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Molecular Cloning and Characterization of Oryzacystatin-III, a Novel Member of Phytocystatin in Rice (*Oryza sativa* L. *japonica*)

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On the basis of cDNA sequences, we found that the calli of rice encodes an amino acid sequence that shares 56% and 89% identity, respectively, with oryzacystatin-I and oryzacystatin-II. This sequence differs from that of oryzacystatin-II in the N-terminal region ($Gln^{7}-Ala^{19}$ in the oryzacystatin-III numbering), and this region contained a glycine residue (Gly^{14}), which is evolutionarily conserved in the cystatin superfamily. We named this novel protein oryzacystatin-III. Nucleotide sequencing of the 5′-flanking region of the oryzacystatin-III gene showed that it is highly homologous to the oryzacystatin-II gene but distinct from the oryzacystatin-II locus. Oryzacystatin-III inhibited papain, ficin, and human cathepsin B. The inhibition constants for papain and ficin differ from those of oryzacystatin-I and -II, and cathepsin B activity is affected only by oryzacystatin-III, showing differences in the interaction of these inhibitors with enzymes. These data suggest that the above three inhibitors may play unique physiological roles in the regulations of rice cysteine proteinases.

KEYWORDS: Oryzacystatin-III; phytocystatin; cysteine proteinase inhibitor; rice

INTRODUCTION

Phytocystatins that belong to the cystatin superfamily are proteinaceous inhibitors of cysteine proteinase that are found in plants (*1*). Phytocystatins have been found in a variety of plant species; thus, oryzacystatins (-I and -II) have been found in rice (*2*) and muticystatins have been identified in sunflower seeds (*3*), potatoes (*4*), and tomatoes (*5*). While their primary structure shows a high degree of homology with the cystatins found in animals (*6*), they resemble stefins by virtue of the absence of disulfide bonds and cysteine residues (*1*). Phytocystatins are structurally different from the groups of plant cysteine proteinases inhibitors including soybean trypsin inhibitor-like inhibitors of cysteine proteases that are found in potatoes (*7*), the inhibitors of cysteine proteinases that are present in pineapples (*8*, *9*), and clitocypin, which resembles a lectin-like family of proteins found in the mushroom, *Clitocybe nebularis* (*10*).

The best-characterized group of phytocystatins is the oryzacystatins found in rice (*Oryza sativa* L. *japonica*). To date, two kinds of oryzacystatin (OC), OCs-I and -II, have been identified in *O. sativa* L. *japonica* cultivar Nipponbare, which have been well characterized with regard to their gene structure and organization (*11*, *12*), enzyme inhibition (*13*−*15*), molecular structure (*16*), biological controls (*2*), and gene expression (*15*, *17*). OCs-I and -II, which coexist in the seeds, are distinct from each other in two important respects: (1) their specificities for cysteine proteinases and (2) their mRNA expression patterns during the seed maturation (*15*, *17*). These differences suggest that they have somewhat different functions in the seeds, further supporting the notion that they play important roles in rice physiology. However, it is still unclear as to whether OCs-I and -II play a physiological role in rice tissues other than the seeds and whether other cystatins exist in rice.

To delineate the roles of phytocystatins in the early stage of rice development, we have initiated analysis of the cDNA for OCs from the calli of the rice cultivar Koshihikari, the representative cultivar for food staple in Japan, and discovered a third, novel member of the OC family, which we named OC-III. We also produced and characterized recombinant (r-) OCs-I, -II, and -III and indicated that OC-III exhibited properties that were different from OCs-I and -II.

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Table 1. Primers Used in This Study

Primer	Nucleotide sequence (5'-3')
Isolation of cDNA	
OC1.1	ATGTCGAGCGACGGAGGGCC
OC1.2	GATGGGCCTTAGGCATTTGC
OC2.1	ATGGCCGAGGAGGCGCAGAG
OC2.2	CTATGTACGTTTAGGCGGTG
Isolation of genomic gene for OC-III	
Geno1	TCCAACTCCAACATCGCGTTGGCCTTGCTGTTGTG
Geno2	TGGGCGGGTTCATGCACTACCTCACCGTCGAGGTG
Geno3	ATGGCCGAGGAGGCGCAGCA
Geno4	TGTTTTACGTTTAGGCGGTG
Construction of expression plasmids	
GST-OC1.1	ATCGAAGGTCGTATGTCGAGCGACGGAGGGCC
GST-OC1.2	GATGGGCCTTAGGCATTTGC
GST-OC2.1	GGATCCATCGAAGGTCGTATGGCCGAGGAGGCGCAGAGCCACGCG CGTGAAGGTGGGCGGCATCCACGACAGCCGGCCGGGCGCGAGA
GST-OC2.2	TTAGGCGGTGGCGTCGTCGAGGGGCTTGAAATCC
GST-OC3.1	GGATCCATCGAAGGTCGTATGGCCGAGGAGGCGCAGCA
GST-OC3.2	TTAGGCGGTGGCGTCGTCGAGGGGCTTGAAATCC

MATERIALS AND METHODS

Plant Materials. Seed-derived calli were induced by culturing surface-sterilized seeds of the rice cultivar Koshihikari, *Oryza sativa* L. *japonica* using DKN medium (*18*) containing 2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/L of sucrose, and 8 g/L of agarose type I (Sigma-Aldrich, St. Louis, MO) (pH 5.8) at 25 °C in the dark. Calli produced after 1 month were proliferated in liquid DKN medium (pH 5.8) supplemented with 1 mg/L of 2,4-D and 30 g/L of sucrose on a gyratory shaker (90 rpm) at 25 °C under continuous illumination (300 lx). The cultures were maintained for 1 month by subculturing the calli every 7 days to the same fresh medium and by breaking them into small pieces every two weeks using a spoon and stainless steel mesh (20 mesh count per inch) (*19*).

Isolation of cDNA Clones for OC-I and OC-III. Total RNA was isolated from seed-derived calli of rice with ISOGEN (Nippon Gene, Tokyo, Japan). DNA fragments specific to the cDNAs for OCs were amplified by PCR. The cDNAs for OC-I and OC-III were amplified with TaKaRa One Step RNA PCR Kit (Takara Bio Inc., Otsu, Japan) by using the total RNA as the template and the primers shown in **Table 1**. OC1.1 and OC1.2 were designed from the published sequence of OC-I cDNA (*17*). OC2.1 and OC2.2 were designed from the published sequence of OC-II cDNA (*15*). The PCR products were subcloned into the pCR2.1 vector using a TA cloning kit (Invitrogen Co., Carlsbad, CA).

Isolation of Genomic Clone for OC-III. Total genomic DNA was isolated from seed-derived calli of rice with DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA). The 5′- and 3′-flanking regions of the OC-III gene were isolated by inverse PCR (*20*, *21*). The genomic DNA was digested with *Pst*I and ligated to generate circular molecules, the latter of which were used as templates with the primers (Geno1 and Geno2; **Table 1**) to amplify the 5′- and 3′-flanking regions by PCR with TaKaRa LA Taq (Takara Bio Inc.). The PCR products were subcloned into the pCR2.1 vector using TA cloning kit (Invitrogen Co.). After the 5′- and 3′-flanking regions were sequenced, a complete genomic clone of the OC-III gene was isolated by PCR with TaKaRa LA Taq and a primer set, Geno3 and Geno4 (**Table 1**). The PCR products were then subcloned into pCR2.1 vector using TA cloning kit (Invitrogen Co.).

Sequencing. Nucleotide sequences were determined from both directions using the dideoxynucleotide chain-termination method (*22*) with an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, CA). The N-terminal amino acid sequences of the OCs were determined with a PPSQ-21 protein sequencer (Shimadzu Co., Kyoto, Japan).

Construction of Plasmids for Expression of OC-I and OC-III as Fusion Proteins. Plasmids were constructed to express OC-I and -III as fusion proteins consisting of glutathione-S-transferase (GST) sequence and a cleavage site recognized by factor Xa between the COOH terminus of GST and the N-terminus of the OCs (**Figure 1**). The factor Xa recognition site (Ile-Glu-Gly-Arg) was directly attached to the methionine initiation codon for translation of OC-I/-III cDNA by PCR using the cloned cDNA as the template and primer sets GST-OC1.1/GST-OC1.2 for OC-I and GST-OC3.1/GST-OC3.2 for OC-III (**Table 1**). The GST-OC3.1 primer was designed to include a *Bam*HI restriction site in addition to a factor Xa recognition site. The products of PCR were subcloned into the pCR2.1 vector using a TA cloning kit (Invitrogen Co.). For OC-I, the plasmid was digested with *Eco*RI, and the OC-I cDNA attached to a factor Xa recognition site was inserted into the pGEX-4T-2 plasmid (Amersham Biosciences Co., Piscataway, NJ) at the *Eco*RI restriction site downstream from the GST coding sequence. For OC-III, the plasmid was digested with *Bam*HI and *Xho*I, and OC-III cDNA attached to a factor Xa recognition site was inserted between the *Bam*HI and *Xho*I restriction sites downstream from the GST coding sequence of the pGEX-6P-2 plasmid (Amersham Biosciences Co.).

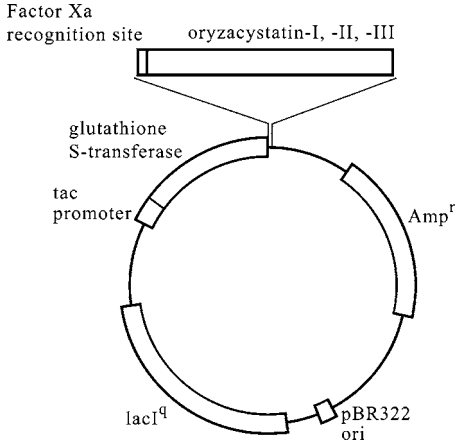

Figure 1. Schematic diagram of the plasmids used to express recombinant oryzacystatins.

Construction of a Plasmid Encoding OC-II for the Expression as a GST Fusion Protein. We synthesized the OC-II cDNA (*15*) with a *Bam*HI restriction site and a factor Xa recognition site directly before the start signal for translation by PCR using the cloned OC-III cDNA as the template and the primers GST-OC2.1 and GST-OC2.2 (**Table 1**). The amplified fragment was subcloned into the pCR2.1 vector using a TA cloning kit (Invitrogen Co.). The plasmid was digested with *Bam*HI and *Xho*I, and the OC-II cDNA attached to a factor Xa

Figure 2. Comparison of the nucleotide sequences encoding oryzacystatin-III (OC-III) and oryzacystatin-II (OC-II). Sequences are numbered from the translation start point of oryzacystatin-III, and gaps were introduced to obtain maximum similarity. The nucleotides of oryzacystatin-II cDNA that are identical to those of oryzacystatin-III cDNA are shaded. This figure was prepared using the Javashade program (http://industry.ebi.ac.uk/JavaShade/).

recognition site was inserted between the *Bam*HI and *Xho*I restriction sites downstream from the GST coding sequence of pGEX-6P-2 (Amersham Biosciences Co.). **Figure 1** illustrates the expression vector for the r-OCs.

Preparation of r-OCs from GST-OC Fusions Expressed in *Escherichia coli*. *E. coli* strain BL21 (Amersham Biosciences Co.) was used to express the GST-OC fusion proteins. BL21 cells harboring the plasmids were cultured at 37 °C in LB-broth containing 100 μg/mL ampicillin with reciprocal shaking (200 cycles/min). When the OD_{600} reached ca. 0.8−0.9, isopropyl-1-thio-β-D-galactoside was added to the medium to a final concentration of 1.0 mM to induce the expression of the fusion proteins. After an additional cultivation for 6 h, the cells were harvested by centrifugation and resuspended in PBS (pH 7.3) containing 1% Triton X-100. The suspended cells were disrupted with a sonicator (INSONATOR, model 201M, Kubota Co., Tokyo, Japan) and centrifuged at 15 000 rpm for 15 min at 4 °C. The clarified cell extract was applied to a column of GSTrap HP (Amersham Biosciences Co.), and GST-OC fusion proteins were eluted with 50 mM Tris-HCl containing 10 mM reduced glutathione (pH 8.0). The pooled fractions containing the fusion proteins were dialyzed against 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 5 mM $CaCl_2$. After dialysis, factor Xa (Novagen, Madison, WI) was added to a final concentration of 5 U/mL and incubated for 2−3 h at 25 °C. The conditions for the cleavage were determined by small-scale optimization. The protein solution digested with factor Xa was passed through a column of HiTrap benzamidine FF (Amersham Biosciences Co.) to remove factor Xa, after which it was applied to a column of GSTrap HP after dialysis against 20 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl. The fractions containing the r-OCs were pooled and concentrated by ultrafiltration (Centriplus YM-3; Millipore, Billerica, MA). Purified r-OCs were stored at −20 °C prior to use.

Analyses. Protein concentration was determined using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA), which is based on the method of Lowry et al. (*23*), using bovine serum albumin as the standard. Denaturating polyacrylamide gel electrophoresis (PAGE) was performed with NuPAGE Novex 4−12% Bis-Tris gel using 2-(*N*-morpholino)ethanesulfonate (MES) as the running buffer (Invitrogen Co.), according to the manufacturer's instructions. The proteins were stained with Coomassie Brilliant Blue R-250, and Mark12 unstained standard (Invitrogen Co.) was used as the electrophoresis marker. The molecular mass of OC-III and its truncated form were determined by Shimadzu Biotech (Tsukuba, Japan) with AXIMA-CFR matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Shimadzu Co.) using sinapinic acid as the matrix.

Enzyme Inhibition Assay and Determination of Inhibition Constant (K_i). Enzyme activity was measured by using fluorogenic substrates purchased from Peptide Institute Inc., Osaka, Japan. Ficin (0.23 nM; Sigma-Aldrich), papain (0.14 nM; Sigma-Aldrich), cathepsin B (0.27 nM from human liver; Calbiochem, San Diego, CA), and cathepsin K (11.6 nM, His-tag, human, recombinant; Calbiochem) were preincubated for 5 min at 40 °C with various concentrations of the r-OCs in a total volume of 2.0 mL of assay buffer. The assay buffer consisted of 0.1 M sodium phosphate buffer (pH 6.8), 1 mM EDTA, 4 mM dithiothreitol (DTT), and 0.05% Briji35 for ficin and papain and 0.1 M MES (pH 6.0), 1 mM EDTA, 4 mM DTT, and 0.05% Briji35 for cathepsin B and cathepsin K. After preincubation, the fluorogenic substrate was added to the reaction mixture at the final concentration of 25−50 μM, after which it was incubated for 1−5 min at 40 °C. Carbobenzoxy (*Z*)-L-Phe-L-Arg-4-methyl-coumaryl-7-amide (MCA) was used as the substrate for ficin, papain, and cathepsin K, and (*Z*)-L-Arg-L-Arg-MCA was used for cathepsin B. The release of 7-amino-4-methyl-coumarin (AMC) was monitored with a thermostated RF-5300PC spectrofluorophotometer (excitation 380 nm; emission 440 nm; Shimadzu Co.). Active concentrations of papain and ficin were determined by titration with E-64 (L-*trans*-epoxysuccinylleucylamido [4-guanidino] butane) (Peptide Institute Inc.) using (*Z*)-Phe-Arg-MCA as the substrate according to the method of Barrett and Kirschke (*24*). K_i values were calculated from residual enzyme activities using Dixon plots (*25*). One unit of inhibition against cathepsin K was defined as the inhibition of the liberation of 1 μmol of AMC from (*Z*)−L-Phe-L-Arg-MCA per minute.

RESULTS AND DISCUSSION

Isolation of the cDNA Clone for a Novel OC, OC-III. We planned to isolate the cDNA clones for OC-I and -II, which had previously been reported to be present in the seeds of rice cultivar Nipponbare (*15*, *17*), from the calli of rice cultivar Koshihikari, and obtained the cDNA encoding OC-I by RT-PCR using a set of primers (OC1.1 and OC1.2), as expected. However, the cDNA that we were able to amplify with our set of primers OC2.1 and OC2.2, which were designed based on the published sequence of the cDNA for OC-II, did not encode OC-II but rather a novel protein with a calculated molecular mass of 12 077. We named this novel protein OC-III. **Figure 2** compares the nucleotide sequences of the cDNAs for OC-II and OC-III. The sequence of the open reading frame (ORF) (327-bp) of cDNA for OC-III showed 98% identity to that of OC-II, and its deduced amino acid sequence (108 residues) showed 56% and 89% identity, respectively, to that of OC-I and OC-II (**Figure 3**).

```
                    10        20        30        40        50
OC-III  MAEEAQQPRGVKVGGIHDA-PAGRENDLTTVELARFAVAEHNSKANAMLE
OC-II   MAEEAQSH--AREGGRHPRQPAGRENDLTTVELARFAVAEHNSKANAMLE

                    60        70        80        90       100
OC-III  LERVVKVRQQVVGGFMHYLTVEVKEPGGANKLYEAKVWERAWENFKQLQD
OC-II   LERVVKVRQQVVGGFMHYLTVEVKEPGGANKLYEAKVWERAWENFKQLQD

OC-III  FKPLDDATA
OC-II   FKPLDDATA
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Figure 3. Comparison of the amino acid sequences of oryzacystatin-III (OC-III) and oryzacystatin-II (OC-II). Sequences are numbered from the first methionine of oryzacystatin-III, and gaps were introduced to obtain maximum similarity. The residues of oryzacystatin-II that are identical to those of oryzacystatin-III are shaded. The figure was prepared using the Javashade program (http://industry.ebi.ac.uk/JavaShade/).

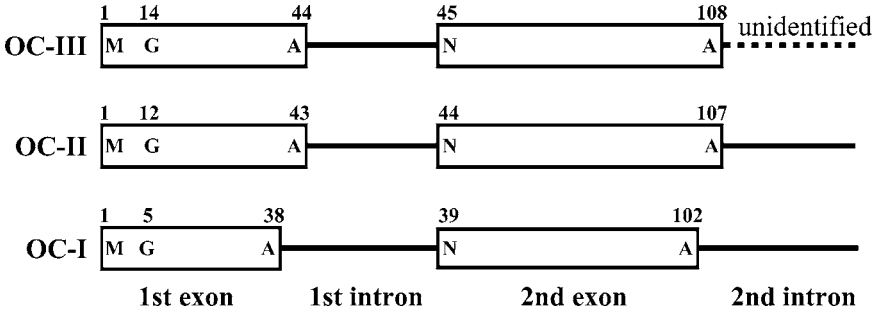

Figure 4. Comparison of the exon–intron organization of oryzacystatin (OC)-I, -II, and -III genes. Open boxes and solid lines indicate the coding regions and introns, respectively. The numbering starts at the initial methionine residue. The glycine residue in the N-terminal region that is conserved in the cystatin superfamily is indicated. The second intron of the OC-III gene could not been identified (see text).

We searched for cDNA sequences that were homologous to OC-III by BLAST (http://www.ddbj.nig.ac.jp/search/blast-j.html) and found one clone (accession no. AK059563) with a sequence that perfectly matched with that of OC-III ORF. This clone was obtained as a full-length cDNA from the cultivar Nipponbare, from which OCs-I and -II had been isolated (*11*, *12*), by the rice full-length cDNA project (http://cdna01.dna.affrc.go.jp/cDNA/). The nucleotide sequence of the cDNA for OC-III was found to be distinct from that of OC-II at three deletions and one transition mutation (**Figure 2**). These findings suggest that OC-III would be encoded by an allele at the OC-II locus or by a third OC gene at a different locus in the rice genome.

Figure 3 compares the amino acid sequences deduced from the cDNA clones of OC-II and OC-III with each other. The differences in amino acid sequence between OCs-II and -III clustered around a glycine residue (Gly^{14}) on the N-terminus (from Gln^{7} to Ala^{19} in the OC-III numbering scheme) and the sequence of OC-III in the remaining region was identical to that of OC-II. This glycine residue is known to be evolutionally conserved in the cystatin superfamily (*26*, *27*). Docking experiments based on the crystal structure of chicken egg white cystatin suggested that one function of this conserved residue could be to provide flexibility of the N-terminal region, allowing it to adopt a conformation that provides maximal binding of cystatin molecule to its target enzyme (*28*). Also, it has been reported that the mutations in the region around this glycine residue influence the enzyme inhibitory activities of cystatins (*26*, *27*, *29*), leading to the speculation that OC-II and OC-III have different target specificities.

Genomic DNA Encoding OC-III. We cloned the 2152-bp genomic DNA fragment that carries the 5′-flanking region and the first and the second exons of the OC-III gene. **Figure 4** compares the exon−intron organization of the OC-I, OC-II, and OC-III genes. An intervening sequence (424-bp) interrupts the first exon (132 bp) and second exon (195 bp) of the OC-III gene. It has been demonstrated that the OC-I and OC-II genes are composed of three exons and two introns and that the second intron in both of the genes interrupts the second exon and the 3′-noncoding region following the termination codon (third exon) (*2*, *3*). We were unable to identify the second intron of the OC-III gene, since the cloned cDNA did not contain the 3′-noncoding region. However, the first intron of the OC-III gene, which showed 95.5% of sequence identity with that of the OC-II gene, was found to intervene between Ala^{44} and Asn^{45}. An identical interruption was observed in both the OC-I and OC-II gene. Therefore, it is highly probable that the exon−intron organization of the OC-III gene is essentially identical to that of the OC-I and OC-II genes, as summarized in **Figure 4**.

Figure 5 compares the nucleotide sequences of the 5′-flanking regions of the OC-II and OC-III genes with each other. The DNA sequence of the region from the initiation codon to the position at −339 nt was found to share 96.5% identity with that of the OC-II gene. This region contains the putative CAAT box (−245 to −242 nt) and TATA box (−169 to −165 nt) sequences. In addition, the DNA sequence from the position −1 to −176 nt of the OC-III gene matched perfectly with that of the OC-II gene, in which the transcriptional starting point of OC-II is present at the position of −134 nt. This information notwithstanding, we speculate that OC-III is encoded by a gene located at a different locus from that of the OC-II gene, since there is minimal nucleotide sequence identity between OC-II and OC-III upstream from position −337 (**Figure 5**).

Preparation and Characterization of r-OCs. To obtain recombinant proteins that carried the identical amino acid sequences to those of authentic OCs, we expressed and purified r-OCs as GST fusions, after which they were digested with factor Xa (see the Materials and Methods section). Following digestion under the appropriate conditions, the N-terminal sequences of the purified OCs were confirmed by automated Edman degradation to be identical to those of the proteins predicted from the cDNA sequences (data not shown). **Figure 6** shows molecular masses and purity of the r-OCs by denaturing PAGE. Prolonged digestion with factor Xa resulted in the intramolecular cleavages of the r-OC-II (data not shown) and

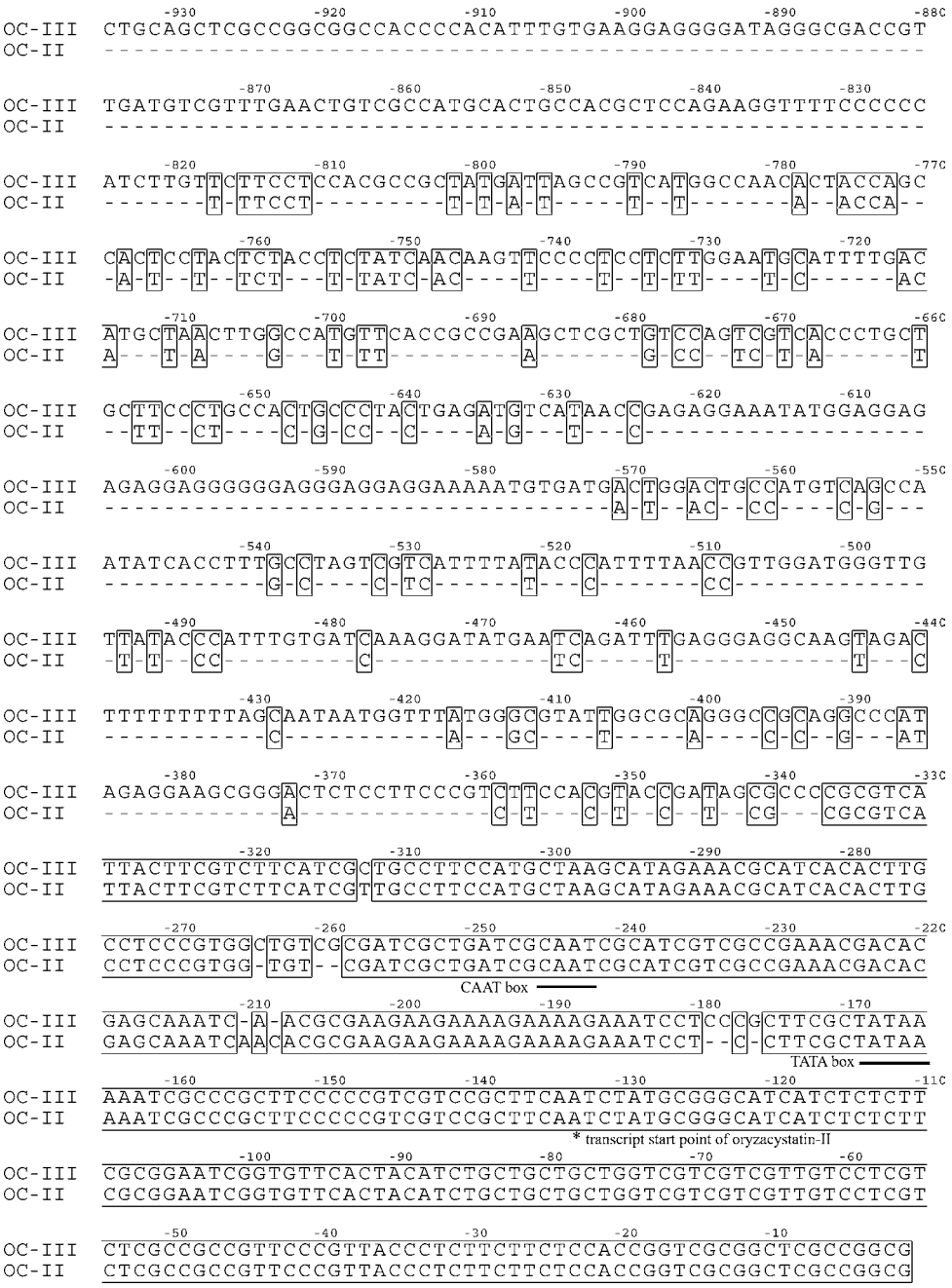

Figure 5. Similarity of the 5′-upstream regions of oryzacystatin-III (OC-III) and oryzacystatin-II (OC-II) genes. Alignment was generated using GENETYX-WIN software (ver.3; GENETYX Corp., Tokyo, Japan). The sequence is numbered from the translation start of oryzacystatin-III, and gaps were introduced to obtain maximum similarity. The nucleotides of oryzacystatin-II that are identical to those of oryzacystatin-III are boxed. The figure was prepared using the Javashade program (http://industry.ebi.ac.uk/JavaShade/).

r-OC-III. To further confirm this limited-proteolysis by factor Xa in the GST-OC fusion protein molecules, the molecular mass of r-OC-III and its truncated form were examined by MALDI-TOF MS. The molecular masses of OC-III and truncated r-OC-III were determined, respectively, to be 12 084 and 11 035, respectively, corresponding to the values that were calculated based on their amino acid sequences, 12 077 for OC-III and 11 085 for the truncated form. This finding suggests that the intramolecular cleavage by factor Xa occurred only at the Arg^9-Gly^{10} peptide bond in the r-OC-III molecule. This type of proteolytic cleavage is unusual since the sequence (Gln^6-Gln^7-Pro^8-Arg^9) around the cleaved site of r-OC-III is different from the recognition site for factor Xa (Ile-Glu-Gly-Arg). This observation supports the notion that the N-terminal region of cystatin molecules is highly sensitive to proteolytic cleavage (*30*).

Table 2 summarizes the K_i values of the r-OCs for papain (80% active), ficin (70% active), and cathepsin B. For all of the OC species, the K_i values for papain were comparable to those for ficin. Kondo et al. (*15*) estimated that the K_i values of OC-I and OC-II for papain were 3.02×10^{-8} and 8.3×10^{-7} M, respectively, using *N*-benzoyl-DL-arginine-2-naphthylamide as the enzyme substrate. The K_i value of r-OC-II for papain that we obtained was consistent with that previously reported for OC-II (*15*). However, the K_i value that we obtained for the activity of r-OC-I against papain was 100-fold lower than that reported for OC-I by Kondo et al. (*15*). While it is not immediately clear why our results differed, it may have been a

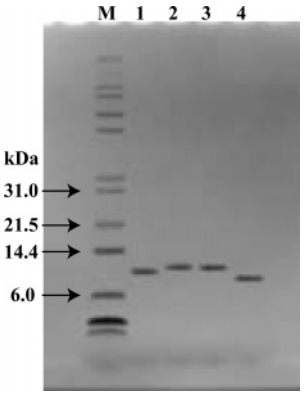

Figure 6. Denaturating polyacrylamide gel electrophoresis of recombinant oryzacystatins. Purified recombinant oryzacystatins (0.5 μg per lane) were loaded. Lane M contains the molecular weight standard with relevant sizes indicated on the left side; lane 1 contains oryzacystatin-I; lane 2 contains oryzacystatin-II; lane 3 contains full-length oryzacystatin-III; lane 4 contains truncated oryzacystatin-III.

Table 2. Enzyme Inhibition by Recombinant Oryzacystatins

	K_i value (M)		
	papain	ficin	cathepsin B
oryzacystatin-I	1.11×10^{-10}	1.30×10^{-10}	*a*
oryzacystatin-II	1.87×10^{-7}	1.70×10^{-7}	*a*
oryzacystatin-III			
full-length form	1.08×10^{-9}	2.48×10^{-9}	1.04×10^{-7}
truncated form[b]	1.16×10^{-8}	2.35×10^{-8}	1.02×10^{-6}

[a] No inhibition. Enzyme inhibition was not observed with oryzacystatin-I or oryzacystatin-II at 0.72 and 0.65 μM, respectively. [b] The truncated form of oryzacystatin-III lacks the N-terminal nine amino acid residues.

reflection of differences in assay conditions. The rank order of OC inhibitory activity against papain and ficin was OC-I > OC-III > OC-II. Interestingly, OC-I and OC-II showed no inhibition of cathepsin B at concentrations of 0.72 and 0.65 μM, respectively, while OC-III effectively inhibited the activity of cathepsin B ($K_i = 1.04 \times 10^{-7}$ M). The difference in amino acid sequence between OC-II and OC-III was restricted to the N-terminal region around the glycine residue (Gly^{14} in the OC-III numbering scheme). Therefore, this region would markedly influence the inhibitory activity of OCs, though it was reported that the N-terminal 21 amino acid residues were not essential for the papain-inhibiting activity of OC-I (*31*). The K_i value of truncated OC-III was found to be 10-fold higher than that of full-length OC-III against each enzyme (see **Table 2**), supporting the notion that the N-terminal region of cystatins was important for their inhibition of enzyme activity (*26*, *27*, *29*, *30*, *32*, *33*). On the basis of our results, we suggest that the N-terminal region around the conserved glycine residue of OCs is important in maximizing their affinity for cysteine proteinases.

All of our r-OCs inhibited the activity of cathepsin K, though with different specific activities. r-OC-I exhibited the highest activity (7.40 U/mg), followed in order by OC-III (1.98 U/mg) and OC-II (8.57×10^{-3} U/mg). Cathepsin K has been reported to play a pivotal role in several pathological conditions, including obesity (*34*), the progression of prostate cancer (*35*), and osteoclastomas (*36*); thus OCs may be applicable to the therapeutic use for these conditions.

In conclusion, we reported a novel rice cystatin, designated OC-III, in calli of *O. sativa* L. *japonica* cultivar Koshihikari that was distinct from OCs-I and -II that were obtained from *O. sativa* L. *japonica* cultivar Nipponbare. The difference of substrate specificity among OCs-I, -II, and -III may reflect the difference of their physiological roles. We are now planning the study to elucidate the physiological roles and the regulation of OC-III in rice.

The nucleotide sequence data reported here are available in the DDBJ, GenBank, EMBL, and GSDB Nucleotide Sequence databases under the following accession numbers, AB125972 (cDNA encoding oryzacystatin-III) and AB125973 (genomic DNA encoding oryzacystatin-III).

ACKNOWLEDGMENT

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