Molecular Identity of Uncoupling Proteins in Thermogenic Skunk Cabbage

Yasuko Ito-Inaba 1,*, Yamato Hida 1, Hitoshi Mori 2 and Takehito Inaba 3

1 Cryobiofrontier Research Center, Faculty of Agriculture, Iwate University, Morioka, 020-8550 Japan
2 Graduate School of Bio-agricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya, 464-8601 Japan
3 The 21st Century Centers of Excellence Program, Cryobiofrontier Research Center, Iwate University, Morioka, 020-8550 Japan

Thermogenic skunk cabbage has been reported to have two types of uncoupling protein (UCP), a typical 6-transmembrane (TM) SrUCPA and an atypical 5-TM SrUCPB. To verify further the role of SrUCPs in thermogenic skunk cabbage, we examined the molecular identity of SrUCPs in more detail. Both mRNA and genomic analyses supported the presence of SrUCPA, but not SrUCPB. Furthermore, SrUCP protein purified from spadix mitochondria was identified as SrUCPA by mass spectrometry. These results clearly indicate that SrUCPA is the major expressed UCP in skunk cabbage, and the presence of atypical SrUCPB is unlikely to be associated with thermogenesis of skunk cabbage.

**Keywords:** mRNA splicing — Plant mitochondria — Reproductive organ — Skunk cabbage — Thermogenesis — Uncoupling protein.

**Abbreviations:** AOX, alternative oxidase; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; RT–PCR, reverse transcription–PCR; TEV, tobacco etch virus; TM, transmembrane; UCP, uncoupling protein; UTR, untranslated region.

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Uncoupling proteins (UCPs), which are known factors for thermogenesis in mammals, are also present in plants. Thermogenesis has been reported from various plant species (Seymour 2001). Among them, skunk cabbage (Symplocarpus renifolius) has been reported to have two types of UCPs, designated as SrUCPA and SrUCPB (Ito 1999). While SrUCPA is a typical 6-transmembrane (TM) UCP, SrUCPB lacks the fifth TM domain and is an atypical UCP. Because cDNA encoding a 5-TM-type UCP has only been isolated from skunk cabbage, it has been speculated that SrUCPB plays a major role in regulating the thermogenesis in skunk cabbage (Onda et al. 2008). In contrast, we also identified the typical 6-TM UCP, SrUCPA, as the major UCP in thermogenic tissue of skunk cabbage (Ito-Inaba et al. 2008). To solve the riddle of UCPs in thermogenic skunk cabbage, we investigated the genomic organization and the molecular identity of SrUCP by various approaches.

The spadix, which is the thermogenic organ of skunk cabbage, can produce enough heat to keep its internal temperature at around 20°C, even if the air temperature falls below freezing (Seymour and Blaylock 1999). Because of its ability to generate massive amounts of heat, the atypical SrUCPB has been speculated to be involved in the thermogenesis in skunk cabbage (Ito 1999, Onda et al. 2008). However, whether or not SrUCPB is functional has been debated for years. In rice, a number of aberrant UCP mRNAs were identified such that it has been postulated that the mis-splicing of typical UCP transcripts might account for the appearance of SrUCPB (Watanabe and Hirai 2002). Furthermore, there is no evidence that 5-TM UCP functions in vivo in any other organisms including thermogenic plants (Vercesi et al. 2006). One of the strongest arguments against the major role of SrUCPB in skunk cabbage is that the electrophoretic mobility of mitochondrial SrUCP is identical to that of in vitro translated SrUCPA, but not in vitro translated SrUCPB (Ito-Inaba et al. 2008).

To verify further which type of UCP (5-TM vs. 6-TM) is the major UCP in skunk cabbage, we first raised antibodies against SrUCPA, which is supposed to detect both SrUCPA and SrUCPB. To obtain this, the SrUCPA fusion gene carrying NusA and the tobacco etch virus (TEV) protease cleavage site was constructed (Fig. 1A) and was expressed in *Escherichia coli* (Fig. 1B, lane 1). After purification by Ni-NTA chromatography, the fusion protein was cleaved into NusA and SrUCPA (Fig. 1B, lane 4). Either SrUCPA excised from the gel or the mixture of NusA and SrUCPA was used as the antigen. SrUCP-specific antibodies were affinity purified using NusA–Sepharose as well as NusA–SrUCPA–Sepharose to deplete antibodies against NusA. As shown in Fig. 1C, affinity-purified antibodies cross-reacted with SrUCPB in addition...
to SrUCPA synthesized by in vitro translation. Consistent with our previous observation (Ito-Inaba et al. 2008), the electrophoretic mobility of mitochondrial SrUCP was identical to that of SrUCPA. To confirm that the electrophoretic mobility of SrUCP is similar to that of a typical 6-TM UCP, we also examined the electrophoretic mobility of potato UCP. Because potato UCP is ~80% identical to SrUCPA, SrUCP antibodies also recognized it (Fig. 1C, lane 4). The identical electrophoretic mobility of potato UCP and SrUCP further supported the idea that mitochondrial SrUCP was the typical 6-TM UCP. Consistent with this idea, SrUCP antibodies failed to detect the 5-TM-type UCP of skunk cabbage mitochondria (Fig. 1C, compare lanes 2 and 3).

A previous study showed by Northern blotting that SrUCPB was the major UCP transcript (Onda et al. 2008). However, these results were not reproducible in reverse transcription–PCR (RT–PCR) for UCP transcripts. Instead, we found that SrUCPA is the major UCP transcript in thermogenic tissue of skunk cabbage (Ito-Inaba et al. 2008). To address the discrepancy between these two analyses, we investigated the unusually long 3′-untranslated region (UTR) of SrUCPB and found that this region was distantly similar to cysteine protease and PSI-E in other organisms (Fig. 2A). Because the SrUCPB probe used in the previous study also contained the 3′-UTR, it was possible that the probe hybridized with genes other than SrUCPB in the Northern blotting experiment (Onda et al. 2008). Furthermore, it is possible that SrUCPB is a cloning artifact as it contains nucleotide sequences similar to other genes in its 3′-UTR. Therefore, we examined whether the previously cloned SrUCPB is indeed expressed or not. To study this, we carried out RT–PCR using several different primers, shown in Fig. 2B, and compared PCR fragments amplified from cDNAs with those from plasmids containing full-length cDNA of SrUCPA or SrUCPB (Fig. 2C).

However, when primer pairs spanning the poly(A)- and poly(T)-rich region between the coding region and the 3′-UTR were used for RT–PCR, we failed to amplify the PCR products using the cDNA template (Fig. 2C, lanes 4–7). When we used primers that amplify the 3′-UTR, the amplified PCR products and the length of the amplified fragments were similar to those of the SrUCPB plasmid control (Fig. 2C, lanes 8–11). Consistent with the previous observation (Ito-Inaba et al. 2008), the primer pairs spanning the fifth TM domain only amplify SrUCPA but not SrUCPB (Fig. 2C, lanes 1–3). These data suggest that SrUCPB mRNA that was cloned previously is undetectable in thermogenic tissue of skunk cabbage.

We next examined if the genomic clone of the SrUCP genes contained the fifth TM domain or not. To this end, we performed PCR using primer pairs spanning the fifth TM domain of SrUCPA (F1 and R1 primers in Fig. 2) and skunk cabbage genomic DNA as the template. Previously, it was reported that there were multiple copies of the SrUCPA gene and a single copy of the SrUCPB gene in the skunk cabbage genome (Ito 1999). Because primers used for this PCR completely matched both SrUCPA and SrUCPB, we anticipated the amplification of multiple PCR fragments if both SrUCPA and SrUCPB genes exist. However, the major PCR product we obtained was a single ~2,000 bp fragment (Fig. 3A, lanes 2 and 4). We cloned this PCR fragment and found that this was a partial genomic clone of the SrUCPA gene (accession No. AB459551). Clarification of the exon–intron structure revealed that this fragment appeared to contain the fifth TM segment (Fig. 3B). Intriguingly, the portion missing in SrUCPB is exactly identical to one of the exons we identified (Fig. 3B).

As suggested previously (Watanabe and Hirai 2002),
this exon VI was entirely conserved among all plant UCP genes whose genomic organization has been revealed to date (Fig. 3C). These data suggest that the loss of the fifth TM domain is probably due to mis-splicing of the SrUCP A gene.

To confirm further that the SrUCP detected by immunogenic reaction is indeed the 6TM UCP, we purified the SrUCPs using anti-SrUCP IgG–Sepharose. Affinity-purified antibodies against SrUCP were covalently cross-linked to CNBr-activated Sepharose to generate anti-SrUCP IgG–Sepharose. As shown in Fig. 4A (lane 3, arrow), we were able to purify an ~30 kDa protein from skunk cabbage mitochondria. We also confirmed that SrUCP is highly enriched in this eluate fraction by immunoblotting (Fig. 4B). The major band in Fig. 4A was excised from the gel and digested with chymotrypsin. Nano-liquid chromatography followed by MALDI-TOF-TOF (matrix-assisted laser desorption ionization tandem time of flight) mass spectrometric (MS) analysis revealed that the band was indeed SrUCPA. As shown in Fig. 4C, one of these peptide fragments appeared to correspond to the sequence only found in SrUCPA but not in SrUCPB.

Taking together all our molecular and biochemical data, we provide convincing evidence that the typical 6-TM UCP A, but not the atypical 5-TM UCP B, is the major expressed UCP in skunk cabbage. The presence of a 6TM UCP in non-thermogenic plants and the almost identical structure of this gene in two different species raises the question of whether UCP is indeed involved in regulating heat production in thermogenic plants. UCPs in non-thermogenic plants have been considered to be involved in the regulation of energy metabolism or in the reduction of reactive oxygen species in mitochondria (Considine et al. 2003, Smith et al. 2004). Recently, AtUCP1 in Arabidopsis was shown to be related to photosynthetic metabolism (Sweetlove et al. 2006). In contrast, not much work has been done to unveil the role of 6-TM UCP in regulating the thermogenic process in thermogenic plants. In fact, to date, we have no direct evidence showing that this 6-TM UCP is involved in generating heat in thermogenic plants.

Besides the UCPs, alternative oxidase (AOX) is another candidate that has been proposed to play an important role in thermogenesis, because there is a strong correlation between heat generation and cyanide-resistant respiration in thermogenic plants (Watling et al. 2006). In voodoo lily (Sauromatum guttatum), AOX activities and AOX protein levels increased during the development of the thermogenic appendix (Rhoads and McIntosh 1992). Recently, similar results were obtained from other arum species and sacred lotus (Grant et al. 2008, Wagner et al. 2008). Since there are also sets of AOX genes expressed in non-thermogenic plants and these AOX proteins have been proposed to prevent the production of reactive oxygen species (Fiorani et al. 2005, Umbach et al. 2005), the
regulatory mechanism of the AOX activity and protein level in thermogenic species may be critical for heat production.

The presence of a 6-TM UCP and AOX in thermogenic tissues implies that these two proteins may play important roles in regulating the thermogenic mechanism. However, the direct evidence supporting this notion is lacking. Also, it is still unclear whether these two proteins act in concert or independently. To address these questions directly, a transgenic approach should be utilized. Introduction of AOX or UCP genes isolated from thermogenic plants into model organisms, such as Arabidopsis, and the investigation of transgenic plants may potentially address this question. The existence of both AOX and UCPs in non-thermogenic plants also suggests that another factor may have a regulatory function for AOX and UCP activity in thermogenic plants (Sluse et al. 1998, Sluse and Jarmuszkiewicz 2000, Considine et al. 2001, Almeida et al. 2002, Clifton et al. 2005, Borecky et al. 2006).

In summary, our data clearly suggest that the primary structure of UCP from thermogenic plants, such as skunk cabbage, to date do not exhibit a significant difference compared with UCPs in non-thermogenic species. The results also imply that the thermogenesis is less likely to result from the presence of an atypical gene product. Rather, it is more intriguing to hypothesize that the thermogenesis is achieved by the interplay between multiple proteins and their unique regulation in thermogenic plants.

Materials and Methods

The skunk cabbage (S. renifolius) used in this study was harvested from marshlands of Shizukuishi town and Nishiwaga town in Iwate, Japan. Potato tubers (Solanum tuberosum L. cv. Danshakaiu) were purchased from a local market. Mitochondria were isolated as described previously (Ito-Inaba et al. 2008).

For construction of pET-NusA-SrUCPA, SrUCPA cDNA was amplified by KOD-plus DNA polymerase (Toyobo, Osaka, Japan) using skunk cabbage cDNA as the template. The amplified DNA fragments were cloned into the pET-NusA vector (Novagen, Madison, WI) using the NheI and XhoI restriction sites. The recombinant plasmid was then transformed into the Escherichia coli strain BL21 (DE3) (Novagen, Madison, WI) and the expression of SrUCPA was induced by IPTG at 30°C. The expressed protein was then purified by Ni-NTA affinity chromatography and analyzed by SDS-PAGE and Western blotting.

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NusA–SrUCPA fusion protein was expressed in pET43.1a vector (Novagen) to generate pET-NusA-SrUCPA. The ExTaq (TAKARA, Shiga, Japan) DNA polymerase. F1 and R1 SrUCP the Sepharose and NusA–SrUCPA–Sepharose. antibodies. SrUCP-specific antibodies were purified using NusA–SrUCPA was used as the antigen to produce rabbit polyclonal SrUCP excised from the gel or the mixture of NusA and then cleaved into NusA and SrUCPA by TEV protease. Either and purified by Ni-NTA chromatography. The fusion protein was fragment was then cloned into the SnaI and XhoI sites of pET43.1a vector (Novagen) to generate pET-NusA-SrUCPA. The NusA–SrUCPA fusion protein was expressed in E. coli BL21(DE3) and purified by Ni-NTA chromatography. The fusion protein was then cleaved into NusA and SrUCPA by TEV protease. Either SrUCPA excised from the gel or the mixture of NusA and SrUCPA was used as the antigen to produce rabbit polyclonal antibodies. SrUCPA-specific antibodies were purified using NusA–Sepharose and NusA–SrUCPA–Sepharose.

PCR was performed to amplify genomic DNA containing the SrUCP genes using KOD-plus (Toyobo, Osaka, Japan) or ExTaq (TAKARA, Shiga, Japan) DNA polymerase. F1 and R1 primers shown in Fig. 2B were used for the reaction. The amplified DNA fragment was then cloned into the EcoRV site of the plasmid pZero2.1 (Invitrogen).

Immunoaffinity purification was carried out as described previously (Nakayama et al. 2007) with some modification. Mitochondria isolated from the thermogenic spadix of skunk cabbage were fractionated into membrane and soluble fractions. The membrane fraction was then solubilized with 1% Triton X-100 and applied to pre-immune IgG-Sepharose. The flow-through from the pre-immune IgG-Sepharose was further applied to anti-SrUCP IgG-Sepharose and the bound proteins were eluted with 0.2 M glycine-HCl, pH 2.2. The starting material (1% of the total solubilized membrane fractions) and the eluates were then resolved by SDS-PAGE, and proteins were stained with Coomassie Brilliant Blue.

Excised gel pieces were washed with 30% acetonitrile solution containing 50 mM NH4HCO3 three times followed by treatment with 0.1% RapigestSM SF (Waters, MA, USA) at 55˚C for 30 min. The gel pieces were incubated with chymotrypsin (Roche, Basel, Switzerland) at 25˚C for 12 h. Recovered peptides were then resolved by reverse-phase nano-liquid chromatography (DiNa Nano LC system, KYA TECH Corporation, Tokyo, Japan) and were directly fractionated onto a MALDI target plate with α-cyano-4-hydroxycinnamic acid by a spotter (DiNa Map system). MALDI-MS and MS/MS were performed on a 4700 Proteomics Analyzer with version 3.6 software (Applied Biosystems, CA, USA). MS/MS data were analyzed by MASCOT.

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References


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