Isolation of two distinct cold-inducible cDNAs encoding plant uncoupling proteins from the spadix of skunk cabbage (Symplocarpus foetidus)  
Kikukatsu Ito *

Division of Biosystem and Bioresource Technology, Cryobiosystem Research Center, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan

Received 7 May 1999; received in revised form 2 August 1999; accepted 3 August 1999

Abstract

To know the involvement of mitochondrial uncoupling protein (UCP) in thermogenesis in plants, cDNA cloning of UCP-like gene was performed using cDNA library prepared from the spadix of skunk cabbage (Symplocarpus foetidus). Two novel cDNAs (SfUCPa and SfUCPh) encoding uncoupling proteins were isolated. The SfUCPa cDNA contained an open reading frame of 303 amino acids (predicted molecular weight 32.6 kDa) while the SfUCPh cDNA encoded 268 amino acids (predicted molecular weight 29.0 kDa). While the SfUCPa protein had six transmembrane domains and three mitochondrial energy-transfer protein signatures that are characteristic of all known UCPs, SfUCPh lacks the fifth transmembrane domain and the third energy-transfer protein signature, suggesting an altered topology in the inner membrane of the mitochondria. The expression of SfUCPa and SfUCPh mRNAs was detected only in the spadix but not in the leaf. Further, the extents of the expression of SfUCPa and SfUCPh mRNAs were induced by low-temperature treatment. These data suggest that SfUCPa and SfUCPh may have an important role in thermogenesis in the spadix of skunk cabbage. © 1999 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Skunk cabbage; Low temperature; Thermogenesis; Mitochondria; Uncoupling protein

1. Introduction

Low temperature, drought and salinity are common adverse environmental factors encountered by land plants. Among these stresses, low temperatures that cause chilling or freezing injury seem to be the most important factor limiting crop productivity [1]. To cope with these low temperature stresses, cold-tolerant plants such as wheat and rye carry out a number of physiological and metabolic responses that lead to cold acclimation [2–5]. In contrast, it is well-known that some plants, including skunk cabbage, have developed specialized mechanism to avoid cooling by thermogenesis [6–8].

* Tel./fax: + 81-19-6216253.  
E-mail address: kikuito@iwate-u.ac.jp (K. Ito)

The temperature of the flowers on the spadices of skunk cabbage that appear in early spring remain above +10°C, even if the air temperature drops to −15°C [7]. This is achieved by doubling the respiration rate from 12°C to subfreezing temperature levels. The heat produced by a thermogenic plant has been believed to be associated with a large increase in the activity of the mitochondrial cyanide-insensitive, nonphosphorylating electron transport pathway regulated by alternative oxidase (AOX), which is unique to plant mitochondria [9–12].

In mammals, on the contrary, a mitochondrial protein called uncoupling protein (UCP) has been shown to play an important role in heat production. The UCPs that are found in the inner membrane of the mitochondria, allow for a transmembrane H⁺ influx, which uncouples respiration from ATP synthesis permitting the dissipa-
tion of chemical energy into metabolic heat [13–15]. In animals, three UCPs have been found: UCP1 is distributed mainly in brown adipose tissue [16]; UCP2 is ubiquitously found in many tissues [17]; and UCP3 is highly specific for skeletal muscle [18]. Like other mitochondrial carrier proteins, mammalian UCPs seem to be composed of six transmembrane segments whose net hydrophobic character derives from paired amphiphilic helices [19,20]. Furthermore, the activity of these UCPs is regulated negatively by binding purine nucleotides (ATP, GTP, GDP and ADP) at the C-terminal region, whereas free fatty acids increase the activity [21–25].

Recently, two cDNAs encoding UCP-like protein have been isolated from potato (StUCP) [26] and Arabidopsis (AtPUMP) [20]. Because the expression of StUCP was detected mainly in flowers and fruit, it has been hypothesized that StUCP can be associated with a burst of respiration in flowering and fruit ripening in combination with AOX [26].

Although potato and Arabidopsis are thought to be non-thermogenic plants, cold-inducible expressions of StUCP and AtPUMP have been suspected their involvement in thermogenesis [20,26]. In thermogenic plants such as skunk cabbage, however, molecular mechanism underlying UCP-mediated heat production remains to be determined.

The present report describes the isolation of two distinct spadix-specific and cold-inducible cDNAs belonging to a novel UCP gene family from skunk cabbage. This is the first report showing the presence of a UCP-like gene in thermogenic plants.

2. Materials and methods

2.1. Plant material and treatments

Skunk cabbage plants (Symplocarpus foetidus) were collected at Ikoino-Mori, a public park in Omonogawa Town located in Akita Prefecture, Japan. The plantlets were subjected to cold treatment by letting them in the cold room (4°C) for 12 h under continuous dim light.

2.2. cDNA library construction and screening

Total RNA was extracted from the spadix of skunk cabbage and the integrity of isolated RNA was determined by 1.0% agarose gel electrophoresis [27]. Poly(A)$^+$ RNA purified using an mRNA isolation kit (Pharmacia) was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) to isolate the related clones of the UCP gene family. For the first-strand cDNA synthesis, poly(A)$^+$ RNA (0.1 μg) was annealed with 20 pmol of cDNA priming primer [27] and then extended using 10 units of reverse transcriptase (New England Biolab) at 37°C for 30 min in 20 μl of 1 × RT buffer containing 10 mM 1,4-dithiothreitol and 0.5 mM dNTPs. The reaction mixture consisted of 10 mM Tris–HCl at pH 8.0, 50 mM KCl, 1.5 mM MgCl$_2$, 4 mM dNTPs, 0.2 unit of EX Taq polymerase (Takara) and 10 pmol of two degenerated primers, ZF1 (5’-CCYITGYACIG-CIAAR-3’) and ZR1 (5’-ACWTCAISYIC-CIAWIC-3’) that correspond to the conserved amino acid sequences of the UCP family. A major PCR product of approximately 0.8 kb was obtained after 35 cycles of amplification as follows: 0.5 min at 94°C; 1 min at 50°C and 1 min at 72°C. The amplified cDNA fragments were cloned into the T-vector, and one clone (p2-1) giving the UCP-related sequence was used as a probe for the following library screening. Poly(A)$^+$ RNA (5 μg) prepared from the spadix was processed to construct the λgt11 phage library by standard DNA techniques [28]. The positive clones were excised and subcloned into the pBluescript SK plasmid (Stratagene). Two plasmids, pz8-1 and pz8-2 that carry full length $SfUCPa$ and $SfUCPb$ cDNAs, respectively, were sequenced on both strands using the BcaBest sequencing kit (Takara) and ABI373A automated sequencer with T3, T7 and gene-specific primers. Sequence data were assembled and analyzed using a GENETYX-Homology software system, version 2.2.0 (Software Development).

2.3. In vitro transcription and translation

Plasmids (pz8-1 and pz8-2) carrying full length $SfUCPa$ and $SfUCPb$, respectively, were linearized, and sense or anti-sense RNA was transcribed in vitro using the protocol of the MAXiSscript transcription kit (Ambion) using T7 or T3 RNA polymerase. Equal amounts of RNA (4 μg) were used in the in vitro translation reaction, using wheat germ extracts (Promega) in the presence of $^{35}$S-methionine (Amersham) as de-
scribed by the manufacturer. The translation products were analyzed with SDS-PAGE. Before fluorography, the gels were fixed, incubated in Amplify (Amersham) and then dried.

2.4. Preparation of total RNA and Northern blot analysis

Total RNA was extracted as described by Ito et al. [27]. Northern blotting was performed as described previously [29].

3. Results

3.1. Isolation and nucleotide sequence analyses of SfUCPa and SfUCPb cDNA

To isolate a UCP-related gene in skunk cabbage, RT-PCR was initially performed using two degenerated primers, ZF1 and ZR1, that correspond to the conserved amino acid sequences of human and plant UCPs. One main product of 0.8 kb was detected using poly(A)$^+$ RNA from the spadix of skunk cabbage. Sequencing of the amplified fragment showed that the deduced amino acid sequence of one reading frame had a fairly high similarity with that of UCP genes. With this fragment as a probe, eight positive clones were isolated from the cDNA library. After determining the DNA sequences, it was found that they originated from two distinct genes. Two cDNAs, pz8-1 and pz8-2, carrying the full length of UCP-like gene were designated SfUCPa and SfUCPb, respectively, and were analyzed further.

The sizes of SfUCPa and SfUCPb cDNA inserts were 1525 and 2991 bp, respectively (Fig. 1). A putative polyadenylation signal (AATAAA) was found 236 bp upstream from the poly(A) sequence in SfUCPa cDNA, while SfUCPb cDNA contained two polyadenylation signals at the position of 1171 and 1243 bp. The fact should be noted that SfUCPb cDNA contains a long 3′-untranslated region compared with that of SfUCPa. The SfUCPa cDNA contained a single open frame of 303 amino acids that encodes a putative protein with a predicted molecular mass of 32.6 kDa (Fig. 1). The SfUCPb cDNA contained an open reading frame of 268 amino acids that encodes a putative protein with a predicted molecular mass of 29.0 kDa (Fig. 1). Southern blot analyses showed that the genome of skunk cabbage contains a multi-copy for the SfUCPa gene and a single copy for the SfUCPb gene (data not shown).

3.2. In vitro transcription/translation analysis of SfUCPa and SfUCPb

To confirm the molecular mass of each protein, in vitro transcription/translation were conducted. The translation products of $^{35}$S-labeled SfUCPA or SfUCPB gave one main band corresponding to the estimated molecular mass (ca. 32 kDa for SfUCPA, ca. 29 kDa for SfUCPB) only when sense RNA was used as a template (Fig. 2).

3.3. Amino acid sequence analyses of SfUCPa and SfUCPb

The amino acid sequences of SfUCPA and SfUCPB show higher similarity to plant UCPs than to human UCPs (Fig. 3). It should be noted that one region corresponding to the amino acid sequences between Thr 204 and Val 238 of SfUCPA was completely deleted in SfUCPB (Fig. 3). In addition, Leu at position of 265 in SfUCPa was substituted by Pro in SfUCPB (Fig. 3). Like other mitochondrial UCP proteins, SfUCPA contained six predicted transmembrane domains and three typical mitochondrial energy-transfer-protein signature domains [18,20], while SfUCPB lacked the fifth transmembrane domain and the third mitochondrial energy-transfer protein signature domain (Fig. 3). Potential purine nucleotide binding domains (PNBD) were located at the C-terminal of both SfUCPA and SfUCPB proteins (Fig. 3).

3.4. Spadix-specific and low temperature-induced expressions of SfUCPa and SfUCPb mRNAs

Northern blot analyses demonstrated that both SfUCPa and SfUCPb genes were detected in the spadix, not in the leaf, at room temperature (15°C) (Fig. 4). Further, the expressions of both SfUCPa and SfUCPb in the spadix was shown to be induced by the cold treatment. Again, there was no detectable band in the leaf under the cold treatment.
4. Discussion

In this study, two novel cDNA clones named SfUCPa and SfUCPb encoding putative mitochondrial uncoupling proteins were isolated and characterized using skunk cabbage, a typical thermogenic plant species. The predicted polypeptides of SfUCPA and SfUCPB shared a high sequence similarity with potato UCP (StUCP) whose uncoupling activity has been shown in yeast mitochondria [26]. Although the activity of SfUCPA and SfUCPB is not yet defined in molecular detail, amino acid sequence similarities between skunk cabbage and potato UCPs support the view that the SfUCPA and SfUCPB proteins have at least in part UCP-like function.

The structure-function relationship of mammalian UCP1 has been extensively studied. The polypeptide chain has been shown to consist of three tandemly related domains of about 100 amino acids in length [23]. Each domain is characterized by the presence of two transmembrane regions and one mitochondrial energy transfer motif. Topological studies showed that both the C- and N-terminal ends of UCP1 are oriented towards the cytosolic side of the inner mitochondrial membrane [30]. Further, the binding of purine nucleotide to the C-terminal region induces a conformational change that leads to inhibition of H+ permeability [14].

Although SfUCPA has six predicted transmembrane domains, three mitochondrial energy trans-

Fig. 1. Nucleotide and deduced amino acid sequences of SfUCPa and SfUCPb cDNAs. The putative polyadenylation signals are underlined. The asterisk indicates the stop codon. The SfUCPa and SfUCPb cDNA sequences have been submitted to the GenBank, EMBL, and DDBJ data bases with accession numbers AB024733 and AB024734, respectively.
Fig. 2. In vitro translation of SfUCPa and SfUCPb genes. Sense or anti-sense RNA was transcribed in vitro using SfUCPa or SfUCPb cDNA templates as described in Section 2. Four micrograms of sense (S) or anti-sense (AS) RNA were subjected to in vitro translation using wheat germ extracts in the presence of 35S-methionine. Control reaction without RNA temperate was also performed (−). Positions of SfUCPA and SfUCPB are indicated by arrows. Bands indicated by asterisks are non-specific products. Open circle denotes the position of low-molecular-weight translation artifacts synthesized by small ORFs.

The discovery of genes encoding SfUCPa and SfUCPb from the spadix of skunk cabbage seems to support the idea that the existence of UCPs, as first described in brown adipocytes in mammals, represents a general phenomenon in thermogenesis that also occur outside the mammalian. Especially, cold-inducible and spadix-specific mRNA expressions of SfUCPa and SfUCPb seem to indicate their direct involvement in the thermogenesis in skunk cabbage. However, considering that non-thermogenic plants such as Arabidopsis and potato also contain UCP-like genes (StUCP and AtPUMP, respectively), molecular mechanism gov-

Fig. 3. Alignment of the deduced amino acid sequences of the putative SfUCPa and SfUCPb proteins with the potato (StUCP), Arabidopsis (AtPUMP) and human UCPs. The sequences are in single letter codes. Asterisks indicate perfect matches and dot represents conservative changes within all sequences. Bold letters depict identical sequences between SfUCPa and SfUCPb. The gaps introduced to optimize the sequence alignment are represented by dashes. The alignment was performed with the CLUSTAL W program. The typical mitochondrial energy-transfer protein signature domains are boxed. Six predicted transmembrane regions (I–VI) are indicated by horizontal shadow lines. The potential purine-nucleotide binding domain (PNBD) is overlined. Accession numbers are: StUCP (Y11220); human UCP1 (P25874); human UCP2 (P55851); human UCP3 (P55916).
Fig. 4. Spadix-specific and low-temperature-inducible expressions of SfUCPa and SfUCPb mRNAs. Expression profiles of SfUCPa and SfUCPb transcripts in the spadix and leaves of skunk cabbage at room temperature (RT) and cold condition (4°C, 12 h). Each lane was loaded with 20 μg of total RNA. [32P]-labeled cDNA probe for SfUCPa (A) or SfUCPb (B) was used as a probe. Positions of SfUCPa and SfUCPb transcripts are indicated by arrows. An ethidium bromide-stained gel pictures showing undegraded rRNA are also shown at the bottom of plates.

Concerning plant thermogenesis seen in skunk cabbage seems to be more complex than those of animals.

One could postulate that two energy-dissipating systems, both AOX and UCP, are involved in heat production in plants. In accordance with this, partial purification of AOX from the spadix of skunk cabbage has been succeeded [9]. However, it has been recently shown that AOX and UCP never seem to work simultaneously at their maximal activity [24]. Namely, high levels of free fatty acid-induced UCP activity excludes AOX activity. Therefore, it seems to be necessary to assign each functional relationship during the thermogenesis in the spadix of skunk cabbage.

In conclusion, identification of two distinct genes encoding UCP from skunk cabbage provides novel insights on the molecular mechanism of heat production in plants. Especially, SfUCPB, topologically different isofrom in the UCP family, may have an important role in the thermogenesis, presumably being escaped from the negative regulation by the cytosolic purine nucleotide. Functional analyses of SfUCPA and SfUCPB are in progress.

Acknowledgements

This work was supported by the Kuribayashi Foundation. I am grateful to Koji Tomita, Mayor of Omonogawa Town, for his kind permission to sample skunk cabbage at the ‘Ikoino-mori’, a public park in Omonogawa Town. I thank Dr. M. Uemura for his valuable discussions and critical reading of the manuscript, Y. Konishi for his kind advice.

References