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A Novel Chitinase in Rice (*Oryza sativa* L.) Detected from Husk Proteins and its Gene Locus

Tetsuya Nakazaki[1], Yoshiteru Tomimoto[1], Hiroshi Ikehashi[1], Yasuo Kowyama[2], Masahiro Yano[3], Kimiko Yamamoto[3] and Takuji Sasaki[3]

[1] *Graduate School of Agriculture, Kyoto University,* Kyoto 606-01, Japan

[2] *Faculty of Bioresources, Mie University,* Tsu, Mie 514, Japan

[3] *Rice Genome Research Program (RGP), National Institute of Agrobiological Resources (NIAR)/Institute of Society for Techno-innovation of Agriculture, Forestry and Fisheries (STAFF),* Tsukuba, Ibaraki 305, Japan

Summary

A unique protein which was induced in husk of rice strongly under high temperature (in greenhouse) and weakly under low temperature (out-of-doors) was found in a survey with two-dimensional electrophoresis. The N-terminal 16-amino-acid sequence of this rice husk protein (RHP) showed a significant homology with chitinases in other plant species and a complete identity with a peptide deduced from a partial sequence of registered cDNA clone SS2594 in the library of the Rice Genome Research Program, Japan. Thus, the deduced amino-acid sequence for SS2594 was identified as a kind of chitinases. The amino-acid sequence for SS2594 has a unique structure among other chitinases reported for rice. It is concluded that RHP is translated from the gene for SS2594 and a product of a new locus, *Chi-4* (S12594) in rice. On the basis of RFLP linkage analysis, the gene *Chi-4* was located on chromosome 4.

Key Words: *Oryza sativa* L., chitinase, husk protein, RFLP mapping, cDNA clone.

Introduction

Chitinases are considered to be important in the biochemical defense of plants against chitin-containing fungal pathogens. Indeed, Lin *et al.* (1995b) reported that the resistance to pathogen, *Rhizoctonia solani,* in the transgenic rice plants with chitinase gene under the control of CaMV 35S promoter was correlated with the level of chitinase expression.

To search for a dormancy-related protein we have surveyed a group of proteins in rice husk using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) after growing plants during maturation at high temperature condition or at low temperature condition, as a high temperature is known to induce strong dormancy (Ikehashi 1972). From this experiment, a protein was found which was strongly induced in husk formed under high temperature condition. In an analysis of N-terminal amino-acid sequences, this protein (RHP) showed a significant homology with other known chitinases. At the same time, a rice cDNA clone with an identical amino acid sequence to RHP N-terminal was found from a DNA database and a deduced amino-acid sequence of this cDNA clone is more homologous with chitinases in other plants than rice chitinases reported so far.

We herein report the expression of RHP with 2D-PAGE analysis, the structure of protein deduced from the partial cDNA sequence and chromosomal location of the novel chitinase gene in rice.

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Materials and Methods

Plant materials

Four rice varieties were used: Koshihikari, Ginmasari, Nanjing 11 and IR 36. Koshihikari is a Japonica variety showing a relatively high level of seed dormancy. Ginmasari is also a Japonica variety, but shows a low level of dormancy as do most Japanese rice varieties (Ikehashi 1972). Koshihikari and Ginmasari were planted in pots out-of-doors at Kyoto University until flowering. To create differential temperature conditions during maturation, half of the plants of each variety were transferred to a greenhouse and the rest were kept outside during maturation. Temperature conditions in the greenhouse were higher by about 5 °C than the temperature outside which averaged 22 °C during maturation. Temperature data were provided by courtesy of the Laboratory of Irrigation and Drainage, Faculty of Agriculture, Kyoto University.

IR 36 and Nanjing 11 are Indica varieties which display high and low levels of dormancy, respectively (Wan *et al.* 1997). IR 36 and Nanjing 11 were planted at the farm of Kyoto University and kept out-of-doors during maturation. Seeds obtained from plants grown in this condition during maturation are expected to possess different levels of dormancy between the two cultivars (Wan *et al.* 1997).

The panicles of each variety were harvested at about 30 days after heading, rapidly frozen in liquid nitrogen and stored at −80 °C until use.

2D-PAGE

The husk were dissected from stored panicles and were processed into acetone powder according to the method

of Damerval *et al.* (1986). Proteins were extracted from the acetone powder with lysis buffer (O'Farrell 1975) which contains 9.5 M urea, 2 % (w/v) NP-40, 5 % (w/v) PVP-40, 2 % Ampholine (pH 3.5-10) and 5 % 2-mercaptoethanol. The supernatant was recovered by centrifugation at 15,000 rpm for 10 min. and subjected to 2D-PAGE. Proteins were separated by non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and by SDS-PAGE in the second dimension as described by Sassa *et al.* (1993). Proteins in the gels were detected by silver staining using the Sil-Best Stain for Protein/PAGE (Nacalai Tesque, Kyoto).

Analysis of amino acid sequence and homology search

Proteins separated by 2D-PAGE were electroblotted onto a PVDF membrane and detected by Ponceau S staining (Salinovich and Montelaro 1986). PVDF membrane sections carrying the proteins were cut out and placed in the upper blot cartridge of a reaction chamber in a protein sequencer (476A, Applied Biosystems, Foster City, CA., USA). Edman degradation was performed according to the standard program obtained from Applied Biosystems and a sequence of 16 amino acids were determined.

Analyses of amino-acid sequence homology were performed by basic local alignment search tool (BLAST, Altschul *et al.* 1990) of National Center for Biotechnology Information (NCBI) non-redundant protein sequence databases. A cDNA sequence corresponding to the husk protein of rice was found from DNA Data Bank of Japan (DDBJ) in the Institute of Genetics, Mishima, Japan.

Sequencing and RFLP mapping

As the registered DNA sequence of a cDNA that was detected from the database in this study seemed to contain some errors, the cDNA clone was again sequenced for correction. The experimental procedures for preparation of DNA and sequencing were the same method in the report of Sasaki *et al.* (1994).

An F_2 population derived from a cross of rice cultivars Nipponbare/Kasalath was used for mapping. The procedure of DNA extraction, blotting, hybridization with RFLP markers and linkage analyses were the same as those reported in Kurata *et al.* (1994).

Results

Identification of proteins in rice husk

Husk proteins obtained from the two Japonica varieties, Koshihikari and Ginmasari, each of which matured at high and low temperature conditions, were surveyed by 2D-PAGE. The resulting protein profiles from the four samples were almost identical to each other. However, one protein spot, which had a size of about 23 kDa and somewhat high pIs (around pH 9), showed a significant difference in the amount of protein detected. The protein spots obtained from the high temperature condition were significantly larger than those from the low temperature condition (Fig. 1). The differentiated induction levels in the two conditions were found in both varieties.

The 2D-PAGE protein profiles for Nanjing 11 and IR 36 were also similar to each other. With respect to the spot mentioned above, IR 36 showed a larger spot than did Nanjing 11 (Fig. 2). The unique protein was designated as RHP (rice husk protein).

N-terminal amino-acid sequence of RHP

We determined a sequence of 16 N-terminal amino acids of RHP obtained from Koshihikari grown at high temperature. Subsequently, from the results of BLAST searches of non-redundant protein sequences, the N-terminal sequence of RHP was found to have significant identities with internal regions of ten known chitinases (Fig. 3). Two chitinases from maize (*Zea mays*) and a chitinase from rape (*Brassica napus*) had an amino-acid sequence of 68.8 % homology with RHP at 16 positions. And the positions of residues which varied between the sequences of RHP and those of other chitinases are limited and also variable among other known chitinases. The segments of chitinases having homology with N-terminal of RHP are all the segments in N-terminal side of the catalytic domain (see below), indicating that the N-terminus of RHP may possess chitinase functions. These findings suggest that RHP is a chitinase of rice.

A rice cDNA clone homologous to RHP encodes a kind of chitinases

On the basis of another BLAST search offered by DDBJ, an amino-acid sequence deduced from rice cDNA clone SS2594 was found to have a segment that is identical with the 16-amino-acid N-terminus of RHP. The cDNA clone SS2594 was isolated from green rice shoot, sequenced and registered in the database of DDBJ (Accession: D47304) in a research at the Rice Genome Research Program, Japan. As the sequence data of D47304 (450 bp) were considered to contain a few erroneous positions, the cDNA clone SS2594 was sequenced again in this study. Then, the sequence of SS2594 (632 bp, Accession: C19108) was determined (Fig. 4). Then, 189 amino acids were deduced out of a complete protein for the cDNA clone.

In BLAST searches for proteins similar to the 189 amino-acid residues deduced from SS2594 a set of chitinases including the chitinases in Fig. 3 were identified. The alignments of amino-acid sequence for SS2594 and the sequences of other homologous chitinases obtained from the database were summarized in Fig. 5. The amino-acid sequence for SS2594 covered about four fifths of an entire protein assumed by other homologous chitinases. Four cysteine residues in the sequence for SS2594 were conserved perfectly (at positions 48, 97,110 and 119, Fig. 4), which are related to three dimensional structure of proteins by disulfide bonds (Hart *et al.* 1993, Yamagami and Funatsu 1993).

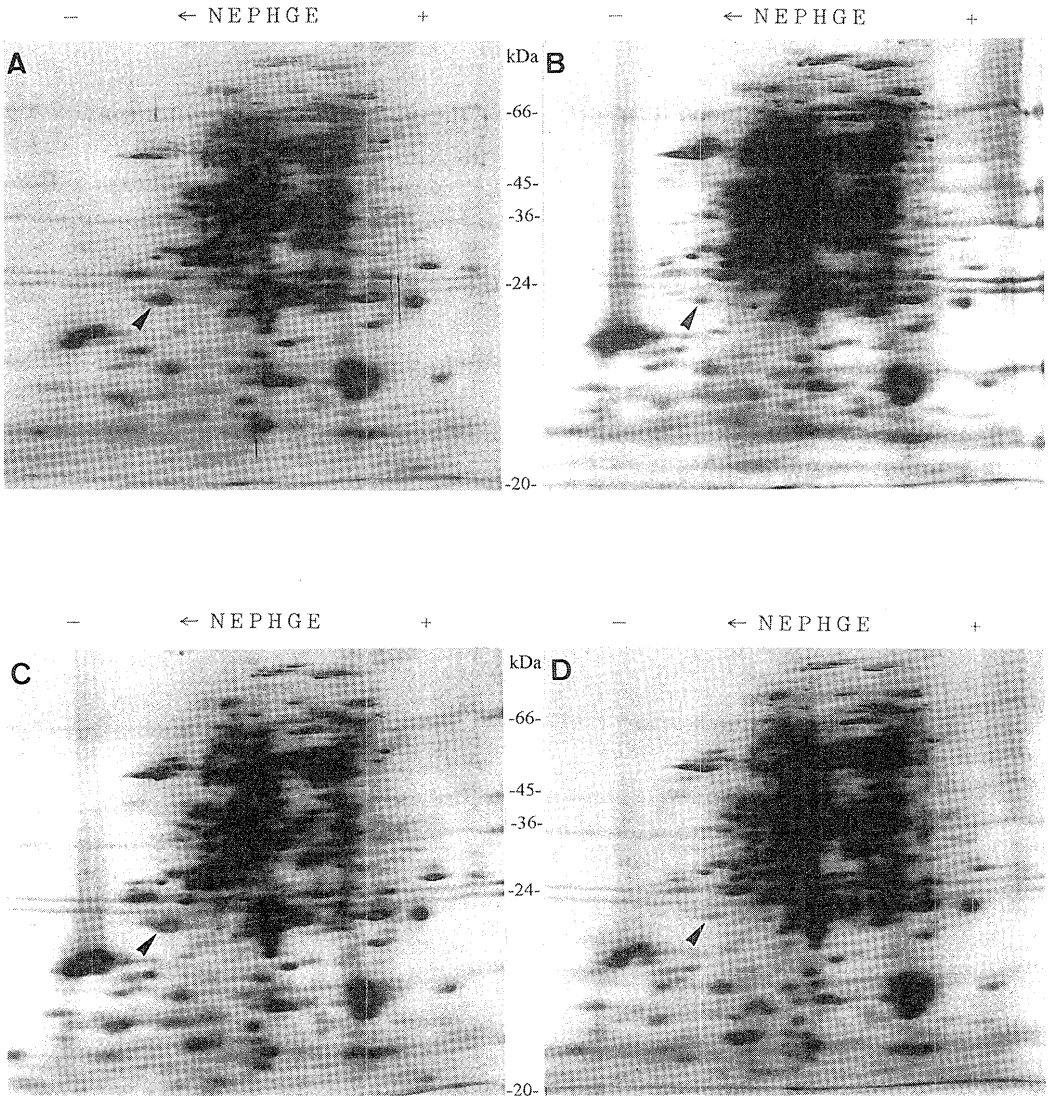

Fig. 1. 2D-PAGE profiles of rice husk extracts from Koshihikari (A and B) and Ginmasari (C and D), each of which matured under high (A and C) and low (B and D) temperature conditions. The size of RHP (marked with *arrows*) was significantly different between the two temperature conditions.

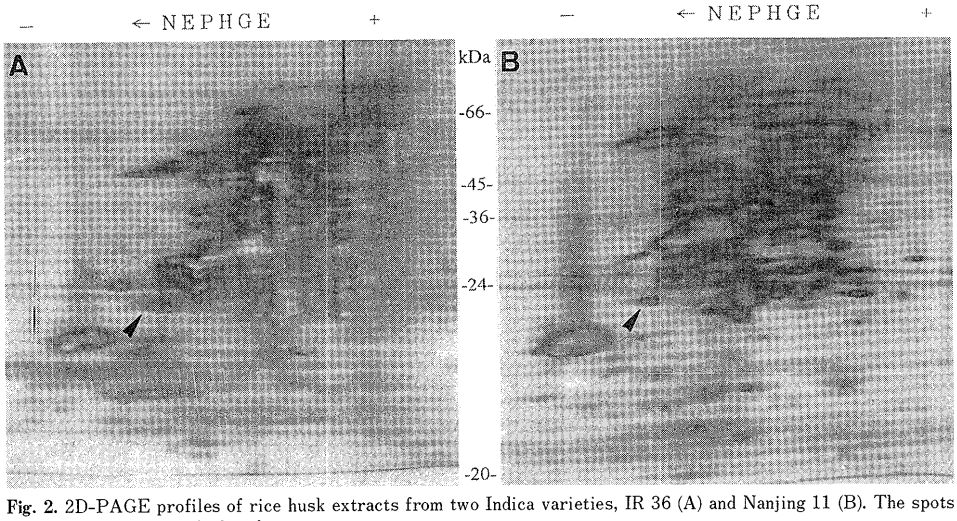

Fig. 2. 2D-PAGE profiles of rice husk extracts from two Indica varieties, IR 36 (A) and Nanjing 11 (B). The spots of RHP are marked with *arrows*.

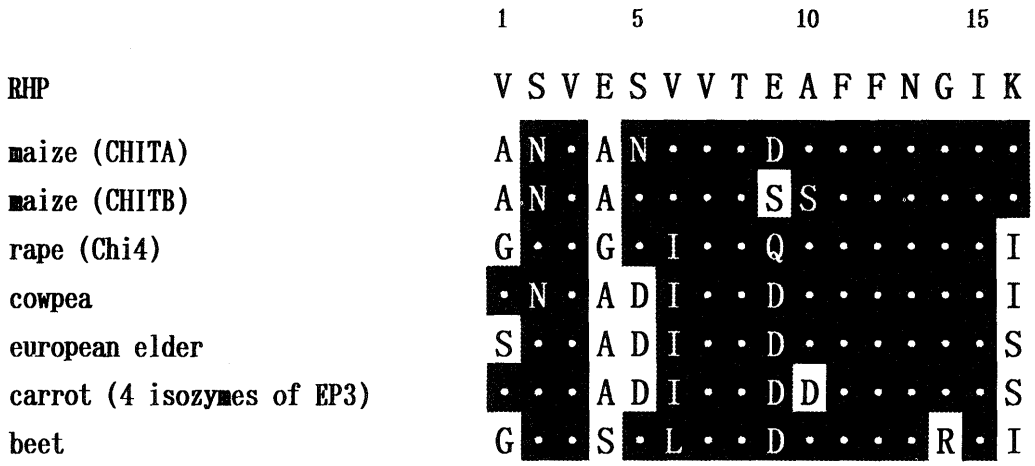

Fig. 3. Alignments of the N-terminal amino-acid sequence of RHP with those of homologous sequences of known chitinases. Amino-acid residues identical to those of RHP are represented by dots. Black boxes indicate the sequences where the residues are identical and similar to those of RHP. Sources: maize (CHITA and CHITB, Huyuh *et al.* 1992), rape (Chi4, Rasmussen *et al.* 1992), cowpea (PIR: S57476), European elder (PIR: S51645) carrot (4 isozymes of EP3, Kragh *et al.* 1996, and nr: U52846, U52847, U52848 in the DDBJ/EMBL/GenBank International Nucleotide Sequence Database) and beet (nr: A23392).

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a  ACCTCTCCCACACGGGGCGTACGTGCTAAGCTGCAGCACAACCCAAAGCCATATATCT                58
a  CCGATAATGGCGAACTCACCGACGCTGACAATGCTGGTGTTCCTAGCTATTGGGCTATCT            118
b            M  A  N  S  P  T  L  T  M  L  V  F  L  A  I  G  L  S          18
a  CTCGTCCTCTCCGCAGCCGGCGTGTCTGTGGAGAGCGTGGTCACCGAGGCGTTCTTCAAC            178
b     L  V  L  S  A  A  G  V  S  V  E  S  V  V  T  E  A  F  F  N          38
a  GGGATCAAGAACCAGGCCCCGAACGGCTGCGCCGGTAAGAGCTTCTACACACGGCAATCG            238
b     G  I  K  N  Q  A  P  N  G  C  A  V  K  S  F  Y  T  R  Q  S          58
a  TTCCTGAACGCCGCCCGCTCCTACTCGGGCTTCGCCAATGACCGCACCAACGACGACTCC            298
b     F  L  N  A  A  R  S  Y  S  G  F  A  N  N  R  T  N  D  D  S          78
a  AAGCGCGAGATCGCTGCCTTCTTCGCGCATGTCACACACGAGACTGGACATATGTGCTAC            358
b     K  R  E  I  A  A  F  F  A  H  V  T  H  E  T  G  H  M  C  Y          98
a  ATCAATGAGATAAATGGGGCAAACATGGACTATTGTGACAAGAGCAACAAACAGTGGCCG            418
b     I  N  E  I  N  G  A  N  M  D  Y  C  D  K  S  N  K  Q  W  P          118
a  TGCCAGCCGGGCAAGAAGTACTACGGACGCGGGCCGCTGCAGATCTCATGGAACTTCAAC            478
b     C  Q  P  G  K  K  Y  Y  G  R  G  P  L  Q  I  S  W  N  F  N          138
a  TACGGGCCGGCGGGAAAGAACATCGGGTTCGACGGGCTAAGGGACCCAGACAAGGTGGCG            538
b     Y  G  P  A  G  K  N  I  G  F  D  G  L  R  D  P  D  K  V  A          158
a  CAGGACCCGACAATCTCTTTCAAGACGGCGCTCTGGTTCTGGATGAACAACGTGCACCAG            598
b     Q  D  P  T  I  S  F  K  T  A  L  W  F  W  M  N  N  V  H  Q          178
a  GTGATGTCGCAAGGGTTTGGCGCCAACATCCGGG  632
b     V  M  S  Q  G  F  G  A  N  I  R     189
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Fig. 4. Nucleotide sequence (a) and the deduced amino-acid sequence (b) of rice cDNA clone, SS2594. The amino-acid sequence identical with N-terminal of RHP is under-lined. Cysteine residues related to disulfide bonds (Hart *et al.* 1993, Yamagami and Funatsu 1993) are indicated with black boxes.

Chitinases homologous with the sequence for SS2594 included three types of chitinases, class I, II and IV, according to the classification of chitinases based on primary structures by Collinge *et al.* (1993). But the protein for SS2594 lacked both the cysteine-rich domain and the hinge region harboring in class I and IV chitinases. In addition, two regions of class I and II chitinases in catalytic domain are deleted in the sequence for SS2594 as in the class IV chitinases. The deletions extended over positions 95-96 and 178-183 in the alignment (Fig. 5). The degree of identity of amino acid residues in the alignment of sequence for SS2594 is higher with class IV chitinases than with chitinases of other two classes. For example, in catalytic domain (164 residues, excluding signal peptide region) identity of the sequence for SS2594 to maize class IV chitinase, CHITA is 70.1 %, as compared with 48.8 % to class II chitinase from peanut (*Arachis hypogaea,* Kellmann *et al.*

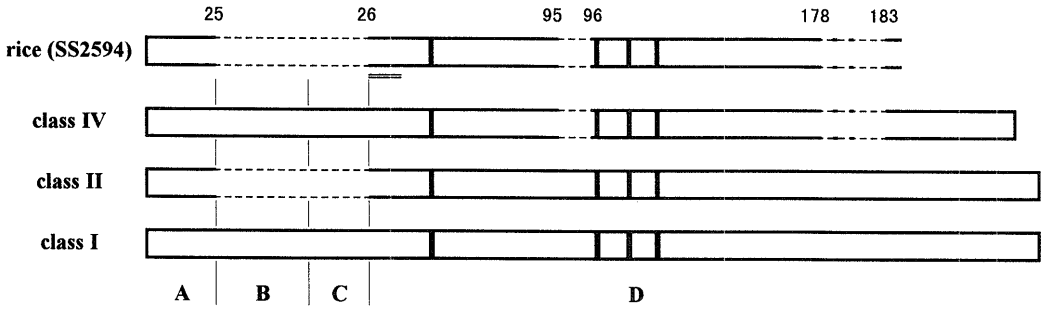

Fig. 5. Structure of the protein deduced from the DNA sequence of SS2594 in comparison with other chitinases homologous with the sequence for SS2594. The bold vertical lines indicate cysteine residues related to disulfide bonds. The position numbers of deleted regions in the sequence for SS2594 (see text) are shown upper side of the box. Comparative position of signal peptide (A), cysteine-rich domain (B), hinge region (C) and catalytic domain (D) are shown under boxes. The double underline indicates N-terminal 16 amino acid of RHP identical with the sequence for SS2594.

1996) and 48.2 % to class I chitinase from wheat (*Triticum aestivum,* PIR: S38670 in PIR protein sequence database).

Xu *et al.* (1996) compared the primary structures among ten rice chitinases reported so far, and the obtained sequence similarities and identities ranged from 79.7 % to 98.7 % and 62.4 % to 98.1 %, respectively. All of them belonged to class I possessing cysteine-rich domain and hinge region. Whereas, the primary sequence similarities and identities between the protein for SS2594 and other rice chitinases in the catalytic domain ranged from 54.9 % to 62.2 % and 40.2 % to 47.0 %, respectively. Therefore, the protein for SS2594 is a novel chitinase in rice.

From the comparison between amino-acid sequence for SS2594 and other chitinases, it is found that the protein for SS2594 includes a putative signal peptide of 25-amino acid at 5' end and that the amino-acid sequence identical with N-terminal of RHP is in the N-terminal side of catalytic domain in other chitinases. Based on these and subsequent data, RHP is considered to be a chitinase protein produced from the same locus of SS2594.

RFLP analysis and mapping of a new locus of chitinase

RFLP linkage analysis with SS2594 as a probe showed that the assumed locus was closely linked with marker R1849 (0.0 cM) and mapped between markers C1399 and R896 with a distance of 0.3 cM and 1.4 cM, respectively, on chromosome 4 (Fig 6). Chitinase loci reported so far are three; *Chi-1* and *Chi-3* on chromosome 6 and *Chi-2* on chromosome 5 (Nishizawa *et al.,* 1993). This supports our assumption that the product for SS2594 is encoded at a new locus in rice. We, therefore, designated it as *Chi-4* (RFLP locus: S12594).

There were a few minor flanking bands in the RFLP analysis, but all of them cosegrigated with the band of SS2594. This further indicates that RHP is very likely the chitinase protein encoded by *Chi-4,* the locus of SS2594.

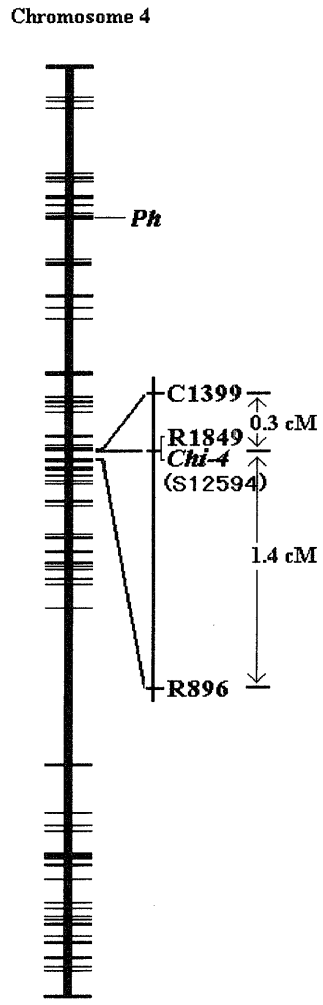

Fig. 6. Location of chitinase gene, *Chi-4* (S12594) on rice chromosome 4. The location of *Chi-4* in chromosome 4 was determined from RFLP linkage analysis. Unlabeled horizontal bars indicate other RFLP loci on the map.

Discussion

The N-terminus of RHP showed an appreciable homology with the catalytic domain of chitinases and had an identical amino-acid sequence with the sequence deduced from rice cDNA clone SS2594. In addition, hybridization signals in the RFLP analysis indicated that there is no DNA segment homologous with SS2594, at least on other regions in the genome. Thus, it is very likely that RHP is encoded by the new locus *Chi-4* (S12594) assigned herein to chromosome 4.

RHP was identified as a strongly expressed protein in husk of rice, and might be related to high levels of seed dormancy determined by either high maturation temperatures or varietal source. Seed dormancy in rice is controlled by factors present in the seed husk which is maternal tissue (Takahashi 1960, Ikehashi 1972). Also, a high level of dormancy can be induced at high temperature during maturation (Ikehashi 1972). These findings suggest that expression levels of proteins responsible for germination inhibitors may be affected by temperature during maturation. The induction of RHP was intensified under the conditions which may induce high levels of seed dormancy.

On the other hand, four loci for seed dormancy were detected by isozyme markers using some varieties including IR 36 and Nanjing 11 (Wan *et al.* 1997). Also, Lin *et al.* (1995a) reported five loci responsible for seed dormancy by applying RFLP markers in a hybrid between Indica and Japonica cultivars. These loci are located on other chromosomes but chromosome 4. Therefore, it is difficult to consider that the gene of RHP directly controls seed dormancy.

Momilactones, which are diterpenes, were previously isolated from husks of dormant rice seed (Kato *et al.* 1973, Takahashi *et al.* 1976), and identified as major growth inhibitors and factors controlling seed dormancy in rice. Later, momilactones were identified as major rice phytoalexins (Cartwright *et al.* 1980). In suspension-cultured rice cells, Ren and West (1992) reported that chitin could induce diterpene hydrocarbon synthase, one of the enzymes proposed for the biosynthetic pathway of diterpenoid phytoalexine. Yamada *et al.* (1993) found that N-acetylchito-oligosaccharides at very low concentrations induced formation of the both momilactone A and B and chitinase. In the light of these facts, RHP and momilactones may be induced by a common factor, or one of the two can be induced by the other, since it seems reasonable for seed to possess antifungal proteins during dormant phase.

Although physiological role of RHP is not yet clear, it is a new rice chitinase identified from this study. It was also found that this chitinase has a unique structure; two deleted regions in catalytic domain like as class IV and lacking cysteine-rich domain and hinge region like as class II (Fig. 5). Its primary structure not only differs from known other chitinase in rice but is rare among other chitinases in database. So far, a chitinase from European elder (PIR: S51645) shows the same structure according to a sequence information available with registration to database. For characterization of this unique structure, further studies to obtain the total sequence are necessary.

Chitinases are considered as important enzymes in the area of breeding for disease resistance. In addition, its other functions are also interesting. Plant resistance against a kind of stress by some chitinases was reported as non-defensive ones; relating to cold acclimatization (Gatschet *et al.* 1996) or to a rescue effect at embryogenesis (Kragh *et al.* 1996). The findings reported here may contribute to further studies in these areas.

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