Pergamon

Insect Biochemistry and Molecular Biology 33 (2003) 649–658

Insect Biochemistry and Molecular Biology

www.elsevier.com/locate/ibmb

# Sterol biosynthesis by symbiotes: cytochrome P450 sterol C-22 desaturase genes from yeastlike symbiotes of rice planthoppers and anobiid beetles

Hiroaki Noda *, Yoko Koizumi

*National Institute of Agrobiological Sciences, Owashi, Tsukuba, Ibaraki 305-8634, Japan*

Received 17 December 2002; received in revised form 10 March 2003; accepted 19 March 2003

**Abstract**

Rice planthoppers and anobiid beetles harbor intracellular yeastlike symbiotes (YLS), whose sterols are nutritionally advantageous for the host insects that cannot synthesize sterols. YLS of anobiid beetles synthesize ergosterol, whereas YLS of planthoppers produce ergosta-5,7,24(28)-trienol, which is a metabolic intermediate in the ergosterol biosynthetic pathway in yeasts. Since sterol C-22 desaturase (ERG5p, CYP61) metabolizes ergosta-5,7,24(28)-trienol into ergosta-5,7,22,24(28)-tetraenol, which is the penultimate compound in the ergosterol biosynthesis, we examined the gene of this enzyme to determine whether this enzyme works in the planthopper YLS. C-22 desaturase genes (*ERG5*) of YLS of the planthoppers and beetles had four introns in identical positions; such introns are not found in the reported genes of yeasts. Cytochrome P450 cysteine heme-iron ligand signature motif was well conserved among the putative amino acid sequences. The gene expression of the planthopper YLS were strongly suppressed, and the genes possessed nonsense mutations. The accumulation of ergosta-5,7,24(28)-trienol in the planthopper YLS was attributed to the inability of the planthopper YLS to produce functional ERG5p.


*Keywords:* ERG5; Yeastlike symbiotes; Planthopper; Anobiid beetle; Sterol biosynthesis; Ergosterol

## 1. Introduction

Cholesterol is an essential component of cell membranes, a precursor for molting hormone ecdysone (Koolman, 1990; Grieneisen, 1994), and a component in signal transduction pathways (Porter et al., 1996; McMahon, 2000) in insects. Insects are not able to synthesize sterols and have to ingest exogenous sterols from food (Clark and Bloch, 1959; Clayton, 1964; Robbins et al., 1971). Phytophagous insects obtain C-24 alkylated phytosterols, such as sitosterol, campesterol, and/or stigmasterol, from their host plant. These $C_{28}$ and $C_{29}$ sterols are converted into cholesterols ($C_{27}$ sterol) in the insects (Svoboda et al., 1975; Kircher, 1982; Svoboda, 1999). Blood-sucking insects can directly ingest cholesterol from their host animals.

Some insects have another sterol source, i.e. their associated yeasts or fungi. The leaf-cutting ant *Acromyrmex octospinosus* possesses $\triangle^{5,7}$-sterols, such as ergosterol, which originate in its fungal symbiotes (Maurer et al., 1992). The ambrosia beetle *Xyleborus ferrugineus* requires ergosterol, which is presumably supplied by the associated fungus (Norris et al., 1969; Chu et al., 1970). Intracellular eukaryotic symbiotes in insects also supply sterols to the host insects. The anobiid beetles, *Lasioderma serricorne* (the cigarette beetle) and *Stegobium paniceum* (the drugstore beetle), possess yeastlike symbiotes (YLS), *Symbiotaphrina kochii* and *S. buchneri*, in the mycetome on the alimentary canal (Jurzitza, 1979). They are ascomycetous fungi (Jones and Blackwell, 1996; Noda and Kodama, 1996; Jones et al., 1999), which propagate by budding in the beetles. YLS of the beetles synthesize ergosterol, which occupied four fifths of the total volume of sterols in the YLS. The beetles metabolize YLS sterols into 7-dehydrocholesterol and cholesterol (Nasir and Noda, 2003). Sterols are also synthesized in YLS of planthoppers. The planthopper YLS,

* Corresponding author: Tel.: +81-29-838-6109; fax: +81-29-838-6028.

*E-mail address:* hnada@affrc.go.jp (H. Noda).

0965-1748/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0965-1748(03)00056-0

which are also ascomycetes (Euascomycetes: Hypocreales: Clavicipitaceae) (Noda et al., 1995; Suh et al., 2001), live in the fat body cells of the host planthopper (Noda, 1977; Cheng and Hou, 1996). YLS of *Laodelphax striatellus* (the small brown planthopper) and *Nilaparvata lugens* (the brown planthopper) synthesize ergosta-5,7,24(28)-trienol, which is the only dominant sterol in the YLS directly isolated from planthoppers (Wetzel et al., 1992). On the other hand, Eya et al. (1989) reported that cultivated YLS in artificial medium (Kusumi et al., 1979, 1980; Nasu et al., 1981) synthesize ergosterol. Wetzel et al. (1992) interpreted this inconsistency in the final sterol products in the YLS as follows: the YLS synthesized ergosta-5,7,24(28)-trienol under anaerobic conditions (in the planthopper) and ergosterol under aerobic conditions (in the culture medium).

Ergosterol (methylcholesta-5,7,22-trienol, ergosta-5,7,22-trienol) is a common sterol synthesized by most yeasts and fungi (Weete, 1973, 1989). The ergosterol biosynthetic pathway in baker's yeast *Saccharomyces cerevisiae* has been well investigated (Fryberg et al., 1973; Parks and Casey, 1995), and enzymes in the ergosterol biosynthetic pathway of the yeast are all cloned and characterized (Lees et al., 1995; Daum et al., 1998). Ergosta-5,7,24(28)-trienol, which is produced by YLS of the planthopper, is an intermediate in the late pathway of ergosterol biosynthesis in yeasts and fungi (Fryberg et al., 1973; Parks and Casey, 1995) (Fig. 1). The YLS are unable to metabolize ergosta-5,7,24(28)-trienol in the planthoppers and this sterol accumulates as a final product in the sterol biosynthetic pathway. This trienol is apparently converted into 24-methylenecholesterol and thereafter into cholesterol (Wetzel et al., 1992) (Fig. 1). Ergosta-5,7,24(28)-trienol in *S. cerevisiae* is metabolized into ergosta-5,7,22,24(28)-tetraenol by a P450 enzyme ERG5p (C22-sterol desaturase, CYP61), introducing a C22(23) double bond in the sterol side chain. Ergosta-5,7,22,24(28)-tetraenol is then metabolized into ergosterol by ERG4p (C24=28 methylene reductase), which works during the final step in the ergosterol biosynthetic pathway (Parks and Casey, 1995; Palermo et al., 1997). These two enzymes do not apparently work in the YLS in the internal milieu of planthoppers.

We analyzed *ERG5* genes of YLS of the planthoppers and anobiid beetles, and compared the genes at the sequence level with those reported in the yeasts to clarify why YLS of the planthoppers do not synthesize ergosterol in the planthoppers and to explore the significance of producing the ergosta-5,7,24(28)-trienol in the sterol metabolism of YLS and its host planthoppers.

## 2. Materials and methods

### 2.1. Insects and YLS

The anobiid beetles *L. serricorne* and *S. paniceum* were reared on wheat bran with 5% (w/w) yeast extract

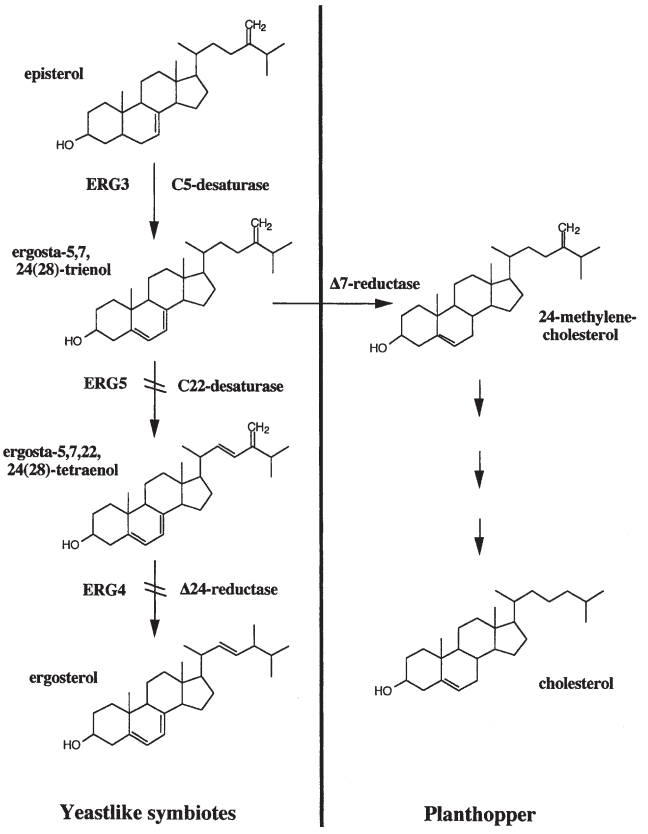



Fig. 1. Simplified late stages of the ergosterol biosynthetic pathway of planthopper YLS (based on the pathway by Parks and Casey (1995)) and sterol metabolism in planthopper.

(Oriental Yeast). The rice planthoppers, *N. lugens*, *L. striatellus*, and *Sogatella furcifera* (the white-backed planthopper), were reared on rice seedlings. Insects were all kept at 26 °C under 16 h daily illumination. YLS of the anobiid beetles were isolated from midgut ceca of adults (Noda and Kawahara, 1995) and grown on YPD medium (dextrose 40 g, peptone 10 g, yeast extract 5 g, $KH_2PO_4$ 5 g, $MgSO_4/7H_2O$ 2 g in 1 l). YLS of the planthoppers were isolated by Percoll density gradient centrifugation (Noda and Omura, 1992).

### 2.2. Genomic DNA and mRNA preparation

Isolated YLS (as a small pellet at the bottom of a centrifuge tube, ca. 30–50 μl ) were dissolved in 200 μl of STE buffer (100 mM NaCl, 1 mM EDTA [pH 8.0], and 10 mM Tris–HCl [pH 8.0]), mixed with the same volume of phenol/chloroform (1:1), and vortexed vigorously with glass beads (425–600 μm, Sigma, St Louis). Aqueous phase was recovered by ethanol precipitation and the pellet was redissolved in water. This solution was used as PCR templates. mRNA was purified from this solution with QuickPrep micro mRNA Purification Kit (Amersham Pharmacia Biotech).

### 2.3. PCR of genomic DNA

Amplifications were performed in 20 μl of buffer (10 mM Tris–HCl [pH8.3], 50 mM KCl, 1.5 mM $MgCl_2$) with 0.15 mM each dNTP, 10 pmol primers and 1 U r*Taq* DNA polymerase (TaKaRa, Tokyo). The PCR thermal conditions were usually as follows: 1 cycle of 95 °C for 1 min; 35 cycles of 95 °C for 30 s, 52–58 °C for 30 s, and 72 °C for 2 min; and final extension at 72 °C for 5 min. Primers used in this study are shown in Table 1.

We designed six degenerate primers (erg5f1- erg5r3) based on the nucleotide sequences of three *ERG5* genes from *S. cerevisiae* (DNA database accession number, U34636, Z49211), *Schizosaccharomyces pombe* (Z98974), and *Candida albicans* (AL033396). We amplified the *ERG5* gene of YLS of *L. striatellus* (LstYLS), *N. lugens* (NLYLS), *L. serricorne* (LseYLS), and *S. paniceum* (SPYLS) using one of the primer pairs (erg5f2 and erg5r1). Genomic 5′ and 3′ flanking regions of the obtained PCR products were also amplified by vectorette PCR (LA PCR in vitro Cloning Kit, TaKaRa,

Table 1
Primers used in this study[a]

| | | | |
|---|---|---|---|
| *PCR primers for genomic gene analysis* | | | |
| Degenerate primers, erg5f2 and erg5r1, amplified ca. 610 bp product of *ERG5* gene | | | |
| | erg5f1 | TGYGTNTCNRTNTTYCAYAARTTYGT | 26 bp |
| | erg5f2 | AAYTGGGTNTTYYTNGAYGG | 20 bp |
| | erg5f3 | GARYTNGTNAAYTTYCCNAT | 20 bp |
| | erg5r1 | GCRTCYTGNGANGCRAANARRAA | 23 bp |
| | erg5r2 | ATNGGRAARTTNACNARYTC | 20 bp |
| | erg5r3 | CCRTCNARRAANACCCARTT | 20 bp |
| Vectorette PCR for genomic ERG5 genes | | | |
| | Cassette primers used in the LA PCR in vitro Cloning Kit | | |
| | CPC1 | GTACATATTGTCGTTAGAACGCGTAATACGACTCA | 35 bp |
| | CPC2 | CGTTAGAACGCGTAATACGACTCACTATAGGGAGA | 35 bp |
| For 5′ franking region | | | |
| | r1NL (S1 for NLYLS) | GCGAAACTGGGGCATGAAGGGGACAGG | 27 bp |
| | r1Lst (S1 for LstYLS) | CTCGCGAAACTTGGGCATGAAGGGGACAGA | 30 bp |
| | r2 (S2 for NLYLS and LstYLS) | TTGCCGTCGTTGTCCTTGGTGATCTGGATG | 30 bp |
| | r3 (S1 for LseYLS and SPYLS) | GATATRGCACAATTYAKCTCTCGGAATTC | 29 bp |
| | r4Lse (S2 for LseYLS) | GCTCTAGGGCTTTCCGAGTGAAGAGAC | 27 bp |
| | r4SP (S2 for SPYLS) | GCTCCAGCGCCTTGCGGGTGAAGAGAC | 27 bp |
| For 3′ flanking region | | | |
| | f1 (S1 for NLYLS and LstYLS) | GCAAATGATCAACTCTGAACGCTGGCGGGA | 30 bp |
| | f2 (S2 for NLYLS and LstYLS) | TGTCAACACAAGGACCATGCCACGGAGTGC | 30 bp |
| | f3 (S1 for LseYLS and SPYLS) | CATGGAYGGYTGGATCAARTCTATGATCG | 29 bp |
| | f4Lse (S2 for LseYLS) | GCCTGCCATGATCATCAGAGAATTCAC | 27 bp |
| | f4SP (S2 for SPYLS) | ACCGGCCATGATGCTCAGGGAATTCAC | 27 bp |
| Additional primers for 5′ and 3′ regions of the gene of LseYLS | | | |
| | f5Lse | GACGCAGTGCTCCTTCTCCA | 20 bp |
| | r5Lse | TCGATGAGGGTTGTACTCATTTGCT | 25 bp |
| Primers for ERG5 gene of SFYLS | | | |
| | f1SF | AGGCTTGTCAGGCTTGGCG | 19 bp |
| | r1SF | CATGGCGGAGAGACTCGTAC | 20 bp |
| | f2SF | GACCGATATTATTCAGGAGACG | 22 bp |
| | r2SF | ACAGTCACAACTCCTTGTGATC | 22 bp |
| | f3SF | TTTGAGGAATACTACGCCAAATGG | 24 bp |
| | r3SF | TAGTGAGGGCCAGTACCAAATAC | 23 bp |
| *Sequencing primers for the products by the vectorette PCR* | | | |
| | Af | CCATTCAGATTCTGTACAAC | 20 bp |
| | Br | GCACTGGAGAGCCAACGTG | 19 bp |
| | Cf | TATTTGGTACTGGCCCTCAC | 20 bp |
| *PCR primers for cDNA analysis* | | | |
| Primers in SMART RACE cDNA Amplification Kit (Clontech) | | | |
| | SMART II Oligonucleotide | AAGCAGTGGTAACAACGCAGAGTACGCGGG | 30 bp |
| | 3′-RACE cDNA synthesis primer | AAGCAGTGGTAACAACGCAGAGTAC$(T)_{30}$VN | 57 bp |

(*continued on next page*)

Table 1 (*continued*)

| | | |
|---|---|---|
| Universal primer mix (UPM) | Long CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT | 45 bp |
| | Short CTAATACGACTCACTATAGGGC | 22 bp |
| Nested universal primer (NUP) | AAGCAGTGGTAACAACGCAGAGT | 23 bp |
| RT–PCR for ERG5 genes of anobiid beetle YLS for the internal region of cDNA | | |
| fRTLse (for LseYLS) | GTCGTCATTGCCTCAACTCG | 20 bp |
| fRTSP (for SPYLS) | CATACGTAACAAAGGCCGGA | 20 bp |
| rRT (for LseYLS and SPYLS) | GGAAAGATTGTTGCGAAGACCTTGAT | 26 bp |
| For 3′-RACE (3′-RACE cDNA synthesis primer was used for cDNA synthesis) | | |
| fRT3′Lse (for LseYLS) | GGCAAGTCTGCATCTTGACTG | 21 bp |
| fRT3′SP (for SPYLS) | GGCCTCTCTACACCTTGACTG | 21 bp |
| For 5'RACE (r4Lse or r4SP were used for cDNA synthesis) | | |
| rRT5′ Lse (for LseYLS) | GAGTTGAAGATTTTGCGAGCCA | 22 bp |
| rRT5′ SP (for SPYLS) | GGCCCATAAAGGGGATCTTGA | 21 bp |
| RT-PCR for ERG5 genes of planthopper YLS (375 bp DNA is expected to be amplified) | | |
| RT-f1 | CGATACCAGCCTCCGGTGCT | 20 bp |
| RT-r1 | GCCACATACCTTGGGAAATATAGTCGC | 27 bp |
| RT–PCR for uricase gene of planthopper YLS (409 bp DNA is expected to be amplified; designed from Hongoh et al., 2000) | | |
| uriRTf | AAGAGCACGGGTTCCGCCTT | 20 bp |
| uriRTr | ACTTCGCATTTGATGAGACCATTGG | 25 bp |

[a] The left side of the sequences is 5′. 'f' and 'r' indicate forward and reverse primers. S1 and S2 designate gene-specific primers in the vectorette PCR using LA PCR in vitro Cloning Kit (TaKaRa) and were used for first and second PCR in combination with CPC1 and CPC2.

Tokyo). The YLS genomic DNA was digested with one of the restriction enzymes, *Eco* RI, *Pst* I, *Hin*d III, or *Xba* I, and the digested products were ligated with each cassette DNA of about 50 bp in the Kit, and amplified with a cassette primer CPC1 and *ERG5* gene-specific primer S1. We performed nested PCR using CPC2 primer and gene-specific primer S2. The *ERG5* gene of LseYLS was further amplified toward the 5′ and 3′ flanking regions after the vectorette PCR. The DNA toward the 5′ flanking region was obtained by PCR using primers f5Lse and rRT5′Lse and that toward the 3′ flanking region using r5Lse and fRT3′Lse. These primers, f5Lse and r5Lse, were designed based on the sequence results of 5′RACE (rapid amplification of cDNA ends) and 3′RACE for LseYLS, as described below. The *ERG5* gene of YLS of *S. furcifera* (SFYLS) was obtained by PCR using three primer pairs, f1SF/r1SF, f2SF/r2SF, and f3SF/r3SF, which were designed based on the sequences of *ERG5* genes of YLS of two other planthopper species, and which amplified 5′, 3′ and central regions of *ERG5* gene of SFYLS, respectively.

### 2.4. cDNA amplification

cDNA was synthesized from the purified mRNA by a reverse transcriptase SuperScript II RT (GibcoBRL, Gaithersburg, MD) using 3′RACE cDNA Synthesis Primer (SMART™ RACE cDNA Amplification Kit, CLONTECH, Palo Alto). ERG5 genes of YLS of two anobiid beetles were amplified in three parts. The internal region of the cDNA was amplified using primer pairs fRTLse/rRT and fRTSP/rRT for LseYLS and SPYLS, respectively. The 3′region of the gene was amplified by 3′-RACE using Nested Universal primer (NUP) and fRT3′Lse for LseYLS, and NUP and fRT3′SP for SPYLS. For 5′-RACE, cDNA was synthesized with SMART II Oligonucleotide using primer r4Lse for LseYLS and r4SP for SPYLS, according to the manufacturer's recommendation. The first PCR for 5′-RACE was performed using the universal primer mix (UPM) in the Kit and r4Lse for LseYLS, and UPM and r4SP for SPYLS. The second PCR was done using primer pairs NUP/rRT5′Lse for LseYLS and NUP/rRT5'SP for SPYLS.

In planthopper YLS, cDNA was synthesized using 3′-RACE cDNA Synthesis Primer. ERG5 genes were amplified using primer pairs RT-f1/RT-r1, and uricase genes (Hongoh et al., 2000) using uriRTf/uriRTr as a control.

### 2.5. DNA sequencing and analysis of genes

We cloned the amplified products into pGEM-T (Promega, Madison) by TA cloning method. Templates for sequencing were prepared by colony PCR under conditions similar to those mentioned above using M13 and reverse primers. Sequencing reactions were done by BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA) using T7 or SP6 primers. We used primers Af and Br as sequence primers for sequencing the 5′ region of the *ERG5* gene of LstYLS, and Cf for the 3′ region of the *ERG5* gene of NLYLS and LstYLS (Table 1).

The sequences were determined with a DNA sequence System (model 377) or a DNA analyzer (model 3700) (Applied Biosystems). We sequenced at least three clones of the insert and eliminated polymerase errors in

PCR. The sequences were analyzed with GENETYX-MAC ver.11 (Software Development Co., Tokyo) and Clustal X (Thompson et al., 1997).

## 3. Results

### 3.1. Cloning and sequencing of ERG5 genes from YLS

Based on the nucleotide sequences of *ERG5* genes of three yeasts, we designed three each of forward and reverse primers and tested six different combinations of the primers (erg5f1/r1, erg5f1/r2, erg5f1/r3, ergf2/r1, ergf2/r2, and ergf3/r1) for PCR amplification of the *ERG5* gene from YLS of *L. striatellus* (LstYLS). The PCR product was clearly observed in one primer pair, erg5f2 and erg5r1 (Table 1). The DNA product of the 562 bp fragment possessed high homology to the expected internal region of *ERG5* genes of the three yeasts (54% for *S. cerevisiae*, 54% for *S. pombe*, and 53% for *C. albicans*). We also amplified *ERG5* genes in other samples using this primer pair and determined nucleotide sequences in YLS of three insect species, *N. lugens* (NLYLS), *L. serricorne* (LseYLS), and *S. paniceum* (SPYLS).

Genomic flanking region of the obtained *ERG5* genes were amplified by vectorette PCR. We also performed further PCR in LseYLS toward the 5′ flanking region using primers f5Lse and rRT5′Lse, and toward the 3′ flanking region using r5Lse and fRT3′Lse. The *ERG5* gene of YLS of *S. furcifera* (SFYLS) was obtained by PCR using three primer pairs, f1SF/r1SF, f2SF/r2SF, and f3SF/r3SF. Finally, sequences were determined in the genomic regions that include the whole *ERG5* gene from LseYLS (1988 bp), SPYLS (2385 bp), NLYLS (3244 bp), LstYLS (3005 bp), and SFYLS (2825 bp) (Database accession numbers, AB086890 to AB086894).

### 3.2. cDNA cloning and sequencing of ERG5 gene from YLS of the anobiid beetles

We synthesized, amplified and cloned cDNA of ERG5 genes of LseYLS and SPYLS into the bacteria. Three parts of the ERG5 cDNA, 5′, internal and 3′ regions, were amplified by PCR. The nucleotide sequences (excluding polyA+ tail) were determined in cDNA of ERG5 genes of LseYLS (2050 bp) and SPYLS (2019 bp) (Database accession numbers, AB086895 and AB086896). ERG5 cDNA of LseYLS (SPYLS) encodes a putative protein of 534 (535) amino acids. The amino acid sequence sizes of ERG5p of these YLS were close to those of *S. cerevisiae* (538-aa), *S. pombe* (551-aa), and *C. albicans* (517-aa). The ERG5 genes contained cytochrome P450 cysteine heme-iron ligand signature motif of ‘FGTGPHYCLG’ (amino acid positions 473-482 and 474-483 for LseYLS and SPYLS, respectively), where cysteine is involved in binding the heme iron (Fig. 3).

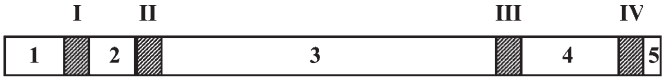
Sequence comparison of genomic *ERG5* genes and the cDNA revealed that the former contains four introns in the ORF of the gene in both LseYLS and SPYLS (Fig. 2).

### 3.3. RT–PCR of the ERG5 genes from YLS of the planthoppers

We amplified cDNA of ERG5 genes of YLS of the planthoppers by RT-PCR. Primer pairs RT-f1/RT-r1 were tested for amplifying a part of the 3′ region of ERG5 cDNA. We postulated some introns based on a comparison of putative amino acid sequences deduced from the genomic sequences of the *ERG5* genes with those of anobiid beetles. The primers were therefore designed to include intron III (Fig. 2). At the same time, cDNA of uricase genes of NLYLS and LstYLS was amplified using the primers uriRTf and uriRTr, which amplify 515 bp of genomic DNA and 409 bp of cDNA in NLYLS (Hongoh et al., 2000). Expected size of the uricase cDNA product was amplified. The sequence analysis confirmed that the product comes from the cDNA of the uricase gene. We tested cDNA from NLYLS and LstYLS under the same PCR conditions but did not observe PCR products of the expected size (375 bp excluding postulated intron III sequence) in agarose gel electrophoresis. We also performed RT–PCR using various combinations of primers shown in Table 1 but did not observe the expected PCR bands. The solutions amplified using the primer pairs RT-f1 and RT-r1 were subjected to TA-vector cloning, though no visible expected-sized DNA was observed. Six clones from NLYLS and seven clones from LstYLS were analyzed, and one clone from NLYLS had a sequence identical to that of the ERG5 gene. This sequence apparently originated in cDNA/mRNA, because it had no intron III sequence. No other clones were related to the ERG5 genes.

### 3.4. Putative amino acid sequences and exon/intron sequences of ERG5 genes from YLS of the planthoppers

We predicted the putative amino acid and exon/intron sequences from the genomic nucleotide sequences of the *ERG5* gene of planthopper YLS, comparing them with those of YLS of the anobiid beetles and the three yeasts (Fig. 2). We found cytochrome P450 cysteine heme-iron ligand signature motif ‘FSTGPHYCLG’ for NLYLS (amino acid position 477–486) and ‘FGTGPHYCLG’ for LstYLS (476–485) and SFYLS (477–486), which well match with the consensus sequence [FW]-[SGNH]-x-[GD]-x-[RKHPT]-x-C-[LIVMFAP]-[GAD] (PROSITE). The alignment of the predicted amino acid sequences




| host | 1 | I | 2 | II | 3 | III | 4 | IV | 5 | exon | intron | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LseYLS | 169 | 55 | 151 | 57 | 968 | 50 | 281 | 55 | 36 | 1605 | 217 | 1822 |
| SPYLS | 169 | 69 | 151 | 59 | 971 | 62 | 281 | 49 | 36 | 1608 | 239 | 1847 |
| NLYLS | 178 | 76 | 151 | 75 | 968 | 60 | 284 | 74 | 38 | 1619 | 285 | 1904 |
| LstYLS | 175 | 76 | 151 | 75 | 968 | 60 | 284 | 74 | 39 | 1617 | 285 | 1902 |
| SFYLS | 178 | 76 | 151 | 75 | 968 | 60 | 284 | 74 | 39 | 1620 | 285 | 1905 |

Fig. 2. Schematic drawing of the structure of *ERG5* genes between putative start and stop codons in five yeastlike symbiotes. Open and shaded boxes represent exons (Arabic numerals) and introns (Roman numerals), respectively. The numbers in the table represent the size (bp) of each exon and intron.

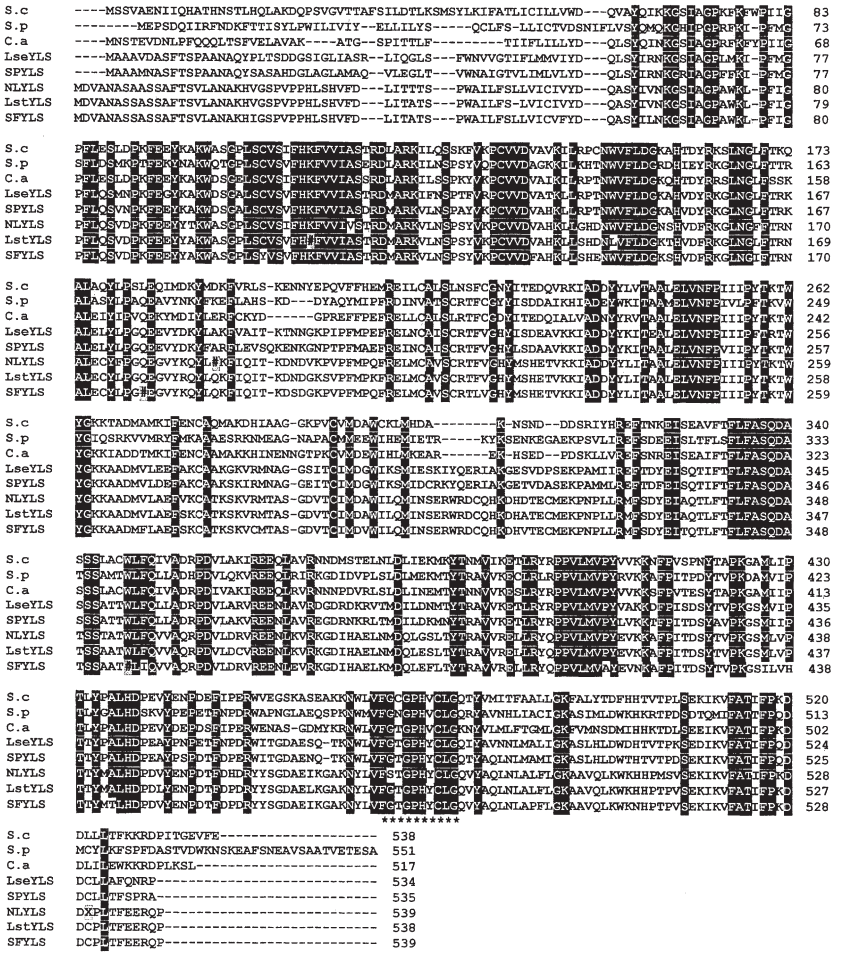



Fig. 3. Comparison of the deduced amino acid sequences from ERG5 genes among three species of yeasts and five yeastlike symbiotes. Amino acid residues matched in all eight (or seven) species are highlighted using a white character on a black background. Asterisks indicate cytochrome P450 cysteine heme-iron ligand signature motif. '#' represents stop codons in YLS of planthoppers, and 'X' a one-nucleotide deletion in NLYLS.

from three yeasts and five YLS demonstrated highly conserved amino acid sequences, especially at a position about 1/5 from the N-terminal [LSCVS(VI) FHKFVVIASXRD(ML)ARK] (amino acid position 105–126 in *S. cerevisiae*) and at a position about 2/3 from the N-terminal [(FL)(TS)FLFASQDA(TS)SS] (331–343 in *S. cerevisiae*) (Fig. 3). The alignment also disclosed four introns in the genomic *ERG5* genes of the planthopper YLS. The postulated intron positions in the *ERG5* gene of the planthopper YLS were exactly the same as those in YLS of the anobiid beetles based on the consensus sequence for RNA splicing in eukaryotes (5′GU-----AG3′).

The ERG5 genes of YLS of the planthoppers had non-

sense mutations: one for NLYLS (amino acid position 188), one for LstYLS (109), and two for SFYLS (179 and 355). The sites of the mutations were all different among the YLS. A one-nucleotide deletion was also predictable at the 3′ end in NLYLS (amino acid position 530) (Fig. 3).

### 3.5. Conservation of nucleotide sequences in ERG5 genes of YLS of the planthoppers

One remarkable phenomenon was a nucleotide sequence similarity among *ERG5* genes of planthopper YLS in both the exons and introns. Throughout the entire putative exon, whose sizes were 1614 to 1617 bp, only 46–58 bp were different each other for planthopper YLS (Table 2). In contrast, 444 bp out of 1605 exon nucleotides differed between *ERG5* genes of LseYLS and SPYLS.

Intron sequences of the genes were also conserved among planthopper YLS. In the total 285 bp intron sequence of planthopper YLS, only two nucleotides differed between NLYLS and LstYLS, nine between NLYLS and SFYLS, and seven between LstYLS and SFYLS. In contrast, the intron sequences of *ERG5* genes of the anobiid beetle YLS could not be confidently aligned because of the many nucleotide substitutions and/or deletions.

## 4. Discussion

### 4.1. Do YLS of planthoppers synthesize ergosterol?

Three reports have dealt with sterols in the planthoppers and their YLS. Noda et al. (1979) detected cholesterol, 24-methylencholesterol, and small amount of ß-sitosterol from *L. striatellus*. The ß-sitosterol was apparently ingested by the planthoppers from host rice plant. Apo-symbiotic (YLS-destroyed) planthoppers greatly reduced the relative amount of 24-methylenecholesterol. Eya et al. (1989) reported three sterols, cholesterol, 24-methylenecholesterol, and ergosterol from *N. lugens* and *L. striatellus*. They also detected ergosterol as only sterol found in the culture broth of the cultivated YLS (Kusumi et al., 1979, 1980; Nasu et al., 1981) and concluded that ergosterol was provided from YLS to host planthoppers. In contrast, Wetzel et al. (1992) detected cholesterol, 24-methylenecholesterol and ergosta-5,7,24(28)-trienol and neither planthopper species did not possess ergosterol. Ergosta-5,7,24(28)-trienol was also detected as the only sterol from the YLS directly isolated from the planthopper bodies by Percoll buoyant density gradient centrifugation (Noda and Omura, 1992). They also pointed out that ergosta-5,7,24(28)-trienol was incorrectly assigned to ergosterol in the planthopper samples in their previous report (Eya et al., 1989). Therefore, the sterols in the planthoppers are cholesterol, 24-methylenecholesterol, ergosta-5,7,24(28)-trienol, and occasional small amount of plant sterols that come from the food. Our recent analysis, that cholesterol, 24 methylenecholesterol, and ergosta-5,7,24(28)-trienol were detected from *N. lugens* at the ratio of 23:56:21, respectively (Nasir and Noda, unpublished data), also supports this conclusion.

However, there is an inconsistency in sterols of YLS between two reports. Eya et al. (1989) reported that YLS synthesize ergosterol, because ergosterol from the cultivated YLS was correctly assigned in GC-MS analyses (Wetzel et al., 1992), whereas Wetzel et al. (1992) detected ergosta-5,7,24(28)-trienol in the biochemically isolated YLS directly from planthopper bodies. Wetzel et al. (1992) interpreted this inconsistency as that the introduction of the C22(23) double bond and reduction of the C24(28) double bond are inhibited in the symbiotic environment of YLS (anaerobic in planthoppers) or that the cultivated YLS underwent mutation under artificial culture condition to produce ergosterol. However, many attempts including the present authors to cultivated YLS of planthoppers have failed. These cultivated YLS are reported to be the members of the genus *Candida* (Eya et al., 1989), but phylogenetic analysis of 18S rRNA genes of YLS of *N. lugens*, *L. striatellus*, and

Table 2
Nucleotide substitutions in ERG5 genes between two species of YLS[a]

| Two YLS compared | Position in triplet codon | | | Total exon | Total intron |
|---|---|---|---|---|---|
| | First | Second | Third | | |
| LseYLS–SPYLS | 79 | 43 | 322 | 444/1605 | —[b] |
| NLYLS–LstYLS | 19 | 9 | 18 | 46/1614 | 2/285 |
| NLYLS–SFYLS | 26 | 13 | 19 | 58/1617 | 9/285 |
| SFYLS–LstYLS | 24 | 9 | 19 | 52/1617 | 7/285 |

[a] The numbers indicate the different nucleotides between ERG5 genes of the two YLS. The number after '/' is the length compared. Two nucleotides that form a triplet codon with one nucleotide deleted were excluded from the comparison in NLYLS.

[b] We could not confidently align the intron sequences of LseYLS and SPYLS.

*S. furcifera* indicated that they are the members of the family Clavicipitaceae in the class Pyrenomycetes (Noda et al., 1995; Suh et al., 2001), which are phylogenetically far from the genus *Candida*. These results suggest that the experimental results using the cultivated YLS should not be consulted and we should only consider the results from the isolated YLS directly from the planthopper bodies. The isolated YLS possessed only ergosta-5,7,24(28)-trienol and we can safely conclude that YLS of planthoppers synthesize ergosta-5,7,24(28)-trienol as a final product of sterol biosynthesis. The idea that ergosta-5,7,24(28)-trienol is converted into cholesterol through 24-methylenecholesterol (Fig. 1) is strongly suggested by some evidence shown by Noda et al. (1979), Eya et al. (1989), and Wetzel et al. (1992).

### *4.2. YLS of the planthoppers do not have functional ERG5p*

YLS of the anobiid beetles apparently possesses complete ergosterol biosynthetic pathways (Nasir and Noda, 2003), whereas YLS of the rice planthoppers lacks a step in the late stage of ergosterol biosynthesis, in which ERG5p (and probably ERG4p) takes part (Wetzel et al., 1992; Parks and Casey, 1995). The fact that RT–PCR failed to amplify detectable amount of cDNA of ERG5 gene in planthopper YLS indicates that the gene was not actively expressed in them. However, the YLS still retained the ability to make mRNA from the *ERG5* genes in a very low rate because one cDNA clone, whose sequence corresponded to a part of the predicted mature mRNA of the ERG5 gene, was obtained from NLYLS. The *ERG5* genes, however, had nonsense mutations in all three planthopper YLS, and a nucleotide deletion was disclosed in NLYLS (Fig. 3). Therefore, planthopper YLS cannot make a functional enzyme that introduces a double bond at C-22 in the sterol side chain, resulting in accumulating ergosta-5,7,24(28)-trienol as a final product of sterol biosynthesis.

### *4.3. Mutation of eg5 gene does not significantly affect YLS growth and host insect development*

Ergosta-5,7,24(28)-trienol is sometimes detected in filamentous fungi or yeast along with ergosterol (Weete, 1973, 1989). This sterol is detected as a major sterol in *Phycomyces blakesleeanus* in the class Zygomycetes (Goulston and Mercer, 1969). Symbiotic fungi of the leaf-cutting ant *A. octospinosus* possess a considerable amount of ergosta-5,7,24(28)-trienol (15% in gongylidia, the swellings of the hyphae) (Maurer et al., 1992). This sterol apparently fulfills the need of the YLS as a structural component. The *ERG5* gene is not essential for cell viability of *S. cerevisiae* (Skaggs et al., 1996). The study of the mutants in sterol biosynthetic enzymes of *S. cerevisiae* suggests that the late genes in the ergosterol biosynthetic pathways, *ERG6* (C-24 methylase), *ERG2* (△8->7 isomerase), *ERG3* (C-5 desaturase) and *ERG4* (C24(28) reductase), are not essential for the survival of the yeast either (Lees et al., 1995). However, mutants of erg6 have several deficiencies, erg2 mutants are non-viable in an aerobic environment, and *ERG3* is essential in heme-deficient cells (Daum et al., 1998). It is, therefore, generally accepted that the final two steps of the pathway, which are mediated by ERG5p and ERG4p, are not essential for the growth of *S. cerevisiae* (Daum et al., 1998). Although recent reports suggest that inhibition of ERG5p correlates with cell growth arrest in azole-resistant strain of the cereal pathogen *Rhynchosporium secalis* (Hollomon, referred to in Lamb et al., 1999) and that ergosterol is essential for vacuole fusion in *S. cerevisiae* (Kato and Wickner, 2001), the loss of activity in ERG5p probably does not significantly affect the growth of the YLS.

When *Drosophila melanogaster* was tested for their ability to survive on erg mutants of *S. cerevisiae*, growth on erg2 and erg6 mutants led to developmental arrest in the larval stage (Bos et al., 1976; Parkin and Burnet, 1986). The mutants of erg3 and erg5 can satisfy for the sterol requirements of *D. melanogaster*, which suggests that this insect can grow on episterol or ergosta-5,7,24(28)-trienol. Since *Drosophila* species are considered unable to dealkylate phytosterols (Svoboda, 1999), it is unclear how these sterols are used in *Drosophila*. These reports, however, suggest that ergosta-5,7,24(28)-trienol could be a sterol source for insects. Actually, ergosta-5,7,24(28)-trienol is converted into cholesterol in *Tribolium confusum* (Svoboda and Lusby, 1994; Svoboda, 1999), and 24-methylenecholesterol, which has a reduction in the B ring of ergosta-5,7,24(28)-trienol, is metabolized into cholesterol in some insects (Svoboda et al., 1975; Svoboda, 1999) and the planthopper *N. lugens* (Eya et al., 1989).

### *4.4. Introns and exons of ERG5 genes of YLS*

The alignment of amino acids and nucleotides revealed that *ERG5* genes of planthopper YLS have four introns and that the intron insertion positions in the genes were identical to those of the beetle YLS. The third intron was indeed eliminated from mature mRNA in NLYLS. YLS of anobiid beetles and planthoppers were phylogenetically distantly related to each other, though they are all ascomycetous fungi (Jones and Blackwell, 1996; Noda and Kodama, 1996; Suh et al., 2001). The introns in the *ERG5* genes seem to be an ancient origin, because the existence and insertion positions of the introns were well conserved among YLS of anobiid beetles and planthoppers. No intron sequences have been reported in *ERG5* genes of *S. cerevisiae*, *S. pombe*, and *C. albicans*.

Among three planthopper YLS, the nucleotide

sequences in the introns were as well conserved as the exon sequences were. Two to nine out of 285 total intron nucleotides differed in *ERG5* genes among planthopper YLS. *ERG5* genes of the planthopper YLS did not work and are presumably no longer useful. Certain change(s) in the gene expression or in some other processes probably had occurred in old times before the nonsense mutations were deposited in the gene. Generally, useless genes accumulate mutations in the nucleotide sequences. Nevertheless, the *ERG5* genes of planthopper YLS were well conserved in both exons and introns. We have no idea of why the *ERG5* genes of planthopper YLS have been tolerant of mutations.

### *4.5. Preservation of the genes and genome size*

Studies of intracellular symbiotes and parasites reveal that they use the metabolites of the host and have discarded unnecessary genes. Consequently, the genomes of these microorganisms are small. In particular, symbiotic bacteria of aphids, *Buchnera*, apparently discarded many genes, so the genome is quite small, one seventh of that of a close relative *Escherichia coli* (Shigenobu et al., 2000; Tamas et al., 2002). In planthopper YLS, the *ERG5* gene that is probably useless was still retained, and the sequences were quite conserved. In this connection, there may be two opposite opinions. One is that the *ERG5* genes still have a certain function and work in the YLS, though we do not have any idea about the other functions of the *ERG5* gene. The other is that the planthopper YLS are apt to keep the waste gene system. The genomes of YLS are 17 to 20 Mbp and are within the usual sizes of fungal genomes (Noda and Kawahara, 1995). The trend of minimizing the genome, which has been reported in the intracellular bacteria, is probably not the case in eukaryotic symbiotes of planthoppers.

## Acknowledgements

We thank A. Kanauchi of the Department of Biology, Vanderbilt University for his valuable discussion on yeast sterol biosynthesis.

## References

Bos, M., Burnet, B., Farrow, R., Woods, R.A., 1976. Development of *Drosophila* on sterol mutants of the yeast *Saccharomyces cerevisiae*. Genet. Res. 28, 163–176.

Cheng, D.J., Hou, R.F., 1996. Ultrastructure of the yeast-like endocytobiont in the brown planthopper, *Nilaparvata lugens* (Stal) (Homoptera: Delphacidae). Endocyt. Cell Res. 11, 107–117.

Chu, H.-M., Norris, D.M., Kok, L.T., 1970. Pupation requirement of the beetle, *Xyleborus ferrugineus*: sterols other than cholesterol. J. Insect Physiol. 16, 1379–1387.

Clark, A.J., Bloch, K., 1959. The absence of sterol synthesis in insects. J. Biol. Chem. 234, 2578–2582.

Clayton, R.B., 1964. The utilization of sterols by insects. J. Lipid Res. 3, 3–19.

Daum, G., Lees, N.D., Bard, M., Dickson, R., 1998. Biochemistry, cell biology, and molecular biology of lipid of *Saccharomyces cerevisiae*. Yeast 14, 1471–1510.

Eya, B.K., Kenny, P.T.M., Tamura, S.Y., Ohnishi, M., Naya, Y., Nakanishi, K., Sugiura, M., 1989. Chemical association in symbiosis. Sterol donors in planthoppers. J. Chem. Ecol. 15, 373–380.

Fryberg, M., Oehlschlager, A.C., Unrau, A.M., 1973. Biosynthesis of ergosterol in yeast. Evidence for multiple pathways. J. Am. Chem. Soc. 95, 5747–5757.

Grieneisen, M.L., 1994. Recent advances in our knowledge of ecdysteroid biosynthesis in insects and crustaceans. Insect Biochem. Mol. Biol. 24, 115–132.

Goulston, G., Mercer, E.I., 1969. Ergosta-5,7,24(28)-trien-3ß-ol, a new intermediate in ergosterol biosynthesis in *Phycomyces blakesleeanus*. Phytochemistry 8, 1945–1948.

Hongoh, Y., Sasaki, T., Ishikawa, H., 2000. Cloning sequence analysis and expression in *Escherichia coli* of the gene encoding a uricase from the yeast-like symbiont of the brown planthopper, *Nilaparvata lugens*. Insect Biochem. Mol. Biol. 30, 173–182.

Jones, K.G., Blackwell, M., 1996. Ribosomal DNA sequence analysis places the yeast-like genus Symbiotaphrina within filamentous ascomycetes. Mycologia 88, 212–218.

Jones, K.G., Dowd, P.F., Blackwell, M., 1999. Polyphyletic origins of yeast-like endocytobionts from anobiid and cerambycid beetles. Mycol. Res. 103, 543–546.

Jurzitza, G., 1979. The fungi symbiotic with anobiid beetles. In: Batra, L.R. (Ed.), Insect–Fungus Symbiosis. Allanheld, Osmun & Co., Montclair, pp. 65–76.

Kato, M., Wickner, W., 2001. Ergosterol is required for the Sec18/ATP-dependent priming step of homotypic vacuole fusion. EMBO J. 20, 4035–4040.

Kircher, H.W., 1982. Sterols and insects. In: Dupont, J. (Ed.), Cholesterol Systems in Insects and Animals. CRC Press, Boca Raton, FL, pp. 1–50.

Koolman, J., 1990. Ecdysteroids. Zool. Sci. 7, 563–580.

Kusumi, T., Suwa, Y., Kita, H., Nasu, S., 1979. Symbiotes of planthoppers: I. The isolation of intracellular symbiotes from the smaller brown planthopper, *Laodelphax striatellus* Fallen (Hemiptera: Delphacidae). Appl. Entomol. Zool. 14, 459–463.

Kusumi, T., Suwa, Y., Kita, H., Nasu, S., 1980. Properties of intracellular symbiotes from the smaller brown planthopper, *Laodelphax striatellus* Fallen (Hemiptera: Delphacidae). Appl. Entomol. Zool. 15, 129–134.

Lamb, D.C., Maspahy, S., Kelly, D.E., Manning, N.J., Geber, A., Bennett, J.E., Kelly, S.L., 1999. Purification, reconstitution, and inhibition of cytochrome P-450 sterol △22-desaturase from the pathogenic fungus *Candida glabrata*. Antimicrob. Agents Chemother. 43, 1725–1728.

Lees, N.D., Skaggs, B., Kirsch, D.R., Bard, M., 1995. Cloning of the late genes in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae* - a review. Lipids 30, 221–226.

Maurer, P., Debieu, D., Malosse, C., Leroux, P., Riba, G., 1992. Sterols and symbiosis in the leaf-cutting ant *Acromyrmex octospinosus* (Reich) (HymenopteraFormicidae: Attini). Arch. Insect Biochem. Physiol. 20, 13–21.

McMahon, A.P., 2000. More surprises in the hedgehog signaling pathway. Cell 100, 185–188.

Nasir, H., Noda, H., 2003. Yeast-like symbiotes as a sterol source in anobiid beetles (Coleoptera, Anobiidae): metabolic pathways from fungal sterols to 7-dehydrocholesterol. Arch. Insect Biochem. Physiol. 52, 175–182.

Nasu, S., Kusumi, T., Suwa, Y., Kita, H., 1981. Symbiotes of planthoppers: II. Isolation of intracellular symbiotic microorganisms from

the brown planthopper, *Nilaparvata lugens* Stal, and immunological comparison of the symbiotes associated with rice planthoppers (Hemiptera: Delphacidae). Appl. Entomol. Zool. 16, 88–93.

Noda, H., 1977. Histological and histochemical observation of intracellular yeastlike symbiotes in the fat body of the smaller brown planthopper, *Laodelphax striatellus* (Homoptera: Delphacidae). Appl. Entomol. Zool. 12, 134–141.

Noda, H., Wada, K., Saito, T., 1979. Sterols in *Laodelphax striatellus* with special reference to the intracellular yeastlike symbiotes as a sterol source. J. Insect Physiol. 25, 443–447.

Noda, H., Omura, T., 1992. Purification of yeast-like symbiotes of planthoppers. J. Invertebr. Pathol. 59, 104–105.

Noda, H., Kawahara, N., 1995. Electrophoretic karyotype of intracellular yeast-like symbiotes in rice planthoppers and anobiid beetles. J. Invertebr. Pathol. 65, 118–124.

Noda, H., Nakashima, N., Koizumi, M., 1995. Phylogenetic position of yeast-like symbiotes of rice planthoppers based on partial 18S rDNA sequences. Insect Biochem. Molec. Biol. 25, 639–646.

Noda, H., Kodama, K., 1996. Phylogenetic position of yeastlike endosymbionts of anobiid beetles. Appl. Environ. Microbiol. 62, 162–167.

Norris, D.M., Baker, J.M., Chu, H.M., 1969. Symbiotic interrelationships between microbes and ambrosia beetles. III. Ergosterol as the source of sterol to the insect. Ann. Entomol. Soc. Am. 62, 413–414.

Palermo, L.M., Leaks, F.W., Tove, S., Parks, L.W., 1997. Assessment of the essentiality of ERG genes late in ergosterol biosynthesis in *Saccharomyces cerevisiae*. Curr. Genet. 32, 93–99.

Parkin, C.A., Burnet, B., 1986. Growth arrest of *Drosophila melanogaster* on erg-2 and erg-6 sterol mutant strains of *Saccharomyces cerevisiae*. J. Insect Physiol. 32, 463–471.

Parks, L.W., Casey, W.M., 1995. Physiological implications of sterol biosynthesis in yeast. Ann. Rev. Microbiol. 49, 95–116.

Porter, J.A., Young, K.E., Beachy, P.A., 1996. Cholesterol modification of Hedgehog signaling proteins in animal development. Science 274, 255–259.

Robbins, W.E., Kaplanis, J.N., Svoboda, J.A., Thompson, M.J., 1971. Steroid metabolism in insects. Ann. Rev. Entomol. 16, 53–72.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., Ishikawa, H., 2000. Genome sequence of the endocellular bacterial symbiont of aphid *Buchnera* sp. APS. Nature 407, 81–86.

Skaggs, B.A., Alexander, J.F., Pierson, C.A., Schweitzer, K.S., Chun, K.T., Koegel, C., Barbuch, R., Bard, M., 1996. Cloning and characterization of the *Saccharomyces cerevisiae* C-22 sterol desaturase gene, encoding a second cytochrome P-450 involved in ergosterol biosynthesis. Gene 169, 105–109.

Suh, S.-O., Noda, H., Blackwell, M., 2001. Insect symbiosis: derivation of yeast-like endosymbionts within an entomopathogenic filamentous lineage. Mol. Biol. Evol. 18, 995–1000.

Svoboda, J.A., Kaplanis, J.N., Robbins, W.E., Thompson, M.J., 1975. Recent developments in insect steroid metabolism. Ann. Rev. Entomol. 20, 205–220.

Svoboda, J.A., Lusby, W.R., 1994. Variability of sterol utilization in stored-products insects. Experientia 50, 72–74.

Svoboda, J.A., 1999. Variability of metabolism and function of sterols in insects. Crit. Rev. Biochem. Mol. Biol. 34, 49–57.

Tamas, I., Klasson, L., Canback, B., Naslund, A.K., Eriksson, A., Wernegreen, J.J., Sandstrom, J.P., Moran, N.A., Andersson, S.G.E., 2002. 50 million years of genomic stasis in endosymbiotic bacteria. Science 296, 2376–2379.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL—X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25, 4876–4882.

Weete, J.D., 1973. Sterols of the fungi: distribution and biosynthesis. Phytochemistry 12, 1843–1864.

Weete, J.D., 1989. Structure and function of sterols in fungi. Adv. Lipid Res. 23, 115–167.

Wetzel, J.M., Ohnishi, M., Fujita, T., Nakanishi, K., Naya, Y., Noda, H., Sugiura, M., 1992. Diversity in steroidogenesis of symbiotic microorganisms from planthoppers. J. Chem. Ecol. 18, 2083–2094.