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The over-expression of calmodulin from Antarctic notothenioid fish increases cold tolerance in tobacco

Yang Na [a,1], Peng Changlian [a,1], Cheng Deng [b], Huang Qiao [a], Xu Guanghui [b], Gao Fei [b], Chen Liangbiao [b,*]

[a] College of Life Science, Key Laboratory of Ecology and Environmental Science in Guangdong Higher Education, Guangdong Provincial Key Laboratory of Biotechnology for Plant Development, South China Normal University, Guangzhou 510631, China
[b] Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

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ABSTRACT

Genes involved in the calcium signalling pathway have a relationship with cold tolerance in many plants. The primary reaction to many different environmental stresses is an increase in the cytoplasmic Ca^{2+} concentration. Such variations in the Ca^{2+} concentration could change the activity of Ca^{2+}-dependent protein functions, further regulating the expression of stress-related genes; therefore, the Ca^{2+} signalling pathway is involved in the biological stress reaction. The expression of the calcium-modulated protein gene, calmodulin, in Antarctic notothenioid fish (*Dissostichus mawsoni*) accounts for 0.23% of all transcripts, which is a very high level of expression in this cold-water fish. To elucidate the function of calmodulin (*CaM*) from Antarctic notothenioid fishes, we introduced the calmodulin (*CaM*) gene into tobacco plants using a viral vector based on pea early browning virus (PEBV). RT-PCR and Western blot results confirmed that the *CaM* gene was over-expressed in tobacco. Under low-temperature stress, the *CaM* transgenic plants exhibited faster growth than wild-type plants. The physiological and biochemical effects of the high-level expression of *CaM* in tobacco were analysed, and the changes in the electrolyte leakage activity and malondialdehyde content showed that *CaM* over-expression in tobacco increased the cold tolerance of the plants. These results demonstrate that *CaM* can possibly be used to enhance the low-temperature tolerance of plants.

1. Introduction

Low temperature plays a very important role in the growth of plants, limiting their distribution and productivity, and the damage to crops due to low temperature lead to an annual economic loss of a billion dollars (Graham, 1982). However, most plants from temperate regions can acclimate to the cold (Minami et al., 2005); chilling resistance has been characterised as the physiological and biochemical changes that result from the selective increases or decreases in the biosynthesis of a large number of distinct proteins (Quivey et al., 1995). The cold tolerance of cells is widely researched. In particular, it is known that microtubular stability is affected and some calcium channels are activated in a low temperature environment (Wang and Nick, 2001). Many experiments have indicated a cold-induced Ca^{2+} increase in the cytosol, and this increase might be involved in cold-stress signalling (Chinnusamy et al., 2007).

Most plants will increase their cold tolerance when exposed to cold temperatures: a phenomenon known as chilling resistance (Ruelland et al., 2009). Chilling resistance involves many changes in the physiology and biochemistry of cells, including extensive alterations in the composition of lipids, proteins, and the metabolome (Doherty et al., 2009). To date, the best-known regulatory pathway involved in chilling resistance is the CBF cold-response pathway. In this pathway a calmodulin binding transcription activator (CAMTA) acts as a positive regulator for *CBF2* expression. CAMTA is an important factor for integrating low-temperature calcium and calmodulin (*CaM*) signalling with cold-regulated gene expression (Doherty et al., 2009). Other direct evidence shows that the genes involved in the calcium signalling pathway increase cold tolerance. For example, rice plants transformed with gene constructs for the over-expression of calcium-dependent protein kinase 13 (CDPK13) and calreticulin-interacting protein 1 (CRTintP1) possess increased cold tolerance (Komatsu et al., 2007). When the transgenic rice plants with ectopically expressed CDPK13 or CRTintP1 proteins and the wild-type control were incubated at 5 °C for 3 days, the leaves of both types of plants were wilted and curled; however, if the plants were returned to the greenhouse for recovery, the leaves of the wild-type rice died, but the leaves of the transgenic rice plants recovered and resumed growth

Abbreviations: CaM, calmodulin; PEBV, pea early browning virus; RT-PCR, reverse transcription polymerase chain reaction; PCR, polymerase chain reaction; MDA, malondialdehyde; TBA, trichloroacetic thiobarbituric acid; TCA, thiobarbituric acid; CBF, C-repeat binding factor; CAMTA, calmodulin-binding transcription activator; CRTintP1, calreticulin-interacting protein 1; CDPK, calcium-dependent protein kinase.

* Corresponding author at: West Lincui Road, Chaoyang District, Beijing 100101, China. Tel./fax: +86 10 62554807.
E-mail address: lbchen@genetics.ac.cn (L. Chen).

[1] These authors contributed equally to this work.

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(Groenendyk et al., 2004). These results show that transgenic expression of *CDPK13* and *CRTintP1* confers cold tolerance in rice. Additionally, there is published evidence showing that the expression levels of the genes related to the calcium signalling pathway are up-regulated after chilling treatment. For example, a calcium-dependent protein kinase (CDPK) in orchid was activated by low temperature (Tsai et al., 2007). The expression level of calcium/calmodulin-regulated receptor-like kinase 1 (CRLK1), a positive regulator of cold tolerance in plants, can be rapidly increased after cold treatment in *Arabidopsis thaliana* (Yang et al., 2010).

Because many genes involved in the calcium signalling pathway have some relationship with cold tolerance, we focus on the gene for another calcium-binding protein, calcium-modulated protein (calmodulin or *CaM*). We find that the expression of calmodulin in Antarctic notothenioid fish (*Dissostichus mawsoni*) accounts for 0.23%, a very high level of expression in these fishes, suggesting that *CaM* may be involved in chilling resistance in cold-water fishes. When the protein sequences of calmodulin from various species are aligned, a high similarity is found (Fig. 1a). Most importantly, there is no difference between the cold-water fish *D. mawsoni* and the warm-water fishes *Danio rerio*, *Oryzias latipes* and *Takifugu rubripes* (Fig. 1b), suggesting that the expression level of *CaM*, and not the protein itself, may be increased by cold treatment. Many cold-inducible genes have been cloned from various plants (Thomashow, 1999), and some of the products are involved in the stabilisation of membranes and macromolecules, metabolic shifts, scavenging of oxygen radicals, and signal transduction pathways. The function of numerous other cold-inducible proteins, however, remains unknown. Analysis of the genes that are induced during chilling resistance is important for understanding the mechanism of cold tolerance and for use in breeding cold-tolerant plants.

The notothenioid fishes of Antarctica are the predominant fish taxa in the frigid/freezing Antarctic waters. Thrived in the freezing temperatures for millions of years, the fishes have evolved many new genes and molecular mechanisms to cope with the low temperature including the evolution of the Antifreeze glycoproteins (Chen et al., 1997). In previous work (Chen et al., 2008), we have identified a series of genes that are either duplicated or highly expressed in the cold-adapted Antarctic notothenioid fishes. These genes could confer cold-tolerance to fishes or even plants. Using a transient transfection and expression technology developed in the plant, tobacco, we can quickly evaluate the potentials of the Antarctic fish genes in protecting the transgenic tobaccos from the cold damage, and the identified genes can further be studied for its applicability in cold stress tolerance improvements in fishes or plants. From many candidate genes, we report in this study that expression of the notothenioid *CaM* improves cold stress tolerance in transgenic tobacco.

2. Materials and methods

2.1. Plant expression plasmid construction

The full-length cDNA coding for Antarctic notothenioid fish (*D. mawsoni*) calmodulin (*CaM*) was amplified using the following primers: *CaM*-ATG (AAAACGCGTATGGCTGATCAGCTTACAGAAGAGC) and *CaM*-TGA (AAAGAATTCTCACTTCGCCGTCATCATTTGTACG) (the restriction sites of MluI and EcoRI sites are underlined). The PCR conditions were 5 min at 95 °C and 1 min at 94 °C, 1 min at 61 °C and 30 s at 72 °C for 35 cycles, followed by an additional 10 min at 72 °C to ensure the completed extension of the product. The PEBV-based (Constantin et al., 2004) pCAPE vector system including two plasmids, pCAPE1 (assistant plasmid) and pCAPE2-GFP (control plasmid) were used to mediate ectopic *CaM* expression in tobacco plants. pCAPE1 contains the full-length cDNA of RNA1 from the PEBV genome, encoding the viral proteins that are responsible for replication and movement of the virus in plants conferring plant infection capability. RNA2 from PEBV encodes for coat protein and proteins needed for nematode transmission but the latter was substituted by GFP in pCAPE2-GFP vector. For developing a vector pCAPE2 to express *CaM*, GFP in pCAPE2-GFP was

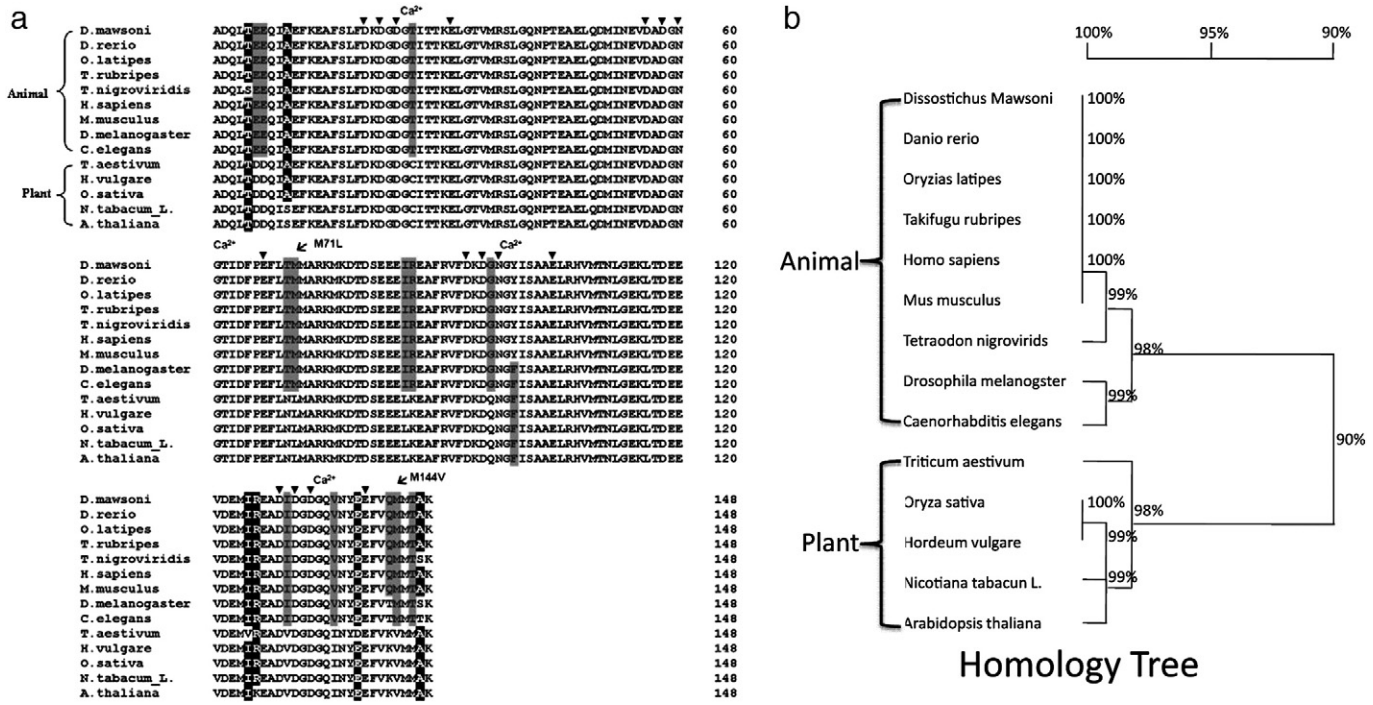

Fig. 1. Comparison of calmodulins from different species. The abbreviations of each species are as follows: *D. mawsoni*, *Dissostichus Mawsoni*; *D. rerio*, *Danio rerio*; *O. latipes*, *Oryzias latipes*; *T. rubripes*, *Takifugu rubripes*; *T. nigroviridis*, *Tetraodon nigroviridis*; *H. sapiens*, *Homo sapiens*; *M. musculus*, *Mus musculus*; *D. melanogaster*, *Drosophila melanogaster*; *C. elegans*, *Caenorhabditis elegans*; *T. aestivum*, *Triticum aestivum*; *H. vulgare*, *Hordeum vulgare*; *O. sativa*, *Oryza sativa*; *N. tabacum L.*, *Nicotiana tabacum L.*; and *A. thalianann*, *Arabidopsis thaliana*.