the transgenic plants, *mCSD2* showed a very high germination rate compared with *CSD2* under high Cu^{2+} stress (Figure 7B). No obvious differences were observed with respect to seedling development between wild-type and transgenic seedlings on medium with up to 100 μM Cu^{2+} (data not shown), but higher Cu^{2+} concentrations adversely affected seedling development of both wild-type and transgenic lines. In the presence of 150 μM Cu^{2+}, wild-type seeds germinated, but their development was significantly retarded, whereas *CSD2* and *mCSD2* transgenic seedlings could develop (Figure 7A). Development of *mCSD2* seedlings was superior compared with *CSD2* transgenic seedlings, as assessed by visual observation (Figure

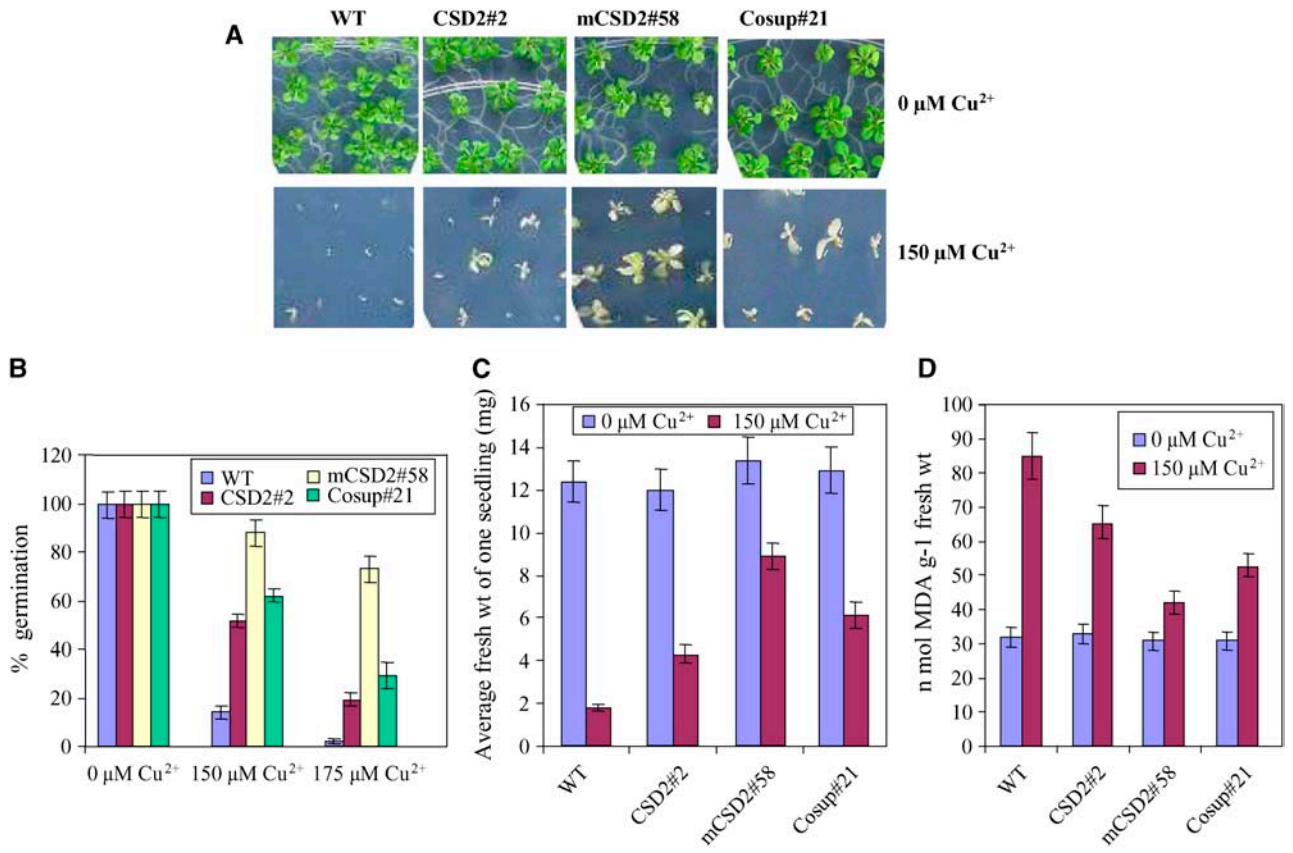

Figure 7. Response of Transgenic and Wild-Type Plants to Cu^{2+}.

(A) Germination and seedling development of *CSD2*, *mCSD2* transgenic, miR398 cosuppression lines, and the wild type exposed to 0 or 150 μM Cu^{2+} stress. Photographs were taken after 18 d of exposure to Cu^{2+}.

(B) Germination was scored when the radicle tips had fully emerged from the seed coats. Data are the means ± SD of three independent experiments (30 seeds/genotype/experiment).

(C) Average fresh weight of seedlings grown on MS-agar plates containing 0 or 150 μM Cu^{2+} for 18 d. For each data point, 30 seedlings were collected and weighed. The results are presented as average fresh weight per seedling. Data are the mean ± SD of three independent experiments.

(D) Lipid peroxidation expressed as MDA content in seedlings of *CSD2*, *mCSD2* transgenic, miR398 cosuppression lines, and the wild type after 18 d of exposure to 150 μM Cu^{2+}. Data are the mean ± SD of three independent experiments.

7A) and biomass accumulation (Figure 7C). MDA levels were elevated in both wild-type and transgenic (*CSD2* and *mCSD2*) seedlings grown in the presence of 150 μM Cu^{2+} (Figure 7D). However, the degree of lipid peroxidation was substantially lower in transgenic plants compared with wild-type plants. Furthermore, the lipid peroxidation was significantly lower in *mCSD2* plants compared with *CSD2* transgenic plants (Figure 7D).

MV exacerbates superoxide and H_2O_2 production (Asada, 1996). When seeds were germinated on MS-agar medium containing different concentrations of MV, germination efficiency did not differ between wild-type and different transgenic lines. However, seedling development was impaired in the wild type to a larger extent than in the *CSD2* and *mCSD2* transgenic lines (Figure 8A). Fresh weight measurements (Figure 8B) showed that 0.25 μM MV interfered with seedling development the most with wild-type plants, less so with *CSD2* plants, and the least with *mCSD2* transgenic plants.

The observation that there was a substantial increase in *CSD1* and *CSD2* transcripts in miR398 cosuppression lines compared with wild-type plants prompted us to evaluate their responses to oxidative stress conditions. As expected, these cosuppression lines displayed an increased tolerance to Cu^{2+} and MV stress, in terms of seedling development and lipid peroxidation rates (Figures 7 and 8).

Possible Feedback Regulation of Endogenous *CSD2* Expression in *mCSD2* Transgenic Plants

The introduced mutations in the miR398 target sequence created an *Msp*I restriction site in the miR398-resistant version of *CSD2* (*mCSD2*), which allowed us to distinguish the transgene (*mCSD2*) mRNA levels from that of endogenous *CSD2* mRNA in *mCSD2* transgenic plants. To determine the endogenous *CSD2* levels in *mCSD2*-overexpressing plants, full-length *CSD2* was amplified by reverse transcription followed by PCR amplification, and the resulting PCR product was digested with *Msp*I restriction enzyme. Endogenous *CSD2* transcript levels were substantially reduced in *mCSD2* transgenic plants compared with wild-type plants (Figures 5D and 5E). To ascertain that the decrease in endogenous *CSD2* levels in *mCSD2* transgenic plants is not due to increased miR398 levels, we analyzed the miR398 levels and found them unaltered in *mCSD2* transgenic lines (data not shown). Therefore, the decrease in endogenous *CSD2* transcript levels in *mCSD2* transgenic lines indicated a possible feedback regulation of *CSD2* gene transcription by CSD2 protein accumulation. In addition, it is possible that the *mCSD2* overexpression driven by the super promoter may have triggered a low level of cosuppression of endogenous *CSD2* in the transgenic plants. Further experiments are required to resolve these different possibilities.

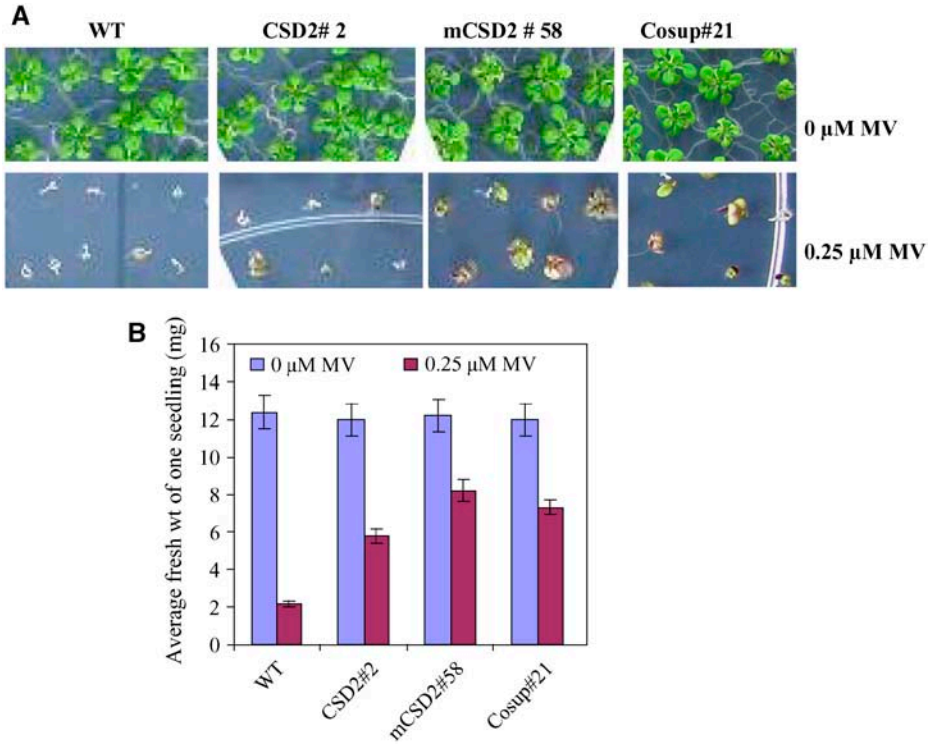

Figure 8. Response of Transgenic and the Wild-Type Plants to MV Treatment.

(A) Germination and seedling development of *CSD2* and *mCSD2* transgenic and miR398 cosuppression lines and the wild type on MS-agar plates containing 0 or 0.25 μM MV. Photographs were taken after 18 d of exposure to MV.

(B) Fresh weight of 30 seedlings grown under indicated concentrations of MV for 18 d. The results are presented as the average fresh weight of 30 seedlings for each data point. Data are the mean ± SD of three independent experiments.

DISCUSSION

Plant miRNAs generally direct their target mRNAs for endonucleolytic cleavage (Llave et al., 2002; Tang et al., 2003; Mallory et al., 2004, 2005; Allen et al., 2005; Axtell and Bartel, 2005; Guo et al., 2005; Schwab et al., 2005; Sunkar et al., 2005). A negative correlation between the expression of a miRNA and its target mRNAs is expected within a given tissue or organ. The expression profile of miR398, *CSD1*, and *CSD2* in the same RNA samples indicated a clear negative correlation and suggested a critical role for miR398 in controlling the *CSD1* and *CSD2* mRNA levels in different tissues, organs, or developmental stages in *Arabidopsis*. Our suggestion that miR398 determines the expression pattern of *CSD1* and *CSD2* is supported by the analysis of miRNA biosynthetic mutant *hen1-1*, in which miR398 expression is impaired (Figure 1B).

Although the precise physiological implication for the differential accumulation of *CSD1* and *CSD2* mRNA in different tissues or organs is not known, some tissues likely require a high level of *CSD1* and *CSD2* expression even under normal growth conditions. This notion is consistent with our finding that constitutively overexpressing miR398 is impossible, probably because such overexpression may lead to a general suppression of *CSD1* and *CSD2* in all tissues, which might be lethal to plants. Rizhsky et al. (2003) have also suggested that a complete knockout of *CSD2* may be lethal.

It is well established that cells regulate the expression of many stress-inducible genes at the level of transcription (Kawasaki et al., 2001; Seki et al., 2001; Fowler and Thomashow, 2002; Zhu, 2002). Also, some of the stress-inducible genes might be regulated at the posttranscriptional levels, although the underlying mechanisms are poorly understood (Derocher and Bohnert, 1993; Cohen et al., 1999; Kawaguchi et al., 2004). In this study, we showed that stress induction of genes can be mediated by the downregulation of a miRNA.

Environmental stress conditions, such as drought, salinity, high light, or heavy metals, cause a rapid and excessive accumulation of ROS in plant cells (Hasegawa et al., 2000; Zhu, 2002; Apel and Hirt, 2004; Bartels and Sunkar, 2005). SODs (EC 1.15.1.1) represent the first line of defense against superoxide accumulation by rapidly converting superoxide to H_2O_2 and molecular oxygen (Fridovich, 1995). Cu/Zn-SODs are arguably the most important SODs, and their roles in plant stress responses are supported by their increased expression under stress and by the phenotypic analysis of a *csd2* knockdown mutant. *CSD1* and *CSD2* mRNA is induced under oxidative stress conditions (Figure 2A; Perl-Treves and Galun, 1991; Tsang et al., 1991; Kurepa et al., 1997; Kliebenstein et al., 1998). Our results suggest that *CSD1* and *CSD2* induction by oxidative stress conditions depends on the suppression of miR398. *CSD1* and *CSD2* transcription did not differ between control and Cu^{2+} or Fe^{3+} treatments as indicated by nuclear run-on assays (Figure 2D). Therefore, *CSD1* and *CSD2* regulation under oxidative stress occurs at the posttranscriptional level and occurs by suppression of miRNA expression, thus relieving its suppressive effect on *CSD1* and *CSD2* mRNAs. This study demonstrates that a plant miRNA is a direct target of oxidative stress signaling, and its expression level and miR398b promoter activity are suppressed under stress conditions.

Several attempts have been made to improve plant stress tolerance by overproduction of Cu/Zn-SODs in transgenic plants (Perl et al., 1993; Sen Gupta et al., 1993a, 1993b; Tepperman and Dunsmuir, 1990). The introduced genes contained the miR398 target sites in their ORFs and as shown here were most likely negatively influenced by the miR398 present in wild-type plants, which may explain why minimal or no increase in stress tolerance was observed in some of the studies (Tepperman and Dunsmuir, 1990; Pitcher et al., 1991). As shown here, in experiments with high light, Cu^{2+}, Fe^{3+}, and MV stress, introducing a *CSD2* gene with the miR398 recognition site destroyed can produce a substantial increase in tolerance. These results suggest that understanding posttranscriptional gene regulation is important for our ability to manipulate stress tolerance in plants. Because miR398 is conserved in crop plants (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Sunkar et al., 2005; Lu et al., 2005), our findings offer an improved strategy to engineer crop plants with enhanced stress tolerance.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Columbia *gl-1* was used as the wild type and is the genetic background for transgenic plants, except for the analysis of the *hen1* mutant, for which the wild type is L*er*.

Constructs and Generation of Transgenic Plants

To generate the pBIB:miR398b construct, a 300-bp fragment surrounding the miRNA sequence that includes the fold-back structure of miR398b was amplified from genomic DNA with the primers indicated (forward 5′-CTAGTCTAGATTTAATCAAGTTTGCAGTACACATGTCC-3′ and reverse 5′-CGGGGTACCACTCATTGTGGGTTTCTTTACTTCCTC-3′; *Xba*I and *Kpn*I sites are underlined). The amplified fragments were digested and cloned into *Xba*I and *Kpn*I sites of pBIB downstream of the super-promoter. To introduce the point mutations into the miR398b precursor, PCR was performed with miR398b containing the pBIB plasmid as a template with the mutagenic primers mut forward (5′-CAGCTCTCGTTTTCATATGTGCCTAAGTCACCCCTGCTGAGCTCTTTCTCTACCGTCCATC-3′) and mut reverse (5′-AGCCGTTGATTACTCGTATGTGCTCAAATCTACGGTGTCGAGATCCACTACCTTCATGAT-3′). The first-round PCR products using primer pairs miR398 forward and mutated reverse and mutated forward and miR398 reverse were gel purified and used as template for second amplification, and the resulting product was digested and cloned into pBIB. This fragment was sequenced to ensure that only the desired mutations were introduced.

To generate the SP:*CSD2* construct, the *CSD2* (At2g28190) ORF was amplified by RT-PCR with the indicated primers (forward 5′-CTAGTCTAGAATGGCTGCCACCAACACAATCC-3′ and reverse 5′-CGGGGTACCTTAGAGCGGCGTCAAGCCAATC-3′). The PCR products were first cloned into pBluescript SK+ and verified by sequencing. Then, the *CSD2* ORF was released by digestion with *Xba*I and *Kpn*I and subcloned into pBIB. To generate a miR398-resistant version of *CSD2* (m*CSD2*), mutagenic primers (mut forward 5′-AGATGAGTGCCGTCATGCCGGAGATTTAGGCAATATAAATGCCAATGCCGATGG-3′ and mut reverse 5′-GCATTGGCATTTATATTGCCTAAATCTCCGGCATGACGGCACTCATCTTCTGGAGC-3′) were used. The first-round PCR products were purified and used as a template for the second amplification, and the resulting product was digested and cloned into pBIB and the clone verified by sequencing.

For miR398b promoter:GUS constructs, 2.0-kb fragments upstream from the predicted fold-back structure were amplified with the forward primer 5′-CCCAAGCTTTTCTAAACCTAAAGAAACCTTAG-3′ and reverse primer 5′-CCGGAATTCTCAACCCTGTCGAGATCCACTACC-3′ (*Hin*dIII and *Eco*RI sites are underlined). The amplified products were digested with *Hin*dIII and *Eco*RI and cloned into a pBI101 plasmid.

All the constructs described were electroporated into *Agrobacterium tumefaciens* GV3101, which was used to transform *Arabidopsis* by the floral dip method (Clough and Bent, 1998). T3 homozygous lines were tested for all experiments presented.

Stress Treatments and RNA Analysis

Seeds were surface-sterilized and sown on plates containing MS media with 3% sucrose and 0.6% agar. Seeds were stratified at 4°C for 2 d and then transferred to 22°C. For high light stress, plates containing 15-d-old seedlings grown under 100 μmol m^{-2} s^{-1} were transferred to 800 μmol m^{-2} s^{-1}. Seedlings were harvested after 8 or 24 h of high light stress. For heavy metal or MV treatments, 15-d-old seedlings were sprayed with 100 μM Cu^{2+}, 100 μM Fe^{3+}, or 10 μM MV. Seedlings were grown under a 16/8-h light/dark cycle of fluorescent light (100 μmol m^{-2} s^{-1}) at 22°C. Seedlings were harvested after 8 or 24 h of stress treatment. Untreated seedlings grown under the same conditions served as controls.

Total RNA was extracted from 15-d-old seedlings with Trizol reagent (Invitrogen). Total RNA was separated on 1.2% formaldehyde-MOPS agarose gels and blotted onto Hybond-N^+ membranes (Amersham Biosciences). Hybridization was performed at 65°C with PerfectHyb Plus buffer (Sigma-Aldrich). Probes were labeled with ^{32}P-dCTP by use of a Ready-To-Go DNA labeling kit (Amersham Biosciences). Blots were washed twice in 2× SSC and 0.1% SDS for 20 min at 65°C and once in 1× SSC and 0.1% SDS.

For analysis of small RNAs, 10 μg of total RNA was separated on a denaturing 15% polyacrylamide gel and transferred electrophoretically to Hybond-N^+ membranes. For detection of siRNAs corresponding to the miR398b precursor, 50 μg of total RNA was transferred to membrane. Hybridization and washings were performed as previously described (Sunkar and Zhu, 2004).

MIR398 Locus-Specific RT-PCR

Total RNA was extracted with Trizol reagent from 2-week-old seedlings. Contaminating DNA was removed with RNase-free DNase (RQ1-DNase; Promega), and reactions were performed in 25 μL using 4 μg of RNA (for *MIR398b* and *MIR398c*) or 6 μg of RNA (*MIR398a*) and the Qiagen one-step RT-PCR kit. Input RNA was normalized for each reaction using actin primers. Mock RT-PCR was performed without reverse transcriptase. RT-PCR conditions for primary *MIR398b* and *MIR398c* transcript amplification were as follows: 50°C for 30 min, 95°C for 15 min, 35 times (94°C for 30 s, 60°C for 30 s, and 72°C for 2 min), 72°C for 10 min. For *MIR398a* amplification, essentially the same conditions were used except the number of PCR cycles was increased to 50. The primer pairs used for RT-PCR and predicted amplicon sizes were as follows: for *MIR398a*, forward 5′-AGAAGAAGAGAAGAACAACAGGAGGTG-3′ and reverse 5′-ATTAG-TAAGGTGAAAAAATGGAACAGG-3′ (130 bp); for *MIR398b*, forward 5′-TAACAAGAAGATATCAATATATCATG-3′ and reverse 5′-ACCATTT-GGTAAATGAGTAAAAGCCAGCC-3′ (180 bp); for MIR398c, forward 5′-TCGAAACTCAAACTGTAACAGTCC-3′ and reverse 5′-ATTTGGTAA-ATGAATAGAAGCCACG-3′ (240 bp). Primers used for *Actin2* were as follows: forward 5′-TCTTCCGCTCTTTCTTTCCA-3′ and reverse 5′-GAG-AGAACAGCTTGGATGGC-3′ (440 bp).

Nuclear Run-On Assay

Nuclei were isolated from 2-week-old seedlings sprayed with Cu^{2+} or Fe^{3+} (100 μM) for 24 h. The nuclei isolation and in vitro transcription reactions were performed as described (Dorweiler et al., 2000). Comparable amounts of labeled RNA were used for filter hybridization. Slot blots on nitrocellulose membrane were prepared with 100 ng of denatured purified *CSD1*, *CSD2*, At *APX1*, and tubulin fragments obtained by PCR. For comparison, two to three slots were used for each probe. Prehybridization and hybridization were performed as described (Dorweiler et al., 2000). Following hybridization, the strips were washed for 15 min with 5× SSC and 0.1% SDS at 42°C and then with 2× SSC and 0.1% SDS for 15 min at room temperature. The strips were visualized using a Typhoon phosphor imager.

Stress Tolerance Assays

For agar plate–based assays of Cu^{2+} or MV tolerance, seeds were surface-sterilized and sown on plates containing MS media with 3% sucrose and 0.6% agar. Seeds were stratified at 4°C for 3 d and then transferred to 22°C. For Cu^{2+} or MV tolerance assays, seedlings were germinated directly on Cu^{2+} (0, 100, 150, and 175 μM) or MV (0 and 0.25 μM) containing media. Seedlings were grown under a 16/8-h light/dark cycle of fluorescent light (100 μmol m^{-2} s^{-1}) at 22°C for 18 d.

For pot-grown plants to test high light stress treatments, seeds were first germinated and grown on MS-agar plates for 10 d and then transferred to pots and grown at 100 μmol m^{-2} s^{-1} for another 10 d. These pots were maintained in a growth chamber with continuous light (100 μmol m^{-2} s^{-1}) and served as controls or were exposed to continuous high light (800 μmol m^{-2} s^{-1}) for 8 d, and photographs were taken.

Histochemical Detection of GUS Activity

Histochemical localization of GUS activities in the transgenic seedlings or different tissues were analyzed after incubating the transgenic plants overnight at 37°C in 1 mg/mL 5-bromo-4-chloro-3-indolyl-glucuronic acid, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.03% Triton X-100, and 0.1 M sodium phosphate buffer, pH 7.0. Tissue was cleared with 70% ethanol and samples.

GUS Activity Assay

GUS activity was assayed in protein extracts by a fluorescence method with 4-methylumbelliferyl glucuronide used as a substrate (Jefferson, 1987). The fluorescent product 4-methylumbelliferone (MU) was quantified using a fluorometer. Standard solutions of MU in 0.2 M Na_2CO_3 were used for calibration. To prepare protein extracts, the frozen tissue was ground in liquid nitrogen, extracted with buffer (50 mM sodium phosphate, pH 7.0, 1 mM EDTA, 0.1% [v/v] Triton X-100, and 10 mM 2-mercaptoethanol), and centrifuged for 10 min at 4°C in a microcentrifuge. The fluorogenic reaction was performed in a 1-mL volume with 1 mM 4-methylumbelliferyl-β-D-glucuronide (Duchefa Biochemie) in the extraction buffer supplemented with a 0.1 mL aliquot of protein extract supernatants. Protein concentration was determined according to the Bio-Rad protocol provided with the protein assay kit. GUS activity was calculated as picomoles MU per minute per milligram of protein.

Gene-Specific RT-PCR and Digestion with *Msp*I

Total RNA was isolated from 15-d-old seedlings of mCSD2 transgenic lines and the wild type with Trizol reagent. Two micrograms of total RNA was used for oligo(dT) primed first-strand cDNA synthesis in 20 μL with use of Superscript II RNase H reverse transcriptase (Invitrogen). Two microliters of this assay was used in a 50-μL PCR reaction, which contained 5 μL of 10× PCR buffer, 1.5 μL of 50 mM $MgCl_2$, 1 μL of 10 mM deoxynucleotide triphosphate, 1 μL each of the gene-specific primers (10 pmol μL^{-1}), and 2.5 units of Taq polymerase. The reaction (94°C, 30 s;

55°C, 45 s; 72°C, 60 s) was run for 25 cycles. To monitor that equal amounts of cDNA were synthesized, a cDNA fragment of the constitutively expressed *Actin2* gene was amplified simultaneously in 25 cycles. The primer sequences and predicted amplicon sizes were as follows: for *CSD2*, forward 5′-ATGGCTGCCACCAACACAATCC-3′ and reverse 5′-TTAGAGCGGCGTCAAGCCAATC-3′ (651 bp); and for *actin2*, forward 5′-TCTTCCGCTCTTTCTTTCCA-3′ and reverse 5′-GAGAGAACAGC-TTGGATGGC-3′ (440 bp). Endogenous *CSD2* and miRNA-resistant (*mCSD2*) transcripts were distinguished by digestion with the restriction enzyme *Msp*I, which cuts only the mutant form. Agarose gel separation and ethidium bromide staining revealed the full-length *CSD2* product (651 bp) and the *Msp*I digestion fragments (428 and 223 bp). The relative expression level of endogenous *CSD2* was estimated using Typhoon and the ImageQuant software.

Estimation of Anthocyanin

Anthocyanin levels were measured as described previously (Rabino and Mancinelli, 1986). In brief, whole leaf tissue from three plants per assay was weighed and then extracted with 99:1 methanol:HCl (v/v) at 4°C. The OD_{530} and OD_{657} for each sample were measured and relative anthocyanin levels determined with the equation $OD_{530} - (0.25 \times OD_{657}) \times$ extraction volume (mL) $\times$ 1/weight of tissue sample (g) = relative units of anthocyanin/g fresh weight of tissue.

Lipid Peroxidation Assay

The thiobarbituric acid (TBA) test, which determines MDA as an end product, was used to analyze lipid peroxidation (Heath and Packer, 1968; Hodges et al., 1999). Briefly, 0.2 g plant material was homogenized in 4 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution on ice. The suspension was rinsed into a centrifuge tube with an additional 1 mL of TCA. The homogenate was centrifuged at 10,000*g* for 5 min, and the supernatant was collected. One milliliter of 20% (w/v) TCA containing 0.5% (w/v) TBA was added to a 0.5-mL aliquot of the supernatant. The mixture was kept in a boiling water bath for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000*g* for 10 min, the absorbance of the supernatant was measured at 532 and 600 nm. The absorbance at 600 nm was subtracted from that at 532 nm, and the MDA concentration was calculated with its extinction coefficient 155 mM^{-1} cm^{-1} (Heath and Packer, 1968; Hodges et al., 1999). No readings of note were obtained without the addition of the reactive TBA.

Transient Expression in *Nicotiana benthamiana*

For transient expression assay, the designated constructs were transformed into *Agrobacterium* strain 3301. Overnight cultures grown in the presence of 30 μM acetosyringone were harvested by centrifugation, and cells were resuspended in 10 mM $MgCl_2$, 10 mM MES, pH 5.6, and 150 μM acetosyringone to an OD_{600} of 1.0. After 2 h of incubation at room temperature, the *Agrobacterium* suspension was infiltrated into expanding leaves of *N. benthamiana* using a needleless syringe (Llave et al., 2002). Leaves were harvested 2 d after infiltration and total RNA extraction and blotting performed as described above.

Accession Numbers

The Arabidopsis Genome Initiative numbers for *CSD1* and *CSD2* are At1g08830 and At2g28190, respectively.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. miR398 Targets *CSD1* and *CSD2* mRNA for Degradation.

Supplemental Figure 2. Alignment of Nucelotide Sequences Surrounding miR398a, miR398b, and miR398c Loci.

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