Expression and Localization of Cry1Ac22 Crystal Protein from Bacillus thuringiensis W015-1 in Yeast (Saccharomyces cerevisiae)

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Bt Research 2010, Vol 1 No 3 DOI: 10.5376/bt.2010.01.0003
Received: 27 Aug., 2010
Accepted: 21 Dec., 2010
Published: 30 Dec., 2010

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Preferred citation for this article:
Liu et al., 2010, Expression and Localization of Cry1Ac22 Crystal Protein from Bacillus thuringiensis W015-1 in Yeast (Saccharomyces cerevisiae), Bt Research (online), Vol.1 No.3 (DOI: 10.5376/bt.2010.01.0003)

Abstract

In order to explore the expression and sub-cellular localization of Bt Cry1Ac22 insecticidal crystal protein in eukaryotic organism, we constructed eukaryotic expression vector pYES2-Cry1Ac22-GFP by fusing Cry1Ac22 and green fluorescent protein (GFP) based on the initiative pYES2 vector. The construct was transformed into yeast (Saccharomyces cerevisiae) strain INVSc1 to express the fusion gene promoted by GAL1. With the induction of 20% galactose, the fusion protein of Cry1Ac22-GFP was expressed as a mass of fluorescence particles in the yeast cell. SDS-PAGE analysis showed that the fusion proteins were expressed in the yeast crude succus about 130 kD in size. The fusion proteins can generate intense fluorescence light alone with the yeast cell membrane observed under the fluorescent microscope, which indicated that Cry1Ac22 proteins were anchored in the cell membrane. This research might provide the insights and approach for studying the function of Bt insecticidal proteins as well as for tracing the trail of transgenic proteins.

Keywords
Bt (Bacillus thuringiensis); Yeast (Saccharomyces cerevisiae); Cry1Ac22; Green fluorescent protein (GFP); Eukaryotic expression; Subcellular localization

Background

Bacillus thuringiensis (Bt) is a ubiquitous gram-positive bacterium, which produces parasporal crystal protein during sporulation that has insecticidal action (δ-endotoxin). It attracts increasing attention due to its insecticidal properties to agricultural pests (Li et al., 1991; Knowles and Ellar, 1986). Bt toxin Cry1Ac22 insecticidal crystal proteins are isolated from Bacillus thuringiensis W015-1 in the intestines of diapausing larvae of silkworm (Bombyx mori). It is known that Bt W015-1 has higher insecticidal activity than HD73 on some lepidopteran insects such as Clanis bilineata Walker, Helicoverpa armigera, Spodoptera litura, Plutella xylostella (Xie et al., 2010).

Cry1Ac22 insecticidal crystal protein, encoded by cry1Ac22 gene, is constituted by 1 178 amino acids. It is different from Cry1Ac1 protein (isolated from HD73 (Adang et al., 1985)) in amino acid sequence at sites of 233 (T/R), 448 (M/I) and 1158 (K/E) (Xie et al., 2010). cry1Ac22 gene can express 133 kD proteins in Escherichia coli, and the purified proteins show high insecticidal activity to Plutella xylostella (Xie et al., 2010; Liu et al., 2010). Taking into account that W015-1 was isolated from the intestines of diapausing larvae of silkworm and its cry gene shows apparent diversity with HD73, the strain and its insecticidal proteins could be used as candidate strain for integrated control and resistance
management of agricultural pests.

Figuring out the expression and localization of Cry1Ac22 in Eukaryotic cell is of great significance to the study regarding the expression of cry1Ac22 in higher plant. In this research, we constructed GFP-labeled Cry1Ac22 fusion protein by applying the GFP of *Aequorea victoria* as reporter molecules, and investigated preliminarily the expression and localization distribution of the fusion protein in yeast, expecting to provide theoretical guidance for the research of Bt transgenic plants utilizing cry1Ac22 gene.

1 Results and Analysis

1.1 Construction and identification of pGFP-Cry1Ac22 vector

The GFP gene used in this study is from mammalian expression vector pGFP. *Bam* II and *Kpn* I were inserted into the amphi of the linear vector through the enzyme digestion and ligation. pMD18-T-Cry1Ac22 was constructed with the primers designed according to the cry1Ac22 gene sequence, digested by *Bam* H I and *Kpn* I, and obtained a 2178 bp fragment (Figure 1B); pGFP vector is digested by the *Bam* H I and *Kpn* I and obtained a 3400 bp pGFP linear fragment (Figure 1A). The targeted fragments were recycled, ligated at 16°C, and then transformed into JM109 competent cells. Identification and screening of the positive clones were performed by *Bam* H I and *Kpn* I digestion (Figure 2). From figure 2, we can see that lane No. 1,2,3,4,5,6,7,9,10 are positive recombinants showing a 2178 bp cry1Ac22 target fragment and a 3400 bp pGFP vector fragment. The results indicated that the pGFP-Cry1Ac22 is constructed successfully.

1.2 Construction and identification of pYES2-Cry1Ac22-GFP yeast expression vector

Positive clones was digested by *Bam* H I and *Not* I and obtained a 3000 bp gene fragment, which is a fusion gene of cry1Ac22 gene and GFP gene (Figure 3). pYES2 vector was digested by BamHI and NotI and obtained a 5.9 kb pYES2 vector fragment (Figure 3). The targeted fragments were recycled, ligated at 16°C, and then transformed into JM109 competent cells. The recombinant was digested with *Bam* H I and *Not* I, and obtained a 3000 bp target gene fragment and a 5.9 kb pYES2 vector fragment (Figure 4). These results indicated that No. 1,2,3,4 were positive recombinants and the yeast expression vector pYES2–Cry1Ac22–EGFP was constructed successfully.
1.3 Transformation of pYES2-Cry1Ac22-GFP vector

In order to study the expression and localization of Cry1Ac22-GFP in Eukaryotic cell, transformation into yeast was carried out. Theoretically, GFP-Cry1Ac22 would be expressed in yeast after transformation. pYES2-Cry1Ac22-GFP vector was transformed into the Saccharomyces cerevisiae INVSc1 strains. Observation by fluorescence microscope shows that fluorescence was distributed in the form of puncta, which indicated that Cry1Ac22-GFP protein can be expressed in yeast cells (Figure 5).

1.4 Expression of Cry1Ac22-EGFP fusion gene in yeast

The recombinant yeast induced by galactose was collected at different times, and detected by SDS-PAGE (Figure 6). A new protein was detected in the yeast crude protein induced by the galactose, with a molecular weight of about 130 kD. However, no such protein was detected in the crude protein that is not induced by the galactose (lane one). The results primarily indicate that the recombinant pYES2-Cry1Ac22-GFP expressed Cry1Ac22-GFP fusion protein under the induction of galactose, and the expression was of high performance. The expression level tends to be stable as the elongation of induction.

1.5 Localization of cry1Ac22 gene in yeast

PYES2-Cry1Ac22-GFP vector was transformed into the yeast, induced by the galactose, and directly observed under confocal laser scanning microscopy. After induction for 12 h, 60% yeast cells are observed with green fluorescence under inversion fluorescence microscope. Fluorescent proteins were distributed in the form of dispersion in the whole yeast cell transformed with pYES2-GFP vector (Figure 7), while in the yeast transformed with pYES2-Cry1Ac22-GFP, fluorescent proteins were distributed in plasma membrane and cytoplasm, but not in nucleus. Domain I in the N-terminal of cry1Ac22 gene is constituted by a group of α helical bundle formed by six or seven amphiphilic α helixes surrounding a hydrophobic α helix. The hydrophobic part acts as the function domain of specific virulence and participates in the perforation of cell membrane. The result is in accordance with the localization prediction.

2 Discussion

PYES2 (5 900 bp) used in this study is a yeast-
Figure 7 Localization of Cry1Ac22 protein in yeast
Note: A,B,C,D represent yeast localization in different views; Cry1Ac22: pYES2 is ligated with Cry1Ac22-GFP fusion protein; GFP: The control (pYES2 is ligated with GFP protein); 1/4, 2/5, 3/6 indicates GFP, merged, and white conditions, respectively; The shortest scale is 5.0 μm

Escherichia coli multicopy shuttle plasmid. It is a secretion type expression vector controlled by T7 promoter, and can replicate autonomously in S. cerevisiae and E. coli. When multicloned was inserted to recombinant vector, it can be screened by the insertional inactivation of LacZ gene, which also contains resistance gene.

PYES2 contains yeast URA gene, and can act as the auxotroph screening tag. Taking the auxotroph saccharomyces cerevisiae as model organism, pYES2-Cry1Ac22-EGFP shuttle vector containing reporter gene and enhanced green fluorescent protein (EGFP) gene was constructed successfully (Misteli and Spector, 1997). The vector can replicate in the E. coli and express in the S. cerevisiae. It forms fusion protein in the same open reading fragment. EGFP proteins display green fluorescence under the excitation of ultraviolet light. And its ligation with target gene will not affect the expression of target protein. Besides, it could fuse with target gene and label the expression status of target gene.

After the transformation of recombinant plasmid into the auxotroph S. cerevisiae, the expression of yeast in different times was observed under fluorescence microscope. If the recombinant plasmid can replicate in the cell, the EGFP will be expressed displaying green fluorescence under the excitation of ultraviolet light. The result indicated that green fluorescence can be observed in the transformed yeast under fluorescence microscope, which tended to be most powerful after induction for 12 h. No fluorescence was detected in the control yeast.

In this study, we constructed the Cry1Ac22 fusion protein marked with green fluorescent protein (GFP), and investigate the expression and localization of this fusion protein in Saccharomyces cerevisiae. This study would provide the theoretical guidance for studying the function of Bt insecticidal proteins, and presents insights for the trail of tracing transgenes protein.
3 Materials and Method

3.1 Plasmid and Strains
Strains and plasmids used in this study were stored in Haide Institute of Tropical Agricultural Resources (HITAR) (Table 1).

Table 1 Strains and plasmids used in this research

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Characterization</th>
<th>Origin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMD18–T-Cry1Ac22</td>
<td>AmpR, pMD18–T carrying cry1Ac22 gene</td>
<td>This research</td>
<td>Xie et al., 2010</td>
</tr>
<tr>
<td>pGFP</td>
<td>AmpR, pUC ori, Plac, MCS</td>
<td>HITAR</td>
<td>Unpublished</td>
</tr>
<tr>
<td>pYES2</td>
<td>AmpR, GAL1 promoter, T7 promoter, MCS, CYC1 transcription terminator, URA3 gene F1 origin</td>
<td>HITAR</td>
<td>Unpublished</td>
</tr>
<tr>
<td>E. coli JM109</td>
<td>E. coli recA1 supE44 endA1 hsdR17 gyr A96 relA1 thiΔ(lac-proAB)</td>
<td>HITAR</td>
<td>Sambrook et al., 2002</td>
</tr>
<tr>
<td>INVScl yeast strains</td>
<td>Genotype: MATa his3Δ1 leu2 trp1–289 ura3–52 Phenotype: His-, Leu-, Trp-, Ura-</td>
<td>HITAR</td>
<td>Unpublished</td>
</tr>
</tbody>
</table>

3.2 Culture media
The culture media for yeast growth is SD: yeast nitrogen-containing bases 0.67%, glucose 2.00%, compounds derived from an-uracil amine acid 0.13%; The culture media for inducing the expression of GFP is SC (yeast nitrogen-containing bases 0.67%, Galactose 2.00%, compounds derives from an-uracil amine acid 0.13%). LB culture media was used for E. coli, according to Sambrook et al (2002).

3.3 Reagents and devices
Yeast nitrogen base without amino acids is from Difco. Restriction enzymes, Taq DNA polymerase, denatured characinlike Single-Stranded DNA, dNTP were bought from TaKaRa. Lysozyme, RNaseA, Recycling kit of DNA agarose gel, Amino Acids, DMSO, PEG3350, Raffinose, Galactose were bought from Amresco. Tris (pH 8.0) phenol, chloroform and such kind of reagents were national A.R. PCR instrument used in this study is ABI 9600. Perpendicular-plate-electrophoretic apparatus is products of USA Bio-Rad firm. Microscope Olympus BX50 is used for fluorescence observation.

3.4 The construction of recombinant plasmid pGFP–Cry1Ac22
The pGFP vector and pMD18–T-Cry1Ac22 vector were digested by BamH I and Kpn I. The products were separated by 1.0% agarose gel electrophoresis. The target fragments were recycled by DNA agarose gel recycling kit, and were ligated with vector at 16°C for overnight. 4 μL of the ligation products were added into E. coli JM109 competent cells (kept on ice). The tube was flicked slightly for uniform distribution, kept on ice for 30 min, then kept at 42°C for 90 s for thermal shock, and then add into 400 μL LB culture media, followed by Shaking culture at 37°C for 1h. Then 100 μL broth was spread on LB screening plate (containing 100 μg/mL ampicillin), and culture at 37°C for overnight. The colony plasmid was extracted using boiling method and the identification of positive clones was performed through enzyme digestion.

3.5 The construction of recombinant plasmid pYES2–Cry1Ac22–EGFP
The pGFP-Cry1Ac22 identified positive and pYES2 were digested by BamH I and Not I for 3 h at 37°C. Separated the products by 1.0% agarose gel electrophoresis, recycled the target fragments using DNA agarose gel recycling kit, and ligated the two target fragments with vector at 4°C for overnight. Insert the ligation products into E. coli JM109 competent cells, and choose the positive bacterial colony for expanding and extracting recombinant plasmid. The identification of positive clones was performed by enzyme digestion.
3.6 Transformation of pYES2-Cry1Ac22-EGFP into S. Cerevisiae

The Saccharomyces cerevisiae competent cells were obtained through the method of LiAc/SS2 DNA/PEG according to Invitrogen. Add 500 μL competent cells into 100 μL Buffer 1, mixed with 1–2 μg recombinant plasmids identified positive and 2 μL characinlike DNA, shake lightly. Then add 600 μL Buffer 2 and shaked for 30 min at 30°C. After that, add 70 μL DMSO and treated at 42°C for 15min. Centrifugated the solution for 10 s, add 300–500 μL TE Buffer to wash and precipitate for 30 min at 30°C, spread on SD-ura plate, and cultured for 3 days at 30°C. The bacterial colony become transformant recon; The bacterial colony was picked and observed under microscope for further identification.

3.7 The induced expression of recombinant plasmid in Saccharomyces Cerevisiae

The positive colony was vaccinated into SC-U fluid medium with 1% raffinose and cultured at 170 r/min and 30°C for overnight. The product was centrifuged by 12 000 r/min at 4°C for 5 min. The thallus were precipitated by SC-U fluid medium with 2% galactose until its OD 600 becomes 0.4, then cultured under the condition of 30°C,170 r/min. Collect galactose to culture bacterial liquid for 6 h, 12 h, 24 h, then grind it by liquid nitrogen. After that, collect supernatant fluid for SDS-PAGE, and identify it by 10% PAGE.

3.8 The localization of cry1Ac22 gene in yeast cell

The bacterial colony was transferred from plate into SC screening medium, cultured at 30°C for overnight. 50% of the solution was washed 3 times, then transferred to SC induction medium (20% galactose instead of glucose) and cultured for 20 h. Collect induced and non-induced thallus, wash 3 times, add 2% low-gelling temperature agarose, shake slightly, and placed on microscope slide pretreated at 50°C, and then placed in dark for 5 min, followed by observation under fluorescence microscope.

Authors’ contributions
Shenkui Liu and Zhuoming Liu design and execute this experiment. Youzhi Li participates the experiment design and data analysis; Xuanjun Fang is the person in charge of this project, conducting experiment design, data analysis, writing and modifying of the manuscript. All authors have read and approved the final manuscript.

Acknowledgements
This research is funded by the project of China National Bt Collection Initiative and National 863 plans (Project No. 2004AA211112). Authors appreciate Dr Xinxin Zhang from the ASNESC of Northeast Forestry University, Mr Wenfei Zhang and Liu Xie from HITAR for technological supports and helpful advice on the experiment. Thanks for peer reviewers for there useful advice and revising suggestion to this paper. And we mentioned some reagent suppliers and sequencing suppliers in this work, that doesn’t mean we would like to recommend or endorse their products and services.

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