

## Transfer and expression of the genes of *Bacillus* branched chain alpha-oxo acid decarboxylase in *Lycopersicon esculentum*

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**Engineering of higher plants for increased cold tolerance requires a chemical modification of membrane fluidity in both organelles and cytoplasm of plant cells. A small number of microorganisms use branched chain fatty acids as their membrane constituents to maintain membrane fluidity, instead of unsaturated fatty acids. One of the key enzymes facilitating synthesis of branched chain fatty acids in *Bacillus subtilis* 168s is the branched chain alpha-oxo acid decarboxylase. To examine the role of branched chain fatty acids in plants and the potential for low temperature tolerance, the A and B genes encoding the alpha and beta polypeptides, respectively, of the branched chain alpha-oxo acid decarboxylase were introduced into the genome of tomato plants. The *mas* promoters in the plant expression vector system facilitated the expression of the A and B genes. Transgenic plants were regenerated and shown to produce both the alpha and beta polypeptides. Comparative analysis of a small number of transgenic tomato plants and non-transformed control plants grown at 4°C showed enhanced cold tolerance in the transformed plants. These findings, if confirmed by a larger scale analysis, suggest a potential role for branched chain fatty acids as a protective mechanism for growth of plants under sub optimal temperatures.**

Physiological manifestations of environmental stress in plants often implicate cell membranes as the primary site of injury (Orvar et al. 2000). At low temperatures, a decrease

in membrane fluidity leads to a decrease in the activity of membrane-bound enzymes and loss of semi permeable membrane properties (McMurchie and Raison, 1979; Levitt, 1980). This is due to the transition of membrane lipids from a fluid liquid-crystalline phase to a viscous gel crystalline phase (Cropp et al. 2000). The acyl chain region, comprising the hydrophobic domain of most membrane lipids, determines membrane fluidity. This domain contains fatty acids, either straight chain or branched chain families. The branched chain fatty acid family contains iso-, anteiso- and omega-alicyclic fatty acids with or without a substitution (unsaturation and hydroxylation) (Kromovar et al. 1994; van der Hoeven and Steffens, 2000; Graham and Eastmond, 2002). Branched chain fatty acids in cell membranes contribute to the control of membrane fluidity (Kromovar et al. 1994). In the genus *Bacillus*, the majority of fatty acids contributing to membrane fluidity are branched chain fatty acids (Jackowski et al. 1991). Iso-acyl phosphatidylcholine, one group of branched fatty acids, has a melting point (T<sub>m</sub>) ranging from 18-28°C below that of the corresponding normal saturated acyl phosphatidylcholine (Silvius, 1982). Studies of cold tolerant microorganisms suggest that the fatty acid composition of their cell membranes differs from that of their counterparts adapted to warmer ecological niches. Membrane fatty acid analysis of Antarctic bacteria showed that branched chain fatty acids were predominant in the membrane fatty acids in the psychrophiles (Rotert et al. 1993). Deep-sea barotolerant bacteria contain branched chain fatty acids as a major component of membrane lipids

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(Kaminura et al. 1993; Nichols et al. 2000). *Legionella shakespearei* sp. isolated from cooling tower water contained branched chain fatty acids as the major membrane lipid component (Verma et al. 1992). Other reports show that some *Listeria* and *Legionella* cells are resistant to cold temperature due to the increased branched chain fatty acid concentration (Benson et al. 1996; Juneja et al. 1998). The proportion of anteiso- and iso- branched chain acids is increased by reduced temperature, rather than induction of fatty acid unsaturation. These findings suggest that membrane fluidity may be enhanced at low temperatures by increasing the ratio of branched chain fatty acids in cell membranes.

The biosynthetic pathway of straight and branched chain fatty acid catabolism in higher plants has been recently reviewed (Graham and Eastmond, 2002). This review pointed out the advances in the understanding of branched chain amino acid catabolism have provided convincing evidence that mitochondria play an important role in this process. The biosynthetic pathway of branched chain fatty acids in bacteria is better understood (Kaneda, 1991). Only some bacteria, a few protozoa, and a few fungi use branched chain fatty acids as their membrane components (Kaneda, 1977; Kaneda, 1991). These organisms use branched chain alpha-oxo acids related to valine, isoleucine and leucine as precursors. These alpha-oxo acid substrates are decarboxylated to yield the precursor compounds for fatty acid synthesis reactions. The decarboxylase, named as branched chain alpha-oxo acid decarboxylase (BCOADc), has been found to be essential in fatty acid synthesis from alpha-oxo acids (Oku and Kaneda, 1988). BCOADc is a heterodimer consisting of two alpha subunits and two beta subunits, with a molecular weight of 144.4 KDa. Previously, three genes encoding branched chain alpha-oxo acid dehydrogenase complex (EC1.2.1.25) were cloned and sequenced from *B. subtilis* 168s (Wang et al. 1993). The first two genes encode alpha subunit (36310 Da) and beta subunits (35893 Da) of the BCOADc; the third gene encodes the dihydrolipoyl acyltransferase and is considered to be a part of the dehydrogenase complex (Wang et al. 1993). The BCOADc essential in branched chain fatty acid synthesis from alpha-oxo acid may be a free form of the dehydrogenase of the branched chain alpha-oxo acid dehydrogenase complex (Wang et al. 1993). In this report, we present the transfer and expression of the first two genes that encode branched chain alpha-oxo decarboxylase (BCOADc) in the tomato plant, *L. esculentum*, and the potential for enhanced biosynthesis of branched chain fatty acids in plants to increase cold tolerance capability.

The mannopine synthase (*mas*) promoter is a dual promoter containing two promoter (P1, P2) activities in opposite directions (Velten et al. 1984; Langridge et al. 1989). In this study, two genes, the A gene, encoding the alpha subunit, and the B gene, encoding the beta subunit of the decarboxylase, were placed under the P1 and P2 promoters, which permit the induction and expression of both genes in the same plant cell.

Using the *Agrobacterium* mediated T-DNA transfer method, we generated tomato plants which carried single or multiple copies of the foreign genes. Transformed plants were shown to produce the correct transcripts, and the alpha and beta polypeptides of the BCOADc enzyme. Comparative analysis of a smaller number of transgenic tomato plants grown at 4°C showed these plants possessed enhanced cold tolerance compared to non-transformed control plants. These initial studies suggest that the introduction of genes essential for branched chain fatty acids biosynthesis into plants may provide a new approach in generating low temperature tolerant plants.

## Materials and Methods

### Experimental materials

Plasmid pPCV701 containing mannopine synthase gene promoters, the dual *mas* promoters (Koncz et al. 1987), was used to drive the expression of the BCOADc A and B genes in tomato plants. The new construct was named as pPCV701-AB (Figure 1a). Plasmid pBluescript II KS +/- was used as a cloning vector for cloning of the PCR products and for over expression of the alpha and beta polypeptides of BCOADc in *E. coli* DLT101. *E. coli* DLT101 (LE392  $\Delta$ uncBD::Tn10recA56F' hsdRgal' Snl::Tn10rec56  $\nabla$  $\lambda$  D69lacUV5-T7 gene1) strain was used for over expression of the alpha and beta polypeptides. This strain is a *recA* derivative of BL21 (DE3), which overproduces T7 RNA polymerase following induction with IPTG (Studier et al. 1986). *Agrobacterium tumefaciens* GV3101 (pMP90RK) was used for T-DNA mediated gene transfer into tomato plants. *E. coli* S17-1 [RP4-2(Tc::Mu)(Km::T7) TpSmProres-mod<sup>+</sup> recA<sup>+</sup>] was used as the donor strain for conjugal transfer of genes to *Agrobacterium tumefaciens* (Horsch et al. 1985). Seeds of *Lycopersicon esculentum* var. Roma were a gift from Dr. W. Langridge.

### Cloning primers

The primer sequences for gene cloning and primer extension experiments are listed as following.

A1: 5'-CGGTCGACATGAGTACAAACCGACATCA-3'  
A2: 5'-CGGTCGACCTACTTTCGCATAAACATAAT-3'

B1: 5'-CGGGATCCATGTCAGTAATGTCATATAT-3'  
B2: 5'-CGGGATCCTTAAACTCCGCTAATTCTC-3'

A<sub>SD</sub>: 5'-CGGTCGACAAGGAGGGCTTGAATGAGTA-3'  
B<sub>SD</sub>: 5'-CGGGATCCAGGGAGGAAGAACAAATGT-3'

Primer 14: 5'-TACGGCAATACATAATCC-3'  
Primer 21: 5'-GTTTCATAGAGTCCCGCT-3'

The A and B genes encoding the BCOADc enzyme were amplified by PCR using primer A1 and A2, B1 and B2,

respectively, from the genomic DNA of *B. subtilis* 168s. Primer A1 and A2 were designed to include a Sall restriction cutting site. Primer B1 and B2 were designed to include a BamHI cutting site. For over expression of the alpha and beta polypeptides in *E. coli* DLT101, the A and B genes were amplified by PCR using primer A<sub>SD</sub> and A2, and primer B<sub>SD</sub> and B2, respectively. Both the A<sub>SD</sub> and B<sub>SD</sub> primers contained Shine-Dalgarno (SD) sequences. The Primer 14, designed based on the DNA sequence in the A gene, was used for the detection of the mRNA transcripts of the A gene; Primer 21, the sequence from the B gene, was used for the detection of the mRNA transcripts of the B gene.

### Transformation of tomato plant var. Roma

*Agrobacterium* mediated cotyledon transformation was used for introducing genes into tomato plants. Surface sterilized seeds of tomato *L. esculentum* were germinated on 1/2 MSO medium (2.3 g Gibco M8 salts, 3% sucrose, 1 H B5 vitamins). Before appearance of true leaves, the cotyledons were collected, cross sectioned and immediately floated in 5 ml of MSO liquid medium with 5 µl cultured *Agrobacterium* (OD = 1.0) and 375 µM acetosyringone. After incubation for 5 min, they were placed upside down on D1 medium [1 x MS salts (Sigma), 3% glucose, 1 H B5 vitamins, 1 mg/L zeatin, 0.22% gelrite, pH 5.8]. Two days later, these cotyledons were transferred to D1 medium containing 100 mg/L kanamycin and 300 mg/L claforan. After three to four weeks, shoots with true meristems were cut from the cotyledon tissues and transferred to rooting medium (1 x MS, 2% glucose, 0.22% gelrite). Well-rooted plantlets were then transferred into wet soilless media (Metro-Mix #290), and shaded for five days before moving to the greenhouse.

### Detection of gene integration and gene expression

Plant genomic DNA was isolated from leaf tissues (Doyle and Doyle, 1990), digested with selected restriction enzymes and separated on 0.6% agarose gels. Southern hybridisation was followed by transferring the DNA to nylon membranes (MagnaGraph, MSI) and hybridised with alpha-<sup>32</sup>P-dCTP labelled B gene fragments as probes.

Detection of mRNA transcripts was achieved by primer extension. The primer extension method was used to map and quantitate the 5' termini of RNA and to detect mRNA. The test RNA was hybridised with an excess of an end-labelled single-stranded DNA primer. Reverse transcriptase was then extended the primer to produce cDNA complementary to the RNA template. The length of the resulting end-labelled cDNA, as measured by electrophoresis through a polyacrylamide gel under denaturing conditions, reflected the distance between the end-labelled nucleotide of the primers and the 5' terminus of the RNA. The yield of cDNA was proportional to the concentration of the target mRNA sequences in the total preparation. In this study, oligonucleotide primers 14 and

21 were end-labelled by gamma-<sup>32</sup>P-ATP using T4 polynucleotide kinase. The quality of the labelled primers was checked using gel electrophoresis (15% minipolyacrylamide gel). To initiate the reverse transcription reaction, 5 µg of total RNA, isolated from leaf tissues of the plants, grown in greenhouse for four weeks after transplanting, was dissolved in 3 µl of water and mixed with 1 µl (5 pmoles) labelled primer. The mixture was denatured in a 90°C water bath for 1 min, then quickly transferred to a 42°C heat block. Buffer I, 4 µl, containing 140 mM beta-mercaptoethanol, 100 mM Tris-HCl, pH 8.3, and 0.7 M KCl, was added to the tube and the mixture incubated at 42°C for an additional 15 min. When this incubation was completed, the extension mix (12 µl of Buffer II with 10-20 units of AMV reverse transcriptase) was added and incubated for 30-40 min at 42°C. Buffer II contains 133 mM Tris-HCl, pH 8.3, 16 mM MgCl<sub>2</sub>, and 1.3 mM dNTPs. The reaction was stopped by adding 1/10 volume of DNA loading buffer, and the DNA samples were denatured in boiling water, and 1/3 of the reaction was loaded on a 5% polyacrylamide gel. The gel was dried and placed under X-ray film for exposure at -70°C for 1-2 days.

### Production of anti-beta peptide antibody

The A and B genes were amplified by PCR from genomic DNA of *B. subtilis* 168s using primer A<sub>SD</sub> and A2 for the A gene, primer B<sub>SD</sub> and B2 for the B gene, respectively. Amplified gene fragments were ligated into *E. coli* expression vector pBluescript II KS +/-, and resulted in pBluescript II-A and pBluescript II-B. Both the pBluescript II-A and pBluescript II-B plasmid constructs were transformed into *E. coli* DLT101. IPTG 0.2 mg/ml was added into the *E. coli* DLT101 culture (100 ml, OD<sub>600</sub> = 1.0) and incubated for an additional 1 hr. The induced cells were collected and lysed in protein sample buffer (50 mM Tris-HCl, pH 6.8; 10% glycerol, 2% SDS, 2mg/ml bromophenol blue, 1% beta-mercaptoethanol). The supernatant was boiled and loaded onto a 7.5-25% gradient SDS-polyacrylamide gel and separated at 35 mA for 16 hrs in Tris-glycine buffer. [Figure 3a](#) demonstrates the induction of the beta polypeptide in *E. coli* DLT101 cells. After completion of the electrophoresis, gels were stained in 0.2% Coomassie brilliant blue for 10 min. Protein bands were identified and the band of interest was cut out from the gel, crushed into small pieces and loaded into a glass tube (8 x 100 mm). Dialysis tubing was fixed at the bottom end of the glass tube and proteins were eluted from the gel by electrophoresis. The purified protein was confirmed by SDS-PAGE and was directly used for the preparation of antibodies in rabbits. New Zealand white females, 3 months old and approximately 3-4 kg in weight, were used for the preparation of antibodies. The immunization process was divided into four steps: antigen injection, first boost, second boost, and the third boost. Sera were collected 15 days after each boost. To collect the sera, blood samples were centrifuged at 3,000 rpm for 30 min, and the clear serum was harvested. Twenty-five ml of saturated ammonium sulphate solution (pH 7.8 by 2 N NaOH) was added (drop



















