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Potentiality of Oligodeoxynucleotides as An Inducer for Antifungal Peptide in Two Lepidopteran Insects, *Bombyx mori* and *Galleria mellonella*

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Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG dinucleotides in particular base contexts are known to induce immunity in vertebrate cells. In insect, however, it was recent to find out that ODNs induces insect immunity as other immune inducer such as lipopolysaccharide. However, the finding was solely based on one lepidopteran insect, *Bombyx mori*, and the expression of insect immunity was neither dependent on numbers of CpG repeats nor methylation of CpG repeats within ODNs. Instead, foreignness of DNA has been suggested to be a key factor governing induction of antibacterial peptide. In this study, we expanded our previous understanding to the potentiality of ODNs as an immune inducer for antifungal peptide in *Galleria mellonella* and *B. mori*. To do this, a defensin-type antifungal peptide gene, reported from *G. mellonella* was cloned and partially sequenced from *G. mellonella* and *B. mori* successfully and utilized as a probe in the Northern blot analysis. We found out that ODNs also work as an immune inducer for antifungal peptide in the fat body and midgut of *G. mellonella* and *B. mori* larvae. Also, induction pattern of antifungal peptide was irrelevant to the numbers of CpG repeats within ODNs as previously reported on the induction pattern of antibacterial peptides.

Key words: Oligodeoxynucleotides, ODNs, CpG, *Bombyx mori*, *Galleria mellonella*, Antifungal peptide, Insect immunity

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Introduction

It has been known that synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG dinucleotides induce immunity in mammalian cells (Krieg *et al.*, 1995, 1998; Ballas *et al.*, 1996; Klinman *et al.*, 1996). Stimulatory effect of CpG dinucleotides has been fully proved by substituting 5'-cytosines of CpG to 5-methyl-cytosine, the statue of which is the one presented in the vertebrate DNA or by substituting CpG to GpC dinucleotides, by which induction of the immune stimulation was failed (Hacker *et al.*, 1998).

In the case of insects, we firstly reported the possibility of ODNs as a new immune inducer in the silkworm, *Bombyx mori*. However, the induction pattern was new in insect: the expression pattern determined by two antibacterial peptides, attacin and cecropin, was neither dependent on numbers of CpG motifs nor methylation of CpGs in ODNs (Kim *et al.*, 2004). Instead, the foreignness of ODNs was ascribed to a key factor in the induction of insect immunity (Kim *et al.*, 2004). Induction of insect immunity by ODN was further confirmed in the induction experiment of a cytokine-like molecule in the *B. mori*: Western blot analysis using rabbit anti-human cytokines showed the presence of IL-6-like molecule with an approximate molecular weight of 45 kDa in the ODN-stimulated *B. mori* hemolymph (Kim *et al.*, 2003).

Although the effect of ODNs on immune stimulation of insect was carried out in terms of antibacterial peptides and cytokines, the possibility to induce antifungal peptides by ODN has never been attempted. Further, the pos-

sible role of ODNs as an immune inducer was determined exclusively by the data obtained from single insect species. Thus, in the present study, we tested the role of ODNs on the induction of antifungal peptide in the *B. mori*, and the great wax moth, *Galleria mellonella*, to expand our understanding about ODNs in the insect immunity.

Materials and Methods

Insects

The silkworm, *Bombyx mori*, used was hybrid Jam-113 supplied by Department of Sericulture & Entomology, The National Institute of Agricultural Science & Technology, Korea. Silkworms were reared on fresh mulberry leaves at 26°C, 65 ± 5% of relative humidity, and a 12 hrs light : 12 hrs dark photoperiod as usual. The great wax moth, *Galleria mellonella*, was reared on an artificial diet at 30°C in the dark as usual.

Total RNA extraction, cloning of antifungal peptide, and cDNA sequencing

In order to clone a cDNA encoding an antifungal peptide to be utilized as a probe, total RNAs were isolated from fat bodies of both *G. mellonella* and *B. mori* larvae at 16 hrs after immunization with lipopolysaccharide (LPS) using the Total RNA Extraction Kit (Promega). The first-strand cDNA was synthesized by PCR at the condition of 37°C for 1 hr and 93°C for 5 min using a Reverse Transcription System (Promega, Madison, WI, USA). A primer set was designed on the basis of an antifungal peptide, originated from the great wax moth (Lee *et al.*, 2004). The peptide has been reported to be a member of insect defensins, exerting strong antifungal activities (Lee *et al.*, 2004). The primer sequences for forward direction was 5'-ATGGCGAAAAATTTCCAGTCCG-3 and for reverse direction was 5'-TAGGCAGATTAAACAAAACAAATCC-3 (Fig 1A). PCR was conducted under the following conditions: one cycle of 94°C for 7 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and one cycle of 72°C for 7 min. The PCR product with an approximate size of 520 bp was identified on 1.0% agarose gel and cloned into pGEM-T Easy vector (Promega). Plasmids were extracted using the Plasmid Extraction Kit (Quiagen, USA) and sequenced using an automatic sequencer (Applied Biosystems, model 310, Foster City, USA).

Injection of ODNs

Six ODNs were synthesized at a commercial company (Bioneer, Korea), and their nucleotide sequences and number of CpG repeat are described in Table 1. Based on the previous study (Kim *et al.*, 2004), the purchased ODNs were dissolved in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at a concentration of one μg/μl. Ten micro-liters of each ODN were injected to the silkworm larvae on the 3rd day of the 5th instar or two micro-liters of the ODN was injected to the final larval staged wax moth. Non-injected and saline-injected (150 mM NaCl, 5 mM KCl) larvae were used as negative controls. LPS (Sigma, St. Louis, MO, USA) was dissolved in the ringer solution (128 mM NaCl, 1.8 mM $CaCl_2$, 1.3 mM KCl, 2.3 mM $NaHCO_3$, pH 6.2) at a concentration of one μg /μl, and two micro-liters to the silkworm larvae and one micro-liter to the wax moth were injected for the positive controls.

Table 1. Synthetic ODNs containing variable number of CpG motif utilized for the induction of immunization in the silkworm and great wax moth

Name	Sequences (5' 3')	Characteristics
33H	TCGTCGTTTTGTCGTTTTGTCGTT	24 mer, 4CpG
34H	TCGTCGTTCCCCCCCCCCCC	20 mer, 2CpG
36H	TGCTGCTTCCCCCCCCCCCC	20 mer, 0CpG
37H	TCCATGACGTTCCTGATGCT	20 mer, 1CpG
38H	TCTCCCAGCGTGCGCCAT	18 mer, 2CpG
39H	GAGAACGCTCGACCTTCGAT	20 mer, 3CpG

Northern blot analysis

To test expression of antifungal peptide gene by ODNs at the transcriptional level, total RNA was separately isolated from the whole body, fat body, midgut, and epidermis of the silkworm and wax moth using the Total RNA Extraction Kit (Promega). Total RNAs (10 μg/lane) from the *G. mellonella* and *B. mori* were separated on glyoxalation gel (McMaster and Carmichael, 1977), transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42°C with a probe in a buffer containing 2 × PIPES, 50% formamide, 1% sodium dodecyl sulphate (SDS) and blocking agent (Boehringer Mannheim, Mannheim, Germany). The probe used to detect the antifungal peptide was 517-bp of *G. mellonella* antifungal peptide cDNA cloned in this study (Fig. 1B). The probe was labeled with [α-^{32}P] dCTP (Amersham, Arlington Heights, IL) using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA). After hybridization, the membrane filter was washed three times for 30 min each in 0.1% SDS and 0.2 × SSC at 65°C, and finally exposed to autoradiography film.

Results and Discussion

cDNA sequencing

To obtain a probe for Northern blot analysis, the partial

cDNA encoding the antifungal peptide gene was PCR-amplified using a primer set designed on the basis of an antifungal peptide cDNA originated from *G. mellonella* (Fig. 1A). Both from *B. mori* and *G. mellonella*, PCR products were obtained with the expected molecular size of ~520 bp (Fig. 1B).

The nucleotide sequences of the partial products were analyzed. As expected, the cDNA sequence of the antifungal peptide from *G. mellonella* was identical to known cDNA sequence of the *G. mellonella* antifungal peptide gene (Lee *et al.*, 2004). In the case of *B. mori*, the partial cDNA sequence of the peptide revealed several substitutions on various sites at downstream region of 3' end (Fig. 1A). It is surprising that the coding region of the peptide is conserved in such diverged insect species such as *B. mori* and *G. mellonella*, although both species belong to Lepidoptera. On the other hand, the mature peptide sequence of the defensin type-antifungal peptide originated from *G. mellonella* (Lee *et al.*, 2004) was 86.4% protein identity (38 among 44 amino acids) to the previously reported insect defensin (named as heliomicin) originated from lepidopteran *Heliothis virescens* (Lamberty *et al.*, 1999). Thus, it seems somewhat unusual that *G. mellonella* and *B. mori* are highly similar in the nucleotide sequences. Therefore, further researches on the complete

A

ATGGCGAAAAATTTCCAGTCCGTTTTGTTGTTGGTCTGCCTATCATTTTTAGTGATCGTA
TCGTCACCGCAAAATGCTGTACAAGCGGATACGCTGATAGGGAGGTGCGTGTGGGGTGCG
ACGAATTACACCTCTGATTGCAATGCTGAGTGCAAACGTCGTGGGTATAAAGGTGGTCAT
TGCGGCAGCTTCTTGAACGTCAATTGTTGGTGCCAGTAATAATTTTTTTTTTTACTACAA
ATTCAGTTTTTAAGATTATAAAATAACTGCTGTTTCACCGCATCAGCGGACTACCACTGC
AGCCTAGAATTCTTGGATTAGTTCCATTCAGAAGCTGCAAAAGTAACATTTTTTTTATTG
GATTTTCCCAAAGAATGATGCATTTTTAATTGGACGCTATTATTTTTATACTTTGATAAT
GTCTTTGTTTATATTTAAAAAAATTGTACCTGTTGACTAACTTGGCAGCATTTGAATGTA
TACAAATTGTTAAGGATTTGTTTTGTTTAATCTGCCTA

B

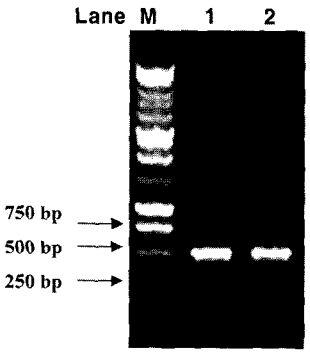
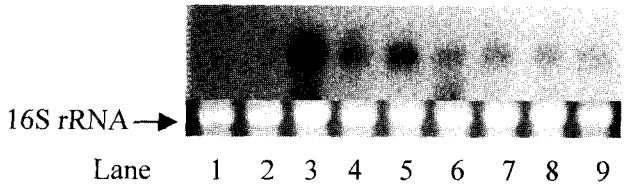
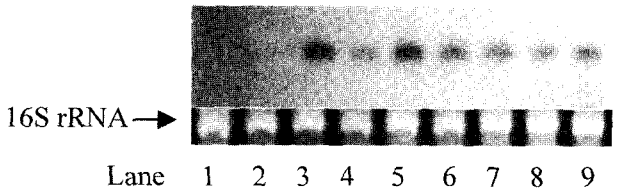


Fig. 1. Nucleotide sequences of the antifungal peptide utilized as a probe and PCR product of the peptide. **A**, Primer site and nucleotide sequence of the partial cDNA encoding antifungal peptide gene. The start codon ATG and termination codon TAA are underlined and primer sites are written in bold-faced nucleotides. The bold and italicized nucleotides are variations found in *B. mori* cDNA. **B**, PCR amplification of the partial antifungal peptide. M, 1 kb DNA size marker; lane 1, *B. mori*; and lane 2, *G. mellonella*.

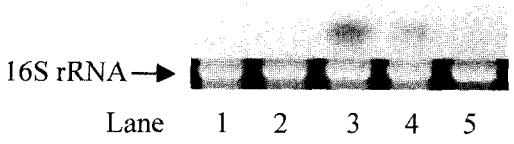
A, Wax moth fat body



B, Wax moth midgut



C, Silkworm fat body



D, Silkworm midgut

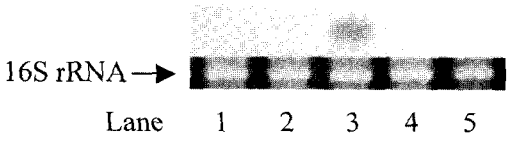


Fig. 2. Northern blot analysis for the detection of immunization by ODNs in the wax moth fat body (A) and midgut (B) and the silkworm fat body (C) and midgut (D) by using 517-bp of antigungal peptide obtained in this study as a probe. In wax moth (A and B), samples were collected from naive wax moth larvae (lane 1), injected ones with autoclaved-saline (lane 2), with LPS (lane 3), 33H-ODN (lane 4), 34H-ODN (lane 5), 36H-ODN (lane 6), 37H-ODN (lane 7), 38H-ODN (lane 8), and 39H-ODN (land 9), respectively. In silkworm (C and D), samples were collected from naive silkworm larvae (lane 1), injected ones with autoclaved-saline (lane 2), with LPS (lane 3), 34H-ODN (lane 4), and 35H-ODN (lane 5), respectively. The total RNA extracted at 24 hrs post-injection was separated by 1.2% formaldehyde agarose gel electrophoresis, trasnsferred on to a nylon membrane, and hybridized with the radiolabelled probe. The 16S rRNA gene was used as an internal loading control.

cDNA and genomic DNA sequences are urgent to fully understand the extension of conservancy of the peptide in insect species.

Induction of antifungal peptide gene by ODNs

To confirm the expression of antifungal peptide gene at the transcriptional level, Northern blot analysis was carried out using total RNA prepared from the wax moth and silkworm larvae injected with several sources of CpG dinucleotide-containing ODNs (Fig. 2). In the wax moth, the hybridization signal was detected as a single band in total RNAs from most of the ODN-injected fat body and midgut, and this event was irrelevant to the number of CpG repeats in ODNs (Fig. 2A, B). In the silkworm, the hybridization signal was very weak or undetectable even though two representative ODNs were selectively utilized, but still discernable from negative controls (Fig. 2C, D). Taken these together, the result here suggest that all types of ODNs used in this study serve as immune inducer in insect, although overall signal was weak compared with LPS. Also, our result suggests that number of CpG repeats in ODN is not a sole factor to determine the induction of antifungal gene.

Previously, we found that ODNs also induce antibacterial peptide such as attacin and cecropin in the *B. mori* larvae as other immune inducer such as LPS and the expression was neither dependent on numbers of CpG motifs nor methylation of CpGs within ODNs (Kim *et al.*, 2004). Thus, our finding here is consistent with the previous finding (Kim *et al.*, 2004), but reverse to the finding that CpG repeats in the bacterial DNA and synthetic ODN are essential for vertebrate immune induction (Krieg *et al.*, 1995).

To know the tissues involved in the expression of the antifungal peptide gene at transcriptional level, Northern blot analysis was performed using total RNA prepared from whole body, fat body, midgut, and epidermis of *G. mellonella* and *B. mori* (Fig. 3). In the case of *G. mellonella*, signals were detected in the whole body, fat body,

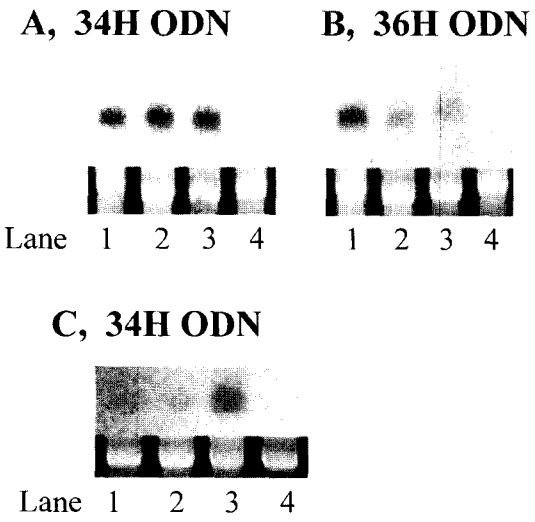


Fig. 3. Northern blot analysis of the antifungal peptide gene in the different tissues for the detection of immunization by 34H ODN (A) and 36H ODN (B) in the wax moth larvae and 34H ODN (C) in the silkworm larvae by using 517-bp of antifungal peptide obtained in this study as a probe. Total RNA was extracted from the whole body (lane 1), fat body (lane 2), midgut (lane 3), and epidermis (lane 4) at 24 hrs after infection of the larvae with 34H ODN (A, C) or 36H ODN (B), respectively. The 16S rRNA gene was used as an internal loading control.

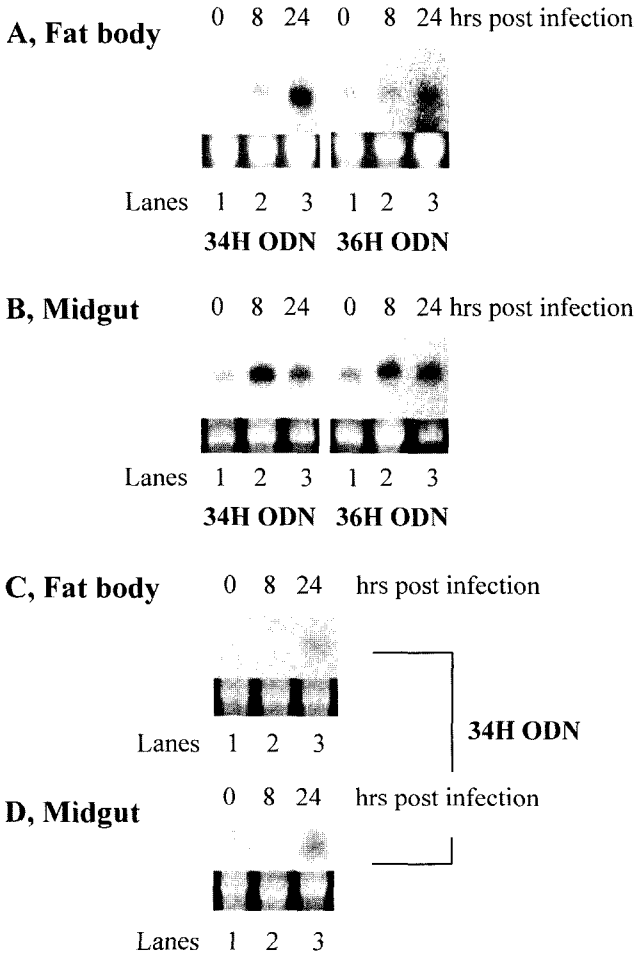


Fig. 4. Northern blot analysis of the antifungal peptide gene in the different post-injection time. Total RNA was extracted from the fat body (A) and midgut (B) of the wax moth larvae without injection (lane 1), at 8 (lane 2), and 24 (lane 3) hrs post-injection with 34H ODN and 36H ODN, respectively. Also, total RNA was extracted from the fat body (C) and midgut (D) of the silkworm larvae without injection (lane 1), at 8 (lane 2), and 24 (lane 3) hrs post-injection with 34H ODN. The 16S rRNA gene was used as an internal loading control.

and midgut, but not in the epidermis by injection of both 34H ODN and 36H ODN, indicating that the gene is expressed mainly in the fat body and midgut of the wax moth larvae in response to the injection of the ODNs containing varying number of CpG repeats. In the case of *B. mori*, signals were overall weak, but still detectable in the whole body and fat body and strong in the midgut. Lee *et al.* (2004) cloned a cDNA of the defensin-type antifungal peptide in the fat body of the *G. mellonella* larvae immunized with *E. coli* and reported induction of the peptide in the fat body and midgut, but not in the integument epithelial cells or trachea. Based on the observation they concluded that the major sites for the synthesis of the defensin are the fat body and the midgut (Lee *et al.*, 2004). Thus, this result is consistent with the expression pattern observed in our study of *G. mellonella* and *B. mori*.

The induction of antuifungal peptide gene was detected at different times after ODN injection (Fig. 4). In the case of *G. mellonella*, the antifungal peptide gene was detected at 8 hrs in both tissues and by both ODNs, and reached its maximum at 24 hrs in the fat body or 8 hrs in the midgut. In the case of *B. mori*, overall signal was weak, but detectable at 24 hrs both in the fat body and midgut. Thus, the data suggest that ODNs also induce antifungal peptide and induction occurs roughly within 8 ~ 24 hrs after injection with ODN.

In conclusion, the present study on the induction of insect immunity by ODNs confirms the previous finding that ODNs truly induce insect immunity, regardless of number of CpG repeats within ODNs. Further, this study expanded our understanding of the ODNs as immune inducers in that *G. mellonella* as well as *B. mori* are responsive to the injection of ODNs. Although limited, the finding that ODN truly induces insect immunity such as induction of antibacterial peptides (Kim *et al.*, 2004), cytokine-like molecules (Kim *et al.*, 2003), and antifungal peptide (in this study) is hoped to understand the mechanism by which insect react with the foreign non-self.

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