Expression profile of arylphorin gene during diapause and cold acclimation in the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Crambidae)

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Abstract
A storage protein gene, *CsSP2*, was cloned and sequenced from the rice stem borer, *Chilo suppressalis*. Analysis of the cDNA sequence revealed an open reading frame of 2,118 bp in length which encodes an arylphorin-like protein with a calculated molecular weight of 83.7 kDa. The expression level of *CsSP2* was higher in non-diapausing larvae than in diapausing larvae at 20°C. When diapausing larvae were acclimatized in a stepwise fashion from 20°C to 5°C, *CsSP2* expression was up-regulated five days after acclimation at 15°C. The level was maintained for 10 days after acclimation at 5°C and down-regulated thereafter. Some up-regulation in *CsSP2* expression was detected in the post-diapause state. In non-diapausing larvae, *CsSP2* expression was down-regulated in the course of cold acclimation. Protein products of *CsSP2* might have an important role as an amino acid reservoir or a cellular defense mechanism during diapause.

Key words: Arylphorin; cold acclimation; diapause; hexamerin; rice stem borer

INTRODUCTION
Insect storage proteins (SPs) consist of six identical or similar subunits of ca. 80 kDa, so called hexamerins. Hexamerins are synthesized mainly during larval development, stored in the hemolymph and also sequestered in fat body where they serve as a source of nitrogen and amino acids for use by pupae and adults during metamorphosis and reproduction, respectively (Kanost et al., 1990; Telfer and Kunkel, 1991). The functions of those proteins have not been fully understood, but recent studies indicate that they functionally diverge both within and among insect species (Burmester, 2001). There are several different groups of SPs; in Lepidoptera, at least two representative groups of SPs, aromatic amino acid-rich arylphorin and methionine-rich SP, are known.

The rice stem borer, *Chilo suppressalis* Walker, is known as a rice pest in Japan. In fields, *C. suppressalis* enters larval diapause under short day-lengths below 14 h in autumn (Inoue and Kamano, 1957; Kishino, 1974). In *C. suppressalis*, cold tolerance has been correlated with increased glycerol content in the hemolymph (Tsumuki, 1990). When final instar larvae were reared at 27°C, more than 90% of the larvae entered diapause under a short day-length (10L:14D) (Tsumuki, 1990); however, in diapausing larvae, no glycerol production occurred (Tsumuki, 1990). High levels of glycerol were produced only when diapausing larvae were transferred to low temperatures below 10°C (Tsumuki and Kanehisa, 1978); therefore, cold acclimation in a diapause state is essential to elicit cold tolerance in *C. suppressalis*.

In the previous study, we cloned and examined a methionine-rich SP gene, *CsSP1*, from *C. suppressalis*. The expression level of *CsSP1* was lower in diapausing larvae than in non-diapausing larvae; however, *CsSP1* expression was up-regulated in diapausing larvae acclimatized below 10°C (Sonoda et al., 2006). In the present study, we cloned another SP gene encoding an arylphorin-like protein, *CsSP2*, and examined its expression profile during larval diapause and cold acclimation.
MATERIALS AND METHODS

Insects. Female adults of the rice stem borer, C. suppressalis, were collected from paddy fields in Kurashiki City, Okayama Pref. in August 2004. Egg masses were obtained from collected female adults and newly hatched larvae were reared on rice seedlings at 20°C under long (16L : 8D) and short (10L : 14D) day-lengths. Diapause was induced by rearing larvae under a short day-length. About 10 days after molting into the final instar larvae were selected as diapausing and exposed to cold acclimation. Newly molted non-diapausing final-instar larvae reared under a long day-length were also exposed to cold acclimation. Cold acclimation was performed by lowering the temperature in a stepwise fashion from 20°C to 5°C in 5°C increments over five days. The larvae were kept at 5°C for 10, 40 and 120 days. After incubation at 5°C for 120 days, the larvae were transferred to 25°C to allow pupation. After the temperature shift from 5°C to 25°C, the larvae molted into pupae within 10 days (Izumi, unpublished data).

RNA extraction, cDNA cloning and sequencing. Total RNA extraction and cDNA synthesis were described previously (Sonoda et al., 2004). To obtain a partial clone for the C. suppressalis arylphorin gene, rapid amplification of cDNA ends (RACE) (Frohmann et al., 1988) was carried out using the degenerate primer 1, 5'-GCHTTGACT-TCTACCAGAC3'- (nucleotides 1,258–1,277) (Fig. 1), and the M4 adaptor primer (M4) (Takara Bio Inc., Japan). Subsequently, nested polymerase chain reaction (PCR) was performed using the degenerate primer 2, 5'-CTTCAYTWGGTGC-GTAAA3'- (nucleotides 1,375–1,394) (Fig. 1), and M4. Both degenerate primers were designed based on consensus sequences from several lepidopteran insects obtained from the DNA Data Bank of Japan (DDBJ).

A 5' portion of cDNA was also obtained by RACE. cDNA was constructed from 1 μg of total RNA using a smart RACE cDNA amplification kit (Clontech, USA). Gene-specific primer (GSP) 1, 5'-GATTGCAGTTCAAGCGAGGCTGC-3' (complementary to nucleotides 1,521–1,543) (Fig. 1), and GSP 2, 5'-CGGAGATCAATCCTTGGTTCTGG-3' (complementary to nucleotides 1,473–1,495)
The PCR conditions were 30 cycles of 30 s at 94°C, 1 min at 55°C and 2 min at 72°C followed by a final extension at 72°C for 7 min. The amplified PCR products were size-fractionated on a 1.0% agarose gel, cut out from the gel and purified using a Qiaex II gel extraction kit (Qiagen Inc., Germany). The purified DNA fragments were cloned into pGEM-T Easy (Promega Corp., USA).

The nucleotide sequence was determined using a dye terminator cycle sequencing kit (Applied Biosystems, USA) with M13 forward and reverse primers and employing a DNA sequencer (3100 Avant Genetic Analyzer, Applied Biosystems). The nucleotide and deduced amino acid sequences were analyzed using Genetyx-Mac Ver. 10.1 (Software Development Co. Ltd., Japan).

**R**RNA gel blot analysis. Total RNA (20 μg) extracted from final-instar larvae was size-separated in a 1.2% agarose gel containing 0.66 M formaldehyde and transferred to a Biodyne PLUS membrane (Pall Corp., USA). The blot was hybridized with a random-primed 32P-labeled fragment containing the cloned cDNA sequence (Sambrook et al., 1989).

**RESULTS**

**cDNA sequence analysis of CsSP2**

The complete nucleotide sequences of CsSP2 comprised 2,179 bp with the initiation codon (ATG) at nucleotides 1–3 and the termination codon (TAA) at nucleotides 2,119–2,121 (Fig. 1) (GenBank/EMBL/DDBJ accession no. AB248058). The polyadenylation signal (AATAAA) occurred in the 3′-untranslated region at nucleotides 2,155–2,160 (Fig. 1). The nucleotide sequence had an open reading frame of 2,118 bp which produced a putative protein of 706 amino acids with a calculated molecular weight of 83.7 kDa (Fig. 1).

The hydropathy profile of the deduced amino acid sequence of CsSP2 suggested a signal peptide of 16 amino acids that ends in alanine and satisfies the (-3, -1)-rule for a cleavage site of a signal peptide (von Heijne, 1986) (Figs. 1 and 2). Two highly conserved SP signature motifs, the SP signature 1, Y(F/Y/W)XED(L/I/V/M)XXNXXXXXXXHXXXP, and the SP signature 2, TXXRDPX(F/Y)(F/Y/W), (Burmester, 1999; Zhu et al., 2002) were present in the deduced amino acid sequence of CsSP2. The SP signature-1 motif, YLTEDIGAHYSFHT-VMP, was located at amino acid positions 228–247 (Fig. 2). The SP signature-2 motif, TSLRDPAFY, was located at amino acid positions 426–434 (Fig. 2).

Based on a homology search using the Blast program provided by DDBJ, the deduced amino acid sequence of CsSP2 was found to be related closely (47–52% identity) to those of arylphorin genes from other lepidopteran insects (Fig. 2 and Table 1).

**Expression of CsSP2 during diapause and cold acclimation**

The expression of CsSP2 was examined using RNA gel blot analysis with diapausing and non-diapausing larvae at 20°C (Fig. 3A). The expression level of CsSP2 was higher in non-diapausing larvae than in diapausing larvae.

The effects of cold acclimation on CsSP2 expression were examined using non-diapausing larvae (Fig. 3B, right panel). A high level of CsSP2 expression was observed at 20°C before cold acclimation. That level was decreased in the course of cold acclimation. At 10 days after acclimation at 5°C, little CsSP2 expression was detected.

The effects of cold acclimation on CsSP2 expression were examined using diapausing larvae (Fig. 3B, left panel). A detectable level of CsSP2 expression observed at 20°C before cold acclimation was up-regulated five days after acclimation at 15°C. That level was maintained for 10 days after acclimation at 5°C and decreased thereafter (Fig. 3B and C). Little CsSP2 expression was observed at 40 and 120 days after incubation at 5°C (Fig. 3C). When larvae were transferred from 5°C to 25°C and incubated for five days, some up-regulation in CsSP2 expression was detected (Fig. 3C). In pupae, little CsSP2 expression was detected (Fig. 3C).

**DISCUSSION**

In the present study, cDNA encoding the SP of C. suppressalis, CsSP2, was isolated and sequenced. Insect SPs are generally classified into
Fig. 2. Alignment of *Chilo suppressalis* CsSP2 amino acid sequence with eight other lepidopteran storage protein amino acid sequences. The highly conserved storage protein signature sequences are boxed. (*) identical residue, (:) strong positive residue, (.) weaker positive residue, (-) insertion or deletion. Cs, *Chilo suppressalis* (GenBank/EMBL/DDBJ accession no. AB248058); Gm, *Galleria mellonella* (accession no. M73793); Ms1, *Manduca sexta* (accession no. M28396); Cc, *Corcyra cephalonica* (accession no. AF294808); Ms2, *M. sexta* (accession no. M28397); Hc, *Hyalophora cecropia* (accession no. AF032396); Ap, *Antheraea pernyi* (accession no. AY278025); Sl, *Spodoptera litura* (accession no. AJ249471); Bm, *Bombyx mori* (accession no. AB019209).
two groups, aromatic-amino acid-rich arylphorin and methionine-rich SPs. In general, arylphorin contains 1–3% methionine and 16–21% aromatic amino acids, and methionine-rich storage protein contains 4–11% methionine and 9–13% aromatic amino acids in lepidopteran insects (Telfer and Kunkel, 1991). CsSP2 encodes a protein containing 3% methionine and 18% aromatic amino acids. Furthermore, the protein showed high sequence identity to several lepidopteran arylphorins. These results suggest that the protein is classifiable as an arylphorin.

Storage protein genes associated with diapause have been cloned from various insects, including the Colorado potato beetle, Leptinotarsa decemlineata (de Kort and Koopmanschap, 1994), the spruce budworm, Choristoneura fumiferana (Palli et al., 1998), the greater wax moth, G. mellonella (Godlewski et al., 2001), and the boll weevil, Anthonomus grandis (Lewis et al., 2002). High levels of expression in CfDAP1 and CfDAP2 were detected one day after hatching and maintained throughout the first instar as well as the second instar before entering diapause in C. fumiferana (Palli et al., 1998). During diapause and at diapause termination, only a trace amount of the transcripts was detected. On the other hand, Lhp82 and Lhp76 were expressed during diapause in G. mellonella (Godlewski et al., 2001). A detectable level of CsSP2 expression was observed in diapausing larvae before cold acclimation; however, the level was lower than that in non-diapausing larvae. These results suggest that CsSP2 is not expressed as part of the diapause program in C. suppressalis.

In the previous study, it was shown that the expression of CsSP1 in diapausing larvae was up-regulated 10 days after cold acclimation at 5°C (Sonoda et al., 2006). The expression was detectable 40 days after incubation at 5°C (Sonoda et al., 2006). In the case of CsSP2, the expression level was up-regulated five days after acclimation at 15°C in diapausing larvae. The level was maintained for 10 days after acclimation at 5°C; however, little CsSP2 expression was detected at 40 days after incubation at 5°C. It is plausible that larvae at 40 days after incubation at 5°C remained in a diapause state (Sonoda et al., 2006), suggesting that the expression of CsSP2 is terminated during diapause.

Storage proteins are present in different stages

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Fig. 3. RNA gel blot analysis of CsSP2 from Chilo suppressalis. (A) Expression of CsSP2 was examined using diapausing and non-diapausing larvae before cold acclimation. (B) Expression of CsSP2 was examined in the course of cold acclimation performed by lowering temperature in a stepwise fashion by 5°C over five days from 20°C to 5°C using diapausing and non-diapausing larvae. Numbers on the photographs show temperatures during cold acclimation. (C) Acclimatized diapausing larvae were incubated at 5°C for 120 days and then transferred to 25°C to allow pupation. CsSP1 expression was examined using larvae incubated at 5°C for 10 days (lane 1), 40 days (lane 2) and 120 days (lane 3), larvae at five days after temperature shift from 5°C to 25°C (lane 4) and pupae (lane 5). Total RNA (20 μg) was subjected to denaturing gel electrophoresis, transferred to a nylon membrane and hybridized with a 32P-labeled probe. Photographs of the ethidium bromide-stained RNA gel before transfer are also shown. DP, diapausing larvae; ND, non-diapausing larvae.
of development and have diverged functions. Arylphorins are thought to supply certain amino acids, especially aromatic amino acids, for the production of the adult cuticle (Kanost et al., 1990) and eggs (Pan and Telfer, 1996). Protein products of CsSP2 might be an important amino acid reserve during diapause. Cho et al. (1999) injected positively charged DEAE-Sepharose beads into the abdomen of Tenebrio molitor and isolated an 86 kDa protein enriched on the beads as an early-stage encapsulation-relating protein. The deduced amino acid sequence of the 86 kDa protein showed strong similarity with that of diapause protein 1 from L. decemlineata (Cho et al., 1999). Since diapause protein 1 belongs to the family of arylphorin-type SP genes, CsSP2 might participate in the defense mechanism during diapause. Gene suppression experiments using an RNA interference technique will be required to investigate the functional roles of CsSP2 during diapause.

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