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ARTICLE ADDENDUM

The broad roles of *CBF* genes: From development to abiotic stress

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ABSTRACT

Cold acclimation is an important adaptive response of plants from temperate regions to increase their freezing tolerance after being exposed to low nonfreezing temperatures. The three *CBF* genes are well known to be involved in cold acclimation. As the 3 *CBF* genes are linked tandemly in the Arabidopsis genome, it is almost impossible to obtain *cbf* triple mutants using traditional genetic methods. Recently, using the CRISPR/Cas9 technology, we generated *cbf* single, double, and triple mutants. Our results showed that the *cbf* triple mutants are extremely sensitive to freezing stress. In addition, the *cbf* triple mutants are defective in early development and salt tolerance. Interestingly, the *cbf1 cbf3* double mutants show increased expression of the *CBF2* gene and some downstream cold-responsive genes and display increased freezing tolerance, compared to the wild type, revealing that CBF1 and CBF3 negatively regulate *CBF2* expression.

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The three *CBF* genes have been well known to encode AP2/ERF (APETALA2/Ethylene-Responsive Factor) transcription factors involved in cold acclimation.[1-3] When plants are exposed to low temperatures, *CBF* genes are rapidly up-regulated and the encoded proteins promote the transcription of downstream cold-responsive (COR) genes, which in turn increase the freezing tolerance of plants.[4-6] The expression of *CBF* genes is tightly regulated, usually reaching maximal expression levels at 1–2 h, and beginning to decrease at 3 h after low temperature treatment.[1] Experimental data have shown that the 3 *CBF* genes are regulated by 3 upstream transcription factors, ICE1, ICE2 and CAMTA3,[7-9] and the CBF proteins also regulate the expression of a number of downstream transcription factors.[4,10,11] These results suggest that transcriptional cascades are used by plants to magnify cold responses. Even though the role of *CBF* genes in cold acclimation had been supported by many studies,[12,13] due to the lack of stable loss-of-function of *cbf* mutants, the extent of their contribution to freezing tolerance and cold acclimation was still not fully understood, and also little was known about the functions of *CBF* genes in development and other abiotic stress responses.

Recently, by using the newly developed CRISPR/Cas9 technology, we generated 2 independent *cbf* triple mutants, in each of which a large fragment, including the CDS regions of *CBF1* and *CBF3*, the partial CDS region of *CBF2* and the intergenic regions among these 3 genes, was deleted, which leads to the abolishment of the expression of all 3 *CBF* genes.[4] Both electrolyte leakage and survival assays showed that the *cbf* triple mutants are extremely sensitive to freezing after cold acclimation, indicating that the 3 *CBF* genes are essential for cold acclimation. By analyzing the RNA-Seq data, we found a novel, transcriptionally active region between the *CBF1* and *CBF3* genes. Interestingly, the expression of this region was also induced by low temperature treatment (Fig. 1A). By analyzing this novel transcribed sequence using BLASTn, we found that this region has already been annotated as a putative long intergenic non-coding RNA (ncRNA),[14] although the biological function of this ncRNA is still unknown. This region was deleted in both *cbf123-1* and *cbf123-2* mutants. To exclude the possibility that the freezing-hypersensitive phenotype of the *cbf* triple mutants is caused by the absence of this ncRNA, we generated a third line, named as *cbf123-3*, in which the whole CDS region of *CBF1* was deleted, but only point mutations occurred in the *CBF2* and *CBF3* genes (Fig. 1B). So in the *cbf123-3* mutant, the intergenic regions are still kept intact. Quantitative RT-PCR showed that the expression of this ncRNA was still induced in the *cbf123-3* mutant after cold treatment (Fig. 1C), which confirms that this ncRNA was not disrupted in the *cbf123-3* mutants. Freezing survival assays showed that, similar to *cbf123-1*, all seedlings of *cbf123-3* were dead after freezing treatment, whereas many of the wild type seedlings survived (Fig. 1D), indicating that the 3 *CBF* genes, but not the ncRNA, are responsible for the observed difference in freezing tolerance between the triple mutants and wild type. In addition to the freezing-hypersensitive phenotype, our previous study also showed that the *cbf* triple mutants are defective in early development and salt stress tolerance.[4] Further, our data showed unexpectedly that the *cbf1 cbf3* double mutants had increased freezing tolerance compared to the wild type after cold acclimation, which was probably due to an increased expression of *CBF2* and some of the downstream COR genes.[4]

Analyzing the phenotypes of *cbf* single, double, and triple mutants in development, freezing tolerance, and other abiotic stresses enabled us to demonstrate the biological functions

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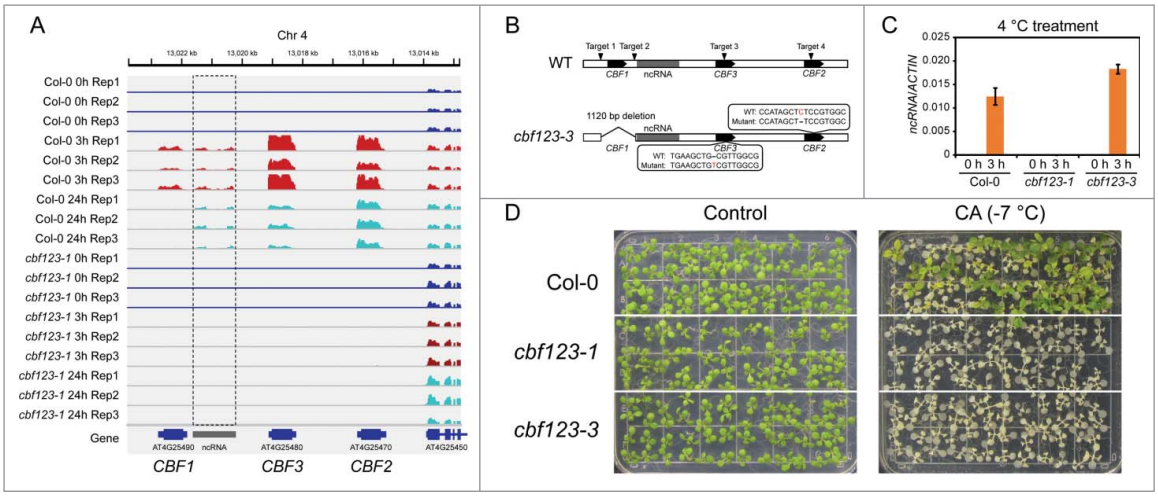

Figure 1. The *cbf* triple mutants are sensitive to freezing. (A) RNA-Seq data showing the expression of the 3 *CBF* genes in the wild type and *cbf123-1* mutant after low temperature treatment for 0, 3, and 24 h. The dashed box indicates a long intergenic non-coding RNA that was also induced by low temperature treatment. (B) A diagram showing that 4 sgRNAs targets were used in the CRISPR/Cas9 system to generate the *cbf123-3* mutant, in which the whole CDS region of *CBF1* was deleted and point mutations occurred in both *CBF2* and *CBF3* genes. The ncRNA was not disrupted in the *cbf123-3* mutant. (C) The expression of the ncRNA was examined in the wild type and *cbf* triple mutants that were treated with low temperature (4°C) for 3 h. Transcript accumulation was assessed by quantitative real-time RT-PCR, and *ACTIN8* was used as the internal control. Error bars indicate the standard deviation of 3 biological replicates. (D) Freezing survival assay. Col-0, *cbf123-1*, and *cbf123-3* seedlings were grown on MS medium for 12 d and then transferred to 4°C for cold acclimation. The acclimated plants were subjected to −7°C for 1 h before they were transferred to 23°C for recovery. The photographs show the seedlings before (left) and after (right) freezing treatment.

and the redundancy of the 3 *CBF* genes. However, there are important questions remain to be answered. Firstly, freezing assays showed that the 3 *CBF* genes are very important for cold acclimation, but it is also obvious that the *cbf* triple mutants do not completely lose the ability to acclimate to cold, implying that other components or pathways are also involved in cold acclimation. These other components most likely include the other "first-wave" transcription factors, such as HSFC1, ZAT12 and CZF1.[4,10] To confirm this hypothesis in the future, an Arabidopsis line carrying mutations in all 3 *CBF* genes and other first-wave transcription factors need to be generated to determine whether the other first-wave transcription factors contribute to the remaining part of cold acclimation in the *cbf* triple mutant. Secondly, the *cbf* triple mutants were sensitive to salt stress and NaCl-induced expression of *RD29A* was down-regulated in the *cbf* triple mutant.[4] How CBFs contribute to salt tolerance needs further investigation. Thirdly, a striking result of our earlier study is that the *cbf1 cbf3* double mutants showed increased freezing tolerance after cold acclimation compared with the wild type plants. RNA-Seq data showed that *CBF2* gene was upregulated in the *cbf1 cbf3* double mutants,[4] which could be one of the reasons for the increased freezing tolerance of *cbf1 cbf3* double mutants. Alternatively, the CBF2 protein may have a higher transcriptional activity than CBF1 and CBF3, so in the *cbf1 cbf3* double mutant, only CBF2 protein binds to the promoter regions of COR genes, which leads to the higher expression of some of the COR genes and increased freezing tolerance. Whether CBF2 has a higher transcriptional activity than CBF1 and CBF3 needs experimental evidence. Finally, whether and how the cold-inducible ncRNA may be involved in cold acclimation needs to be investigated.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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