SHORT COMMUNICATION

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Downregulation of *CSD2* by a heat-inducible *miR398* is required for thermotolerance in *Arabidopsis*

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Abbreviations: CSD, copper/zinc superoxide dismutase; CCS, copper chaperone of CSD1 and CSD2; HSF, heat stress transcription factor; HSP, heat shock protein; qRT-PCR, real-time quantitative RT-PCR

MicroRNAs (miRNAs) play important roles in plant growth and development and abiotic stress responses. We report here that heat stress rapidly induces *miR398* and reduces transcript of its target gene *CSD2*. Transgenic plants overexpressing the *miR398*-resistant form of *CSD2* are more sensitive to heat stress than transgenic plants overexpressing normal coding sequence of *CSD2*. Expression of heat stress transcription factors (*HSFs*) and heat shock proteins (*HSPs*) is reduced in the heat-sensitive transgenic plants overexpressing *miR398*-resistant form of *CSD2*. Our results suggest that downregulation of *CSD2* by the heat-inducible *miR398* is required for thermotolerance in *Arabidopsis*.

Results and Discussion

MicroRNAs (miRNAs) are a class of small non-protein encoding regulatory RNAs ranging from 20 to 24 nucleotides in size that recognize endogenous target mRNAs for degradation or translational repression.[1-4] Many plant miRNAs are important for growth and development.[5-10] Accumulating evidence showed that miRNAs play essential roles in plant responses to biotic and abiotic stresses.[11]

We showed that *miR398* is heat-inducible and its target *CSD2* is downregulated under heat stress.[12] We then generated transgenic *Arabidopsis* plants overexpressing the *miR398*-resistant form of *CSD2* or normal coding sequence of *CSD2* (**Fig. 1A**). Three-week-old soil-grown transgenic plants overexpressing the *miR398*-resistant form of *CSD2* are more sensitive to heat stress at 37°C compared with wild-type or transgenic plants overexpressing normal coding sequence of *CSD2* (**Fig. 1B**). Relative to wild-type or transgenic plants overexpressing normal coding sequence of *CSD2*, the transgenic plants overexpressing the *miR398*-resistant form of *CSD2* displayed substantially stunted growth and development in shoot and significantly reduced accumulation of chlorophyll pigments required for photosynthesis (**Fig. 1C and D**). Plants are especially sensitive to heat stress at reproductive developmental stage. Thus, we examined thermotolerance of flowers of the *CSD2* transgenic plants. Flowers of the transgenic plants overexpressing the *miR398*-resistant form of *CSD2* are hypersensitive to heat stress compared with wild-type or transgenic plants overexpressing normal coding sequence of *CSD2* (**Fig. 1E**). These results suggest that heat tolerance requires the downregulation of *CSD2*.

We subsequently analyzed the expression of heat stress-responsive genes in these transgenic plants by qRT-PCR analysis. Compared with their expression in wild-type or transgenic plants overexpressing normal coding sequence of *CSD2*, expression of *HSFA2*, *HSFA3* and *HSFA7b* is reduced markedly in transgenic plants overexpressing the *miR398*-resistant form of *CSD2* (**Fig. 2A–C**). Expression levels of *HSP17.6*, *HSP70B* and *HSP90.1* are dramatically decreased (relative to their expression in wild-type or transgenic plants overexpressing normal coding sequence of *CSD2*) in transgenic plants overexpressing the *miR398*-resistant form of *CSD2* (**Fig. 2D–F**). Effects of *CSD2* on expression of *HSPs* are much stronger in transgenic plants overexpressing the *miR398*-resistant form of *CSD2* (**Fig. 2D–F**) than transgenic plants expressing the *miR398*-resistant form of *CSD2* under the control of the *CSD2* native promoter as described.[12] These results indicate that reduced thermotolerance of transgenic plants overexpressing the *miR398*-resistant form of *CSD2* is associated with decreased expression levels of heat stress-responsive genes.

miR398 also targets *CSD1* and *CCS* (encoding copper chaperone for CSD1 and CSD2) for degradation under heat stress.[12] Therefore, we attempted to generate transgenic plants overexpressing the *miR398*-resistant forms of *CSD1* or *CCS*. However, these two transgenes (*CSD1* and *CCS*) are silenced in the T_2 and

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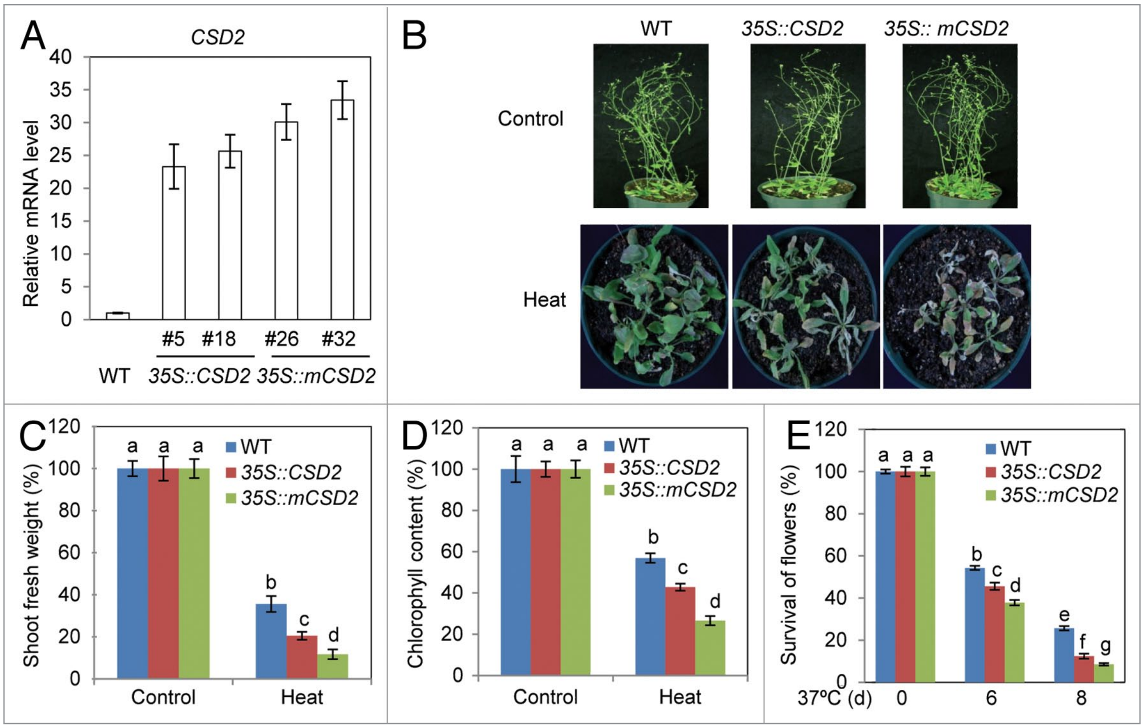

Figure 1. Thermotolerance of *CSD2* transgenic plants. (**A**) *CSD2* expression in transgenic plants expressing normal coding sequence of *CSD2* or the *miR398*-resistant form of *CSD2* (*mCSD2*) under the control of the 35S promoter (these transgenic plants are referred to as *CSD2* transgenic plants hereafter). (**B**) Thermotolerance of wild-type (WT) and *CSD2* transgenic plants. Three-week-old soil-grown seedlings were subjected to 0 (control) or 8 d (heat) at 37°C. (**C**) Shoot fresh weight of WT and *CSD2* transgenic plants shown in (**B**). (**D**) Chlorophyll content of WT and *CSD2* transgenic plants shown in (**B**). (**E**) Survival rates of flowers of separate batches of 1-mo-old of WT and *CSD2* transgenic plants under heat stress (37°C for 0, 6 or 8 d). Data presented in (**B–E**) are from one representative individual transgenic line of each transgene. Error bars represent the standard deviation [n = 4 in (**A**); n = 50–80 in (**C–E**)]. One-way ANOVA (Tukey-Kramer test) was performed for data in (**C–E**) and statistically significant differences are indicated by different lowercase letters ($p < 0.008$).

subsequent generations because of potential unknown posttranscriptional regulation mechanisms.

In summary, our data presented here clearly demonstrate that downregulation of *CSD2* by heat-inducible *miR398* is required for heat stress-responsive gene expression and thermotolerance in *Arabidopsis*. Because *miR398* family members and their target genes are highly conserved among many eukaryotic plant species,[2,12-14] downregulation of *CSD2* by heat-inducible *miR398* might be a common mechanism by which plants cope with the detrimental effects of heat stress. As a matter of fact, we found that *miR398* is induced by heat stress in corn plants.[12] Therefore, manipulation of *miR398* and/or its target genes might be viable strategies for improving the thermotolerance and yield stability of corn.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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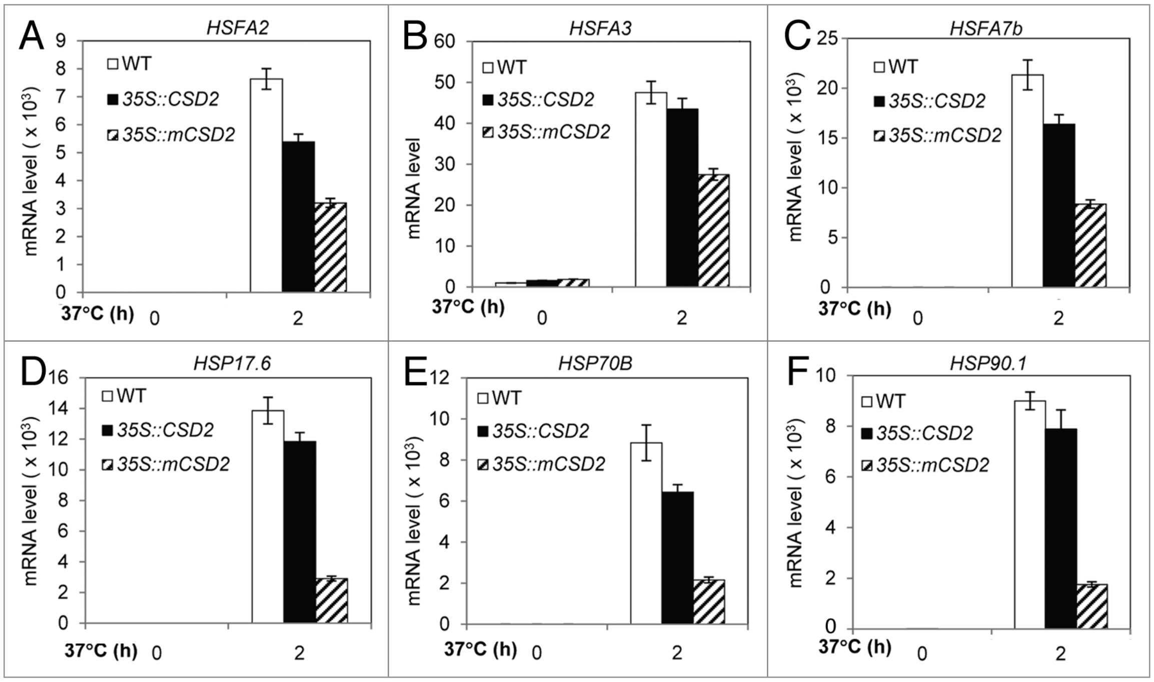

Figure 2. Expression patterns of heat stress-responsive genes in wild-type (WT) and *CSD2* transgenic plants. (**A–F**) Expression of *HSFs* and *HSPs* in WT and *CSD2* transgenic plants subjected to 0 or 2 h at 37°C. Error bars represent the standard deviation (n = 4). Data in **Figure 2** are from one representative individual transgenic line of each transgene. There are two independent transgenic lines per transgene (**Fig. 1A**).

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