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

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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 Ca2+ concentration. Such variations in the Ca2+ concentration could change the activity of Ca2+-dependent protein functions, further regulating the expression of stress-related genes; therefore, the Ca2+ 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.

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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 Ca2+ 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 plants 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.
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(Groenendyk et al., 2004). These results show that transgenic expression of CDPK13 and CTR1ptideP1 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 (CRKL1), 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) protein (CaM) was amplified using the following primers: CaM-ATG (AAAACCCTATGGCCTGATCTACCAAGAC) and CaM-TGA (AAAGAATTCATCCGCCTGATCATTGTACG) (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, Tetrarodon 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.; A. thaliana, Arabidopsis thaliana.
replaced by the CaM gene from the Antarctic fish. The construct was transformed into competent Escherichia Coli DH5α for plasmid preparation, and the inserted fragment was re-sequenced to confirm the correct open reading frame (Fig. 2).

2.2. The transient transfection of tobacco

Assistant vector pCAPE1, control vector pCAPE2-GFP, and the re-combinant vector pCAPE2-CaM were transformed into Agrobacterium tumefaciens strain GV3101 by the liquid nitrogen freeze–thaw method (An, 1987). Agrobacterium cultures harbouring pCAPE1 and a pCAPE2-CaM derivative were mixed 1:1 prior to infiltration. The mixed culture was infiltrated into the abaxial side of the third pair of leaves on 5-week old tobacco plants using a 1 ml syringe without needle. Detailed operation was referenced as described by Jia et al. (2008).

The transgenic and wild-type tobacco were grown for one day in the dark at 25 °C, and then the plants were grown for 2 weeks in the greenhouse (25 °C) in preparation for the cold treatment.

2.3. Reverse transcription–PCR (RT-PCR) analysis

In accordance with the method of Wassenegger et al. (1994), genomic DNA was extracted from the leaves and used as the template for PCR with the above primers. After 2 weeks in the greenhouse (25 °C), the total RNA of the plant leaves was isolated using Trizol reagent (Tiangen, China) according to the supplier’s protocol. The reverse transcription–PCR reaction was performed according to the TAKARA standard protocol, and agarose gel electrophoresis was used to evaluate the RT-PCR product.

2.4. Western blot analysis

An anti-CaM antibody was purchased from CW Biotechnology, Beijing, China. The total proteins were extracted from the tobacco leaves, and the protein concentration was measured using the BCA protein assay reagent (CW Biotechnology, Beijing, China). Equal amounts of total protein (25–30 mg) were separated on a 15% SDS-PAGE gel and blotted onto a PVDF membrane. The blots were incubated overnight at 4 °C with the anti-CaM antibody and then HRP-labelled goat anti-rabbit IgG secondary antibody for 2 h. The blots were exposed to X-ray film for visualisation (Kodak).

2.5. Electrolyte leakage measurements

Three transgenic and wild-type tobacco plants were chosen at random to be subjected to cold treatment; the electrolyte leakage and MDA content were then measured. The percentage of electrolyte leakage shows the degree of injury of plants under cold stress. Both the wild-type and transgenic tobacco plants were subjected to 4 °C in a cold chamber for various time periods (0, 3, 8, and 14 d), and the phenotypes were examined. We washed the sample leaves with de-ionised water and then immersed them in 10 ml de-ionised water. After vacuum infiltration, we detected the electric conductivity of the supernatant (S1) using a DDS-11A detector (Shanghai Dapu Instrument, China). We boiled the mixture for 15 min, and then cooled it to room temperature. After centrifugation at 12,000 × g for 5 min, the supernatant was analysed using a spectrophotometer. We calculated the MDA content as follows: MDA (μmol/l) = 6.452 × (D532 − D450) − 0.599 × D450. For the statistical analysis, we repeated the test three times, and the mean value was used in comparing the transformed and wild-type plants.

2.6. MDA content

We detected the relative rate of lipid peroxidation of the leaves of seedlings to determine the level of MDA at 4 °C using a TBA (trichloroacetic thiobarbituric acid) reaction (Fryer et al., 1998). The leaves were homogenised by adding 3 ml of 10% TCA (thiobarbituric acid). After centrifugation at 12,000 × g for 5 min at 4 °C, a 2-ml aliquot of the supernatant was mixed with 2 ml of 0.6% TBA freshly prepared with 10% TCA. We boiled the mixture for 15 min, and then cooled it to room temperature. After centrifugation at 12,000 × g for 5 min, the supernatant was analysed using a spectrophotometer. We calculated the MDA content as follows: MDA (μmol/l) = 6.452 × (D532 − D450) − 0.599 × D450. For the statistical analysis, we repeated the test three times, and the mean value was used in comparing the transformed and wild-type plants.

3. Results

3.1. Characterisation of CaM in D. mawsoni

CaM has four EF-hand structures (Fig. 3) that are highly conservative in all eukaryotes. The cDNA of D. mawsoni CaM encoded a polypeptide of 148 amino acids. Residues 71 and 144 are different between Antarctic notothenioids and tobacco plants, being Mets in the notothenioids and Leu and Val, respectively, in tobacco. These amino acids are located in the termini of CaM (Fig. 3) and are closely related with the potential substrate-binding site (Aoyagi et al., 2003; Meador et al., 1993; Osawa et al., 1999).

3.2. Identification of transgenic plants

When the tobacco plants had been grown for 2 weeks in the greenhouse (25 °C), RT-PCR was performed using the top leaves. The cDNA from the transgenic tobacco generated amplification products of the correct size (450 bp), which indicated that the CaM gene was transcribed. No bands were visible using cDNA from wild-type plants or the no-enzyme negative control (Fig. 4a). These results show that the CaM mRNA was expressed in tobacco after two weeks of growth in the greenhouse. To prove that the CaM protein was also expressed in tobacco, we extracted total protein from the same tobacco leaves that were used for the RT-PCR experiment; the Western blotting for CaM (17 kD) demonstrated CaM protein expression (Fig. 4b).

![Fig. 2. Expression construct of the notothenioicd CaM used to transform tobacco. The expression was driven by the 3SS promoter from the cauliflower mosaic virus and terminated by the NOS terminator (T). CP: coat protein coding region. CaM: the notothenioid calmodulin gene. RB: right border, LB: left border. Two restriction enzymes, MluI and EcoRI, were used for cloning the CaM ORF.](image-url)
3.3. Cold resistance analysis of transgenic tobacco

Transgenic and wild-type tobacco seedlings at similar growth stages showed the same phenotype before chilling treatment (Fig. 5a). Two-week-old CaM transgenic tobaccos and wild-type tobacco plants were grown in a 25 °C chamber under a long-day photoperiod (16 h light, 8 h dark). The leaves of the wild-type all exhibited dehydration (Fig. 5b) after exposure to 4 °C for 2 weeks, but only the bottom leaves of the transgenic plants exhibited dehydration (Fig. 5b). The differences between the wild-type and CaM-expressing tobacco were also evident during recovery from stress. After being returned to room temperature for 2 weeks, the CaM-expressing plants overcame the dehydration and completely recovered (Fig. 5c). In contrast, the wild-type tobacco plants displayed severe chlorosis and wilting, suffering some irreversible damage. These results showed that the transgenic tobaccos carrying the CaM gene could increase their cold tolerance and enhance their recovery from a cold treatment.

3.4. Ion leakage and MDA content

It is known that the semi-permeability of tobacco plasma membranes is disturbed under low temperature, resulting in increased electrical conductivity in various tissues. Transgenic and wild-type tobacco plants were placed at 4 °C for 0, 3, 8 and 14 days, and ion leakage was detected (based on three treatments), showing significantly increasing differences between the wildtype and transgenic plants over time (Fig. 6a). Although the ion efflux was similar between the transgenic and wild-type tobacco plants after 3 days of treatment, a clear difference in the ion leakage was found after 8 days exposure to 4 °C: the electrolyte leakage reached 49% for the wild-type tobacco, 28% for K1 and 30% for K2. On day 14, the ion outflow was increased to 61, 32 and 33% for the wild-type tobacco plants and K1 and K2 lines, respectively.

We also assessed damage by evaluating the lipid peroxidation in the wild-type and transgenic plants at different temperatures by measuring the accumulation of malondialdehyde (MDA), a product of lipid peroxidation, after continuous cold treatment for 14 days. The levels of MDA were significantly different between the wild-type and transgenic plants (Fig. 6b). The MDA content was the lowest under normal conditions but gradually rose with the duration of the cold treatment, reaching the highest level after 14 days, whereas the MDA content of the transgenic plants was comparatively lower at each treatment time. Overall, a significant reduction in the accumulation of lipid peroxidation was detected in the transgenic plants in comparison to the wild-type plants.

4. Discussion

Subcellular structures can change under cold stress, and specific Ca^{2+} channels are activated (Reddy, 2001). The primary response to various environmental stresses is a change of the concentration of Ca^{2+}, and such a change can affect the activity of Ca^{2+}-dependent proteins, and these, in turn, regulate the expression of stress-related genes. Therefore, the stress responses of many organisms involve a Ca^{2+} signalling pathway (Plieth, 2001; Reddy, 2001). Because CaM is one of the most important Ca^{2+}-binding proteins in plants (Plieth, 2001; Reddy, 2001), we hypothesised that CaM from Antarctic notothenioid fishes may enhance the cold-tolerance mechanism of plants.

The existence of a cold-resistance gene has not been clearly demonstrated to date in fishes. However, when comparing gene expression, Antarctic notothenioid fishes over-express 177 genes versus warm-water fishes. The expression of calmodulin in Antarctic notothenioid fishes accounts for 0.23%, which represents a very high level in these fishes (Chen et al., 2008). We chose to clone the CaM gene into the pCAPE2 plasmid and transform it into tobacco plants to assess their cold tolerance. Based on our results of transgenic tobacco plants treated at 4 °C for two weeks, the transgenic CaM indeed improved cold tolerance (Fig. 5). It is possible that the observations...
could be due the synergetic action of multiple genes. Our results will contribute to the understanding of cold resistance in plants.

In this report, we demonstrated the CaM gene function by transferring it into tobacco. We found that low-temperature stress caused significant increases in electrolyte leakage in both the wild-type and transgenic lines (Fig. 6a). However, the transgenic lines displayed significantly ameliorated damage levels under low-temperature stress (Fig. 6a). Although the molecular mechanism by which CaM decreased the electrolyte leakage level under cold treatment remains to be elucidated, we clearly demonstrate over-expression of CaM in the transgenic tobacco plants.

The accumulation of MDA is an indicator of lipid peroxidation (Smirnoff, 1995), and thus indicates damage to major cellular components (Monk LS and Crawford, 1989). The MDA content was increased under low-temperature stress, but the increase was greater in the wild-type plants than in the transgenic plants (Fig. 6b). The reduction of cold injury to tobacco caused by CaM may be related to the inhibition of lipid peroxidation.

The reduced electrolyte leakage and MDA levels in the transgenic tobacco plants suggests that the lower amount of membrane damage under cold stress was due to the over-expression of the CaM gene, indicating that CaM could confer protection to the cell membrane of tobacco under cold stress. However, it is possible that the CaM protein became associated with the cell membranes due to the changes in the cellular architecture induced by cold and contributed to the protection of normal membrane-associated processes during cold stress. Further experiments are necessary to clarify the relationship between CaM and membranes and the effect of CaM on cell signalling or downstream gene expression during cold stress.

Our results showed that the fish calmodulin is a positive regulator in cold stress tolerance in tobacco, which is seemingly contradictory with the Townley and Knight (2002) report that over-expression of calmodulin in Arabidopsis causes inhibition of some COR (cold on regulated) genes when the samples was cold treated for 14 h at 4 °C. In fact, the results are not completely conflict to ours for that in Townley and Knight’s study they checked the two COR genes (KIN1/2 and LTI78) only at one time point (14 h), but we actually tested the whole cold tolerance for a long time. As we known, under cold stress, the wildtype plants will activate the cold tolerance system quickly at the beginning, and COR gene expression is up-regulated and peaked at an early time (Maghuly et al., 2009), but decreased rapidly thereafter. To the contrast, in the calmodulin-transgenic plants accumulation of COR gene expression is a relatively continuous process, it takes a prolonged time to reach the bigger peak (Gilmour et al., 1998). So it is easy to understand the inverse correlation between CaM expression and COR gene expression at one time point (Townley and Knight, 2002). In a long term, CaM over-expression would increase cold tolerance of the plants.

We conclude that over-expression of the CaM gene from Antarctic notothenioid fishes in tobacco contributes to the accumulation of defence proteins by regulating stress-related gene expression in tobacco under cold stress. Further research should be conducted to obtain additional information about the function or pathway of CaM in plant stress responses.
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