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Characterization of cathepsin B-like gene from rice

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In this study, a rice cDNA clone encoding a putative cysteine protease with sequence homology to mammalian cathepsin B has been isolated and characterized. Sequence analysis revealed that the gene designated *OsCatB* encodes a cysteine protease that belongs to the papain family without the consensus motif, ERFNIN. The result suggests that the *OsCatB* is the cathepsin B-like protein in rice. Among the cathepsin B-like proteins in plants, the highest homology was occurred to the sequence of the barley cathepsin B. DNA blot analysis showed that the *OsCatB* gene belongs to a multiple gene family in the rice genome. Expression analysis of the *OsCatB* revealed that the gene is expressed in all organs of rice including the germinating seed. To elucidate *OsCatB* function, we generated knockout mutants by RNAi system. Microscopic analysis revealed that knockout of the *OsCatB* gene caused an excessive cell death and suggested that the gene functions as an anti-cell-death component.

Key words: cathepsin B, cysteine protease, OsCatB, rice, anti-cell death.

Cysteine proteases belong to a family of enzymes that play important role in intracellular protein degradation in widely distributed systems; animals, plants, and microorganisms (Barrett, 1986). In higher plant, the cysteine proteases have been extensively studied in seed (Shutov and Vaintrub, 1987; Ryan and Walker-Simmons, 1991; Ho et al., 2000). Nutrient supply for seed germination can be achieved by hydrolyzing starch and storage proteins preserved in the endosperm and therefore, the cysteine proteases have been reported as the major enzymes responsible for hydrolysis of the major storage proteins, hordeins and glutelin in barley and rice, respectively (Rostogi and Oaks, 1986; Kato and Minamikawa, 1996). Recently, a rice cysteine protease, *OsCP1*, has been reported that the gene expression is highly preferential to the anther, suggesting that the cysteine proteases can be active in a tissue-specific manner (Lee et al., 2004). Cysteine proteases also contribute to cells undergoing programmed cell death (PCD) (Solomon et al., 1999).

Cathepsins, such as capthesin B, H, and L, are cysteine proteases which belong to the papain superfamily of proteases (Turk et al., 1997). In mammalian system, the proteases are known to be mainly associated with autophage and necrosis, promoting direct cell degradation (Boya et al., 2003). Recently, however, it has been reported that the cathpesin B activates caspases, the central players of apoptosis, suggesting its involvement in apoptosis (Benchoua et al., 2004). Yet, in plant, only a few cathepsin B-like proteases have been studied. A cDNA clone that showed a sequence homology to the mammalian cathepsin B was isolated and characterized in wheat (Cejudo et al., 1992). Expression analysis of the cathepsin B-like cysteine protease genes were also done in *Nicotiana rustica* and *Hordeum vulgare* (Lidgett et al., 1995 and Martinez et al., 2003, respectively). These genes were expressed in the aleurone layer of germinating seeds under the regulation of GA and ABA, suggesting that the cysteine protease plays a role in the germination process. However, no cathepsin B-like proteases have been studied in rice.

In this study, we isolated and characterized a cDNA clone that showed the sequence homology to the mammalian cathepsin B. Like the cathepsin B-like cysteine protease from barley, the gene, *OsCatB*, was expressed ubiquitously including the germinating seed. Using RNAi strategy, we have identified that rice CatB like protease functions as an anti-cell death component.

MATERIALS AND METHODS

Isolation and sequence analysis of *BcXTH1* gene

The *OsCatB* clone was isolated from the rice cDNA library. Sequence analysis was done by using the BLASTX at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequence comparisons were conducted by using Clustal W (Altschul et al. 1990).

Southern blot analysis

Genomic DNA (20 μg) isolated from the rice leaves were digested with *Eco*RI, *Hin*dIII, and *Xba*I restriction enzymes, fractionated by electrophoresis on a 0.8% (w/v) agarose gel, and transferred to Hybond-N membrane (Amersham, Arlington Heights, IL). The membrane blot was then hybridized with a ^{32}P-labeled probe for overnight in a hybridization solution and washed as previously described (Muthukalianan et al., 2003).

Expression analysis

For expression analysis of the *OsCatB* gene, total RNA was isolated from different organs of the plant by using TRIzol reagent. PCR reaction was carried out in a solution mixture containing 100 ng of cDNA, 50units of taq, 2.5 mM dNTPs, and 10pmol primers, for *OsCatB* (forward primer, 5'-CTA TCA TGG CAT GGC GCT AC -3'; reverse primer, 5'- GCA GCC AGT AAT CCT CGC CAG -3'); for OsActin (forward primer, 5'-TCC ATC TTG GCA TCT CTC AG-3', reverse primer 5'-GTA CCC GCA TCA GGC ATC TG -3') and for *Oryzain β* (forward primer,

5'-TGA CAT CAA CAG GGA AAA TGC T-3', reverse primer, 5'- GTG TTC AGC TTA GCG AGC GTG-3').

Transformation and generation of the transgenic callus

A binary vector, pANDA vector was used for RNAi mediated gene silencing. A 300-bp *OsCatB* fragment was used to make the RNAi construct following the procedure as previously described (Miki and Shimamoto, 2004) *Agrobacterium*-mediated transformation of rice calli (cv Dongjin) was performed as previously described (Gothandam et al., 2005). For control, pANDA vector without insert was also transformed into the rice calli.

Cytological analysis

Rice callus from vector transferred and *OsCatB* RNAi were fixed in a solution containing 4% (w/v) paraformaldehyde, 0.5% (v/v) glutaraldehyde, and 100 mM phosphate buffer (pH 7.0) for overnight at 4°C. The samples were then dehydrated in an ethanol series and embedded in an acrylic resin (London Resin Company, London, UK). The resin-embedded samples were sliced into 1 µm sections with an ultramicrotome (LKB, Bromma 2088) and stained with 0.5% toluidine blue containing 0.1% sodium carbonate. The tissue sections were examined under a light microscope (Zeiss).

RESULTS AND DISCUSSION

Sequence analysis of *OsCatB*

The *OsCatB* cDNA clone was isolated from a rice cDNA library. Nucleotide and deduced amino acid sequences of the *OsCatB* was registered in the GenBank (accession number AY916493). Blast search suggested that the gene encodes a putative cysteine protease with sequence homology to the mammalian cathepsin B which belongs to the papain family of cysteine proteases (Fig. 1A). Amino acid sequence of *OsCatB* revealed that the protein did not contain the consensus sequence of ERFNIN motif which is present in all of the papain family cysteine proteases but does not exist in the cathepsin B, suggesting that the *OsCatB* is the cathepsin B-like protein in rice (Karrer et al., 1993). Among the cathepsin B-like proteins studied in plants, we observed that the highest homology was occurred to the sequence of the barley CatB with 75.3% and 82.1% of identity and similarity, respectively, at the amino acid level (Fig. 1A, Martinez et al., 2003). The result was consistent with the phylogenetic analysis among the cysteine proteases found in plant species (Fig. 1B). Catalytic triad with Cys^{130}-His^{285}-Asn^{309} conserved among members of the papain family was also observed in the *OsCatB* sequence. Taken together, the results suggested that the *OsCatB* gene encodes a cathepsin B-like cysteine protease in rice.

Southern blot and expression analysis of *OsCatB*

DNA blot analysis was performed to determine genomic complexity of the *OsCatB* gene in the rice genome. The rice genome database analysis revealed that the

OsCatB coding sequence was separated by nine introns (Fig. 2A). The genomic DNA was digested with three different restriction endonucleases (*Eco*RI, *Hin*dIII, and *Xba*I). The DNA blot hybridization result showed that the *OsCatB* cDNA probe hybridized to at least more than three DNA fragments, indicating that the gene belongs to a multiple gene family in the rice genome (Fig. 2B). This also supports the reports that Capthepsin B, H, L –like proteases were present as small multi-gene families in most of plant species (Lidgett et al., 1995; Cejudo et al., 1992; Pechan et al., 1999; Li et al., 2000; Ueda et al., 2000). Contrarily, the highest homology to *OsCatB*, the barely CatB gene is a single copy gene (Martinez et al., 2003).

Expression pattern of *OsCatB* gene was examined by RT-PCR analysis. To do this, we isolated total RNAs from different organs of rice (flower, leaf, and root) and used them to synthesize cDNAs for PCR reaction. The result indicated that the gene transcript is present in all organs of rice (Fig. 2C). We also examined *OsCatB* gene expression during seed germination, since *OsCatB* showed homology with the barley *CatB* which was known to be active in the germinating seed (Martinez et al., 2003). Our RT-PCR experiment showed that transcripts of *OsCatB* were highly accumulated during seed germination as expected (Fig. 2D). However, we did not observe a significant regulation of *OsCatB* expression under hormones (i.e, GA and ABA) during the seed germination (data not shown). Taken together, the results support that capthepsin B like proteins are expressed both in seeds and vegetative tissues in plants (Cejudo et al., 1992; Martinez et al., 2003).

Generation of knockout plants by RNAi

In order to study the function of *OsCatB* gene in rice, we made RNAi construct using the binary vector, pANDA (Fig. 3A). RNA interference (RNAi) has been extensively used in various species to suppress gene function and it is becoming a common tool for the functional analysis of the genome (Hannon, 2002). We transformed the construct into rice by *Agrobacterium*-mediated transformation method (Lee et al., 1999) and produced a total of twenty individual transgenic calli. T-DNA integration was then confirmed using the transgenic calli by RT-PCR experiment (Fig. 3B). Among the twenty calli, we found that nine of the transgenic calli did not express *OsCatB* whereas wild-type (or non-transformed callus) showed the transcript accumulation in the callus (Fig. 3B). Surprisingly, we found that all of the nine transgenic calli were unable to continue to plant regeneration, whereas the vector control transgenic callus successfully regenerated into a plant. To gain detailed effect of *OsCatB* gene knockout into the transgenic callus, we performed cytological analysis using the calli that did not undergo regeneration (Fig. C-F). Interestingly, our microscopic observation of the transgenic callus showed that the callus did not survive on the regeneration media, suggesting that the RNAi callus underwent

an excessive cell death instead of the regeneration process (Fig. 3E, F). The vector transformed callus cell contained well organized cytoplasmic contents with nucleus (Fig. 4C, D) whereas the *OsCatB* gene knockout callus showed highly vacuolated cells due to degradation of cytoplasmic contents (Fig. 4E, F).

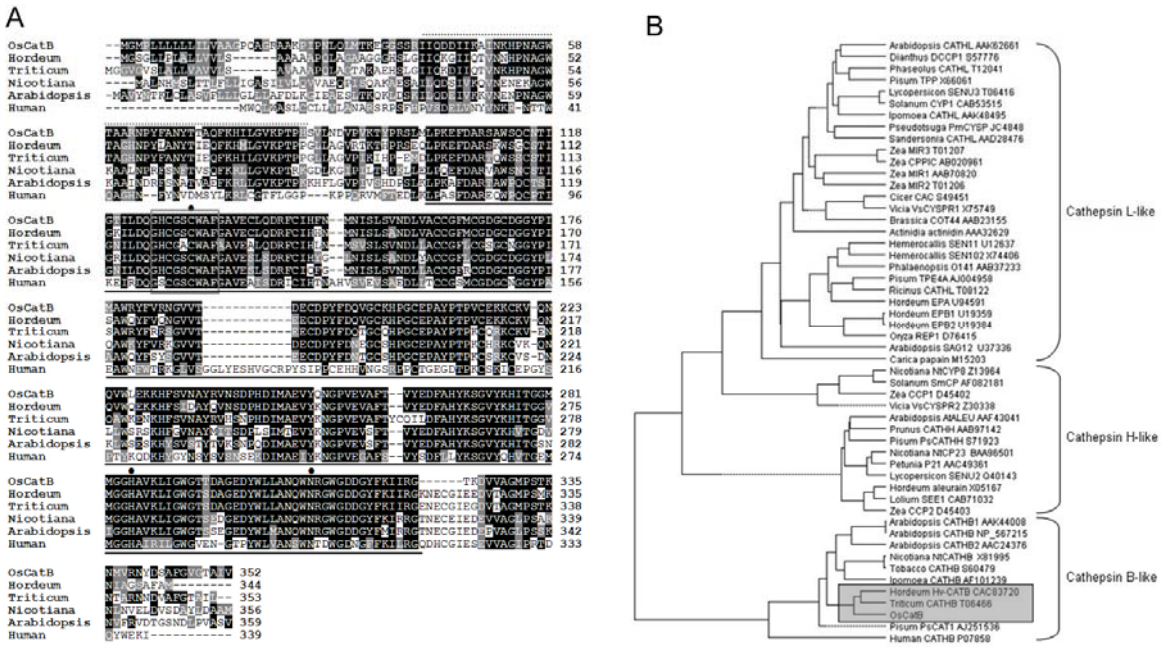

Figure 1. Sequence analysis of *OsCatB*. A. Sequence alignment of *OsCatB*. Identical amino acid residues were black shaded and similar ones were grey shaded. The catalytic cysteine-histidine-asparagine triad of cysteine protease was indicated by filled circles. Peptidase C1 domain of the papin family cysteine protease was underlined. The dotted line at the N terminal region indicates the Propeptide motif of Peptidase C1 family which is involved in activation of the peptidase. The consensus motif, Gly-Cys-Ans-Gly-Gly was boxed. Amino acids are numbered in the right side. Proteins used in the alignment were *OsCatB* (*Oryza sativa*, AY916493), Hordeum (*Hordeum vulgare*, CAC83720), Triticum (*Triticum aestivum*, Q03107), Nicotiana (*Nicotiana rustica*, CAA57522), Arabidopsis (*Arabidopsis thaliana*, AAK44008) and Human (*Homo sapiens*, NP_680093). B. Phylogenetic analysis of rice cathepsin-B like cysteine protease with other plant (cathepsin B, H and L like) proteases. The whole sequences were used for analysis. The GenBank accession numbers are indicated.

Recently, Bozhkov et al., (2005) reported that cysteine protease involves in the programmed cell death during plant embryogenesis. In contrast, our RNAi result suggests that *OsCatB* is required for general cellular functioning and metabolism. Moreover, a recent report indicates that aspartic protease functions as an anti-cell-

death component during embryonic development and reproduction processes (Ge et al., 2005). Several proteases have been implicated in regulating and executing cell death processes. Our result suggests that the cathepsin B like-cysteine protease plays an important role in cell survival.

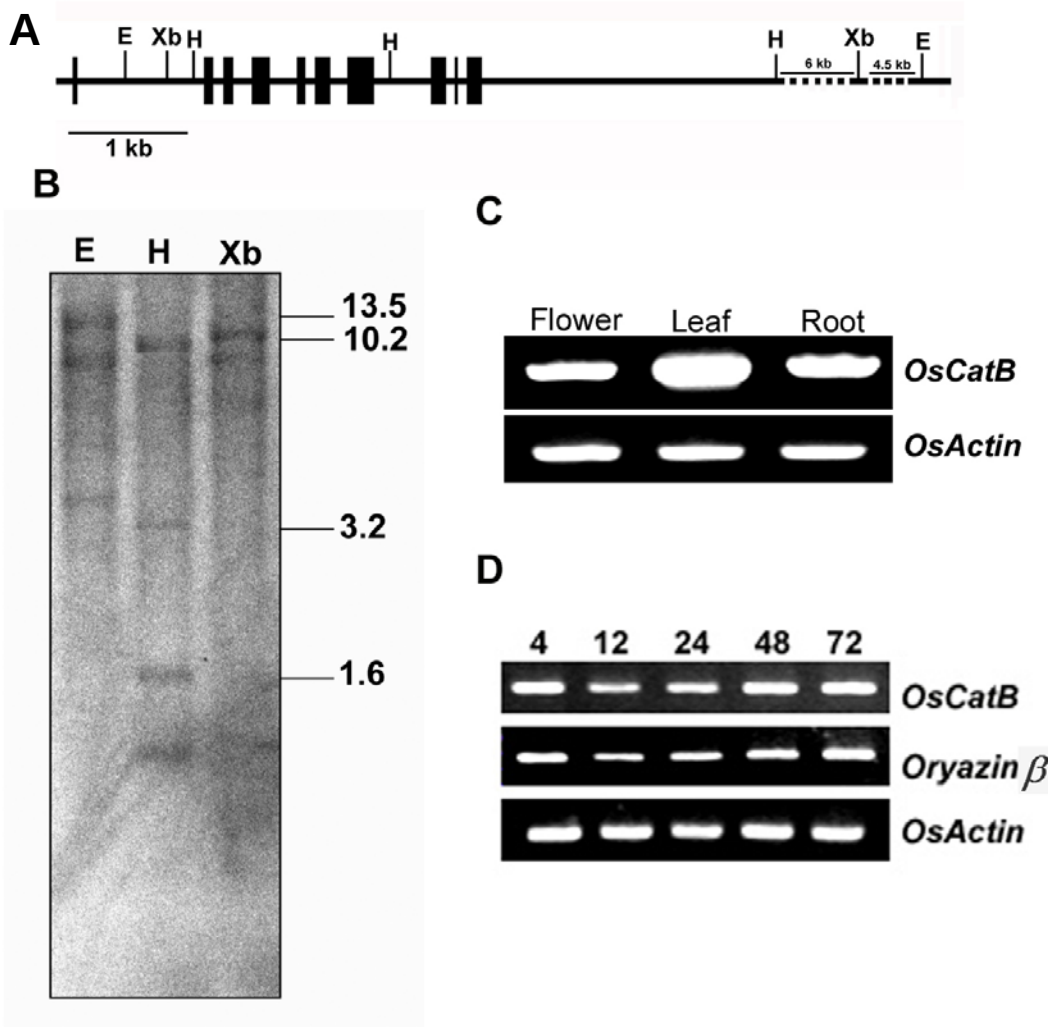

Fig. 2. Southern and expression analysis of *OsCatB*. A. Schematic diagram of *OsCatB* gene structure in the rice genome. The closed black box represents the exons. Position of the restriction enzyme sites is shown with the specific abbreviations; *Eco*RI (E), *Hin*dIII (H), and *Xba*I (Xb). B. Southern blot hybridization of *OsCatB*. Genomic DNA was digested with the restriction enzymes mentioned above and hybridized with *OsCatB* probe. *Eco*RI (E), *Hin*dIII (H), and *Xba*I (Xb). C. Transcript accumulation of *OsCatB* in rice flower, leaf and root. D. Transcript accumulation of *OsCatB* in germinating seed of rice during a time course (in hours). *Ozyzain β* and *OsActin* as a positive control.

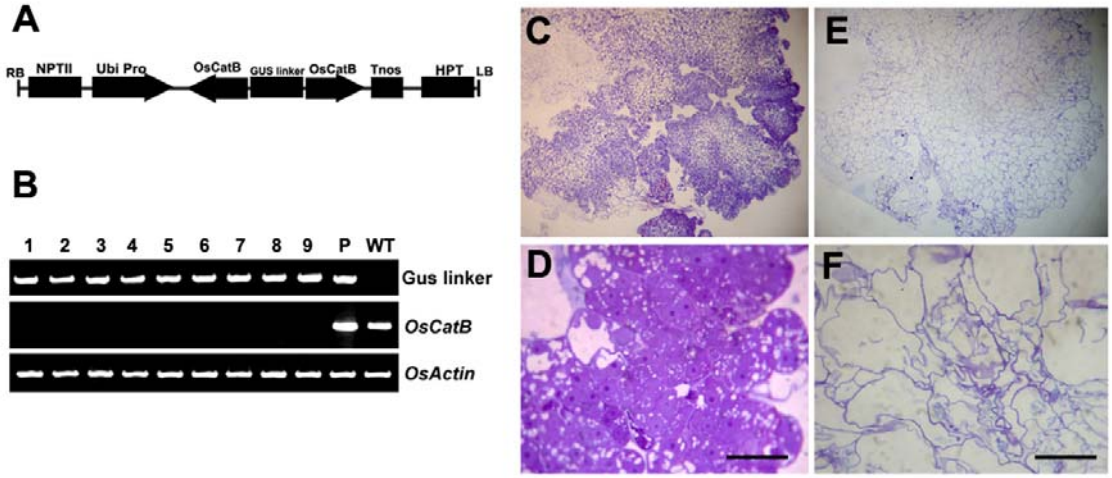

Fig. 3. Transformation and expression analysis. A. Diagram of RNAi vector construct. RB and LB, the right and left border of the T-DNA, respectively. The *OsCatB* cDNA clone was subcloned into both sides of the gus linker in antisense and sense orientations. Ubi Pro, Ubiquitin promoter; Tnos, nos terminator; NPTII, Kanamycin resistance gene; HPT, hygromycin resistance gene. B. Transcript accumulation of *OsCatB* and Gus linker in the transgenic callus. 1-9, individual transgenic callus; P, pANDA vector transformed into callus as a positive control; WT, non-transferred callus. *OsActin* used as a control. C – F. Cytological analysis of callus. Light microscopic observation of vector transformed callus (C, D) and *OsCatB* gene (RNAi) transformed callus (E, F). Scale bar = 20μm.

In conclusion, the data showed an ubiquitous expression of *OsCatB* gene in rice. We have done an overexpression study of the gene in rice. However, we did not observe any phenotypic alteration in the transgenic rice (data not shown). Our study using a RNAi approach has suggested that *OsCatB* may play an important role in determining of cell survival. Future identification of physiological substrates will give a clue on the precise role of *OsCatB* in regulating the cell survival or cell death in plants.

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