

# Expression and Preliminary Functional Analysis of Rice Oryzacystatin-II Protein

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**Abstract:** Cystatins are inhibitors of cysteine proteinases that were found in a number of plants, and demonstrated to play a role in defense against pathogens and pests. Although it was reported that there are twelve cystatin genes present in rice (*Oryza sativa* L.), their expression pattern and biological functions still remain unclear. In this study, a gene, *oryzacystatin-II* (*OC-II*) encoding a cysteine proteinase inhibitor from rice was cloned, which can inhibit papain (cysteine proteinase) to some extent. The transcriptional analysis showed that *OC-II* was expressed in root, leaf and mature panicle. *OC-II* protein was expressed in rice leaves in normal growth condition and up to a higher level at 8h when rice leaves inoculated with incompatible *Xoo* strain. Perhaps, the induction of *OC-II* protein was involved in the defense mechanism.

**Keywords:** Rice, Cystatin, OC-II, XA21K, Binding

## 1. INTRODUCTION

Cystatins play crucial roles in regulation of normal physiological processes by inhibiting the potentially destructive activity of their target proteases belonging to the papain (C1) family, such as cysteine cathepsins [1]-[2]. Furthermore, it was found that some members of cystatins also have the activity in inhibiting the legumain (C13) family of enzymes [3]. Studies of the plant protease papain and human cysteine cathepsins were crucial for the discovery of the cystatins. Failures in biological mechanisms to controlling protease activities result in many diseases such as neurodegeneration, cardiovascular diseases, osteoporosis, arthritis, and cancer [4]-[6]. The isolation and characterization of the chicken egg-white protein inhibitor, which inhibits ficin, papain, cathepsins B and H, stimulated further studies of cysteine protease inhibitors. As a result, abundant information has been accumulated on the cystatin superfamily over the last two decades.

Cystatins not only have the capacity to regulate normal body processes and perhaps cause disease when down-regulated, but may also participate in the defense response against pests and pathogens [7]-[9]. It was reported that there are 12 cystatin genes in the rice genome (I to XII) [10]. Heterogeneous expression of oryzacystatin in other plants that possess cysteine proteinases may improve their resistance against viruses [11], nematodes [12] and coleopteran pests [13]. In barley, the recombinant cystatin Hv-CPI efficiently inhibited the growth of three phytopathogenic fungi as well as the saprotrophic fungus [14].

*Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a rice (*Oryza sativa*) pathogenic bacterium that has an extensive race

differentiation. *Xa21* is the first disease resistance gene cloned from rice, which encodes a receptor kinase and confers broad spectrum resistance against *Xoo* [15]-[16]. So it is intriguing to investigate whether *OC-II* is related with *Xa21* and involved in its interaction with *Xoo*. In this study, we reported the characterization of the recombinant *OC-II*, and analyzed their inhibitory properties and interactive relationship with *Xoo*, a result supporting their putative role in plant disease defense.

## 2. MATERIALS AND METHODS

### A. Plant Material

TP309 is a *Japonica* rice variety, which is susceptible to bacterial leaf blight, 4021 is a homozygous transgenic line contains bacterial leaf blight resistant gene *Xa21* [17].

### B. Methods

1) *Cloning of OC-II gene* Based on the sequence of rice *OC-II* gene, a pair of primers was designed using PrimerCE. The forward primer, which incorporated a *EcoR I* restriction site (underlined), 5' GGAATTCATGGCCGAGGAGGCGCAG 3' was used. 5' CCAAGCTTTTAGCGGTGGCGTCGTCGAGGGGC 3', which contains a *Hind III* site (underlined) was used as the reverse primer. Plasmid DNA isolated from rice cDNA library (A gift from Prof. Lihuang Zhu, The Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, China) was used as PCR template. Reactions consisted of 1 µg of plasmid DNA in a 50 µl reaction with 2.0 mM MgCl<sub>2</sub>, 50 pM each primer, 0.25 mM each dNTP, and 1 unit of *Taq* DNA polymerase (Takara). Reactions were carried out for 30 cycles under 94°C for 30s, 58°C for 1 min, and 72°C for 1 min. The amplification product was firstly cloned into the pET-30a (+) vector and then sequence verified.

2) *Purification of a recombinant OC-II protein expressed in E.coli* The plasmid was introduced into ER2566 strain of *E. coli*. Bacterial cells were harvested after 3 h of induction with IPTG (isopropyl β-D-thiogalactopyranoside) at 37°C. Recombinant proteins were purified by affinity chromatography using Ni-NTA bead (Qiagen). The amount and purity of recombinant proteins were detected by SDS-PAGE.

3) *Measurement of inhibitory activity* Inhibitory activity of the recombinant *OC-II* protein was examined against papain (EC3.4.22.2). The corresponding enzyme was pre-incubated at 37°C for 10 min with the *OC-II* fusion protein in 100mM sodium phosphate buffer, pH 6.0, containing 10mM EDTA, 10mM cysteine and 1mM

DTT. After addition of 2mM BANA ( $N_{\alpha}$ -benzoyl-DL-arginine- $\beta$ -naphthylamide) as substrate, and incubation at 37°C for 20min, the reaction was stopped with 2% HCl in ethanol and the color developed by adding 0.06%  $wv^{-1}$  *p*-dimethyl-aminocinnamaldehyde in ethanol. Absorbance was measured at 540nm after 20min at room temperature. The extent of inhibition was demonstrated in percentage of remaining protease activity as compared with control treatment.

4) *Transcriptional analysis of rice OC-II gene*  
 Based on searching for rice sequences deposited in the MPSS (<http://mpss.udel.edu/rice/>) and dbEST databases (<http://www.ncbi.nlm.nih.gov/projects/dbEST/>), the transcriptional pattern of rice *OC-II* was examined in the tissues of root, stem, leaf, panicle and seed respectively.

5) *Xoo inoculation* Rice varieties TP309 and 4021 were cultivated in the field. The leaves of six-week-old plants were challenged with *Xoo*. *Xoo* was cultured in PSA (0.5% bactopectone, 2% sucrose, 0.05% L-glutamic acid) at 30°C for 48-72 h. *Xoo* was diluted to 10<sup>9</sup> cells per milliliter using distilled water. Rice leaves were inoculated with sterilized scissors dipped from the bacteria, rice leaf cut with sterilized water were used as mock control (M). Samples were collected at 0h, 2h, 8h, 1d, 2d, 3d, 5d, 7d and 10d after inoculation (about 1-2 cm), the materials were frozen by liquid nitrogen and stored in -70°C freezer.

6) *Polyclonal antibody generation* Synthetic polypeptide was conjugated with KLH and used as antigen to generate antibody. To generate OC-II protein-specific antibody, specific epitope in the rice whole proteome was selected using PepDesign software. The generation of polyclonal antibody was carried out by Beijing Protein Innovation Co., Ltd., Beijing, China, with KLH-conjugated synthetic polypeptide as immunogen.

7) *Protein extraction and WB analysis* Frozen rice leaves were ground to fine powder in pre-cooled mortar, then the powder was transferred to pre-cooled eppendorf centrifuge tubes. Extraction buffer (62.5 mmol L<sup>-1</sup> Tris, pH7.4, 10% glycerol, 2% SDS, 1 mmol L<sup>-1</sup> PMSF, 2 mmol L<sup>-1</sup> EDTA and 5%  $\beta$ -mercaptoethanol) were added by 1:2  $wv^{-1}$  ratio (sample: buffer) and mixed thoroughly. The tubes were placed on ice for 10 min and vortexed for every 2 min. The supernatant were recovered by centrifugation for 30 min at 10,000 rpm at 4°C to collect total proteins. The total proteins were separated by SDS-PAGE and transferred to PVDF membrane. WB analysis were carried out according to the protocol published previously. The WB signal generated by anti-heat shock protein (HSP) were used as equal loading control.

8) *WB data collection* Image J software were used to collect signals from scanned  $\chi$ -ray films. The average and standard derivations were calculated from at least three repeats, and their relative signals were compared.

### 3. RESULTS

#### 1) Cloning of OC-II gene from rice

According to the reported sequence [18], a truncated

form of OC-II gene, without the predicted N-terminal signal peptide, was amplified by PCR. Sequence analysis revealed that the amino acid sequence encoded by the amplified fragment is identical with the sequence from rice genome database, however, the sequence is different from that reported by Kondo in about 10 amino acids. We speculated that the difference among OC-II sequence might be due to different rice varieties was used.

#### 2) Expression of OC-II protein in vitro

The recombinant OC-II fused with a 6xhistidine tag was purified as shown in Figure 1. In the un-induced bacterial lysate, the induction of OC-II was observed (Lane U). When bacteria was induced for 3h by IPTG, the target protein was found in supernatant (Lane S) and pellet (Lane P). The recombinant protein was purified to homogeneity from the supernatant (Lane OC-II). The apparent molecular weight of OC-II is about 22.1kD (OC-II 16.6kD + tag 5.5kD = 22.1kD). The quantity of induced protein expression is approximately 1.8 $\mu$ g ml<sup>-1</sup> bacteria.

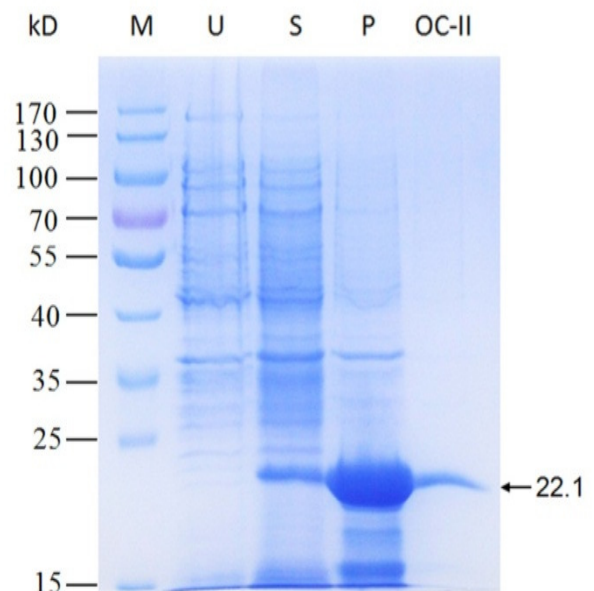


Fig.1. Recombinant OC-II Protein Expressed In *E. Coli* Was Separated By SDS-PAGE.

M: Molecular weight marker; U: Un-induced bacterial lysate; S: Supernatant of bacterial lysate from *E. coli* induced by IPTG; P: Pellet of bacterial lysate from *E. coli* induced by IPTG; OC-II: Recombinant oryzacystatin-II protein purified by affinity column.

#### 3) Inhibitory activity of OC-II against plant cysteine proteinase (papain)

The inhibitory activity of recombinant OC-II cystatin was assayed against plant cysteine proteinase (papain). In the experiments, the commercial inhibitor cystatin from chicken egg white (CEW) was used as a positive control and BSA was used as a negative control. The results indicated that the remaining activity (%) obtained from OC-II against papain was lower than BSA. The activity of papain can be inhibited completely by CEW when its concentration reaches 40 $\mu$ g ml<sup>-1</sup> (Figure 2).

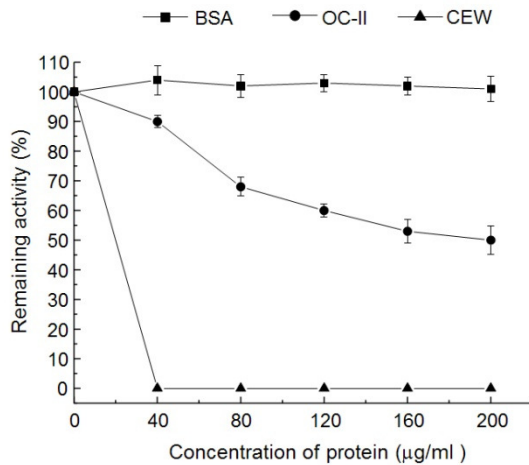


Fig.2 Inhibitory activity of the recombinant OC-II protein.

Inhibitory activity of purified recombinant OC-II protein was measured by pre-incubation with papain (EC3.4.22.2). CEW, cystatin protein isolated from chicken egg white. BSA, Bovine serum albumin.

X-axis designates concentration of proteins; Y-axis designates the remaining activity of papain.

#### 4) Transcriptional analysis of OC-II gene

We performed mRNA profiling using Massively Parallel Signature Sequencing (MPSS) and available Expressed Sequence Tags (ESTs) data. From Figure 3, it was observed that *OC-II* gene was constitutively transcribed in root, leaf and mature panicle, while in young panicle, only a weak expression was detected. Notably, it was found that in the three tissues (root, leaf and mature panicle), the expression of OC-II showed a similar pattern between MPSS and ESTs data. However, the expression pattern in rice stem was differing from each other (Figure 3).

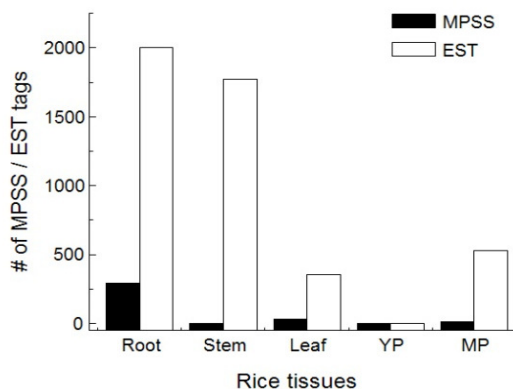


Fig.3 Transcriptional comparison of OC-II among different rice tissues.

YP: Young panicle; MP: Mature panicle X-axis designates different rice tissues; Y-axis designates the number of OC-II transcripts per 10 million MPSS and EST tags.

#### 5) Expression pattern of OC-II proteins in rice-Xoo interactions

To reveal the expression pattern of OC-II proteins in

rice-Xoo interactions, *Xoo* were used to inoculate 4021 (incompatible interaction) plants, water-inoculated TP309 were used as mock control (Figure 4). As shown in Figure 4, OC-II showed non-inducible expression in mock samples, illustrating it may be involved in the process of rice normal growth and development. OC-II protein was up to a higher level at 8 h when rice leaves inoculated with incompatible *Xoo* strain. Perhaps, the induction of OC-II protein was involved in the defense mechanism.

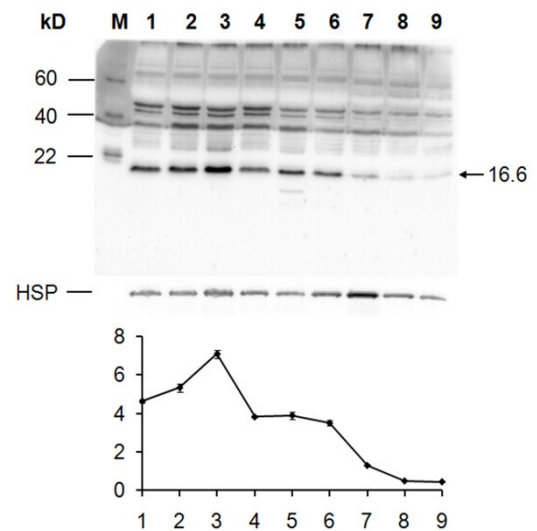


Fig.4 The expression profiling of OC-II proteins in the incompatible interactions between rice and *Xoo*

Rice leaf samples were collected at several time points after inoculation of *Xoo*. 1-9: 0h, 2h, 8h, 1d, 2d, 3d, 5d, 7d and 10d, respectively.

## 4. DISCUSSIONS

The oryzacystatin-II (OC-II) gene was cloned in this study, which shares a high sequence similarity to those of other 11 cystatins, especially to that of OC-I [10]. We further transformed OC-II into *E.coli*, and the expressed clone was used to study its biochemical properties. Previous researches found that each member of the cystatin superfamily has distinct inhibitory capability in barley cysteine-proteases in vitro [19]-[20]. Here, we demonstrated that the recombinant protein is an efficient inhibitor of papain, although the inhibition capacity of OC-II is lower than that of OC-I [21], indicating that OC-II and OC-I may perform different physiological roles in regulation of rice cysteine proteinases. It was proposed that cystatins are located in different cell components, and act on their target substrates specifically.

It was reported that there are seven cystatin genes present in barley. Among them, the barley cystatin (Hv-CPI) was expressed in embryos, developing endosperms, leaves and roots as assessed by northern blot analysis. Western blotting analysis showed that Hv-CPI was expressed in the tissues of endosperm, roots and leaves [22]. Based on bioinformatics analysis using the in silico data of MPSS and ESTs, we found that OC-II was

expressed in root, leaf and mature panicle, but weakly expressed in younger panicle. And we detected the protein expression pattern in leaf tissue of rice by western blotting. The antibody-based rice proteomics (AbRP) strategy could study the target protein [23]. Using the antibody of OC-II, tissue expression pattern of the protein in root, stem, leaf, young panicle, and mature panicle can be investigated. As well, it should be clear whether the protein OC-II will be significantly induced after the invasion of Xoo. These problems deserve to be further explored.

The rice Xa21 acts as a resistance gene, which encodes a protein with predicted leucine-rich repeat (LRR), transmembrane, juxtamembrane, and intracellular kinase domains, conferred resistance to diverse strains of the Gram-negative bacterium Xoo[15]. OC-II belongs to pathogenesis-related gene family 6 (PR6), namely protease inhibitor class in rice genome [24]. Almost all of the changes in PR protein expression appeared at late stages after inoculation [25]. Perhaps the increasing of OC-II expression is passive when rice suffered Xooabotic attack. The detailed physiological function of OC-II remains to be further research.

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 Mapping and molecular cloning of the PCD-related gene in rice
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