Journal of Integrative Agriculture 2017, 16(7): 1601–1608

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RESEARCH ARTICLE

The codon-optimized capsid gene of duck circovirus can be highly expressed in yeast and self-assemble into virus-like particles

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Abstract

The capsid (Cap) protein, which is the only structural protein of duck circovirus (DuCV), is the most important antigen for the development of vaccines against DuCV and the virus's serological diagnostic methods. In order to use yeast expression system to produce a large quantities of DuCV Cap protein which is close to its natural form to display the antigen peptides perfectly, the Cap gene was optimized into the codon-optimized capsid (Opt-Cap) gene towards the preference of yeast firstly. Then, the genes of Cap and Opt-Cap were separately cloned into pPIC9K plasmid and transformed into *Picha pastoris* GS115. The strains that displayed the phenotype of Mut^+ and contained multiple inserts of expression cassette were selected from those colonies. After the induction expression, the secretory type of Cap protein, which was about 43 kDa, was best expressed under 0.5% (v/v) methanol and sorbitol induction. Compared with the Cap gene, the expression level of Opt-Cap gene was much higher. What's more, the purified Cap protein had a good reactivity to its specific polyclone antibody and DuCV-positive serum, and it was able to self-assemble into virus-like particles (VLPs). These VLPs, with a diameter of 15–20 nm and without a nucleic acid structure, showed a high level of similarity to DuCV particles in size and shape. All of the results demonstrated that, based on the codon-optimization, it is suitable to use the *P. pastoris* expression system to produce DuCV VLPs on a large scale. It is the first time that a large amounts of DuCV VLPs were produced successfully in *P. pastoris*, which might be particularly useful for the further studies of serological diagnosis and vaccines of DuCV.

Keywords: capsid gene, codon-optimization, duck circovirus, virus-like particles

1. Introduction

The duck circovirus (DuCV) is a member of genus *Circovirus* within the family *Circoviridae*. It can cause the suppression of immune systems in infected ducks and lead to feathering disorders, poor growth, multiple infections of other pathogens, and even death (Hattermann *et al*. 2003; Soike *et al*. 2004). Histopathological slides of the bursa of Fabricius

Received 8 November, 2016 Accepted 27 March, 2017
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doi: 10.1016/S2095-3119(16)61605-2

from the infected ducks often show lymphocyte depletion, necrosis, and histiocytosis (Soike *et al*. 2004). DuCV is not species specific and it can be transmitted horizontally and vertically (Liu *et al*. 2010; Li *et al*. 2014). DuCV infection has been widely reported from many countries including Germany (Hattermann *et al*. 2003), Hungary (Fringuelli *et al*. 2005), America (Banda *et al*. 2007), South Korea (Cha *et al*. 2013), Poland (Matczuk *et al*. 2015), and China (Chen *et al*. 2006; Wang *et al*. 2011) and the global duck breeding industry has suffered a huge economic loss due to it (Zhang *et al*. 2012). However, despite its widespread prevalence, our knowledge about the molecular biology, pathogenesis, and vaccine development of DuCV is largely limited due to the lack of a suitable cell lines for propagation. DuCV is an icosahedral and non-enveloped virus, with a diameter of 15–16 nm. Its genome is about 1.9 kb, single-standard but ambisense, which contains 3 major open reading frames (ORFs), named ORF1, ORF2 and ORF3 (Hattermann *et al*. 2003; Xiang *et al*. 2012), and can be divided into types 1 and 2, based on the sequence (Wang *et al*. 2011). ORF2 encodes the capsid (Cap) protein which is the main virulence-associated protein eliciting the host's immune response (Xiang *et al*. 2013). Based on the vaccine studies of other virus within the family *Circoviridae* (e.g., PCV_2), the virus-like particles (VLPs), which are self-assembled by Cap protein, have a wide application prospect (Bucarey *et al*. 2009; Liu *et al*. 2001). Many vaccines based on VLPs have been approved for preclinical studies or clinical use (Plummer and Manchester 2011), such as hepatitis B virus (Pumpens and Grens 2001) and human papillomavirus (Kwag *et al*. 2012). More importantly, VLPs have become an important platform for the research of virus structure-function relationship, because they have high similarity with the natural virus in the virus surface structure. For instance, it was reported that the VLPs of PCV_2 can be used in studies of cell death induction, virus adhesion and internalization, as a substitute for PCV_2 virus (Misinzo *et al*. 2005; Walia *et al*. 2014).

It is well known that each system has its own advantages and limitations to produce VLPs. For example, the N-truncated DuCV-Cap has been expressed in *Escherichia coli* (Liu *et al*. 2010), while the expression of full-length Cap gene has just been obtained successfully in baculovirus/insect cells (Xiang 2012), for this protein has a high proportion of arginine residues and rare amino acids to some expression systems (Heath *et al*. 2006; Liu *et al*. 2010). Although the bacterial expression is easy to provide high yields, the capability of correct protein folding and post-translational modification is very weak. The baculovirus/insect cell expression is effective for the formation of VLPs, but the procedure is time-consuming and relatively expensive. By contrast, the methylotrophic yeast *Pichia pastoris*, as an eukaryotic expression system, has the abilities of proper folding and chemical modification (Cereghino and Cregg 2000; Cregg *et al*. 2000). It can highly express foreign proteins and is easy to scale up. There are many kinds of VLPs self-assembling in yeast that enhances the recognition of expressed antigens to host cells and improves the immune response (Allnutt *et al*. 2007; Xia *et al*. 2007; Farnós *et al*. 2009). In this study, we optimized the Cap gene to produce a higher yield of Cap protein and analyzed the features of the produced VLPs. This is the first time to produce DuCV VLPs in *P. pastoris*.

2. Materials and methods

2.1. Sequences of the Cap and Opt-Cap genes

The Cap gene was amplified from DuCV GH01 (JX499186) isolates preserved in our laboratory. Then the Cap sequence was analyzed by an online program, Graphical Codon Usage Analyzer (http://gcua.schoedl.de), followed by codon-optimized on the bias of yeast and synthesized by Sangon Biotech (Shanghai, China), the product was named Opt-Cap gene. The primers of the Cap and Opt-Cap genes were listed as follows:

Cap F: 5′-CCG**GAATTC**ATGCGACGCAGCACCTATC-3′

Cap R: 5′-ACATTT**GCGGCCGC**CTAGAACCCGGT GAACTGACC-3′

Opt-Cap gene F: 5′-CCG**GAATTC**ATGAGAAGAAGCA CATACAGGAGAGC-3′

Opt-Cap gene R: 5′-ACATTT**GCGGCCGC**TTAAAAGC-CG GTGAATTGCCCA-3′

The underlined regions were *Eco*RI and *Not*I restriction enzyme cutting sites, which were designed for further cloning steps.

2.2. Construction of recombinant plasmids

The target genes were digested with *Eco*RI and *Not*I enzymes (TaKaRa, Japan), and cloned into the pPIC9K expression vector, which contained a signal sequence of α-factor mating secretion in yeast. The constructed plasmids, which were named pPIC9K-Cap and pPIC9K-Opt-Cap, were further verified by restriction fragment analysis and DNA sequencing.

2.3. Transformation and phenotypic screening of recombinant clones

In order to insert the recombinant plasmids into the yeast genome, the plasmids were linearized by *Sal*I enzyme, and purified with an ethanol precipitation method. The linearized plasmids were then used to transform into *P. pastoris*