Isolation and Expression of a Pistil-Specific Chitinase Gene in Rice (*Oryza sativa* L.)

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Among the array of pathogenesis-related (PR) proteins, there is a subset which is expressed in some tissues on normal development, while many other PR proteins are induced by a series of elicitors after a fungal or virus infection (Van Loon *et al.* 1994). The transmitting tissue of a pistil secretes a number of proteins into the intercellular matrix, some of which are specifically expressed in the tissue. It is noteworthy that flowers are rarely infected by diseases despite the lack of a protective surface. From this point of view, PR proteins were screened in pistil, and a pistil-specific PR-1-like protein was identified from two species of *Camellia* in our laboratory (Tomimoto *et al.* 1999).

Chitinase, a PR protein, hydrolyzes chitin, a polymer of *N*-acetyl-D-glucosamine which is a primary component of fungal cell walls (Collinge *et al.* 1993). In plants, chitinases are considered to play a role in the biochemical defense against chitin-containing fungal pathogens, because plants have no endogenous substrates for chitinase. So far, two chitinases have been isolated from pistils of two dicot plants, potato (Wemmer *et al.* 1994) and tomato (Harikrishna *et al.* 1996). Although the chitinases expressed in the pistil have not been identified in rice, from the view point mentioned above, we assumed the presence of such a developmentally expressed chitinase. We searched for a chitinase in the pistil of rice using chitinase specific primers designed after conserved sequences, and identified a novel form of class I chitinase which was specifically expressed in pistil just before anthesis.

Cloning and analysis of cDNA encoding chitinase from the pistil of rice

To identify chitinases expressed in the pistil of IR 36, we performed RT-PCR using degenerate primers derived from highly conserved sequences of known chitinases (5'-AAGCGSGAGRTCGCYGCSTTC-3' and 5'-CCAGAACASARCGCCGTCTT-3') and mRNA from rice pistils at the heading stage. Poly(A)⁺RNAs from the

pistil were directly prepared using a Quick Prep Micro mRNA Purification kit (Pharmacia), and cDNAs were prepared using a cDNA Synthesis Kit (Pharmacia). The PCR product in a single prominent signal of the expected size was purified from a gel and cloned into the pCR 2.1 vector of a TA cloning kit (Invitrogen). After sequencing of two clones, the DNA fragment of 294 bp including 42 bp of primers was obtained.

The DNA fragment of the clone for the partial sequence labeled with DIG was used for screening of the cDNA library of the rice pistil, constructed from the same poly(A)⁺RNA as used in RT-PCR using a Lambda ZAP II/*Eco*RI/CIAF cloning kit (Stratagene), and *in vitro* with a Max Plax Lambda Packaging Extract (Epicentre Technologies). Positive phage clones were converted to pBluescript SK⁻ plasmid clones with *in vivo* excision according to the manufacturer's protocol (Stratagene). From the library of c.a. 38000 pfu, 19 clones were obtained after the screening. After PCR-amplification with the degenerate primers, similar fragments were obtained from 18 of the 19 clones. Three of the 18 clones and the remaining clone, which were obtained after the second screening of the library, were used for sequencing analysis. The three clones were shown to have an identical sequence, but, the insert sequence was not detected in the remaining one clone.

The isolated cDNA clones were about 1280 bp and their sequences were estimated to contain an open reading frame (ORF) encoding a protein of 340 amino-acid residues (DDBJ, accession no. AB018248). The deduced amino acid sequence had a high homology with the known chitinases of rice (Fig. 1), so that the protein was named pistil chitinase (PC). The computer-calculated isoelectric point (pI) of the predicted mature protein of PC was 8.0. This pI value showed that the mature protein has a basic nature although the computer-calculated pI may not always be the same as the real pI value because of post translational modifications.

To compare the PC between *indica* and *japonica* a clone of the cDNA from *japonica* cultivar Koshihikari was also obtained by the same method for IR 36. The ORF sequence obtained from IR36 was different from that of the *japonica* cultivar, Koshihikari in three bases, one in the cysteine-rich domain and two in the C-terminal extension (CTE). The single base change replaced an amino-acid residue from glycine (GGC) to aspartic acid (GAC) in the CTE. The deduced catalytic domains of the PC were identical in the two cultivars.

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		Signal peptide		Cysteine-rich domain and Hinge region	
PC		M I A A R A A N L Q V A M K A L A L A V L A L A Y A A A T A R A		E Q C G R Q A G G A R C P N R L C C S R W G W C G L T D	60
Class I	<i>O. sativa</i> (CHT1)		M R A L A V V V V A T A F A V V A V R G	E Q C G S Q A G G A L C P N C L C C S Q Y G W C G S T S	48
Class I	<i>O. sativa</i> (CHT2)		M S T P R A A S L A K K A L A V A L A A L A T A A R A	E Q C G A Q A G G A R C P N C L C C S R W G W C G T T S	60
Class II	<i>O. sativa</i>		M T T T T A R F V Q L A A C A A A A L L A V A S G A A		29
Class IV	<i>O. sativa</i>		M A N S P T L T M L V F L A I G L S L V L S		22
Class IV	<i>Zea mays</i>		M A N A P R I L A L G L L A L L C A A A G P A A A Q N C G		46
Class II	SK2		M R L L V L A W F S V L C L K C V L A		19
Class II	Chi2;1		M R L L V L G L F S V L C L K C V L S		19
			Catalytic domain		
PC		D Y C K G G C Q S Q C R V S R D G G D D D V A A V L L T A P G G G		R A G V A S V V T S D Q F E R M L P H R D D A A C P	119
Class I	<i>O. sativa</i> (CHT1)		A Y C G S G C Q S Q C S G S		101
Class I	<i>O. sativa</i> (CHT2)		D F C G D G C Q S Q C S G		111
Class II	<i>O. sativa</i>				54
Class IV	<i>O. sativa</i>				49
Class IV	<i>Zea mays</i>		A Y C G D G C Q S G P C R S G G G G G		101
Class II	SK2				44
Class II	Chi2;1				44
PC		A R G F Y A Y R A F V A A A G A F P A F A A T G D A D T R		K R E V A A F L A Q T S H A T S G	171
Class I	<i>O. sativa</i> (CHT1)		A K N F Y T Y D A F V A A A N A F P S F A T T G D A A T R		160
Class I	<i>O. sativa</i> (CHT2)		A R G F Y T Y E A F L A A A A F P A F G G T G N T E T R		170
Class II	<i>O. sativa</i>		A R G F Y T Y D A F I A A A N S F P A F G T S G G S A E L I		113
Class IV	<i>O. sativa</i>		G K S F Y T R Q S F L N A A R S Y S G F A N D R T N D S		95
Class IV	<i>Zea mays</i>		G K N F Y T R S A F L S A V N A Y P G F A H G G T E V E G		147
Class II	SK2		A K G F Y T Y E A F I T A T K S F A A F G T T G D T N T R		103
Class II	Chi2;1		A K G F Y T Y E A F I T A T K T F A A F G T T G D T N T R		103
PC		Y C Y K - E V K G A - - - - T S D F C V P N A R - W P C A P G K A Y H A R G P M Q I A Y N Y N Y G A A G E A I G - A D L			224
Class I	<i>O. sativa</i> (CHT1)		Y C F K E E N N G N - - - - V G S D Y C V Q S S Q - W P C A A G K K Y Y G R G P I Q I S Y N Y N Y G P A G Q A I G - S N L		215
Class I	<i>O. sativa</i> (CHT2)		Y C F K Q E Q N P - - - - P S D Y C Q P S P E - W P C A P G R K Y Y G R G P I Q L S F N F N Y G P A G R A I G - V D L		223
Class II	<i>O. sativa</i>		Y C F K E E I N K A - - - - T S - - - - P P Y Y G R G P I Q L T G S N Y Q A A G N A L G - L D L		153
Class IV	<i>O. sativa</i>		M C Y I N E I N G - - - - A N M D Y C D K S N K Q W P C P G K K Y Y G R G P L Q I S W N F N Y G P A G K N I G - F D G		150
Class IV	<i>Zea mays</i>		F C Y I S E I N K - - - - S N - A Y C D A S N R Q W P C A A G Q K Y Y G R G P L Q I S W N Y N Y G P A G R D I G - F N G		201
Class II	SK2		Y C F K Q E Q G S P - - - - G D Y C V A S Q Q - W P C A P G K K Y F G R G P I Q I S Y N Y N Y G P A G R A I G - V N L		156
Class II	Chi2;1		Y C Y K Q E Q G S P - - - - G D Y C A S S Q Q - W P C A P G K K Y F G R G P I Q I S Y N Y N Y G A A G S A I G - V N L		156
PC		L G N P E L V A T D P T V A F K T A L W L W M T A R S P S Q P S P H A V V T G Q W T P T P A D S A A G R A P G Y G L T T			284
Class I	<i>O. sativa</i> (CHT1)		L S N P D L V A S D A T V S F K T A F W F W M T P Q S P - K P S C H A V M T G Q W T P N G N D Q A A G R V P G Y G V V T		274
Class I	<i>O. sativa</i> (CHT2)		L S N P D L V A T D A T V S F K T A L F W M T P Q G N - K P S S H D V I T G R W A P S P A D A A A G R A P G Y G V I T		282
Class II	<i>O. sativa</i>		V G N P D L V S T D A V S F K T A I W F W M T A Q G N - K P S C H D V I L G R W T P S A A D T A A Y R V P G Y D G I T		212
Class IV	<i>O. sativa</i>		L R D P D K V A Q D P T I S F K T A L W F W M N - - - - N V H Q V M S - - - - - Q G F G A T I		188
Class IV	<i>Zea mays</i>		L A D P N R V A Q D A V I A F K T A L W F W M N - - - - N V H G V M P - - - - - Q G F G A T I		239
Class II	SK2		L N N P D L V A N D A V S F K T A L W F W M T P Q Q P - K P S A H D V I T G R W S P S A A D S A A G R V P G F G V I T		215
Class II	Chi2;1		L N N P D L V A N D A V S F K T A L W F W M T A Q Q P - K P S A H D V I T G R W S P S V A D S A A G R V P G F G V I T		215
PC		N I L T G G L Q C A G G - - N G G - - A D R V A F Y K R Y C D V L G V G Y G P N L D C F G Q A P F D G G I M S A S A A K			340
Class I	<i>O. sativa</i> (CHT1)		N I I N G G V E C G H G - - A D S R V A D R I G F Y K R Y C D M L G V S Y G A N L D C Y N O R P F N S - - - - -		323
Class I	<i>O. sativa</i> (CHT2)		N I V N G G L E C G H G - - P D D R V A N R I G F Y Q R Y C G A F G I G T G G N L D C Y N O R P F N S G S S V G L A E Q		340
Class II	<i>O. sativa</i>		N I I N G G I E C G V G - - Q N D A N V D R I G Y K R Y C D M L G T G Y G S N L D C Y N O R N F A S - - - - -		261
Class IV	<i>O. sativa</i>		R A I N G A L E C N G K - N P G A V N A - R V N Y K D Y Q R F G V S P G G N L Y C - - - - -		229
Class IV	<i>Zea mays</i>		R A I N G A L E C N G N - N P A Q M N A - R V G Y K Q Y C Q Q L R V D P G P N L I C - - - - -		280
Class II	SK2		N I I N G G I E C - - S K S N A Q M D N R I G F Y R R Y C Q I L G V D P G N N L D C A N O R P F G Q - - - - -		264
Class II	Chi2;1		N I I N G G M E C - - N S G S N A L M D N R I G F Y R R Y C Q I L G V D P G N N L D C A N O R P F G - - - - -		263

Fig. 1. Alignment of the deduced amino-acid sequence of PC with class I, II and IV chitinases and other developmentally expressed chitinase of dicot plants. Gaps are marked by dashes. Black boxes indicate identical residues with those of PC. Amino-acid residues which are conserved in all cases are marked with asterisks. Sequence data in the alignment are as follows: Class I *Oryza sativa* (CHT1) and (CHT2) (Nishizawa *et al.* 1993), class II *Oryza sativa* (Kim *et al.* 1998), class IV *Oryza sativa* (Nakazaki *et al.* 1997, Nakazaki and Ikehashi 1998), class IV *Zea mays* (Huyuh *et al.* 1992), class II SK2 (Wemmer *et al.* 1994 and class II Chi2; 1 (Harikrishna *et al.* 1996).

Northern blot analysis for the specificity of expression

A Northern blot analysis was performed with each of the total RNAs isolated from pistils, anthers and lemma at the heading stage as well as embryo, endosperm, young leaves and young roots of IR36 and with the Poly(A)⁺ RNAs from pistils at three different developmental stages from cultivar Koshihikari using the cDNA clone of PC as a probe. The procedures for the Northern blot analysis as

follows. A DIG labeled probe was prepared from the pistil chitinase clone using a DIG DNA Labeling Kit (Boehringer Mannheim). A 17S ribosomal RNA clone from rice, which was provided by the courtesy of Dr. Omido at the Hokuriku National Agricultural Experiment Station, was used as a control probe in the Northern blot analysis. Hybridization was performed over night at 71°C. Filters were washed twice with 2×SSC/0.1% SDS at room temperature for 5

min, followed by $0.1 \times \text{SSC}/0.1\%$ SDS at 68°C for 15 min. Hybridization signals were detected with a DIG Labeling and Detection Kit (Boehringer Mannheim), according to the supplier's protocols. Transcripts for PC were specifically accumulated in the pistil just before anthesis (Fig. 2A). No signal was detected in other tissues of the plant. The expression levels of PC in the pistil increased with development (Fig. 2B).

Classification of PC based on its primary structure

Chitinases, whose catalytic domains are homologous to some extent, are classified into three groups by their primary structures (Collinge *et al.* 1993). In rice, all three groups of chitinases have been reported, i.e., class I (Zhu and Lamb 1991, Nishizawa *et al.* 1993 etc.), class II (Kim *et al.* 1998) and class IV (Nakazaki *et al.* 1997, Nakazaki and Ikehashi 1998). An alignment of amino-acid sequences of PC and class I, II and IV chitinases in rice is shown in Fig. 1. Since the primary structure of the rice class IV chitinase is exceptional because of deletion of a chitin-binding domain and a glycine-rich hinge region at N-terminal (Nakazaki *et al.* 1997), which are characteristic of the class I and IV chitinases (Collinge *et al.* 1993), a typical class IV chitinase is added in the alignment. The predicted amino-

acid sequence of PC has a putative signal sequence at the N-terminus. The N-terminal signal sequence of about 32 amino-acid residues contained two methionine residues, the first one followed by 11 residues and the second followed by 19 residues. The signal sequence of PC is longer than that of rice chitinase *Chit-1*, which has 12 residues. The mature protein of PC is started with a cysteine-rich chitin-binding domain and a glycine-rich hinge region at the N-terminal. The predicted catalytic domain had a short deletion of about 7 amino-acid residues at a position between 165 and 166 in amino-acid sequences (Fig. 1). These deletions have not been found among other known class I chitinases. Although the deletion in PC is similar to one of the deletions in class IV chitinase, it is much shorter than those in class IV chitinases. The sequence was found to have a short CTE which was characteristic to class I chitinases and known to function as a vacuole-targeting signal (Neuhaus *et al.* 1991). Thus, PC does not seem to be a member of class IV, but a new and variant member of class I chitinases in rice.

Recently, in two dicot plants, potato and tomato, some dominant basic proteins of the stylar matrix, which were named SK2 and Chi2; 1, respectively, have been shown to be endochitinases and essential for stylar-specific functions (Wemmer *et al.* 1994, Harikrishna *et al.* 1996). This study revealed that a chitinase gene is also expressed developmentally in the pistil of monocot plants.

The two chitinases of potato and tomato are classified into the class II chitinase, which lack the cysteine-rich domain and the hinge region (Fig. 1). However, the catalytic domains in these dicot chitinases are highly homologous to class I chitinase of rice as in PC classified into class I (Fig. 1). The deletion of the 7 amino acids detected in the sequence of PC is a characteristic structure only in PC. PC had the cysteine-rich domain as mentioned above. These facts indicate that the cycteine-rich domain is not an essential character for stylar-specific chitinases.

Many chitinases have been shown to inhibit fungal growth not only *in vitro* (Schlumbaum *et al.* 1986, Leah *et al.* 1991) but also in transgenic plants with a chitinase gene (Brogli *et al.* 1991, Jach *et al.* 1995, Lin *et al.* 1995). The function of the new unique chitinase identified in the pistil of rice should be tested further through genetic transformation.

Fig. 2. Northern blot analysis of RNAs extracted from different organs of the two cultivars (A, IR 36 and B, Koshihikari). A. Total RNAs from pistil (Lane 1), anther (Lane 2), embryo (Lane 3), endosperm (Lane 4), lemma (Lane 5), leaf (Lane 6) and root (Lane 7) of IR36 were used. PC insert (upside) and 17S ribosomal RNA sequence from *Oryza sativa* (downside; control) were used as probes. In each lane 20 μg of total RNA was loaded. B. As RNA materials, 0.3 μg mRNA from pistils (Lane 1 to 3) and anthers (Lane 4 to 6) for three developmental stages (microspore stage (Lane 1 and 4), binucleate stage (Lane 2 and 5) and trinucleate stage (Lane 3 and 6)) of Koshihikari were used. PC cDNA from Koshihikari was used as a probe.

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