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Cloning and sequence analysis of a cDNA encoding rice glutaredoxin

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Abstract

A full-length cDNA clone (RASC8) encoding glutaredoxin (thioltransferase) was isolated from a cDNA library of an aleurone layer prepared from a developing seed of rice (*Oryza sativa* L.). RASC8, 568bp in length, contained an ATG codon and two possible polyadenylation signals, and encoded 112 amino acid residues. Cys-Pro-Phe-Cys, which is the active site and a highly conserved sequence among thioltransferases, was found in the deduced amino acid sequence. RASC8 was introduced into an expression vector pMALc2 and the translated product possessed thioltransferase activity.

Key words: Glutaredoxin; Thioltransferase; Aleurone layer; Rice; *Oryza sativa* L.

1. Introduction

The aleurone is the outer layer of the seed endosperm and is well developed in cerials. However, the aleurone layer is very different from starchy endosperm structurally and physiologically. Since the aleurone layer locates around the starchy endosperm, aleurone cells are prone to be damaged oxidatively. Therefore some genes may be expressed specifically in this layer to scavenge active oxygens.

To clarify the mechanism of the active oxygen scavenging system and the gene expression specific to the aleurone layer, we constructed a cDNA library for this layer from a developing rice seed using PCR technique [1]. In this library, one clone (RASC8) apparently coded for glutaredoxin, which occurs in microorganisms and animals [2–7].

Glutaredoxin, also known as transhydrogenase or thioltransferase, is a heat-stable enzyme with a molecular mass of 12 kDa. This enzyme catalyzes the reduction of disulfides in prokaryotes and eukaryotes, and glutathione (GSH) serves as a cosubstrate. Several reports indicated roles of this enzyme in active oxygen scavenging systems in animals and virus [8–9].

In this paper, we report the cloning of a glutaredoxin cDNA occurring in plant for the first time.

2. Materials and methods

2.1. Cloning and sequencing

A cDNA library for the rice aleurone layer was constructed from a single developing rice grain using PCR technique. One-hundred clones were picked up randomly and selected for length (> 300 bp) of their inserts. Among these size selected clones, five clones were confirmed to be expressed specifically in the aleurone layer by Northern blot analysis. The cDNA inserts were subjected to restriction mapping, and their overlapping fragments were subcloned. Sequence analysis was performed by a dideoxynucleotide dye primer method with a Model 373A DNA Sequencer (Applied Biosystems). The resulting nucleotide and the deduced amino acid sequences were compared with the EMBL database.

2.2. Northern blot analysis

Total RNAs from each tissue and organ were prepared by an SDS-phenol method [10]. After denaturation by formamide and formaldehyde, the total RNAs (5 μg each) were electrophoresed on 1.2% agarose gels, blotted on nylon membranes and probed with ^{32}P-labeled cDNA fragments. The membranes were washed in 3 × SSC and 0.1% SDS and then exposed to X-ray films.

2.3. Construction and preparation of recombinant fusion protein

The cDNA was expressed using 'Protein Fusion and Purification System (New England Biolabs)'. The entire protein-coding region was cloned in expression vector pMALc2 to construct pMAL-RASC8. RASC8 located at the immediate downstream of the *malE* gene, which encoded maltose-binding protein (MBP). *E. coli* TB1 was transformed with pMAL-RASC8, and then treated with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h to induce the cloned cDNA, which treament resulted in the expression of an MBP fusion protein. The fusion protein was purified by affinity chromatography on amylose-resin. The vector also contained the sequence coding the recognition site of the specific protease Factor Xa, and the site located adjacent to the 5′ end of the polylinker insertion site. The fusion protein was cleaved into MBP and RASC8-coding protein (RASC8p) by Factor Xa at 4°C overnight.

2.4. Measurement of thioltransferase activity

Thioltransferase activity was assayed as described elsewhere [11]. In

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brief, the cell extract of transformed *E. coli* was treated with Factor Xa in the reaction mixture consisting of 100 mM sodium phosphate (pH 7.5), 1 mM EDTA, 0.3 mM NADPH, 1 mM GSH, 2 units of GSH reductase (Sigma). The reaction was initiated by the addition of the substrate, 2.5 mM L-cystine, at 16°C and followed by monitoring absorbance at 340 nm. One unit was defined as the amount of enzyme catalyzing oxidation of 1 nmol of NADPH per minute. The extract without Factor Xa treatment was also subjected to this assay.

3. Results

3.1. Isolation and characterization of cDNA clones specific to the rice aleurone layer

Five different clones with longer inserts were selected from the cDNA library for the rice aleurone layer. Their tissue specificity of gene expression was determined by Northern blot analysis using RNA from each tissue and organ (Fig. 1). One clone (RASC8) was specific to the aleurone layer, and possessed high similarity with yeast and animal glutaredoxins on deduced amino acid sequence [2–5,12] (Fig. 2). Among probes from different sources, the gene coded in RASC8 strongly hybridized with RNA from seed, especially from the aleurone layer (Fig. 1).

3.2. DNA sequence analysis of RASC8

The RASC8 cDNA clone contained a 568 bp insert excluding the synthetic *Eco*RI and *Xba*I adaptors and linkers (Fig. 3). Two putative polyadenylation signals were found at 425–436 bp (AATAAA) and 521–526 bp (AATAAT). RASC8 encoded 112 amino acid residues starting from methionine. The sequence of a putative active site for thioltransferase (Cys-Pro-Phe-Cys) [13] was conserved. The sequence alignment of the deduced amino acids of RASC8 with other glutaredoxins [2–5,12] showed 20–41% homology between residues 15 and 92 (Fig. 2).

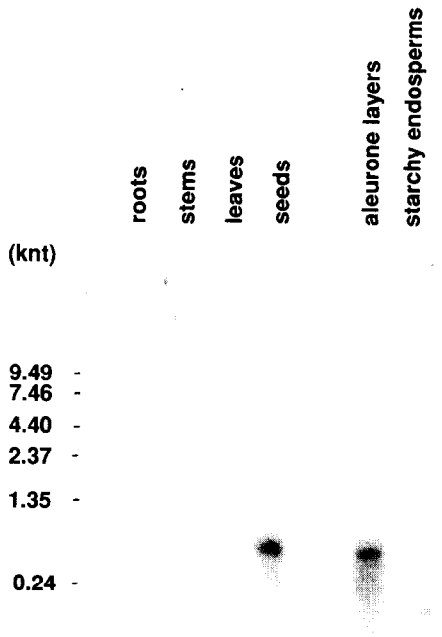

Fig. 1. Tissue specific expression of RASC8 gene. Total RNAs were prepared from roots, stems, leaves and seeds, and also from the aleurone layers and starchy endosperms. The RNA were probed with ^{32}P-labeled cDNA fragment of RASC8. The migration of size markers (in nt) is indicated on the left.

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                10        20        30        40
RASC8     MALAKAKETVASAPVVVYSKSYCPFCVRV-KKLFGQLG--ATF
Yeast   VSQETV.HV.DLIGQKE.F.AA.T...Y.KATLST..QE.NV-PKS
Pig       .Q.FVNSKIQPGK...FI.PT....RKT-QE.LS..PFKEGL
Virus     ..EEFVQQRL.NNK.TIFV.YT....RNA-LDILNKFSFKRGA
E.coli                MQT.IFGR.G..Y...A-.D.AEK.S--NER

                50        60        70
RASC8     KA-I-ELD-GESDGSEL-QSALAEWTGQR--TVPNVFINGKH-
Yeast     ..LVL...-EM.N...I-.D..E.ISG.K--.....Y.....-
Pig       LEFV-DIT-AT..TN.I-.DY.QQL..A.--...R...-..EC
Virus     YEIV-DIK-EFKPEN..-RDYFEQI..G.--...RI.F-..TS
E.coli    DD-F-QYQYVDIRAEGITKED.QQKA.KPVE...QI.VDQQ.-

              80        90        100       110
RASC8     IGGCDDTLALNNEGKLVPLLTEAGAIASSAKTTITA
Yeast     ...NS.LET.KKN...AEI.KPV
Pig       ....T.LESMHKR.E.LTR.QQI..LK
Virus     ...YS.L.EID.MDA.GDI.SSI.VLRTC
E.coli    ...YT.FA.WVK.NLDA
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Fig. 2. Comparison of the amino acid sequences of RASC8 and other glutaredoxins. The sequences are derived from rice; RASC8, yeast [3], pig [5], virus [12], *E. coli* [2]. '.' denotes the amino acid residue identical with that for RASC8. '-' is computer-generated gap which is inserted to improve the alignment. The shadowed amino acid residues denote the conserved active sites of thioltransferase.

3.3. Thioltransferase activity of RASC8 encoded protein

RASC8 was expressed in *E. coli* strain TB1 as a fusion protein MBP-RASC8p. The fusion protein was cleaved with Factor Xa to yield RASC8p and MBP. The extracts containing the cleaved or uncleaved proteins showed thioltransferase activity, which was 10-fold higher than the untransformed TB1 extract (Fig. 4).

4. Discussion

Glutaredoxin affects and regulates various enzymatic activities, such as pyruvate kinase [14], papain [15] and phosphofructokinase [16]. Some glutaredoxins are implicated in the activation of ribonucleotide reductase [17–18]. Glutaredoxin thus may play certain roles on regulating enzymatic activities in cells. Glutaredoxin also has dehydro-L-ascorbate (DHA) reductase activity in some organisms [8,11]. In addition, disulfides in membrane proteins of oxidatively damaged human erythrocyte were efficiently reduced to free-SH form by incubation with glutaredoxin [9].

Compared with other glutaredoxins, RASC8p pos-

sessed a longer C-terminus and less homology in the N-terminal region. It may be due to the lesser importance of the N- and C-terminal region of glutaredoxin relative to its thioltransferase activity. The fusion protein MBP-RASC8p had thioltransferase activity. RASC8p cleaved from MBP-RASC8p with Factor Xa contained excess amino acid residues in its N-terminal, but it showed thioltransferase activity. These thioltransferase activities were at the same level (Fig.4).

Although other glutaredoxins [8,11] have DHA reductase activity in addition to thioltransferase activity, RASC8p did not show this reductase activity (data not shown). However, endogenous rice glutaredoxin may have DHA reductase activity. DHA reductase activity was detected in spinach extract [19] and this activity was attributed to 23 kDa polypeptide. However, further information including amino acid sequence and its relation to thioltransferase activity have not been provided yet.

Glutaredoxin is considered to take part in the superoxide scavenging system [8]. If the endogenous rice glutaredoxin does not possess DHA reductase activity, rice glutaredoxin may play a different role in active oxygen scavenging system. The physiological roles of rice glutaredoxin must be studied further, and it is interesting to

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     TCGACCCCGGAATCCAGGAAGATGGCGCTCGCCAAGGCCA   40
1                          M  A  L  A  K  A  K

     AGGAGACCGTCGCTTCCGCTCCCGTCGTCGTCTACAGCAA   80
8     E  T  V  A  S  A  P  V  V  V  Y  S  K

     GTCTTACTGTCCTTTTTGCGTCCGTGTGAAGAAGTTGTTC  120
21    S  Y  C  P  F  C  V  R  V  K  K  L  F

     GGGCAGCTTGGAGCAACTTTCAAGGCCATTGAGTTGGATG  160
34   G  Q  L  G  A  T  F  K  A  I  E  L  D  G

     GGGAGAGTGATGGATCTGAGCTGCAGTCGGCACTTGCTGA  200
48    E  S  D  G  S  E  L  Q  S  A  L  A  E

     ATGGACTGGACAAAGGACTGTTCCAAATGTCTTCATCAAT  240
61    W  T  G  Q  R  T  V  P  N  V  F  I  N

     GGGAAGCATATTGGTGGCTGTGATGATACTTTGGCATTGA  280
74   G  K  H  I  G  G  C  D  D  T  L  A  L  N

     ACAATGAAGGGAAGCTGGTGCCTCTGCTGACCGAGGCTGG  320
88    N  E  G  K  L  V  P  L  L  T  E  A  G

     AGCAATTGCCAGTTCTGCAAAGACGACAATCACCGCATAG  360
101   A  I  A  S  S  A  K  T  T  I  T  A  *

     TTCTTCGTGGGACACTGGGACTAGCCTTCGTTGACCTCTT  400
     TATACTGCATCCATTCTATTAGATAATAAAGGTGGATGTT  440
     TGTTTGGCAAGACCATTACTTGTTGCCGTCTAGTATCGTG  480
     TGATAGCTATCCTGTGCCCGTGTGAGACTCCTTGGACATC  520
     AATAATATCGTCTTTGTGATAGCAGTTCGCTGAAAAAAAA  560
     AAAAAAAA                                  568
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Fig. 3. Nucleotide and the deduced amino acid sequences of RASC8. The possible polyadenilation signals are underlined. The shadowed residues in the amino acid sequence denote the conserved active site of thioltransferase. '*' indicates the translation stop codon.

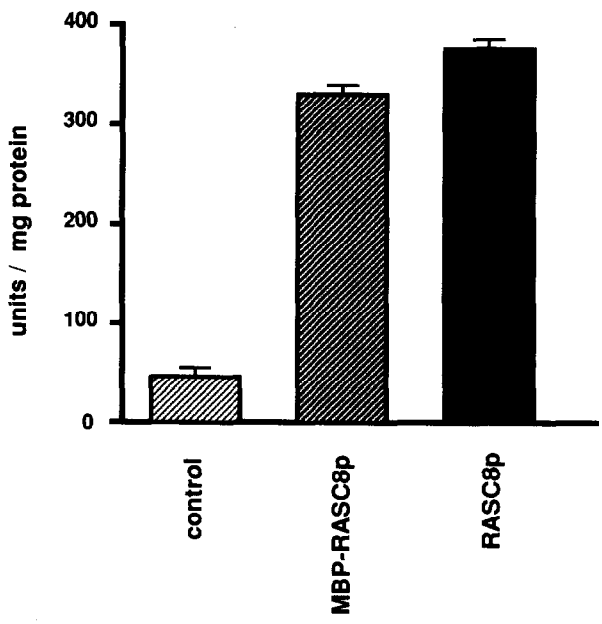

Fig. 4. Thioltransferase activity of RASC8p. Enzyme activities are expressed in units/mg protein. The extract from untransformed TB1 cells was used as control. The fusion protein (MBP-RASC8p) and its cleaved product (RASC8p) were subjected to this assay. The control value indicates the endogenous activity of the *E. coli* strain TB1 cell.

elucidate the relation of glutaredoxin to thioredoxin [20–22], which is similar to glutaredoxin in function, in plants.

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