Genomic Sequence and Polymorphisms of a Rice Chitinase Gene, *Cht4*

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Summary

A novel chitinase was identified in a rice EST library based on a partial polypeptide sequence of a putative dormancy-related protein in rice husk and its genetic locus was detected on chromosome 4. Thereafter, the whole genomic sequence of *Cht4* was determined by using TAIL-PCR based on cDNA sequence data. A full sequence of the protein encoded by *Cht4* was confirmed to be significantly shorter than that of known rice chitinases, and to have a unique primary structure. The 5' flanking sequence of about 700 bp was also determined and compared with that of known chitinases. Besides a common intron in the sequence there is a polymorphism in the 3' flanking region for an inserted sequence which is similar to the *Wanderer* element, one of the transposons. The insertion was observed only in *Indica* cultivars.

Key Words : *Oryza sativa* L., class IV chitinase, genomic sequence, *Wanderer* element insertion, TAIL-PCR.

Introduction

Plant chitinases have been identified as one of the pathogenesis-related proteins (PR proteins) (Van Loon *et al.*, 1994). Chitinases are considered to play an important role in the defense mechanisms against chitin-containing fungi, as there is no chitin in host plants. 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 for the chitinase gene (Brogli *et al.* 1991, Jach *et al.* 1995, Lin *et al.* 1995). So far ten chitinases have been reported in rice (Xu *et al.* 1996). We detected a new chitinase during a survey for dormancy-related proteins in rice husk and identified it in the EST database of rice (Nakazaki *et al.* 1997). The primary structure of the rice husk chitinase (RHP) seemed to be different not only from that of other rice chitinases but also from that of chitinases in many other plant species. However, the full sequence of cDNA remained to be confirmed. In this study we determined the whole genomic sequence of the *Cht4* gene by PCR based on the partial cDNA sequence. The sequences in the 5' and 3' flanking regions of the gene were also determined, in which a polymorphic insertion was found among rice cultivars.

From the view point of gene isolation technology, it is

interesting to note that Liu *et al.* (1995) had applied thermal asymmetric interlaced PCR (TAIL-PCR) to the analysis of flanking regions of T-DNA in chromosomes of a transgenic plant. The TAIL-PCR utilizes a set of nested sequence-specific primers together with a shorter arbitrary primer at a lower annealing temperature, and preferentially amplifies target sequences between a specific primer and the arbitrary primer. They have shown that the method was effective for the analysis of unknown flanking regions of a certain genomic DNA segment. We applied this method to identify the whole sequence of the *Cht4* gene, and were able to determine it. The same method may be applied for the determination of flanking regions of known cDNA sequences.

Materials and Methods

Plant materials and DNA extraction

A Japonica rice cultivar Nipponbare was used to isolate the *Cht4* gene. To analyze the polymorphism in the gene sequence among cultivars, a Japonica cultivar, Koshihikari and three *Indica* cultivars, i.e., IR 36, Nanjing 11 and Kasalath were added. For DNA extraction, total DNA of each cultivar was extracted from fresh leaves, (about 100 - 200 mg) according to the method of Komari (1989). The original method was modified by substituting a 1 : 1 mixture of chloroform and phenol by a 48 : 2 : 25 mixture of chloroform, iso-amylalcohol and TE-saturated phenol.

PCR and DNA sequencing

TAIL-PCR procedures including the preparation of the reaction mixtures were performed according to the method of Liu *et al.* (1995), using BioOven III (Bio Therm Co., USA). The same random primers, i.e., arbitrary degenerate primers (AD primers) as those used by Liu *et al.* (1995) were applied for the TAIL-PCR. The sequences of the AD primers were as follows: AD1; 5'-NTCGA(G/C)T(G/C)G(A/T)GTT-3', AD2; 5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3', and AD3; 5'-(A/T)GTGNAG(A/T)ANCANAGA-3'. Temperatures and duration for each cycle in the TAIL-PCR were basically same as those reported by Liu *et al.* (1995), but slightly modified in this experiment after preliminary tests. Conventional PCRs, in which a pair of specific primers was used, were performed with AmpliTaq (The Perkin - Elmer Co., USA) or rTaq DNA polymerase (Toyobo, Japan) as enzymes for amplifying DNA segments and with PCR Thermal Cycler TP 2000 (Takara Biomedicals, Japan).

Purified tertiary PCR products in the TAIL-PCR

Received April 27, 1998. Accepted September 17, 1998.

were extracted from the gels after electrophoresis, and were directly sequenced with ABI PRISM Cycle Sequencing Kit and ABI 373A DNA sequencer (Applied Biosystems, USA). Other products from the conventional PCR were sequenced by the same procedures.

Results

Sequencing of the 5' flanking region based on partial *Cht4* sequence by TAIL-PCR

To determine the 5' flanking region of the *Cht4* gene, TAIL-PCR was conducted with three specific primers, i.e., B1, B2 and B3, which were constructed based on the partial cDNA sequence for S12594 (Nakazaki *et al.* 1997) as shown in Fig.1A. Of the three AD primers, AD3 was well-matched with the specific primers to amplify DNA segments (Fig.1B). In the primary PCR of TAIL-PCR with B3 as a specific primer, a few amplified signals were detected by electrophoresis, using an ethidium bromide-stained gel. Then, a single amplified product, each band with a length of about 900 bp and about 850 bp, was detected respectively, in the secondary PCR with B2 as a specific primer and tertiary PCR with B1 as a specific primer. The difference in the length between the two bands was approximately 47 bp which was deduced based on the positions of the B1 and B2 primers in the partial cDNA sequence. Therefore, the products obtained by the two PCR procedures were considered to correspond to the segment containing the 5' flanking region of the *Cht4* gene. By sequencing the product from the tertiary PCR with the B1 primer, a sequence of about 470 bp was obtained, as indicated from the ca. -360 to 50 positions in Fig.2. The sequence in the overlapping region was completely identical with the reported partial cDNA sequence.

To analyze the sequence in a more upstream position, two new primers were constructed based on the preceding results. They were B01 (21mer; 5'-CGTGTGGGA GAGGTGAT-GGTC-3') and B02 (22mer; GCTGGC AAGAGAAAGTCATCCT-3'), as shown in Fig.2, and AD3 was used out of the three ADs. As a result, the 5' flanking region of 690 bp was determined together with a part of the reported partial cDNA sequence (Fig.2). In this segment no significant homology was found with the 5' flanking regions of other rice chitinases. Sequences for putative *cis*-elements in the 5' flanking region of plant genes were searched using PLACE Signal Scan Search and PLACE database at the National Institute of Agrobiological Resources, Tsukuba, Japan (PLACE Release 3.0) (Higo *et al.* 1998, Prestridge 1991) by accessing <http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html>. A complementary sequence of prolamin box (P-box) which was detected in the promoters of many cereal seed storage protein genes (Vicente-Carbajosa *et al.* 1997) was identified (sequence from positions -540 to -545 in Fig.2). No other unique or important motifs were detected.

Fig.1. Specific primers for TAIL-PCR to amplify DNA segments in flanking regions of the 632 bp-cDNA sequence (Nakazaki *et al.* 1997) and products subjected to electrophoresis on a 1 % agarose gel.

(A) The position and sequence of specific primers (B1, B2 and B3 for 5' flanking region, and F1, F2 and F3 for 3' flanking region) are indicated by arrow-heads. The numbers under the arrow-heads indicate the position of the 5' end of each primer in the 632 bp-cDNA sequence.

(B) and (C) Electrophoresis of products for 5' flanking region and for 3' flanking region, respectively. Lanes 1 - 3 in (B) and (C): primary PCR, secondary PCR and tertiary PCR of TAIL-PCR, respectively. Lanes 4 and 5 in B: negative controls in the tertiary PCR, without primer AD2 and without F3, respectively. Lanes 4 and 5 in C: negative controls in the tertiary PCR, without primer AD3 and without B1, respectively. Lane M: size marker (mixture of λ DNA, *Hind*III and *Eco*RI double digest and Φ X174 DNA, *Hinc*II-digest).

Amplification and sequencing of the 3' flanking region of the *Cht4* gene

To determine the sequence of the 3' region of *Cht4*, three primers from F1 to F3 were constructed as shown in Fig.1A. Of the three AD primers, AD2 was well-matched with the specific primers, and a DNA segment was amplified (Fig.1C). In the primary PCR of TAIL-PCR with the specific primer F1, more than four amplified signals were detected by electrophoresis using on

Fig.2. Nucleotide sequence and deduced amino acid sequence of the *Chit4* gene in rice.

Position 1 corresponds to the 5' end of the first nucleotide in the reported 632 bp-cDNA sequence (Nakazaki *et al.* 1997). Position numbers exclude the intron sequence. The sequences in black boxes are those reported by Nakazaki *et al.* (1997). Dots in the DNA sequence indicate the abbreviated ones. Arrows indicate the positions of the primers used in several PCR procedures. The sequence obtained by the TAIL-PCR with primers B1, B01 and B02 as specific primers, and the intron sequence enclosed by a bold line are indicated by lower-case letters. Double lines indicate a putative TATA signal, consensus sequence for spliced site in an intron and a putative signal for poly-A signal. The amino-acid residues are indicated under the DNA sequence, i. e., 3' region of *Chit4* alignment of homologous amino-acid sequences of class IV chitinases in the first amino-acid sequence, CHITA from maize (Huyuh *et al.* 1992) in the second amino-acid sequence and ChB4 from rape (Rasmussen *et al.* 1992) in the third amino-acid sequence. Amino-acid residues identical with those for *Chit4* are represented by dots. A deleted amino-acid residue was indicated by a bar. Two nucleotides in the obtained sequence which differed from those in the cDNA sequence are indicated with asterisks. The site of insertion of the *Wanderer* element in *Indica* cultivars (see text) is enclosed by a doubled line.

etidium-bromide-stained gel. Then, a single amplified product was obtained as a band with a length of about 600 bp and about 450 bp, respectively, in the products of the secondary PCR with F2 as a specific primer and in the tertiary PCR with F3 as a specific primer. The difference in the length between the two bands was approximately 145 bp which was deduced from the primer positions in the cDNA sequence. Therefore, the products obtained by the two PCR procedures were considered to correspond to the segment containing the 3' flanking region of the *Cht4* gene. By sequencing the product from the tertiary PCR with the F3 primer, a sequence of about 450 bp was obtained (Fig.2). The 3' region in the sequence was identical with the known partial cDNA sequence except for two bases. To verify the TAIL-PCR-amplified sequence in the 3' region of *Cht4* by conventional PCR procedures, new reverse primers, i.e., L1 (15mer, 5'-CTCCGCCCTAAGATA-3') and L2 (20mer, 5'-TTACTCCGCCCTAAGATAGA-3') were constructed from the 3' flanking region in the determined sequence (Fig.2). Also, a new forward primer, U1 (17mer, 5'-CCACCATAAACCTTCAT-3') was constructed from the 5' flanking region in the determined sequence (Fig.2). With the F2-L2 and F3-L2 primer pairs, a set of conventional PCR was performed using a PCR product as template which was obtained with primer pair U1 - L1 from the total DNA. The length of the nested-PCR products was approximately 559 bp and 414 bp, which was deduced from the sequence determined by sequencing the TAIL-PCR product, and it was confirmed that the products had identical sequences with the known one shown in Fig.2. Therefore, these two sequenced DNA fragments were considered to correspond to the 3' flanking region of the *Cht4* gene. As repeated sequencing using the products from TAIL-PCR and conventional PCR gave the same sequences (Fig.2), it was assumed that no changes in the bases of the nucleotides had occurred during the PCR cycles. The previously reported cDNA sequences (Nakazaki *et al.* 1997) may have contained errors for the two bases.

The length of the amplified product by PCR using the primer pair F1 - L2 was about 1.1 kbp and longer than the cDNA sequence of 671 bp by about 450 bp (Lane 3 in Fig.3). The additional sequence of this amplified product is considered to be an intron segment between the positions of primers F1 and F2. By sequencing this region, an intron sequence of 467 bp with a set of consensus sequences for the splicing site was detected (Fig 2).

Primary amino-acid structure of the C-terminal region

The C-terminal region of this protein was not determined in the previous report on the 189 amino-acid sequence (Nakazaki *et al.* 1997). In the current study, additional 40 residues in the C-terminal region were deduced based on the genomic sequence (Fig.2). The whole sequence was shown to have a significant homol-

Fig.3. Electrophoresis of PCR products amplified with primer pairs F1-L2, F2-L2 and F3-L2 from the PCR products amplified with primer pair U1-L1 as a template for five cultivars on a 1 % agarose gel.

Lanes 1-5: F1-L2. Lanes 6-10: F2-L2. Lanes 11-15: F3-L2. Lane M: size marker, as shown in Fig.1. Lanes 1, 6 and 11: Nanjing 11. Lanes 2, 7 and 12: Koshihikari. Lanes 3, 8 and 13: Nipponbare. Lanes 4, 9 and 14: IR 36. Lanes 5, 10 and 15: Kasalath.

ogy with class IV chitinases. The identity of the newly deduced 40 residues with maize class IV chitinase (CHITA) and that with ChB4 chitinase of rape was 70 % (Fig.2). The two chitinases showed a significant homology with *Cht4* in the N-terminus (Nakazaki *et al.* 1997).

Insertion of *Wanderer* element in the 3' flanking region of *Indica* cultivars

To compare the length of the *Cht4* gene among cultivars, DNA segments between primers F1 - L2, F2 - L2 and F3 - L2 were amplified from the U1 - L1 segments (Fig.3). In the three *Indica* cultivars, PCR products were longer than the determined length (in Fig.2) by about 250 bp. These results indicated the presence of an insertion in the region between the primer positions of F3 and L2, while such an addition was absent in the two *Japonica* cultivars. Thus, a polymorphism was found among the cultivars. By sequencing the inserted region of IR 36, the sequence was found to have a high level of homology with the *Wanderer* element with a 3-bp target site sequence (Fig.2 and Fig.4), which was one of transposons in rice, as reported by Bureau *et al.* (1996).

Discussion

The whole length of the amino-acid sequence was deduced from the nucleotide sequence of the *Cht4* gene. The region around the N-terminus of this protein was already found to be homologous with class IV chitinases (Nakazaki *et al.* 1997). The identity of newly deduced 40 residues in the C-terminal was 70 % with class IV chitinases of maize and rape. At the C-terminal ends of the class IV chitinases seven residues are deleted compared with those of class I or class II (Francine *et al.* 1997). Based on the amino-acid sequence and on the deletion, the C-terminal region of this protein was also homologous with class IV chitinases. Thus, the whole structure of the the protein encoded by *Cht4* appears to be similar to class IV chitinases rather than to class II, although the protein lacks the cysteine-rich domain and the hinge region, which also characterize class IV

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W-Chit4 TAAGGCTGTGTTTGAGAAAGAGGAGATAGAGAA-GATTGGGAAGAT-ACGTAAAACGATGTGA-GCT
W-Os4      *CATTGAGAGCAGAGGAA-AAGAATCGATTGAGAA-TGTACGCAAAAC--GAGCTGAGC
W-Os1 TAAGGCCCAATTAATCTCCACATTT-GGAGATAAATTTTGGT-TGCACGTAAAAC--GAGAAAGCT
W-Os2 TTCGGCCAC-----TCACTGGCT-GGGAACATATATGTTGGCAGCAAAAAA-TAGAGGCGGTC
W-Os11 TTCGGAAGCCAATCTTCCC-----AGCACATG-ATGT-GCGCAGGGAAC-GAA-GCAGTT
W-Os5 TTAGGGTACC--CCCTCCCTCCTG-----GCACGCAAAAC-AAACTCGT-
W-Os3 TAAGGCTGTG-TTCTTGAGTGCTGGT-CAAAACAATCTCTCT-AGTACGAAAAAC-AGAG-TGACT

W-Chit4 -ATTAGTGCATGATTAATTGAGTATTAATTATTTTAAATTTT-A-AACATGAACATAATATGATTTT
W-Os4 CATTAAACGTATAATTAATTGAGTATTAACATTTTAAAA-TTTT-AAAAATAGATTAATATAATTTT
W-Os1 CATTAGCACATAATTAATTAAGTATTAACATTTTATA-CC-TT-CAAAAATGAATCAATATGATTTT
W-Os2 CATTAGCGCGTGATTAGTTAAGTATTAGTT-TTTT-AA-TTTG-AAAAATGGATTTATTTTCTTTT
W-Os11 CATTAGCGCGTGATTAATTAAGTATTTGCTTTT---TT--GCAAAAATGGATTAATATAATTTT
W-Os5 -TTTGTTCATGATTAATTAATATTAATATTTT--T-TTT-ATAAGATAGATTAATATTTT
W-Os3 TATTAGCGCATGATTAATTAAGTATTTGCTAT---AAC-TT-AAAAATGGATTGATATGATTTT

W-Chit4 TAAA-GCATCTTTCTATAGAAAATTTTTT--GCAAAAAA-CGCACCGTTTAATAGTTTGGGAA----
W-Os4 ---AAGTAACTTTCTATAGAAAATTTTTT GCAAAAAATCATACCGTTTAGTAGTTTCAGGAATTGA
W-Os1 TAAGC--AGCTTTTGTATAGAACTTTTTTA-AAAAAT-GTATCATTTAACAATTTGA-AA---A
W-Os2 TAAAA-CACCTTCTATATAGAACTTTTTT--AAAAA--ATACCGTTTAACAGTTTG--AACC-A
W-Os11 TAAGC--AACTTTCGTATAGAACTTTTTT--GCAAAAAA-CACATTGTTTAGCAGTTTGA-AA---A
W-Os5 TAAAA-TATTTTTTATATAGAAAATTTTTT--ATATA-TC--A---TT-A-TA-----A-AA---A
W-Os3 TAAAAGCAACTTTCTATACATTTTTTTTTTGGCAAAAAA-CACAGAGTTTAACAGTTTGGGA----A

W-Chit4 ACGTGCACGCGGAAAAACGAT-GTG-CAATCTATCTCCAATC-TCCCCGAACGAACACTGCC---TAA
W-Os4 ACGTGCAGGTGTAAAACGAGAGAAA--TCTTCTCTTTACACTTGCTGCTGAATTGGTACATGTTG
W-Os1 GCGTGCTAACGGAAAAACAAGGAAG-----TTGAAGAATA--GAACAGGGCC---TAA
W-Os2 ACATATGCGCGGAATACGAGAGAGAGGT-GTTGGGAACCTAGTG-TACT-GAACTGGGG---TTT
W-Os11 GCGTGCGCGCAGAAAAACGAGGGGAGAG-GATGGGAACC-AGC-AATGCC-GAACACAACC---TTG
W-Os5 ACGTGCAGGTGTAAAACGAGAAAGCAA-GTTGGAAAAATGGTGA-TAAA-GAACAAAGC---TT*
W-Os3 GCGTGCTCATGGAAATCAAGAGAG-----TAAACGAATGCAGCC---TAA

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Fig.4. Alignment of insertion sequences in the 3' region of the *Chit4* gene of IR 36 (*W-Chit4*) and known sequences of the *Wanderer* element reported by Bureau *et al.* (1996).

The shaded areas indicate the consensus sequence reported by Bureau *et al.* (1996). Boxed regions indicate the putative target site of insertion. No further sequence is available in the area marked by an asterisk. Broken lines refer to gaps.

chitinases. These findings suggested that the chitinase encoded by *Chit4* is unique among the known chitinases.

The C-terminal region of chitinases is important for their function because a short C-terminal extension (CTE) of about six amino acids controls the subcellular localization of these enzymes (Collinge *et al.* 1993). Francine *et al.* (1997) pointed out that as a general rule, chitinases with CTE are localized in vacuoles and those without CTE are correlated with extracellular targeting, and that these two types appear as two types of class I chitinases in rice and wheat whereas no other types of extracellular chitinases have been identified in these species. The rice class IV chitinase was found to lack CTE for extracellular localization of this enzyme. Therefore, the finding of a new chitinase other than class I in rice would be interesting for the understanding of defense mechanisms involving chitinases.

In rice, so far, 5' flanking sequences of four chitinase genes have been reported, namely *Chit1*, *Chit2* and *Chit3* (Nishizawa *et al.* 1993) and *RC24* (Xu *et al.* 1996). A 5' flanking region of about 700 bp was determined in this study. Compared to other rice chitinases, the 5' region of *Chit4* did not appear to show any significant homology with those in other rice chitinases, and lacked the sequence detected by footprint analysis of PAL promoter or GGCC box which was identified in some other rice chitinases (Nishizawa *et al.* 1993, Xu *et al.* 1996).

Also, putative motifs for *cis*-elements of plant genes were searched using PLACE Signal Scan Search and a putative TATA signal and a complementary sequence of P-box (Vicente-Carbajosa *et al.* 1997) were detected. The role of the P-box which has been reported in the promoters of many cereal seed storage protein genes remains to be clarified in the expression of *Chit4* in rice husk. The protein coded for *Chit4* was first recognized to be endogenously induced in rice husk, and found in cDNA from rice green shoot (Nakazaki *et al.* 1997). The other rice chitinases were identified as pathogenesis-related proteins. Therefore, further studies are necessary to clarify the mode of expression of the *Chit4* gene in the course of development and in other tissues together with the analysis of *cis*-regulatory sequences in the promoter region.

A putative transposable element, *Wanderer* was detected by Bureau *et al.* (1996), and classified in the group of Miniature Inverted-repeat Transposable Elements (MITE). Although *Wanderer* elements were detected in Indica and Japonica cultivars, such as, *W-Os1* in IR 36 (Nelson *et al.* 1994) and *W-Os3* in Nipponbare (Abe *et al.* 1989), the polymorphism has not been related to varietal differentiation. In this study, the *Wanderer* element was detected only in Indica cultivars. Any insertion in a gene can be a good taxonomical marker, which may have marked a branching in the

genealogy of cultivars. Further use of this insertion may provide a tool for the classification of cultivated rice.

From the technical point of view, detection or analysis of flanking regions of a target gene based on cDNA sequences is increasingly important, as such sequences may be playing key roles in gene expression during development or in response to elicitors. In this study, it was demonstrated that the procedures of TAIL-PCR described by Liu *et al.* (1995) are useful in direct sequencing of such flanking regions.

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