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Construction of Gene-Specific Primers for Various Antioxidant Isoenzyme Genes and Their Expressions in Rice (*Oryza sativa* L.) Seedlings Obtained from Gamma-irradiated Seeds

Jin-Hong Kim[1], Byung Yeoup Chung[1*], Jae-Sung Kim[1], Seung Gon Wi[1],
Dae Hwa Yang[1], Choon-Hwan Lee[2], and Myung Chul Lee[3]
[1]Division of Radiation Application Research, Korea Atomic Energy Research Institute, Daejeon 305-353, Korea
[2]Department of Molecular Biology, Pusan National University, Busan 609-735, Korea
[3]National Institute of Agricultural Biotechnology, R.D.A., Suwon 441-707, Korea

Abstract For the expression study of antioxidant isoenzyme genes in rice (*Oryza sativa* L.) plants, extensive searches for genes of superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) isoforms were performed through the GenBank database. The genes for two cytosolic and one plastidic CuZn-SOD, one Fe-SOD, two Mn-SOD, two cytosolic and two chloroplastic (stromal and thylakoid) APX, and three CAT isoforms were available in japonica-type rice. These isoforms were named as cCuZn-SOD1, cCuZn-SOD2, pCuZn-SOD, Fe-SOD, Mn-SOD1, Mn-SOD2, cAPXa, cAPXb, Chl_sAPX, Chl_tAPX, CATa, CATb, and CATc, respectively. Since they shared a high degree of homology in the nucleotide and amino acid sequences, the gene-specific primers for the genes were designed directly from their full-length cDNAs found in the database except for the CATa gene. These primers were used in the RT-PCR analysis to investigate the differential expression of antioxidant isoenzyme genes in rice plants from the seeds irradiated with low doses (2, 4, 8, and 16 Gy) of gamma-radiation. The gamma-irradiation slightly increased the transcripts of pCuZn-SOD, while those of Fe-SOD, cAPXb, and CATb decreased. However, no substantial differences were observed in the expression of all the isoenzyme genes between the control and irradiated groups. In this study, gene specific primers for thirteen SOD, APX and CAT isoenzymes were constructed from the full-length cDNAs. The results of RT-PCR analysis obtained by using these primers suggests that the expression levels of SOD, APX, and CAT isoenzyme genes in rice seedlings were hardly affected by gamma-irradiation at the seed stage.

key words: ascorbate peroxidase, catalase, gamma-irradiation, rice, reactive oxygen species, superoxide dismutase

INTRODUCTION

Reactive oxygen species (ROS) that are inevitably produced in all aerobic cells as byproducts of normal oxygen metabolism have deleterious effects which cause cellular damage. In particular, the level of ROS production elevates in plant cells under environmental stress. However, plants have ROS-scavenging systems to cope with such oxidative stress. The systems consist of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) [1] as well as low-molecular-weight antioxidants including ascorbate, glutathione and phenolic compounds [2].

The antioxidant enzymes exit in various isoforms. SOD is classified by its metal cofactor; copper-zinc (CuZn), manganese (Mn) and iron (Fe). These isoforms are distinguishable by their differential sensitivities to CN^- and H_2O_2, and are distributed in different subcellular locations [1, 3]. In higher plants, CuZn-SOD is found mainly in the chloroplast and cytosol, Mn-SOD chiefly in the mitochondrial matrix, and Fe-SOD in the chloroplast. Unlike the former two isoforms, Fe-SOD was previously regarded to exist only in dicotyledonous plants [3] before the cDNA encoding Fe-SOD was isolated from rice and characterized [4, 5]. In contrast, APX exists in at least four distinct cell compartments in higher plants, stroma (sAPX), thylakoid membrane (tAPX), microbody (mAPX) and cytosol (cAPX), respectively [6, 7, 8].

The genes for SOD and APX isoforms are differentially expressed in plants in response to various oxidative stresses. In spinach, only the expression of cAPX was found to increase significantly at the mRNA level under high light stress, while that of sAPX, tAPX and mAPX were remained relatively unchanged [9]. Kaminaka *et al.* [4] also reported that genes for SOD isoforms in rice were differentially expressed under oxidative and environmental stress conditions such as drought, salinity as well as the treatments of

*To whom correspondence should be addressed.
E-mail : bychung@kaeri.re.kr
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abscisic acid and H_2O_2. However, it remains to be clarified what constitutes the basis of the differential expression of these isoenzymes, although the increase of cAPX mRNA might be induced by H_2O_2 as shown in rice embryos by Morita *et al.* [10]. Moreover, the increase in the cAPX mRNA is not necessarily translated into the parallel change in the protein level [9]. Nevertheless, a study on the expression of antioxidant isoenzymes will be helpful to elucidate the affected cellular compartments and the ROS metabolism within them under oxidative stress conditions.

Recently, it has been reported that low-dose radiation corresponding to several gray (Gy) upon plants not only elevated the stress resistance to environmental stress such as UV-B and high light but also promoted the growth of plants [11-13]. Since ionizing radiation increases activities of antioxidant enzymes such as SOD, APX, CAT and POD in plants [12-15], the enhanced activities of these enzymes may contribute to the improved stress resistance in the irradiated plants. Cellular responses to low dose radiation are generally supposed to occur by the direct cellular signaling participation of ROS produced from water radiolysis [16, 17] or by the involvement of low-molecular-weight signaling factors released from reactions of ROS and neighboring cellular components [18, 19]. Therefore, studies on the gene expression for antioxidant isoenzymes would possibly contribute to elucidate the ROS signaling processes and their metabolism in the irradiated plants.

The present study was intended to analyze the expression patterns of antioxidant isoenzyme genes in rice seedlings from gamma-irradiated seeds. For this purpose, we attempted to design gene-specific primers for RT-PCR analysis based on the cDNA or genomic DNA sequences of all the members belonging to SOD, APX, and CAT isoforms that were obtained in japonica-type rice from GenBank searches.

MATERIALS AND METHODS

Plant material and gamma-irradiation

Rice (Oryza sativa L. cv. Ilpoom-byeo) seeds were irradiated with low doses of gamma-radiation (2, 4, 8, and 16 Gy) generated by a gamma irradiator (^{60}Co, ca. 150 TBq of capacity, Atomic Energy of Canada Limited (AECL)) in Korea Atomic Energy Research Institute (KAERI). After sterilized in 2%(v/v) sodium hypochlorite solution for 30 min at room temperature, the irradiated seeds were sown in a soil mixture of NURSERY MAT TYPE SEEDLING (Bio-media, Korea) and BIOPLUG2 (SeminisKorea, Korea) (4:1). Then, they were grown in a growth chamber with photosynthetic photon flux density (PPFD) at pot level of 330 μmol m^{-2} s^{-1} supplied by two natrium lamps in combination with six fluorescence lamps. The growth chamber was maintained at 28/20°C (day/night) with a 14 h photoperiod. For the extraction of total RNA, seedlings were harvested two weeks after sowing.

RNA extraction and Reverse Transcription (RT)-PCR

Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, USA) according to the supplier's recommendation. One microgram of total RNA was reverse transcribed in AccuPower RT Premix (Bioneer, Daejeon, Korea) for 60 min at 42°C using 0.5 μg oligo$(dT)_{15}$ primers (Promega, Madison, WI, USA). Gene-specific primers for the genes of SOD, APX and CAT isoforms are listed in Table 2. The primer construction will be described in Results and Discussion. The sense / antisense primers for actin and rRNA, used as internal controls, were 5'-TCCATCTTGGCATCTCT CAG-3' / 5'-GTACCCTCATCAGGCATCTG-3' [20] and 5'-CTTCGGGATCGGAGTAATGA-3' / 5'-AACTAAGAACGG CCATGCAC-3', respectively. The primers for rRNA were designed in the same way as those for the genes of SOD, APX and CAT isoforms. PCR was subsequently carried out by adding 3 μL of the synthesized cDNAs in Perfect Premix ver. 2.0 (Takara Korea Biomedical Inc., Seoul, Korea) according to the following protocol: denaturation at 94°C for 5min, 24 or 35 cycles of 94°C (45 s) -58°C (45 s) -72°C (1.5 min) and an extension at 72°C for 7 min. The PCR conditions were optimized empirically by testing various annealing temperatures and cycle reactions using a gradient thermal cycler (TP600, TaKaRa, Shiga, Japan). The resultant RT-PCR products were electrophoresed and analyzed on a 2.0% (w/v) agarose gel after staining with ethidium bromide (EtBr).

Verification of the RT-PCR products

Specificity of each primer pair was examined by the band size and number of the RT-PCR product on an EtBr-stained agarose gel.

RESULTS AND DISCUSSION

Search for the genes of SOD, APX, and CAT isoforms in rice

To construct gene-specific primers for the genes of SOD, APX, and CAT isoforms, we obtained their nucleotide sequences using GenBank searches (http://www.ncbi.nlm.nih.gov/). Genes for two cytosolic and one plastidic CuZn-SOD, one Fe-SOD, two Mn-SOD, two cytosolic and two chloroplastic (stromal and thylakoid) APX, and three CAT isoforms were found to exist in japonica-type rice when the annotated gene names were searched in the GenBank database (Table 1). The nucleotide sequences selected for use in designing the primers were full-length cDNAs, except for CATa whose cDNA was not available in the database (Table 1). In the case of CATa, its cDNA sequence was predicted from the genomic DNA at a gene prediction site (http://www.softberry.com/). Unlike other SOD isoforms, Fe-SOD has been characterized only in dicotyledonous plants [3], but recently its existence in rice was verified by Kaminaka *et al.* [4]. Although mAPX, an APX isoform, was characterized in spinach [8], its existence in rice was not yet experimentally

Table 1. SOD, APX, and CAT isoforms found in japonica-type rice from the GenBank database. Accession numbers in bold represent the nucleotide sequences used to design the gene-specific primers. 1, cDNA; 2, genomic DNA.

Isoenzyme	Gene	Accession Number		Reference
		Nucleotide	Protein	
cCuZn-SOD1	*cuznsod1*	**1. L36320**	AAA33917	
	sodCc1	2. L19435	AAC14464	27,28,29
cCuZn-SOD2		**1. D01000**	BAA00800	27,30
	sodCc2	2. L19434	AAC14465	27,28,31
pCuZn-SOD		**1. D85239**	BAA12745	32
	sodCp	2. AB026724	BAB21760	
Fe-SOD		**1. AB014056**	BAA37131	4
Mn-SOD1	*rmsod1*	**1. L34038**	AAA57130	
	sodAOs1	1. L19436	AAA62657	33
	sodA	2. AB026725	BAA86897	34
Mn-SOD2	*rmsod2*	**1. L34039**	AAA57131	
cAPXa	*APXa*	**1. D45423**	BAA08264	35
cAPXb	*APXb*	**1. AB050724**	BAB17666	
Chl_sAPX		**1. AB114855**	BAC79362	
Chl_tAPX		**1. AB114856**	BAC79363	
CATa	*CatA*	**2. D29966**	BAA06232	36
CATb		**1. D26484**	BAA05494	37
	CatB	2. D64013	BAA34204	38
CATc		**1. AY339372**	AAQ19030	
	CatC	2. D86611	BAA34205	38

clarified. Therefore, the isoenzymes listed in Table 1 encompass all the members of SOD, APX, and CAT isoforms, whose genes were identified and characterized at least in japonica-type rice.

Comparison between the isoforms of the respective SOD, APX, and CAT based on the sequences of nucleotide and amino acids

Alignments of the deduced amino acid sequences from the genes of SOD, APX and CAT isoforms showed that the SOD and APX isoforms are much less conserved in the amino acid sequence than the CAT ones (Figure 1A, B and C). However, the sequence homology was found to be 72.6% between cCuZn-SOD1 and 2, 94.8% between Mn-SOD1 and 2, 74.5% between cAPXa and b, and 73.9% between sAPX and tAPX, respectively. These results are in good agreement with the phylogenetic trees of SOD, APX, and CAT isoforms in view of their cDNA sequences (Figure 1D). Although 3'-untranslated regions (3'-UTRs) in the multi-gene family were useful in designing the gene-specific primers, in some cases, full-length cDNAs were employed for the genes which had high homology in the 3'-UTRs [5, 21]. For example, sAPX and tAPX are encoded, unlike the other APX isoforms, by only one gene and furthermore, their mRNAs are regulated by the

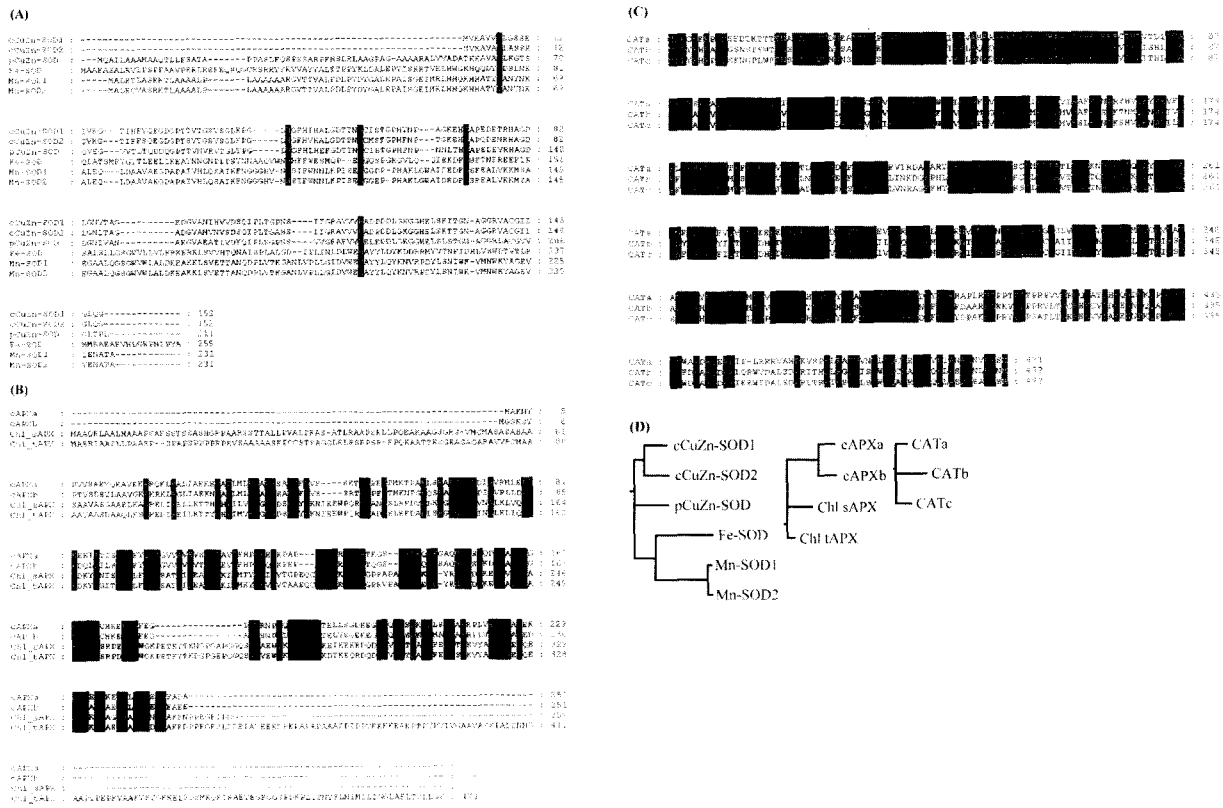

Figure 1. Alignments of the deduced amino acid sequences of SOD (A), APX (B) and CAT (C) isoenzyme genes in japonica-type rice, and phylogenetic trees of the isoenzyme genes based on the cDNA sequences (D). All sequence data were directly obtained from GenBank searches, except for the cDNA sequence of CATa which was predicted from its genomic DNA sequence in a gene prediction site (http://www.softberry.com/). The data were aligned using ClustalX and GeneDoc, and the phylogenetic trees were generated by Phylip. GenBank Accession Nos. of the isoenzyme genes are listed in Table 1. Black boxes indicate identical amino acid residues.

Table 2. Gene-specific primer pairs to the isoenzyme genes listed in Table 1. (L) and (R) represent sense and antisense primers, respectively.

Isoenzyme	Sequence (5'-3')	Size of the whole mRNA and PCR product (bp)
cCuZn-SOD1	(L) GAGATTCCAAACCAGCAGGA	768, 277
	(R) TTGTAGTGTGGCCCAGTTGA	
cCuZn-SOD2	(L) ACAGCCAGATCCCCCTTACT	667, 259
	(R) GCAAACTGCACACTGGTCAT	
pCuZn-SOD	(L) TTTTCCAGTCCCCTTCCTCT	951, 451
	(R) ACGAATGCTCTCCCAACAAC	
Fe-SOD	(L) CTTGATGCCCTGGAACCTTA	1354, 348
	(R) GCCAGACCCCAAAAGTGATA	
Mn-SOD1	(L) GGAGGCCATGTCAATCATTC	951, 504
	(R) CACAAGGTCCAGAAGTGCAA	
Mn-SOD2	(L) ATGCTGGGCAGTTTACCAAC	1352, 400
	(R) GTGGAAACTCAGGCGGTAGT	
cAPXa	(L) GTTTTGAGGGACCTTGGACA	983, 401
	(R) TAACAGCCCACCGAGACATT	
cAPXb	(L) TCTTCCTGATGCCACACAAG	1058, 298
	(R) GTCCTCATCCGCAGCATATT	
Chl_sAPX	(L) CAATTGAGGAAGCTGGTGGT	1379, 560
	(R) ACTTCAGCGATCTGGCTCAT	
Chl_tAPX	(L) GCTAAACTGAGCGACCTTGG	1698, 446
	(R) AGGAGGTCATCAGACCATCG	
CATa	(L) CCACCACAACAACCACTACG	1512, 320
	(R) CCAACGACTCATCACACTGG	
CATb	(L) CCAACAGGCTGGTGAGAGAT	1832, 280
	(R) ATTCACGACTACGGCTGGTC	
CATc	(L) ACCGGTTCATCAAGAGATGG	1861, 304
	(R) ACACGAATTGTGCGGTGATA	

alternative splicing of its two 3'-terminal exons [22, 23.] These two isoforms of the two chloroplastic APX are considered to be undistinguishable in the 3'-UTRs. Therefore, we attempted to design the gene-specific primers of SOD, APX, and CAT isoforms by using the full-length cDNAs.

Construction of the gene-specific primers and confirmation of their specificity

The primer pairs were checked whether they showed 100% homology with the only one gene in the GenBank database which had been used for the primer design. To facilitate the distinction between the isoenzymes by examining the size of RT-PCR products, the primer pairs were carefully designed so that RT-PCR products show different sizes as possible (Table 2). Such an attempt aimed at minimizing any possible cross-contamination caused by nonspecifically amplified PCR products. The specificity of each gene-specific primer pair was confirmed on an EtBr-stained gel after electrophoresis of RT-PCR products (Figure 2). As expected, all the primer pairs were found to produce RT-PCR products with the predicted size.

Expression of the genes for SOD, APX, and CAT isoforms of rice plants grown from gamma-irradiated seeds

Using the gene-specific primers constructed as previously described, the expressions of the genes for SOD, APX, and CAT isoforms were investigated in rice seedlings from the seeds irradiated with low doses of gamma-radiation (2, 4, 8, and 16 Gy) (Figure 3). The gamma irradiation seemingly enhanced the level of transcripts of pCuZn-SOD while

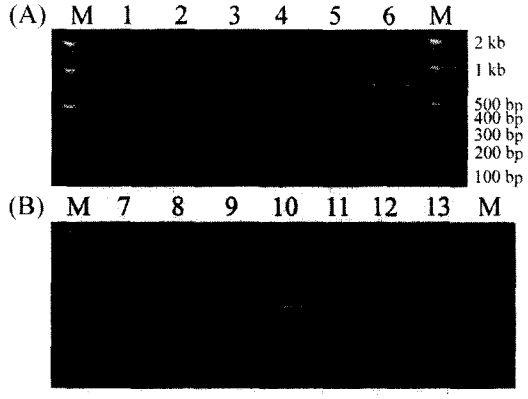

Figure 2. Confirmation of the predicted RT-PCR products of SOD, APX, and CAT isoenzyme genes with gene-specific primer pairs. Lane numbers 1-13: cCuZn-SOD1, cCuZn-SOD2, pCuZn-SOD, Fe-SOD, Mn-SOD1, Mn-SOD2, cAPXa, cAPXb, Chl_sAPX, Chl_tAPX, CATa, CATb, and CATc. M: DNA marker. For the RT, one microgram total RNA was reverse transcribed for 60 min at 42°C using 0.5 μg oligo(dT)$_{15}$primers. The subsequent PCR conditions are as follows: denaturation at 94°C for 5min, cycle reactions of 94°C (45 s) -58°C (45 s) -72°C (1.5 min) and an extension at 72°C for 7 min. All lanes had 24 cycle reactions except 35 in Lanes 3 and 6.

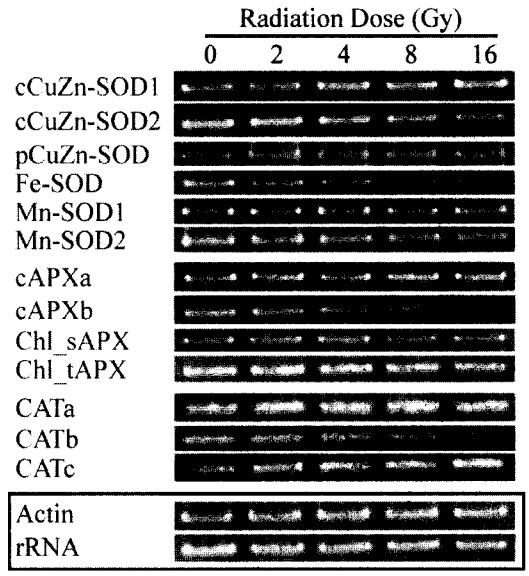

Figure 3. Expression of SOD, APX, and CAT isoenzyme genes in the control and irradiated rice seedlings. The RT-PCR conditions are described in Materials and Methods and Figure 2. Actin and rRNA in a black box imply the internal controls for the comparison of expression of the isoenzyme genes.

decreasing that of Fe-SOD, cAPXb, and CATb. The observed increase in the expression of pCuZn-SOD might be correlated with the high activity of SOD in radish leaves grown from the seeds irradiated with 10 Gy [13]. However, in overall, the irradiation did not induce substantial differences in the expression of all the isoenzyme genes examined in this study. These results imply that increases in the activities of antioxidant enzymes in plants from irradiated seeds may not be due to their increased gene expression. However, when *in planta* irradiation was applied to plants mainly at elevated doses, it could reportedly induce noticeable changes in the gene expression of pCuZn-SOD, POD, CAT, cAPX, and Chl_sAPX in tobacco [24], and the enzyme activities of SOD, APX, CAT, and/or POD in tobacco [14], sweet potato [25], cassava [26], and Poaceae [15]. Therefore, unlike the *in planta* irradiation, it is suggested that the irradiation of seeds might not constitute a sustainable stress factor strong enough to induce the gene expression of antioxidant isoenzymes in the resultant plants.

In conclusion, we designed and tested the gene-specific primers applicable to the expression study on SOD, APX and CAT isoenzyme genes of rice plants with a high homology in the nucleotide sequence. The RT-PCR analysis using these primers demonstrated that the expression levels of SOD, APX, and CAT isoenzyme genes are rarely affected by gamma-radiation, at least when plants were irradiated at the seed stage.

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