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Full Length Research Paper

Cloning and bioinformatics analysis of an ubiquitin gene of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae)

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Ubiquitin which has the function of selective protein degradation may play an important role in the regulation of insect growth and development. The coding sequence of an ubiquitin gene from the larvae of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) named *CsUB* (GenBank Accession No. GU238420) was cloned by RT-PCR and sequenced in this study, with primers according to the sequences of ubiquitin genes from *Homo sapiens*, *Drosophila melanogaster* and Lepidopteran insects. Sequence analysis showed that the length of the coding sequence is 228 bp, encoding 76 amino acids with calculated molecular weight of 8.50 kDa and the theoretical isoeletric point of 5.26. Signal sequence and transmembrane domain had not been found. Multiple sequence alignment indicated that *CsUB* gene sequence with other known gene sequences of invertebrates and vertebrates had a high degree of homology (more than 72% similarity) and a shorter genetic distance (lower than 0.360). During the genetic diversity analysis, the total of 104 polymorphic sites was detected from 18 ubiquitin gene sequences and 18 haplotypes were sorted. Abundant genetic diversity and strong codon usage bias were found by the haplotype diversity (1.000), average number of nucleotide differences (47.475), nucleotide diversity (0.20866), effective number of codons (44.526), codon bias index (0.559) and scaled Chi-square (0.779). The predicated secondary structure composition of CsUB protein had about 32.89% extended strands, 36.84% random colis, 15.79% alpha helixes and 14.47% beta turns. Subcellular localization analysis showed that CsUB protein of cytoplasm, cell nucleus, mitochondrion, cell skeleton and plasma membrane occupied about 47.80, 26.10, 17.40, 4.30 and 4.30%, respectively. Sequence, homology and structural analysis confirmed that *CsUB* gene was highly conserved during evolution and belonged to ubiquitin gene family. The results might provide some fundamental data for further studies on expressed characteristics and physiological functions of *CsUB* gene.

Key words: *Chilo suppressalis* Walker, ubiquitin, gene cloning, bioinformatics.

INTRODUCTION

Ubiquitin is a highly conserved 76 amino acid protein hich is widely distributed in eukaryotic cells and linked to a vast range of protein (Ciechanover, 1998; Goldstein TC, et al; Yamao, 1999). Based on sequencing of either cDNA lones or the protein, the amino acid sequence of ubiquitin proved to be identical in various species (Arribas et al., 1986; Bond and Schlesinger, 1985). In comparison with *Homo sapiens* sequence, there are only 1 to 5 amino acid substitutions among plants, animals, yeast and so on (Gill, 2004). Selective protein degradation is mainly carried out by the ubiquitin system which plays important roles in many cellular functions, including immune regulation, cell cycle control, signal transduction, transcriptional regulation, the nuclear transport process, membrane receptor control by endocytosis and so on

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(Bai J, et al; Hershko, et al; Pickart et al., 2004; Patterson, 2006). Ubiquitin also appear conjugated with certain nuclear, cytoplasmic and cell-surface protein without causing their degradation (Leung et al., 1987). For example, Mezquita et al. (1997) proposed that ubiquitin conjugation plays an essential role in spermatogenesis. Functional ubiquitin is produced from two different types of ubiquitin genes, named polyubiquitin and ubiquitin extension genes (Callis and Vierstra, 1989). Ubiquitin is functionally a very important protein which takes part in mediating intracellular ATP-dependent protein degradation by 26S proteasome (Vierestra, 1996). On the face of it, the authors think that the ubiquitin is useful to investigate the cell physiology of the agricultural important pests and is the main focus of research these years.

Although, many investigations on the coding sequences of ubiquitin gene in *Spodoptera frugiperda* (Guarion, 1990), *Manduca sexta* (Bishoff and Schwartz, 1990), *Drosophila melanogaster* (Barrio et al., 1994), *Spodoptera litura* (Li et al., 2003), *Blattella germanica* (Yu et al., 2004), *Spodoptera exigua* (Niu et al., 2004), *Helicoverpa armigera* (Li et al., 2005), *Bombyx mandarina* (Chen et al., 2007), *Musca domestica* (Jin et al., 2008) and *Haritalodes derogata* (Zhang et al., 2008) and so on have been conducted, there is no report on ubiquitin and ubiquitin gene in the larvae of the rice stem borer, *Chilo suppressalis* Walker. The rice stem borer is a major rice pest around the world, and its density is higher than the locally economic injury level (Zibaee et al., 2008). In China, it has a very broad range distribution from Heilongjiang to Hainan province, and overwinters as diapausing larvae (Shen and Xue, 1988).

Therefore, the main purpose of this study was: (1) to clone the cDNA of ubiquitin gene in the rice stem borer larvae (named *CsUb*) and (2) to predict its characterization and structure using bioinformatics analysis.

MATERIALS AND METHODS

The rice stems including the larvae of the rice stem borer, *C. suppressalis* Walker, were originally collected from the paddy fields in suburbs of Hefei City, China. The larvae were reared at 28°C on the fewflower wildrice, *Zizania latifolia*, as described by Zheng et al. (2009).

Total RNA isolation and RT-PCR

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA, USA) from the rice stem borer larvaes. RNA samples were prepared and stored at -70°C. RNA concentration and purity were assessed spectrophotometrically by measuring their absorbances at 260 and 280 nm in biophotometer (Eppendorf, Germany). 2 µg RNA was used as the template to synthesize first-strand cDNA. Based on multi-alignment of highly conserved sequences of ubiquitin sequences from various insects (*Ostrinia furnacalis*, *D. melanogaste*r, *H. armigera*, *B. mandanina*) available in the GenBank database, one set of degenerate primer was designed corresponding to the conserved sites of the ubiquitins, such as forward: 5'-ATGCARAT HTTYGTNAARAC-3', reverse: 5'-RCCACCNCGVAGNCKVARSAC-3'. Polymerase chain reactions (PCR) temperature profile was 94°C for 5 min followed by 33 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 1 min and a final extension step at 72°C for 10 min.

Cloning and nucleotide sequencing

The PCR products were then purified using the Cycle Pure kit (Omega Bio-Tek, Norcross, GA, USA) and were ligated into a pGEM-T vector (Promega, Madison, WI, USA) according to the manufacter's instructions. Afterwards, plasmids were transformed into competent *Escherichia coli* DH5α competent cells and then plated out on a carbenicillin-containing LB agar plate. After 15 h incubation, formed colonies were checked by colony PCR and several of these positive colonies were then purified using Plasmid mini prep kit (Omega Bio-tek) and sent to Shanghai BioAsia Biotech Company (China) for sequencing.

Bioinformatics analysis

Sequence data were performed using DNAMAN (version 5.2) and the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment was carried out using ClustalX (version1.83) software. Homology and genetic distances were calculated using DNAStar (version 5.01) and MEGA (version 3.1) software. The putative signal peptide was predicted using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). A calculated molecular weight, theoretical isoeletric point, negatively and positively charged residues, grand average of hydropathicity were predicted using ProtParm Tool (http://expasy.org/tools/protparam.html). The genetic diversity analysis was carried out using DnaSP software (version 4.0).The potential protein subcellular localization and transmembrane domains were predicted using PSORTIIPrediction (http://psort.hgc.jp/form2.html) and TMHMM Server (version 2.0) (http://www.cbs.dtu.dk/ services/TMHMM-2.0/), respectively. Three-dimensional structural modeling of CsUB protein was built by the homology-based method using the SWISS-MODEL program (http://swissmodel. expasy.org/). The template used for modeling was *Homo sapiens* ubiquitin. Model was displayed with the Swiss-Pdb Viewer (http://spdbv.vital-it.ch/).

RESULTS

Cloning and sequence analysis of *CsUB* gene

Based on the highly conserved sequences of the ubiquitins, the cDNA designated *CsUB* was isolated from the rice stem borer larvae. Cloning and sequence analysis of *CsUB,* cDNA (GenBank Accession No. GU238420) yielded a 228 bp sequence containing an initial ATG codon and a predicted protein of 76 amino acids (Figure 1), and a calculated molecular weight of 8.50 kDa, the theoretical isoeletric point of 5.26, negatively charged residues of 10, positively charged residues of 12, extinction coefficient of 1490, estimated half-life of 30 h, instability index of 30.86 and grand average of hydropathicity of -0.441. No signal sequence and transmembrane domain were identified in the transcript using the SignalP 3.0 Server and TMHMM 2.0 Server, respectively. In comparison with the length of the

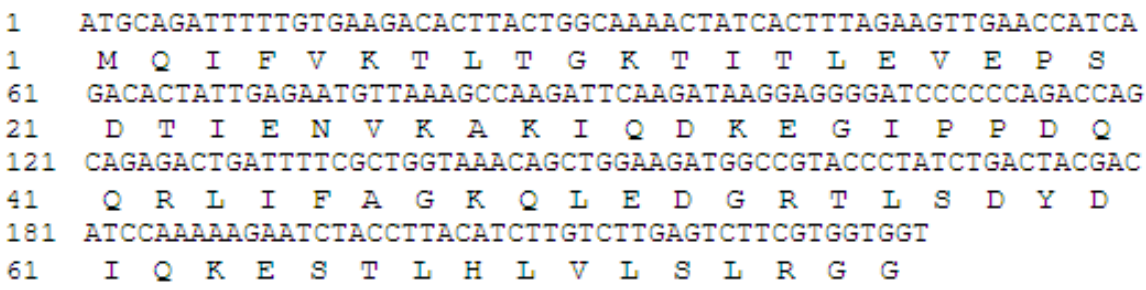

Figure 1. Nucleotide and the deduced amino acid sequence of the coding sequence of *CsUB* gene (GenBank Accession no.GU238420) using DNAMAN (version 5.2) software.

nucleotide and amino acid sequences of cloned *CsUB*, cDNA was in good agreement with earlier reported sizes of other ubiquitins.

Homology analysis of *CsUB* gene

We aligned *CsUB* gene sequence with other known gene sequences of invertebrates and vertebrates using DNAStar (version 5.01) and MEGA (version 3.1) software. The alignments displayed a high degree of homology (more than 72% similarity in all the matches), and a shorter genetic distances (lower than 0.360) (Table 1). Multiple sequence alignment suggested that *CsUB* gene was highly conserved during evolution and belonged to ubiquitin gene family.

One hundred and twenty four (124) monomorphic sites and 104 polymorphic sites were detected from 18 ubiquitin gene sequences by the genetic diversity analysis of DnaSP (version 4.0) software. Singleton variable sites and parsimory informative sites was 15 (amounting to 6.58%) and 89 (amounting to 39.4%), respectively. At the same time, 18 haplotypes were also sorted. Haplotype diversity, average number of nucleotide differences and nucleotide diversity was 1.000, 47.475 and 0.20866, respectively. According to calculation using the total number of mutations, there was no significance (P>0.10). Codon usage analysis showed that the effective number of codons, codon bias index and scaled Chi-square was 44.526, 0.559 and 0.779, respectively. Then a strong codon bias was found among 18 ubiquitin gene sequences. Multiple sequence alignment indicated that synonymous sites and nonsynonymous sites were 51.67 ~ 56.33 and 171.67 ~ 176.33, respectively (Table 2). Furthermore, we also found that nonsynonymous sites were three times as much as synonymous sites.

Structural analysis of CsUB protein

Phosphorylation sites of Ser and Thr occurred in CsUB amino acid residue 57 and 22 by NetPhos 2.0 Server, respectively. The predicated secondary structure composition of CsUB protein had about 32.89% extended strands, 36.84% random coils, 15.79% alpha helixes and 14.47% beta turns in a further study. Subcellular localization analysis demonstrated that CsUB protein of cytoplasm, cell nucleus, mitochondrion, cell skeleton and plasma membrane occupied about 47.80, 26.10, 17.40, 4.30 and 4.30%, respectively.

The three-dimensional structure of CsUB protein was built by the homology-based modeling, based on the structure of *H. sapiens* ubiquitin as template. The resolution based on the template and the E-value was 2.60 Å and 1.13e-32, respectively. The sequence similarity between the CsUB and *H. sapiens* ubiquitin homologue was about 97.33%, indicating that the target sequence was well compatible with the template (Figures 2 and 3). The overall folding pattern contained 3.5 alpha helix (aquamarine blue), 1 310-helix (buff), 5 beta foldings (navy blue, yellow, green, yellow green and sorrel) and 7 reverse angles (Arnold et al., 2006). Evaluation of atomic empirical mean force potential showed that only two amino acid residues of CsUB protein did not yield preferable result for closely related protein.

DISCUSSION

Ubiquitin is a small protein consisting of 76 amino acids that play a major role in both cellular stress response and protein degradation in eukaryotes (Masatoshi et al., 2000). In this study, the cDNA sequence of *CsUB* gene of the rice stem borer larvae was first cloned and reported. The nucleotide sequence was proven to be more than 72% similar to those of other known invertebrates and vertebrates (Table 1). The phenomenon was the same with the reports of Glickman and Ciechanover (2002) and Sharp and Li (1987). The primary structures of these ubiquitins among invertebrates and vertebrates had a high level of similarity and a shorter genetic distance. The result showed that *CsUB* gene was highly conserved during evolution and belonged to ubiquitin gene family. In addition, the very similar three-dimensional molecular modeling of the ubiquitins between the rice stem borer larvae and *H. sapiens* was also observed in *B. mandarina*

Table 1. Homology and genetic distances of 18 ubiquitin gene sequences among invertebrates and vertebrates using DNAStar (version 5.01) and Mega (version3.1) software.

Number	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18
01	***	78.9	74.1	81.1	81.6	72.8	79.4	79.4	78.5	75.4	79.4	81.1	76.3	79.8	75.9	77.6	77.6	79.8
02	0.257	***	77.6	81.1	79.4	77.2	78.5	78.9	75.9	71.9	78.5	77.2	81.1	78.1	76.3	80.7	79.8	77.6
03	0.325	0.270	***	78.9	78.5	72.4	78.1	74.1	76.3	75.9	77.6	74.1	74.6	77.2	75.4	77.6	77.6	77.6
04	0.224	0.221	0.250	***	82.9	76.8	77.6	78.1	77.6	75.0	80.7	82.9	81.1	78.5	77.6	78.9	79.4	79.8
05	0.218	0.247	0.258	0.198	***	79.4	82.9	83.8	82.0	76.8	81.1	81.1	82.0	82.5	80.3	79.4	78.5	82.9
06	0.351	0.282	0.354	0.284	0.242	***	79.8	80.3	78.5	75.4	78.1	76.8	78.5	74.1	74.1	72.4	76.3	76.3
07	0.251	0.262	0.264	0.271	0.197	0.237	***	84.6	81.1	81.6	79.8	80.7	82.5	81.6	83.3	78.5	81.1	80.3
08	0.251	0.261	0.325	0.266	0.184	0.230	0.175	***	83.8	78.9	83.8	83.8	82.0	85.5	82.9	79.4	82.0	82.9
09	0.258	0.304	0.287	0.270	0.206	0.258	0.221	0.184	***	82.9	80.7	80.3	81.6	77.6	79.8	75.0	80.3	77.6
10	0.301	0.359	0.295	0.308	0.280	0.300	0.213	0.248	0.195	***	81.6	78.1	78.1	74.6	76.3	72.4	76.3	72.8
11	0.250	0.265	0.270	0.227	0.220	0.264	0.239	0.185	0.224	0.211	***	78.5	78.9	80.7	75.9	75.4	78.9	75.4
12	0.225	0.282	0.322	0.197	0.223	0.285	0.231	0.186	0.233	0.262	0.256	***	82.5	79.8	80.3	82.9	81.6	80.3
13	0.295	0.223	0.315	0.220	0.208	0.258	0.202	0.209	0.216	0.264	0.252	0.203	***	80.7	81.6	79.8	81.1	79.4
14	0.241	0.274	0.276	0.262	0.204	0.329	0.218	0.163	0.273	0.316	0.228	0.244	0.231	***	84.2	82.9	79.8	79.4
15	0.302	0.296	0.301	0.274	0.234	0.323	0.191	0.198	0.241	0.290	0.299	0.235	0.216	0.180	***	82.9	83.3	82.5
16	0.272	0.232	0.270	0.252	0.246	0.358	0.260	0.250	0.309	0.351	0.309	0.196	0.237	0.199	0.196	***	84.6	80.7
17	0.272	0.244	0.268	0.245	0.259	0.292	0.221	0.210	0.232	0.286	0.252	0.215	0.221	0.242	0.191	0.176	***	82.0
18	0.240	0.275	0.272	0.241	0.199	0.292	0.232	0.198	0.268	0.344	0.309	0.233	0.246	0.247	0.202	0.226	0.210	***

*** Data above diagonal refers to homology, ***data below diagonal refers to genetic distance; 01 ~ 18 presents ubiquitin gene sequence of *Chilo suppressalis* (GenBank Accession no.GU238420, the others are the same), *Musca domestica* (DQ115796), *Bombyx mori* (AF308163), *Blattella germanica* (AY501003), *Ostrinia furnacalis* (DQ307065), *Cluex quinquefasciatus* (XM_001845204), *Trichoplusia ni* (AY267009), *Drosophila melanogaster* (M22428), *Homo sapiens* (NM_021009), *Bombyx mandanina* (DQ839401), *Aedes aegypti* (XM_001664217), *Periplaneta americana* (EF101563), *Helicoverpa armigera* (AY456195), *Spodoptera frugiperda* (M30306), *Spodoptera litura* (AF436066), *Plutella xylostella* (EU428779), *Haritalodes derogata* (EU580145) and *Monochamus alternatus* (EU433567), respectively.

(Zhang et al., 2008). Therefore, it could be concluded that all ubiquitin genes in various species might originate from the same ancestor's gene. Since all the ubiquitin genes of the organisms were so highly conserved, the authors thought that the ubiquitin protein might not be used as a phylogenetic marker for evolutionary clock. But some different relationships that appeared in this study could be due to the association with genetic differentiation to a certain extent of organisms exposed to environmental stress for a long time (Zhang et al., 2008; Li et al., 1998).

This strong sequence conservation suggested that the vast majority of amino acids made up ubiquitin were essential as apparently any mutation that had occurred over evolutionary history had been removed by natural selection (Glickman and Ciechanover, 2002). In this study, 104 polymorphic sites were detected and 18 haplotypes were sorted from 18 ubiquitin gene sequences by the genetic diversity analysis. The results indicated that these ubiquitin genes had a strongly genetic adaptability. For example, Jin et al. (2008) reported that ubiquitin antibody of *M. domestica* had a positive reaction with both ubiquitin fusion proteins of *M. domestica* and *S. litura*, and suggested that it retained the original immunogenicity. Abundant genetic diversity and strong codon usage bias were found by the haplotype diversity (1.000), average number of nucleotide differences (47.475), nucleotide diversity (0.20866), effective number of codons (44.526), codon bias index (0.559) and scaled Chi-square (0.779). Statistical analysis suggested that there was a single major trend in codon usage variation among the genes for encoding specific

Table 2. Synonymous sites and nonsynonymous sites of 18 ubiquitin gene sequences among invertebrates and vertebrates using DnaSP (version 4.0) software.

S/N	Species	Synonymous site	Nonsynonymous site
01	*C.suppressalis*	52.50	175.50
02	*M.domestica*	51.83	176.17
03	*B.mori*	52.33	175.67
04	*B.germanica*	52.67	175.33
05	*O.furnacalis*	53.17	174.83
06	*C.quinquefasciatus*	56.33	171.67
07	*T.ni*	54.17	173.83
08	*D.melanogaster*	54.00	174.00
09	*H.sapiens*	54.17	173.83
10	*B.mandarina*	51.67	176.33
11	*A.aegypti*	52.00	176.00
12	*P.americana*	54.17	173.83
13	*H.armigera*	53.83	174.17
14	*S.frugiperda*	54.17	173.83
15	*S.litura*	52.83	175.17
16	*P.xylostella*	52.67	175.33
17	*H.derogata*	52.67	175.33
18	*M.alternatus*	53.00	175.00

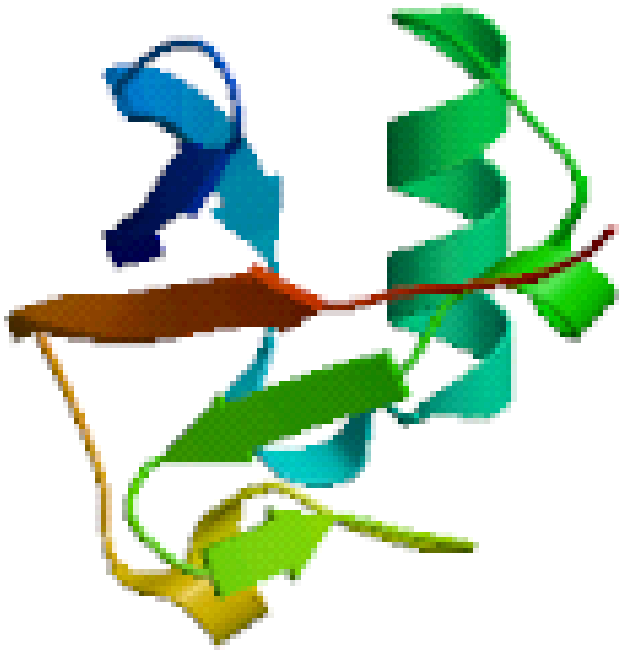

Figure 2. Theoretical three-dimensional-structure modeling of the deduced CsUB protein was based on the crytal structure of *H. sapiens* ubiquitin as template using SWISS-MODEL and WebLab viewer.

proteins (Ghosh et al., 2000). It was presumed that *CsUB* gene might be an important candidate marker gene of signal transduction during immune regulation of *C. suppressalis* larvae. But it also remained to be further studied. In addition, synonymous sites and non-synonymous sites were 51.67 ~ 56.33 and 171.67 ~ 176.33 among 18 ubiquitin gene sequences in this study, respectively (Table 2). And nonsynonymous sites were

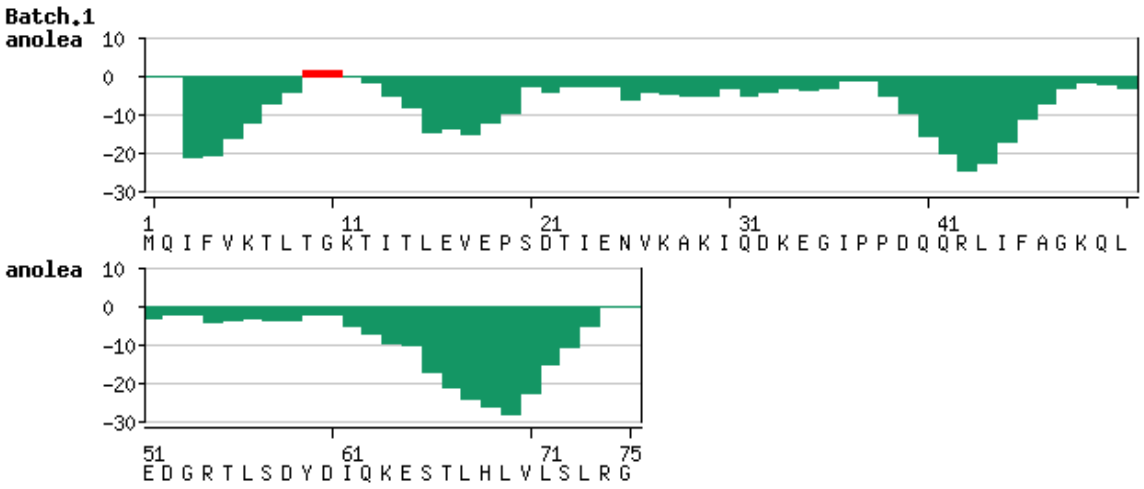

Figure 3. Evaluation of atomic empirical mean force potential.

three times as much as synonymous sites. Thus, we deduced that *CsUB* gene during molecular evolution was under positive selection according to Guo (1993) report.

Many reports had confirmed that ubiquitin gene expression was enriched in the midgut, fat body, malpighian tubule and flight muscle, and ubiquitin played a very important role in insect life activities (Barrio et al., 1994). Since the 1980s, ubiquitin studies have grown enormously and ubiquitin-dependent proteolysis degradation pathways have been shown to play major roles in a legion of biological processes (Varshavsky, 1997). It is well known that some abnormal protein degradation is an integral component of cell physiology. So far, little is known for its physiological functions in insect growth and development, such as insect molting, pupation and metamorphosis. In this study, although we find a novel ubiquitin gene cloned from the rice stem borer larvae, the gene expression in various growth conditions of the larvae and the underlying mechanisms need to be further researched to identify the precise biological properties in the various physiological processes.

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