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Molecular Cloning of Endo-β-D-1,4-Glucanase Genes, *rce1*, *rce2*, and *rce3*, from *Rhizopus oryzae*

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Three endoglucanase genes, designated the *rce1*, *rce2*, and *rce3* genes, were isolated from *Rhizopus oryzae* as the first cellulase genes from the subdivision *Zygomycota*. All the amino acid sequences deduced from the *rce1*, *rce2*, and *rce3* genes consisted of three distinct domains: cellulose binding domains, linker domains, and catalytic domains belonging to glycosyl hydrolase family 45. The *rce3* gene had two tandem repeated sequences of cellulose binding domains, while *rce1* and *rce2* had only one. *rce1*, *rce2*, and *rce3* had various lengths of linker sequences.

Cellulose is the most abundant biological polymer on the earth and is degraded by cellulases in nature (20). It is thought that degradation of cellulose is achieved by the synergistic action of three types of cellulase components: endoglucanases (EC 3.2.1.4, endo-β-D-1,4-glucanases) (EG), cellobiohydrolases (EC 3.2.1.91) (CBH), and β-glucosidases (EC 3.2.1.21) (BGL) (9). Cellulases are known to be produced by a broad range of organisms including fungi (23), bacteria (5), plants (16) and insects such as termites (11). Among them, fungal cellulases have been studied extensively and have been effectively used for industrial purposes, since fungi produce large amounts of cellulases (10).

So far, fungal cellulases have been isolated mainly from the members of the subdivision *Deuteromycotina* (25), which have no sexual reproduction and which mainly reproduce by conidia. Generally, the cellulase systems of the subdivision *Deuteromycotina* contain various cellulase components belonging to different glycosyl hydrolase families. For example, *Trichoderma reesei* produces eight cellulase components belonging to seven different glycosyl hydrolase families (CBHI [family 7], CBHII [family 6], EGI [family 7], EGII [family 5], EGIII [family 12], EGV [family 45], BGLI [family 3], and BGLII [family 1]) (25). These cellulase components have been shown to degrade crystalline cellulose synergistically (9). The variety of cellulase components might arise mainly through multiple gene transfer events rather than gene duplication events, since it is thought that genes encoding cellulase components belonging to the different glycosyl hydrolase families evolved from different ancestral genes (13).

Although many fungal cellulase genes from members of the subdivision *Deuteromycotina* have been isolated and characterized, to our knowledge, the isolation of endoglucanase genes from members of the subdivision *Zygomycota*, which reproduce sexually by zygospores, has not been reported. To determine the cellulase system of the subdivision *Zygomycota*, we screened cellulase-producing fungi belonging to *Zygomycota* from soil. As a result, we obtained *Rhizopus oryzae* FERM BP-6889, a member of the subdivision *Zygomycota*, and purified two major endoglucanases, designated RCE1 and RCE2, from the culture supernatant of *R. oryzae* (14). RCE1 and RCE2 were shown to possess similar hydrolytic properties. Also, both RCE1 and RCE2 possessed homologous N-terminal and internal amino acid sequences, indicating that the endoglucanases were highly homologous, although their molecular masses were different (41 kDa for RCE1 and 61 kDa for RCE2) (14).

To understand the cellulase system of *R. oryzae*, the DNA sequences of *rce1* and *rce2*, encoding RCE1 and RCE2, should provide important information. Therefore, in this study, we cloned and sequenced the *rce1* and the *rce2* genes. Also, we obtained the *rce3* gene, whose derived protein had not been found in the supernatant of *R. oryzae*.

Cloning of *rce1*. To obtain the partial DNA fragment of the *rce1* gene, the primers were designed based on the N-terminal and internal amino acid sequences of the purified RCE1 from *R. oryzae* determined previously (14). PCR was performed with forward primer R1-F (5′-AARAAYTGGAAYGGXCCNAC-3′), corresponding to the N-terminal amino acid sequence of the native RCE1 (KNWNGPT), and with primer R1-R1 (5′-TTRAACCARTTRAANCG-3′) or R1-R2 (5′-TTRAACCA RTTRAAYCT-3′), corresponding to one of the internal amino acid sequences (RFNWFK) determined previously (14). PCR with primers R1-F and R1-R1 and with *R. oryzae* chromosomal DNA as the template yielded no specific band, but PCR with primers R1-F and R1-R2 yielded an amplified band of about 800 bp. The amplified DNA was then subcloned into the pT7Blue-T vector designated pRD05. Nucleotide sequencing of the 800-bp fragment indicated the presence of an open reading frame encoding RCE1. The amino acid sequence deduced from the 800-bp fragment was in perfect agreement with the internal amino acid sequence determined from the purified RCE1 from *R. oryzae* (14).

For cloning the *rce1* gene, the chromosomal DNA isolated

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from *R. oryzae* was partially digested with *Sau*3AI and separated on an 0.8% agarose gel. The DNA fragments in the molecular size range of 9 to 23 kbp were collected and ligated into a *Bam*HI-treated Lambda DASH II vector to prepare the genomic library for cloning the *rce1* gene. pRD05 was digested with *Bam*HI, and a DNA fragment of 800 bp was collected by use of 0.8% agarose gel electrophoresis. Plaque hybridization was performed with this 800-bp fragment as a probe against the *R. oryzae* genomic library for *rce1* by use of an ECL direct DNA/RNA labeling detection system (Amersham). Sixteen positive phage clones were obtained from about 50,000 phage plaques, and one of the strongly hybridized clones was isolated. The phage DNA extracted from the positive clone was digested with *Xba*I, and the resultant 3.5-kbp fragment was subcloned into the *Xba*I site of pUC119.

The nucleotide sequencing of the 3.5-kbp fragment indicated the presence of a single open reading frame encoding a predicted 338-residue protein. This open reading frame was designated the *rce1* gene (Fig. 1). Residues 24 to 39 of the deduced amino acid sequence encoded by *rce1* coincided perfectly with the N-terminal amino acid sequence of the purified RCE1 from *R. oryzae* (14), indicating that residues 1 to 23 constituted the signal peptide. Also, all four internal amino acid sequences of RCE1 determined previously were found in the amino acid sequence deduced from the *rce1* gene (Fig. 1). Therefore, the *rce1* gene was considered to encode RCE1. Based on the deduced amino sequence of RCE1, its mature molecular mass was 32,454 Da.

Cloning of *rce2* and *rce3*. When the genomic DNA of *R. oryzae* digested with *Sac*I was Southern blotted with the *rce1* gene as a probe, one strong signal and two weak signals at about 10 and 3 kbp appeared (data not shown). These results suggested the presence of genes homologous with *rce1*. Therefore, we cloned the *rce1*-homologous genes, which were located on DNA fragments of about 10 and 3 kbp and which were designated the *rce2* and *rce3* genes, respectively.

To clone the *rce2* gene, we performed plaque hybridization with the *rce1* gene as a probe against the genomic library by using the 10-kbp regions of DNA fragments from the *R. oryzae* genomic DNA digested with *Sac*I. Two positive phage clones were obtained from about 10,000 phage plaques, and both of these clones were considered to contain the *rce2* genes as judged by Southern hybridization. The phage DNA extracted from one of positive clones was digested with *Bam*HI, and the resultant 2-kbp fragment was subcloned into the *Bam*HI site of pUC118. The nucleotide sequencing of the 2-kbp fragment indicated the presence of a single open reading frame encoding a predicted 360-amino-acid protein (Fig. 2). Residues 24 to 38 of the amino acid sequence deduced from *rce2* coincided perfectly with the N-terminal amino acid sequence of purified RCE2 from *R. oryzae* determined previously (14), indicating that residues 1 to 23 comprised the signal peptide. Also, all four internal amino acid sequences of RCE2 determined previously (14) were found in the amino acid sequence deduced from the *rce2* gene (Fig. 2). These results suggested that the *rce2* gene encoded RCE2 (14). The molecular mass of the mature RCE2 was calculated to be 35,097 Da.

To clone the *rce3* gene, plaque hybridization was performed with the *rce1* gene as a probe against the genomic library by using the 3-kbp regions of DNA fragments from the *R. oryzae* genomic DNA digested with *Sac*I. Three positive phage clones were obtained from about 10,000 phage plaques, and all these clones were considered to contain the *rce3* genes detected by Southern hybridization. The phage DNA extracted from one of the positive clones was digested with *Sac*I, and the resultant 3-kbp fragment was subcloned into the *Sac*I site of pUC118. The nucleotide sequencing of the 3-kbp fragment indicated the presence of a single open reading frame encoding a predicted 366-amino-acid protein, designated RCE3 (Fig. 3). Since the amino acid sequence up to residue 24 of RCE3 was homologous with the N-terminal sequences of RCE1 and RCE2 (Fig. 4), residues 1 to 23 were considered to comprise the signal peptide (Fig. 3). The calculated molecular mass of mature RCE3 was 35,712 Da. Since we could not find the endoglucanase encoded by *rce3* in the culture supernatant of *R. oryzae* (14), *rce3* is considered to be expressed in only small amounts, if at all.

Domain structures of RCE1, RCE2, and RCE3. FASTA searches for sequences homologous to the amino acid sequences deduced from *rce1*, *rce2*, and *rce3* revealed that RCE1, RCE2, and RCE3 consist of catalytic domains belonging to glycosyl hydrolase family 45, linker domains, and cellulose binding domains (CBDs). Interestingly, RCE3 had two CBDs (CBD1 and CBD2; Fig. 3) repeated at its N terminus; on the other hand, both RCE1 and RCE2 had only one CBD. The two CBDs of RCE3 were connected by a short linker sequence consisting of 10 amino acids (linker 1; Fig. 3). The schematic domain structures of RCE1, RCE2, and RCE3 are shown in Fig. 5.

The N-terminal CBDs of RCE1, RCE2, and RCE3 were highly homologous to the CBDs from fungi, such as CBDs of CBHII from *Trichoderma reesei* (24), CEL3 from *Agaricus bisporus* (2), and XYLB from *Neocallimastix patriciarum* (1) (data not shown). Also, the four CBDs of RCEs were highly homologous to each other as shown in Fig. 4. Especially, the CBD of RCE1 and both CBDs of RCE3 were highly homologous, with 94.6% homology. On the other hand, the homology between the CBD of RCE2 and the CBDs of RCE1 and RCE3 (67.5%) was lower than that between the CBD of RCE1 and both CBDs of RCE3.

The C-terminal catalytic domains of RCE1, RCE2, and RCE3 were homologous to the catalytic domains of endoglucanase type K from *Fusarium oxysporum* (21), EGV from *Humicola insolens* (3), EGL3 from *Humicola grisea* (22), EGI from *Scopulariopsis brevicaulis* (15), EGL4 from *Humicola grisea* (22), EG1 from *Ustilago maydis* (17), and endoglucanase B from *Pseudomonas fluorescens* (6) (data not shown). All these endoglucanases, which are homologous to RCEs, belong to glycosyl hydrolase family 45 (7, 8). Also, the deduced amino acid sequences of RCEs contained the consensus residues of glycosyl hydrolase family 45, (S or T or A)-T-R-Y-(F or Y or W)-D-X-X-X-X-X-(C or A) (7, 8) (Fig. 1 to 3). Therefore, the catalytic domains of the RCEs were considered to belong to family 45. Also, the catalytic domains of RCEs were highly homologous with each other as shown in Fig. 6. Especially, the catalytic domain of RCE1 and that of RCE3 were highly homologous, with 98.6% homology. The homology between the catalytic domain of RCE1 and that of RCE2 was 81.3%, and the homology between those of RCE2 and RCE3 was also 81.3%.

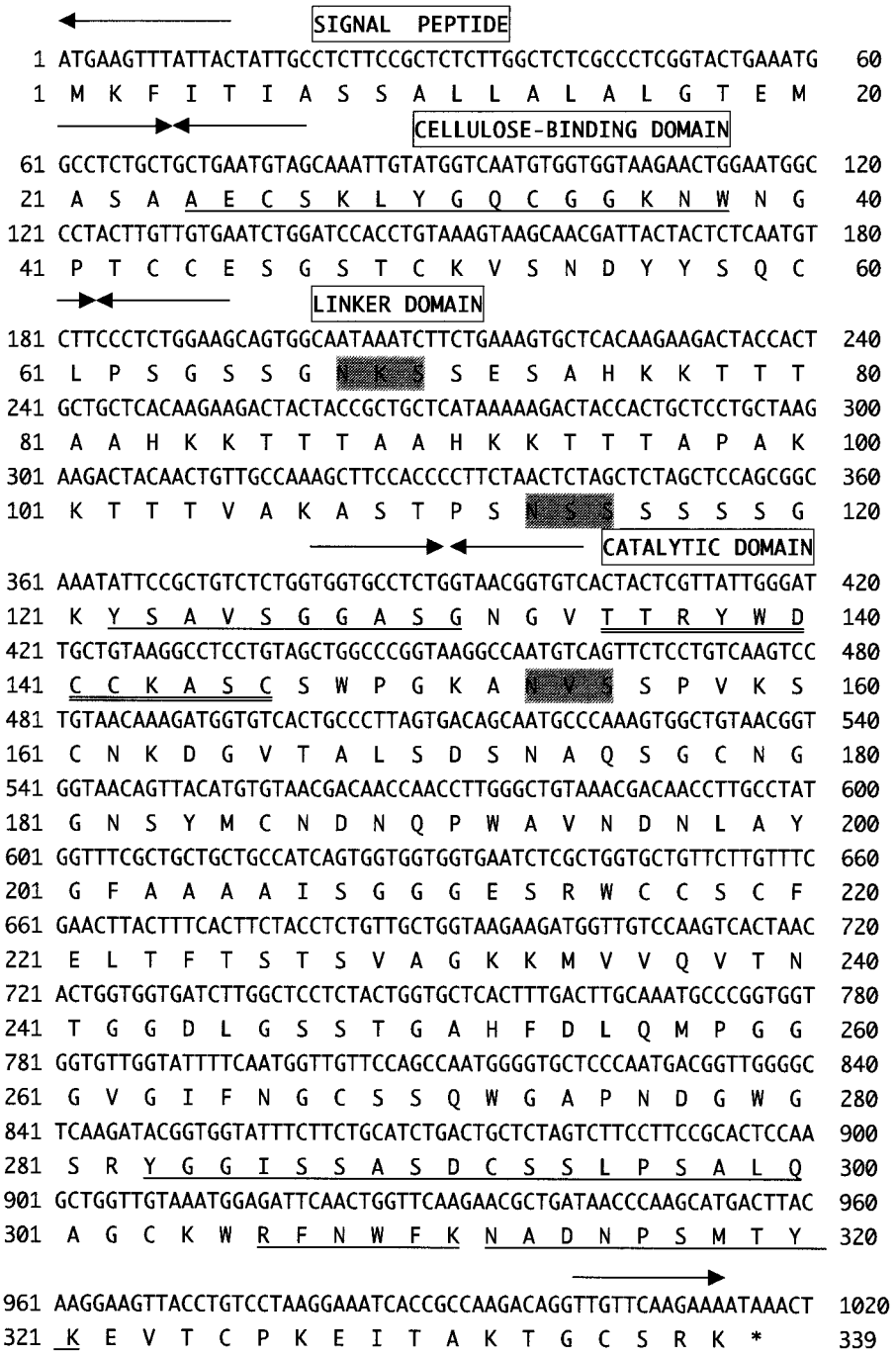

FIG. 1. Nucleotide sequence of the *rce1* gene from *R. oryzae* and deduced amino acid sequence. Arrows, border of each domain: signal peptide, CBD, linker domain, and catalytic domain. The N-terminal and internal amino acid sequences of the purified RCE1 from *R. oryzae*, determined previously (14), are underlined. The consensus amino acid residues of glycosyl hydrolase family 45 are double underlined. Potential N-linked glycosylation sites (N-X-S/T) are shaded.

The deduced amino acid sequences of the RCEs suggested that the cellulolytic system of *R. oryzae* was comparatively simple and consisted mainly of glycosyl hydrolase family 45 endoglucanases, in contrast to the cellulase systems of *Deuteromycotina*, which contain various glycosyl hydrolase families of cellulases (25). Genes encoding different cellulases which belong to different glycosyl hydrolase families probably evolved from different ancestral genes. If so, the fungi belonging to *Deuteromycotina* should have obtained various kinds of cellulase genes by multiple gene transfer events (13). On the other hand, the CBD and catalytic domain sequences of RCEs were highly homologous, suggesting that the *rce1*, *rce2*, and *rce3* genes evolved from the same ancestral *rce* gene by gene duplication rather than by multiple gene transfer events. Further-

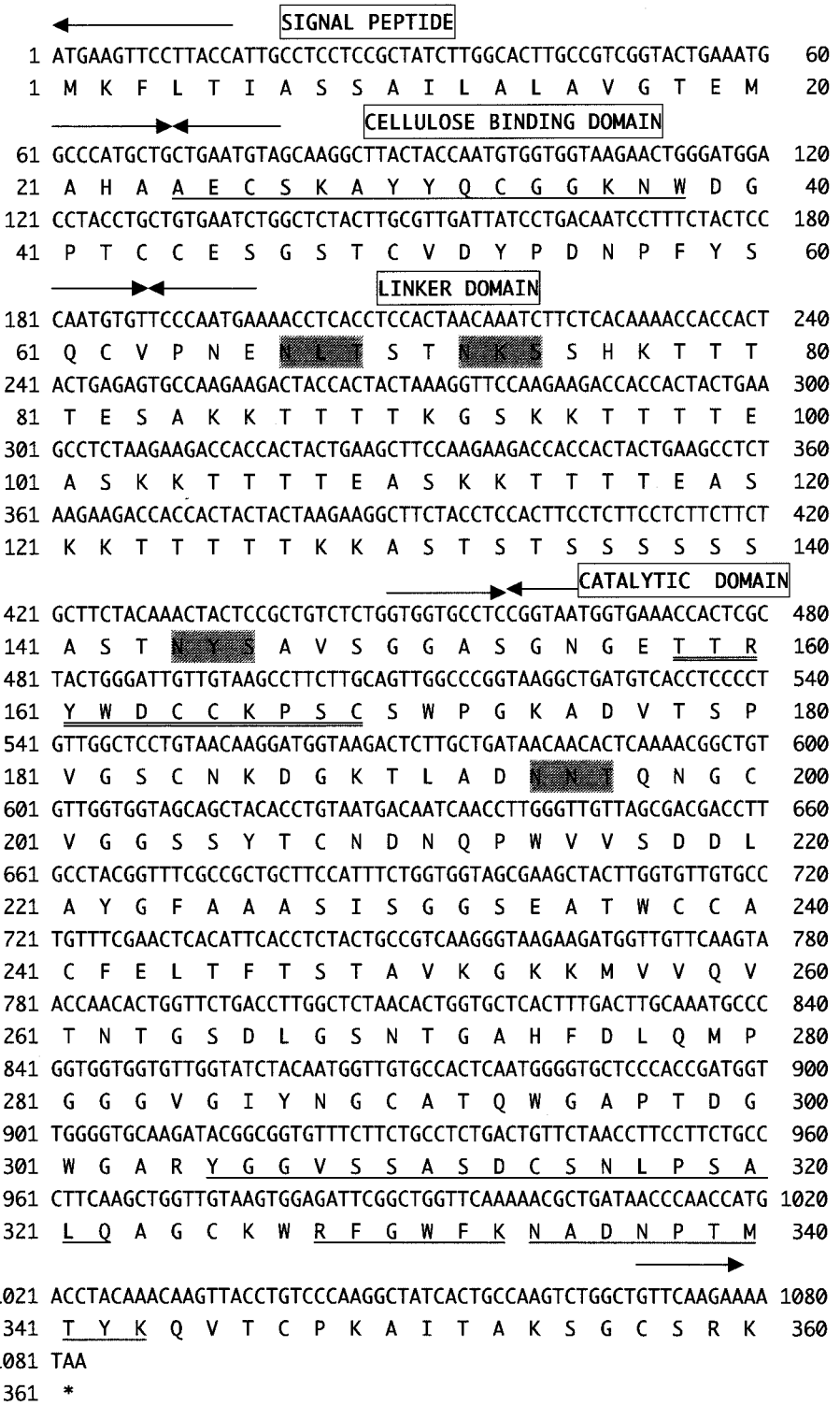

FIG. 2. Nucleotide sequence of the *rce2* gene from *R. oryzae* and deduced amino acid sequence. Arrows, border of each domain: signal peptide, CBD, linker domain, and catalytic domain. The N-terminal and internal amino acid sequences of the purified RCE2 from *R. oryzae*, determined previously (14), are underlined. The consensus amino acid residues of glycosyl hydrolase family 45 are double underlined. Potential N-linked glycosylation sites (N-X-S/T) are shaded.

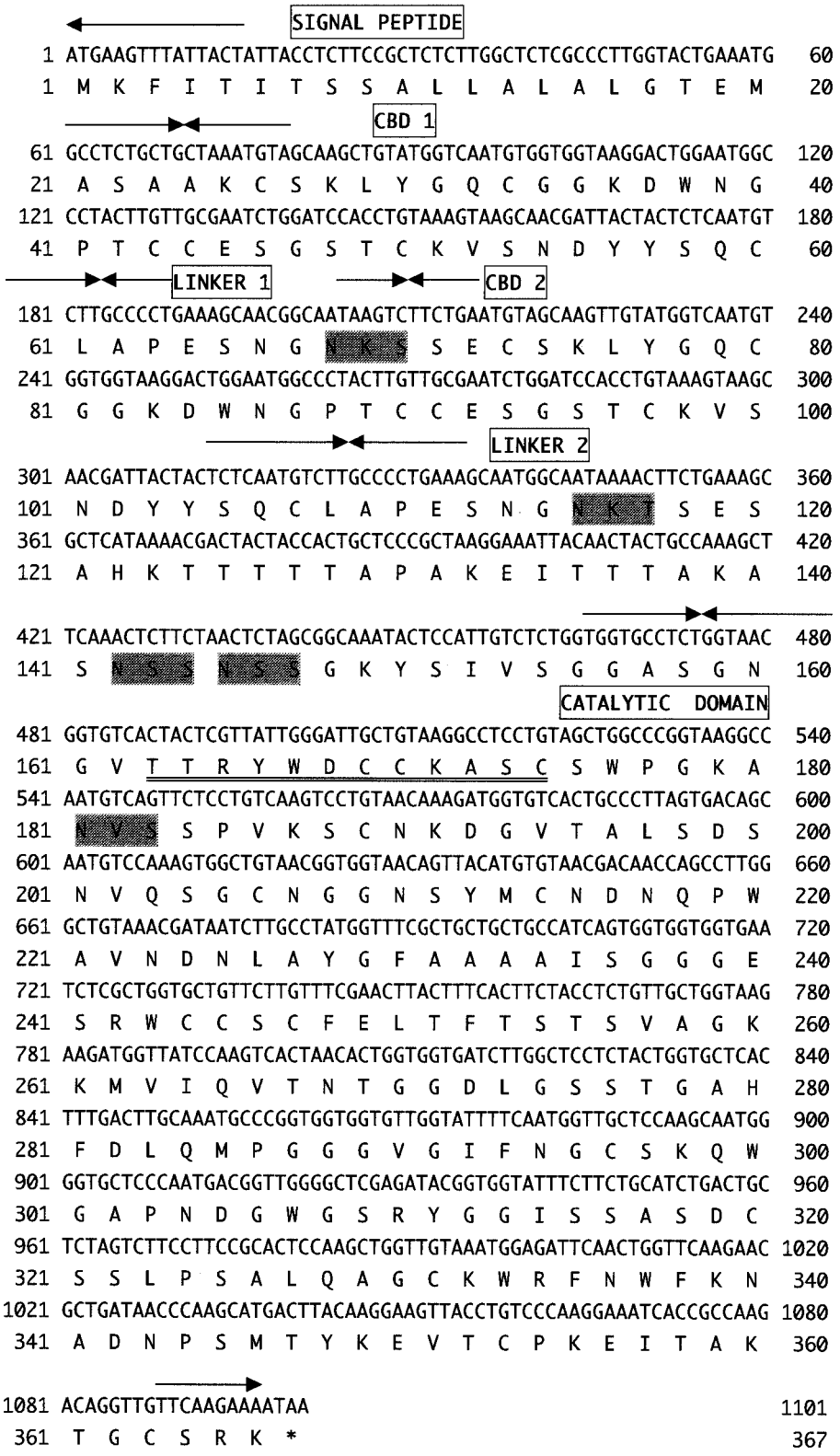

FIG. 3. Nucleotide sequence of the *rce3* gene from *R. oryzae* and deduced amino acid sequence. Arrows, border of each domain: signal peptide, CBDs (CBD 1 and CBD 2), linker domains (linker 1 and linker 2), and catalytic domain. The consensus amino acid residues of glycosyl hydrolase family 45 are double underlined. Potential N-linked glycosylation sites (N-X-S/T) are shaded.

FIG. 4. Alignment of the amino acid sequences of the CBDs of RCE1, RCE2, and RCE3. Amino acids identical to those of the CBD of RCE1 are indicated by white letters in black boxes.

more, the similarity between the CBDs of RCE1 and RCE3 was higher than that between the CBDs of RCE1 and RCE2, and the similarity between the catalytic domains of RCE1 and RCE3 was also higher than that between catalytic domains of RCE1 and RCE2. These results indicate that the *rce1* and *rce3* genes are evolutionarily nearer to each other than to the *rce2* gene.

Comparison of linker domains of RCE1, RCE2, and RCE3. Figure 7 shows the alignment of the linker domains for RCE1, RCE2, and RCE3. This alignment suggests that the linker domains of RCEs were divided into three segments, designated conserved segment 1, repeated segment, and conserved segment 2 (Fig. 7).

Conserved segment 1 was located at the N termini of all linker domains and was highly conserved in the linkers of RCE1, RCE2, and RCE3 (Fig. 7). Conserved segment 2 was located at the C termini of the linkers of RCE1 and RCE2 and at the second linker of RCE3. The amino acid sequences of conserved segment 2 were also highly conserved in RCE1, RCE2 and RCE3. About 50% of the amino acids of conserved segment 2 consisted of serine or threonine.

The repeated segment was located between conserved segments 1 and 2. Interestingly, there were many (H/K)K(K/T)TTT sequences present in this segment as repeated units. The lengths of linkers of RCE1, RCE2, and RCE3 were different from each other because of different numbers of repeated units. The linker of RCE2, which was the longest of the linkers of RCEs, contained the KKTTTT(K/E)(G/A)S sequence four times. The linker of RCE1, which was the second longest, contained the HKKTTTA(A/P) sequence three times. The second linker of RCE3, which was the shortest, contained only one HKTTTTTAP sequence. The linker of RCE2 had another HKTTTT sequence between conserved segment 1 and the repeated segment. The linker domain sequence of xylanase B from the anaerobic fungus *Neocallimastix patriciarum* is known to contain 57 repeats of an octapeptide unit (XSKTLPGG, where X is S, K, or N) (1). The linker domain sequence of the xylanase A from *Ruminococcus flavefaciens* is also known to be composed of some reiteration of the octapeptide QQQN NDWN (28). Based on these facts, the duplication and/or deletion of the repeated peptides might be one of the common evolutionary processes producing diverse linker lengths of plant cell wall-degrading enzymes.

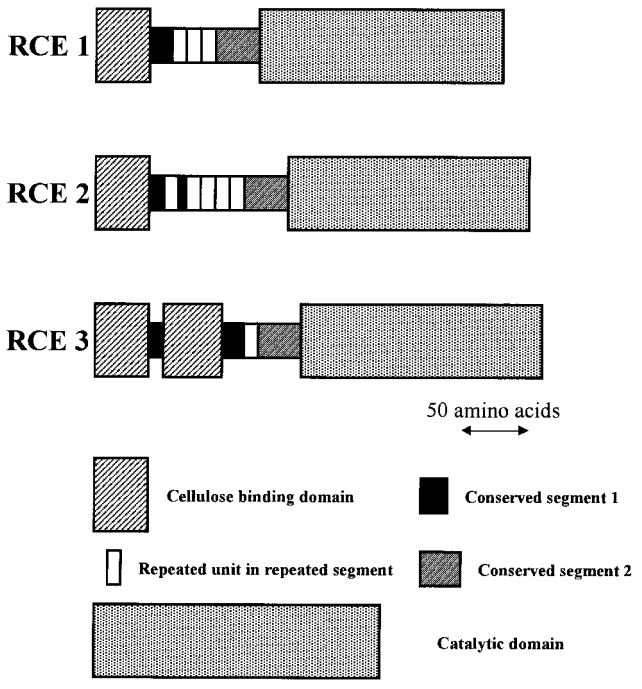

FIG. 5. Modular structures of RCE1, RCE2, and RCE3. CBDs (Fig. 4), conserved segment 1 (Fig. 7), repeated units in repeated segments (Fig. 7), conserved segment 2 (Fig. 7), and catalytic domains (Fig. 6) are shown as boxes to simplify the structure of each domain. Arrow, length of 50 amino acids.

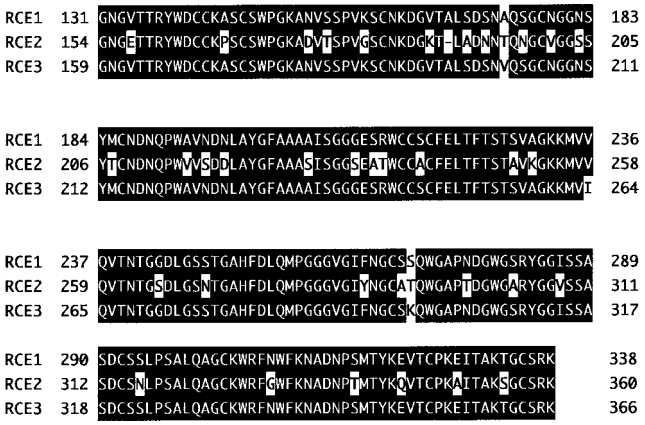

FIG. 6. Alignment of the amino acid sequences of the catalytic domains of RCE1, RCE2, and RCE3. Amino acids identical to those of the catalytic domain of RCE1 are indicated by white letters in black boxes.

Expression of the *rce1*, *rce2*, and *rce3* genes in *Saccharomyces cerevisiae*. To express the *rce1*, *rce2*, and *rce3* genes in *S. cerevisiae*, these *rce* genes were inserted between the GAP promoter and terminator on *S. cerevisiae* expression vector pY2831 (27). To facilitate cloning, *Bgl*II sites were incorporated upstream of the start codons and downstream of the stop codons of the *rce1* and *rce3* genes and the *Bam*HI sites were those of the *rce2* gene. Incorporation was performed by using a Mutagen M13 in vitro mutagenesis kit (Bio-Rad). The primers for mutagenesis were as follows: for *rce1*, 5′-GTAATAAACT TCATAGATCTATGTAAAAAGAATG-3′ (forward primer; underlining indicates the *Bgl*II site) and 5′-GGATGAGTA TAAAAGATCTTATTTTCTTGAAC-3′ (reverse primer; underlining indicates the *Bgl*II site); for *rce2*, 5′-GCGGAT CCATGAAGTTCCTTACCATTGCC-3′ (forward primer; underlining indicates the *Bam*HI site) and 5′-GCGGATCCT TATTTTCTTGAACAGCCAGA-3′ (reverse primer; underlining indicates the *Bam*HI site); for *rce3*, 5′-GTAATAAACT TCATAGATCTATGTAAAAAGAATG-3′ (forward primer;

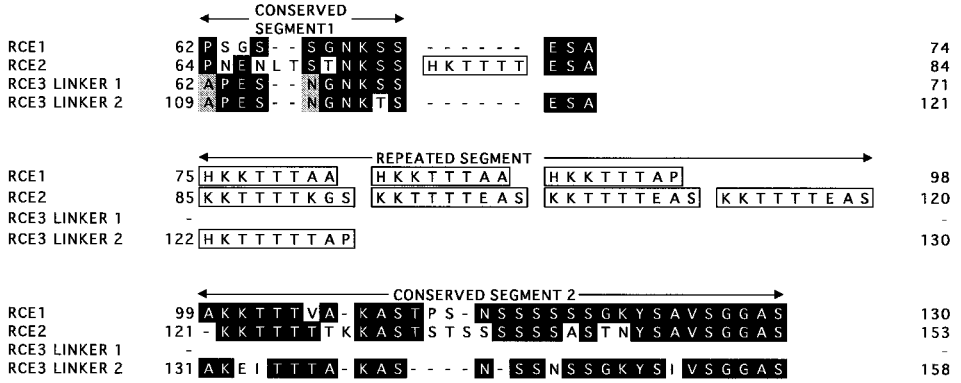

FIG. 7. Alignment of the amino acid sequences of the linker domains of RCE1, RCE2, and RCE3. Conserved segment 1, the repeated segment, and conserved segment 2 are shown. Boxed sequences, repeated units consisting of (H/K)K(K/T)TTT in the repeated segments. Conserved amino acids are indicated by letters in black or gray boxes.

underlining indicates the *Bgl*II site) and 5′-CAAGAAAATA AGATCTTTTATACTCCTACT-3′ (reverse primer; underlining indicates the *Bgl*II site). The resultant plasmids were digested with *Bgl*II or *Bam*HI, and the *rce1*, *rce2*, or *rce3* fragment was subcloned into the *Bam*HI site of pY2831 and placed between the GAP promoter and GAP terminator of *S. cerevisiae.* The resulting plasmids were designated pYRCE1, pYRCE2, and pYRCE3, respectively. *S. cerevisiae* strain MS161 (*MAT***a** *trp1 ura3 Suc*$^-$) was transformed with pYRCE1, pYRCE2, or pYRCE3 by the lithium acetate method described by Ito et al. (12). *S. cerevisiae* transformants were selected on a selective medium containing 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, 0.005% uracil, and 1.5% purified agar (Sigma). For expression of the *rce* genes, the transformants were grown in a production medium containing 0.67% yeast nitrogen base without amino acids, 2% glucose, 0.005% uracil, and 2% Casamino Acids.

To confirm the expression of recombinant RCE1, RCE2, and RCE3, the supernatants of *S. cerevisiae* transformants harboring the plasmid pY2831, pYRCE1, pYRCE2, or pYRCE3 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using ready-made 10% polyacrylamide gels (Tefco). After electrophoresis, the gels were either silver stained or immunostained. For immunostaining, proteins in the gel were transferred to a polyvinylidene difluoride membrane and detected on the membrane by using the antibody against the purified RCE1 from *R. oryzae* (14) in combination with anti-rabbit (goat) antibodies conjugated with horseradish peroxidase as secondary antibodies. Culture supernatant of *S. cerevisiae* harboring the *rce1*, *rce2*, or *rce3* gene showed broad bands around 100, 50, or 100 kDa by Western blotting with anti-RCE1 (data not shown); on the other hand, that of *S. cerevisiae* without the gene did not show any bands. These results indicated that the recombinant RCE1, RCE2, and RCE3 were expressed successfully. The positions of these bands indicated molecular weights much higher than the calculated molecular weights of RCE1, RCE2, and RCE3. The amino acid sequences of RCEs contained potential N-linked glycosylation sites (N-X-S/T) at positions 68, 113, and 153 for the RCE1 (Fig. 1); at 67, 72, 144, and 194 for RCE2 (Fig. 2); and at 68, 115, 142, 145, and 181 for RCE3 (Fig. 3). Therefore, the recombinant RCEs in *S. cerevisiae* were considered to be hyperglycosylated at some of these N-linked glycosylation sites, which are often found in recombinant proteins expressed by the yeast (26).

Characterization of the recombinant RCE1, RCE2, and RCE3. The activities of culture supernatants of *S. cerevisiae* harboring the *rce1*, *rce2*, or *rce3* gene against carboxymethylcellulose (Tokyo Kasei Kogyo Co., Ltd.; for endoglucanase), Avicel (Asahi Chemical Co.; for Avicelase), xylan from birchwood (Sigma), laminarin (Sigma), galactan (Lupin; Megazyme), linear arabinan (Megazyme), and mannan (ivory nut; Megazyme) were determined. Briefly, the activities were measured under standard conditions by using reaction mixtures that contained 10 mg of substrate in 1.0 ml of 50 mM sodium acetate buffer (pH 6.0) and that were incubated for 30 min at 50°C. For Avicelase activity, reaction mixtures were incubated for 24 h at 50°C. One unit of activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per min. All three supernatants of the recombinant RCEs showed much higher activities against soluble cellulose (endoglucanase) than against crystalline cellulose (Avicelase). The culture supernatants of *S. cerevisiae* harboring the *rce1*, *rce2*, or *rce3* gene had endoglucanase activities of 245, 100, and 42 U/liter, respectively, and Avicelase activities of 0.240, 0.185, and 0.188 U/liter, respectively, whereas *S. cerevisiae* without any of the genes did not have any activity. The ratios of Avicelase activity to endoglucanase activity for the culture supernatants of *S. cerevisiae* harboring *rce1*, *rce2*, or *rce3* genes were 0.00098, 0.00185, and 0.00449, respectively. Two tandem CBDs might be more effective for degradation of crystalline cellulose than one CBD, since the recombinant RCE3, which had two tandem CBDs, had higher relative Avicelase activity than the recombinant RCE1, which possessed only one CBD. The culture supernatants of the recombinant RCEs did not show any activities against hemicelluloses. The specific activities of endoglucanase of the native RCE1 and RCE2 purified from the culture supernatant of *R. oryzae* were 272.5 and 110.8 U/mg, respectively (14). Therefore, the calculated amounts of the recombinant RCE1 and RCE2 produced by *S. cerevisiae* harboring the *rce1* or *rce2* gene were 0.90 and 0.91 mg/liter, respectively.

The sugars generated upon hydrolysis of cellooligosaccharides by the recombinant RCEs were also determined by thin-

layer chromatography. The products formed upon hydrolysis of cellotriose (3G), cellotetraose (4G), cellopentaose (5G), and cellohexaose (6G) with the recombinant RCEs were analyzed. The reaction mixtures (1 mg of substrate and 0.25 U of the culture supernatant of RCE1, RCE2, or RCE3 in 1 ml of 50 mM sodium acetate buffer, pH 6.0) were incubated at 50°C for 2 h. The reaction was terminated by boiling the reaction mixtures for 5 min. The hydrolytic products were developed and visualized as described previously (14). The three recombinant RCEs showed similar hydrolytic patterns. The 6G was completely hydrolyzed to produce 4G and cellobiose (2G) with very small amounts of 3G. The 5G was also completely degraded to form 3G and 2G. On the other hand, only small amounts of 4G were hydrolyzed to produce 2G with very faint spots of 3G. The recombinant RCEs did not act on 3G. These hydrolytic patterns were in good agreement with that of EGV from *H. insolens*, which also belongs to family 45 (18). The EGV from *H. insolens* is known to have six subsites in its active site, and the cleavage site is between the fourth and fifth subsites, as determined through substrate specificity studies (19) and crystal structure analysis (4). Based on the similarities of hydrolytic patterns and amino acid sequences between EGV and RCEs, the active sites of RCEs might be similar to that of EGV determined by crystal structure analysis (4).

The effect of temperature was measured under the standard conditions described above by varying the temperature from 30 to 70°C. The optimum temperatures of recombinant RCE1, RCE2, and RCE3 were 55, 50, and 50°C, respectively. The optimum temperatures of recombinant RCE1 and RCE2 were almost same as those of the native RCE1 and RCE2 from culture supernatants of *R. oryzae* (14).

Endoglucanase activity under several different pH conditions was also measured by using 50 mM sodium acetate (pH 3 to 6), sodium phosphate (pH 7 to 8), or glycine-NaOH (pH 9 to 11) buffer. The optimum pH for recombinant RCE1 was 7.0, whereas the optimum pH for the native RCE1 was 6.0. The optimum pH for the recombinant RCE2 was 5.0, and the activity remained at 90% of the highest activity between pH 4 and 7.7. Since the activity of purified RCE2 from culture supernatants of *R. oryzae* remained at 90% of the highest activity between pH 5 and 7 (14), recombinant RCE2 was considered to have higher pH tolerance than purified RCE2 from culture supernatants of *R. oryzae*. The optimum pH of recombinant RCE3 was 7.7, and the high activity was also maintained at a broad pH range (above 90% of the highest activity between pH 5 and 7.7).

Nucleotide sequence accession numbers. The nucleotide sequences of the *rce1* gene from *R. oryzae* FERM BP-6889 have been assigned accession no. AB047927 in the DDBJ database, and the nucleotide sequences of the *rce2* and *rce3* genes have been assigned accession no. AB056668.

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