Structure, Heterologous Expression, and Properties of Rice (*Oryza sativa* L.) Family 19 Chitinases

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We identified four new family 19 chitinases in *Oryza sativa* L. cv. Nipponbare: one class I (OsChia1d), two class II (OsChia2a and OsChia2b), and one class IV (OsChia4a). OsChia2a resembled (about 60% identity) the catalytic domains of class I chitinases, but OsChia2b was almost identical (95% identity) to that of the class IV enzyme. OsChia1c, OsChia1cA(CBD) (a deletion of OsChia1c lacking a chitin-binding domain, CBD), and OsChia2b were separately expressed and purified in *Pichia pastoris*. The activities of these enzymes on chitin polymers were similar, but they acted differently on N-acetylchitooligosaccharides, (GlcNAc)n. OsChia1c slowly hydrolyzed (GlcNAc)6 and very poorly hydrolyzed (GlcNAc)4 and (GlcNAc)5. In contrast, OsChia2b efficiently hydrolyzed these oligosaccharides. The high antifungal activity and low hydrolytic activity of the class I enzyme towards (GlcNAc)n imply that it participates in the generation of N-acetylchitooligosaccharide elicitors from the cell walls of infecting fungi.

Key words: rice (*Oryza sativa* L.); chitinase; N-acetylchitooligosaccharides; antifungal activity; chitin-binding domain

Plant defense against potential pathogens includes a hypersensitive response, the production of reactive oxygen species (oxygen burst), the activation of pathogenesis-related (PR) genes, structural changes in the cell wall, and phytoalexin synthesis.1–3) Elicitors generated by pathogen infection induce the hypersensitive response, oxygen burst, and PR-protein as well as phytoalexin synthesis to destroy plant cells and prevent pathogen growth at the attack site. The hypersensitive response results in the formation of signal substances such as ethylene, as well as salicylic, jasmonic, and nitric acids, which provoke systemic acquired resistance and transient resistance throughout the plant to subsequent attack by pathogens.1,2) As well as elicitors, these signals activate PR protein and phytoalexin synthesis. The PR proteins constitute 11 groups (PR-1 through PR-11).5,6) Among these, chitinases (PR-3, PR-4, PR-8, and PR-11) and β-1,3-glucanase (PR-2) have been more frequently studied because of their ability to inhibit, particularly in combination, fungal growth in *vitro*, through hydrolytic activities on the chitin and β-1,3-glucan that are major components of fungal cell walls.5,6) The participation of these enzymes in plant defense has been substantiated by the increased resistance to fungal pathogens of transgenic plants that constitutively express high levels of chitinase and/or β-1,3-glucanase.7,8) Plant chitinases appear to be responsible for the formation of elicitors (N-acetylchitooligosaccharides) from fungal cell walls to activate their own synthesis and other types of the defense response.9–12)

Plant chitinases belong to structurally unrelated families 18 (PR-8 and PR-11) and 19 (PR-3) of glycosylhydrolases.5,13,14) Family 18 includes classes III (PR-8) and V (PR-11) enzymes. Family 19 consists of three major classes (I, II, and IV). Class I and IV enzymes have a diagnostic cysteine-rich chitin-binding domain (CBD) at the amino terminus,5,14) and class II enzymes lack this domain (Fig. 1A). The CBD domain is dispensable for catalytic and antifungal activities but can increase antifungal function.15) Several enzymes of both families occur in the same plant, allowing the expression of tissue- or developmental stage-specific chitinases or generation of chitinases in response to distinct stimuli.5,16,17) Each
isoform may have distinct catalytic properties in terms of elicitor formation, antifungal spectrum, or synergistic effect with other chitinases or β-1,3-glucanses on antifungal action.19) Like many other plants, *Oryza sativa* L. cv. Nipponbare (japonica ssp.) possesses several family 19 chitinases as predicted by Southern blotting.18,19) Three class I genes (Cht-1, Cht-2, and Cht-3) have been identified in this cultivar (Table 1).18) To avoid confusion with the chitinases of other rice cultivars and other plants, we refer to Cht-1, Cht-2, and Cht-3 as OsChia1a, OsChia1b, and OsChia1c, respectively, according to the recommended nomenclature of plant chitinases.20) These enzymes appear to be expressed at different sites and distinctly regulated,11,18,19) perhaps playing particular roles in the defense mechanism. To further understand the physiological roles of rice chitinases, a complete set of the relevant genes must be identified and the properties of the corresponding products should be understood. We have identified and characterized two family 18 chitinase (OsChib3a and OsChib3b) cDNAs21) in the EST library (about 50,000 clones) of Nipponbare that had been constructed by the Rice Genome Project (http://rgp.dna.affrc.go.jp).

We describe here the structures of four novel family 19 chitinases (one class I, two class II, and one class IV) isolated from Nipponbare and the properties of OsChia1c (a representative of class I), OsChia1cCBD (a class I-type catalytic domain), and OsChia2b (a class IV-type catalytic domain) that are expressed in *P. pastoris*.

**Materials and Methods**

**Strains and media.** We used *Escherichia coli* DH5α (Bethesda Research Laboratory, Bethesda, MA, USA) as the host in plasmid construction, as well as *E. coli* BL21 (DE3) (Novagen; Madison, WI, USA)

### Table 1. Family 19 Chitinases of Nipponbare and Other Cultivars

<table>
<thead>
<tr>
<th>Class</th>
<th>Other cultivars</th>
<th>Expression (Tissue/regulated by)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td>OsChia1a (Cht-1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>RCH10 (94%), RC24 (94%), CH16 (94%)</td>
<td>Leaf/elicitors</td>
</tr>
<tr>
<td>OsChia1b (Cht-2)</td>
<td>CH6 (91%)</td>
<td>Root vacuole/constitutive</td>
<td>18)</td>
</tr>
<tr>
<td>OsChia1c (Cht-3)</td>
<td>OsChia1;175 (100%)</td>
<td>Leaf/elicitors</td>
<td>11, 18, 31)</td>
</tr>
<tr>
<td>OsChia1d</td>
<td>Pestle/tissue-specificity</td>
<td>30)</td>
<td></td>
</tr>
<tr>
<td>Class II</td>
<td>OsChia2a</td>
<td>RCHT2 (95%)</td>
<td>Leaf/elicitors</td>
</tr>
<tr>
<td>OsChia2b (Chi-4)</td>
<td>Husk/tissue-specificity, high temperature</td>
<td>33)</td>
<td></td>
</tr>
<tr>
<td>Class IV</td>
<td>OsChia4a</td>
<td>Floral organ/tissue-specificity</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Amino acid sequence similarities to the corresponding Nipponbare counterparts are shown in parentheses as percent identities. The origins of enzymes are: CH6 and CH16, japonica cv. Naksongbyeo; RCH10 and RC24, indica cv. IR36; OsChia1;175, indica cv. IR24; RCHT2, indica cv. Cheongsongbyeo.

<sup>b</sup> Alternative names of the Nipponbare family 19 chitinases are indicated in parentheses.
and *Pichia pastoris* G115 (Invitrogen; San Diego, CA, USA) for the production of recombinant chitinases. *Escherichia coli* was grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) with ampicillin (100 μg/ml) when necessary. *Pichia pastoris* was cultured in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) for general growth, on MM agar (1.34% yeast nitrogen base, 4×10⁻⁷ M biotin, 1% glucose, and 1.5% agar) for selection of His⁺ transformants, and in BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 4×10⁻⁷ M biotin, and 1% methanol) for the production of recombinant chitinase, as recommended by the manufacturer (Invitrogen).

**Construction of cDNA library and nucleotide sequencing.** We extracted and purified mRNA from various *O. sativa* leaves using the total RNA extraction method as described. The corresponding cDNAs were then synthesized using a SuperScript kit (Bethesda Research Laboratory, Bethesda, MD, USA), ligated to the SalI and NorI sites of plasmid pBluescript II SK+ (Stratagene; La Jolla, CA, USA), and used to transform *E. coli* NM522 (Stratagene). Nucleotides were sequenced as described or using an ABI377 DNA sequencer with Big-dye primer and Big-Dye terminator sequencing kits (Applied Biosystems, Foster City, CA, USA). Amino acid and DNA sequences were analyzed using the Clustal W and Blast programs at the DDBJ (http://www.ddbj.nig.ac.jp).

**Plasmid and strain construction.** We attempted to express class I chitinase OsChia1c, truncated OsChia1c without CBD (OsChia1cΔCBD), and OsChia2b in *E. coli*. The corresponding cDNA regions were amplified by PCR using the primers, P1 (5'-ATGGCCATGACGACGCACCACG-3') and P2 (5'-ACTCGAGGGAAGCGGTAGGCGCTCTGTTG-3') (for OsChia1c), P3 (5'-TTGGGCTATCGAGACGACGCACCACG-3') and P2 (for OsChia1cΔCBD), and P4 (5'-GCCATGGCAGCCGGCGGTGCTGTTGAGA-3') and P5 (5'-ACTCGAGCAGTAGGAGTTCCGCCGCGG-3') (for OsChia2b). After confirming the nucleotides by sequencing, the amplified fragments were cloned between the NcoI and XhoI sites of plasmid pET-22b (+) (Novagen) as MscI (or NcoI) and XhoI fragments to produce pET-OsChia1c, pET-OsChia1cΔCBD and pET-OsChia2b with the chitinase sequences fused in-frame between the pelB leader sequence and the His-tag sequence of the plasmids. Because these enzymes were expressed in *E. coli* BL21 (DE3) as the inactive form in inclusion bodies, we tried to express the chitinases using a *Pichia* expression kit (Invitrogen; San Diego, CA, USA). The chitinase genes in the recombinant plasmids were PCR-amplified using the primers, P6 (5'-ATTACGTAGACGTGCGCGACGGCAGGC-3') and P7 (5'-TGGCAGCGGCTCAAGCTGTCGTTGGCGAGCACCACG-3') (for OsChia1c), P8 (5'-ATTACGTAGACGTGCGCGACGGCAGGGCAACT-3') and P7 (for OsChia1cΔCBD), and P9 (5'-ATTACGTAGACGTGCGCGACGGCAGGGCAACT-3') and P7 (for OsChia2b), and cloned as SnaBI and NotI fragments into plasmid pPIC9 between the corresponding restriction sites. Sequencing the DNA confirmed that the resultant plasmids pPIC-OsChia1c, pPIC-OsChia1cΔCBD, and pPIC-OsChia2b carried the correct chitinase cDNA tagged with histidine codons and that it was fused in-frame downstream of the α-factor signal peptide. The resultant plasmids were linearized with the restriction endonuclease BglII, then used to transform *P. pastoris* G115 (His⁻). The His⁺ transformants, *P. pastoris* OsChia1c-1, OsChia1cΔCBD-1, and OsChia2b-1, were selected on MM agar. We confirmed that the corresponding enzyme was produced by the recombinant strains by measuring chitinase activities in BMMY culture medium.

**Expression and purification of chitinases.** We incubated *P. pastoris* cells harboring a recombinant chitinase cDNA in BMMY medium (50 ml) at 30°C for 36 h to fully express the chitinase as described. The chitinases were purified by passage through a Ni²⁺-affinity column (His-Trap column, Amersham Biotech; Buckinghamshire, UK) according to Park *et al.* Purified enzymes were thoroughly dialyzed against 20 mM phosphate buffer (pH 7.2) containing 1 mM EDTA. The induction and purity of the enzyme were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

**Assays of enzyme, chitin-binding, and antifungal activities.** Chitinase activity was assayed in 0.5 ml of 0.1 M citrate-phosphate buffer (pH 4.8 or pH 5.2) containing 0.2% glycol chitin (or 0.2% colloidal chitin) and enzyme (0.05 ml) according to the modified procedures of Schales. One unit was defined as the amount of enzyme that is required to produce 1 μmol of reducing sugar (as N-acetylglucosamine) per min. *Km* and *Vmax* values were calculated from double reciprocal plots (1/V versus 1/s). To examine heat stability, enzymes were incubated in 20 mM phosphate buffer (pH 7.2) at various temperatures for 10 min and remaining activities were measured. For binding assays, purified chitinase (10 μg) was incubated with 1 mg of colloidal or regenerated chitin as binding substrates in the above citrate-phosphate buffer (0.2 ml) for 1 h on ice with occasional mixing. Thereafter, free enzymes were separated from enzyme-substrate complexes by centrifugation at 15,000×g for 20 min at 0°C and measured using protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Enzyme-substrate complexes in pellets...
were washed with 0.2 ml of the buffer and lyophilized. Bound enzymes were then dissociated from the complexes by boiling in 0.1 ml of sample buffer and 20-μl portions of samples were analyzed by SDS-PAGE. Amounts of the bound proteins were measured by comparing densities of protein bands with that of known amounts of proteins on the gels.

N-acetylchitooligosaccharides (1 mM; Seikagaku Kogyo, Tokyo, Japan) were incubated with 1 mg of enzyme as described above. After the indicated incubation period, reaction products were resolved and detected on silica gel 60 plates (Merck; Darmstadt, Germany) as described. Antifungal activity was assayed by disk-plate diffusion using *Trichoderma reesei* IFO31329 as the test fungus.

**Results**

**Family 19 chitinases of O. sativa L cv. Nipponbare**

Sequencing the cDNA inserts (≥1 kb) with similarity to known class I chitinase in the EST library (48,526 clones) of the Rice Genome Project (http://rgp.affrc.go.jp) discovered four new family 19 chitinases named OsChia1d (AB096139), OsChia2a (AB016497), OsChia2b (AB003194), and OsChia4a (AB096140). A comparison of their deduced amino acid sequences with the sequences of other plant family 19 chitinases showed that OsChia1d and OsChia4a have a CBD at the N-terminal, but OsChia2a and OsChia2b appeared to lack this domain, since a signal peptide was adjacent to the catalytic domain (Fig. 2). The catalytic domains of OsChia1d and OsChia4a resembled (about 60% identity) the corresponding regions of other class I enzymes. OsChia2b and the catalytic domain of OsChia4a have a CBD at the N-terminal, but OsChia2a and OsChia2b appeared to lack this domain, since a signal peptide was adjacent to the catalytic domain (Fig. 2). The catalytic domains of OsChia1d and OsChia2a resembled (about 60% identity) the corresponding regions of other class I enzymes. OsChia2b and the catalytic domain of OsChia4a were very similar (95% identity) (Fig. 1B) and resembled (60–70% identity) the class IV chitinases of other plants rather than the class I enzymes (<60 identity). In addition, three deletions and a cysteine residue located at the carboxyl terminal are features of class IV chitinases.

**Frequency of the family 19 EST clones**

The frequency of the OsChia1a, OsChia1b, OsChia1c, and OsChia2a EST clones in the total EST clones from various tissues, roots (4,079 clones), green shoots (9,212 clones), etiolated shoots (5,103 clones), and panicles (15,128 clones), was low (<0.02%). In contrast, the frequency of OsChia1d EST clones was high in panicles, particularly in flowing panicles (0.35%). OsChia2b and OsChia4a clones also occurred in panicle clones, although at a lower frequency (about 0.04%).

**Heterologous expression and purification**

All efforts to express active OsChia1c, OsChia1cCBD, and OsChia2b in *E. coli* were unsuccessful. The proteins were produced as inactive and insoluble forms in inclusion bodies even when cultures were incubated at 15°C, conditions that we used to produce the barely active class II chitinase in *E. coli*. Attempts by other groups to express rice family 19 chitinases in *E. coli* also met with the formation of inclusion bodies or very low levels of synthesized active enzyme. We accordingly tried to use *P. pastoris* as the host, as eukaryotic proteins have often been produced in this organism. When cultured in BMMY medium, *P. pastoris* cells harboring a recombinant chitinase cDNA produced, extracellularly, satisfactory quantities of the corresponding chitinase. Enzyme formation was maximal by 36 h (about 0.2 mg/50 ml) and remained unchanged for at least up to 72 h (data not shown). The enzymes were purified to apparent homogeneity after Ni²⁺ affinity column chromatography, as judged by SDS-PAGE (Fig. 3A).

**Chitin-binding affinity of CBD and its increase of antifungal activity**

To confirm the binding-affinity of rice CBD to chitin, we bound OsChia1c, OsChia1cCBD, and OsChia2b (10 μg each) to colloidal chitin (1 mg) in 0.2 ml of mixture (see Materials and Methods) and measured the amounts of free and bound proteins by protein assay and by SDS-PAGE, respectively. OsChia1cCBD and OsChia2b (9.2 and 9.4 μg, respectively) were recovered in the supernatant, but only 0.6 μg of OsChia1c was recovered from the supernatant. Association of OsChia1c with the binding substrate was confirmed by SDS-PAGE (Fig. 3B); 9.0, 0.7 and 0.5 μg of OsChia1c, OsChia1cCBD and OsChia2b, respectively, were...
Fig. 3. SDS-Polyacrylamide Gel Electrophoresis of Purified Chitinases (A) and CBD-Dependent Binding to Colloidal Chitin (B).

A: Purified chitinases (1.5–2 μg) were resolved on 12.5% SDS-polyacrylamide gels along with molecular standards (M) and stained with Coomassie brilliant blue. B: Chitinases were incubated with colloidal chitin and bound enzymes were analyzed by SDS-PAGE. 1, OsChia1c; 2, OsChia1cCBD; 3, OsChia2b.

measured in the complexes. The binding-affinity of these proteins to regenerated chitin was essentially the same as that to colloidal chitin (data not shown). Tobacco and bacterial CBD have been shown to increase antifungal activity about three- and 10-fold, respectively.15,25) We examined the effects of rice CBD on antifungal function and whether or not class I and class IV type catalytic domains have similar antifungal activities. Inhibition assays of the hyphal growth of *T. reesei* with purified OsChia1c, OsChia1cCBD, and OsChia2b showed that OsChia1c inhibited fungal growth more than its deletion derivative (Fig. 4). The growth inhibition caused by 10 μg of OsChia1cCBD roughly corresponded to the action of 2 μg of OsChia1c. OsChia2b had inhibitory activity similar to that of OsChia1cCBD. These results showed that rice CBD can stimulate the antifungal activity of the catalytic domain by about 5-fold and that both class I and class IV catalytic domains have similar antifungal properties.

Catalytic properties

OsChia1c and OsChia1cCBD hydrolyzed soluble glycol chitin and insoluble colloidal chitin with similar $K_m$ and $V_{max}$ values and thermal stability (Table 2). The catalytic properties of OsChia2b with the tested substrate were similar, although it was a little more active than class I enzymes toward glycol chitin. However, the activities of the class I and class II enzymes distinctly differed towards $N$-acetylchitoooligosaccharides, (GlcNAc)$_n$. OsChia1c produced mostly (GlcNAc)$_3$ from (GlcNAc)$_6$ and small amounts of (GlcNAc)$_2$ and (GlcNAc)$_4$, and was minimally active towards (GlcNAc)$_4$ and (GlcNAc)$_5$ (Fig. 5). In contrast, OsChia2b actively hydrolyzed all tested chitoooligosaccharides to predominantly yield (GlcNAc)$_3$. To identify their cleavage modes on the $N$-acetylchitoooligosaccharides we analyzed the products of (GlcNAc)$_3$ and (GlcNAc)$_6$ as a function

### Table 2. Properties of OsChia1c, OsChia1cCBD, and OsChia2b Expressed in *P. pastoris*

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Colloidal chitin</th>
<th>Glycol chitin</th>
<th>Optimum pH</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsChia1c</td>
<td>$K_m$ (mg/Wml)</td>
<td>$V_{max}$ (μmol reducing sugars generated/mg protein/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OsChia1cCBD</td>
<td>1.2</td>
<td>1.9</td>
<td>0.2</td>
<td>82.6</td>
</tr>
<tr>
<td>OsChia2b</td>
<td>1.9</td>
<td>2.3</td>
<td>0.4</td>
<td>116.3</td>
</tr>
</tbody>
</table>

$K_m$ (mg/ml) and $V_{max}$ (μmol reducing sugars generated/mg protein/min) values are averages of two measurements. Temperatures that caused 50% reduction of enzyme activity are presented.
of the incubation period. The activity of OsChia1c towards \((\text{GlcNAC})_5\) was very weak, yielding very small amounts of \((\text{GlcNAC})_2\) and \((\text{GlcNAC})_3\) over 20 min (Fig. 6A). OsChia1c hydrolyzed \((\text{GlcNAC})_6\) more actively and generated \((\text{GlcNAC})_3\) as the major product as well as very small amounts of \((\text{GlcNAC})_2\) and \((\text{GlcNAC})_4\). These products were not further hydrolyzed after a longer incubation or by larger amounts of the enzyme. OsChia1c similariy cleaved \((\text{GlcNAC})_5\) and \((\text{GlcNAC})_6\) as OsChia1c (data not shown). In contrast, OsChia2b hydrolyzed \((\text{GlcNAC})_5\) and \((\text{GlcNAC})_6\) at comparable rates and both chitooligosaccharides were completely hydrolyzed within 10 min into \((\text{GlcNAC})_2\) and \((\text{GlcNAC})_3\) (Fig. 6B). \((\text{GlcNAC})_4\), which had been formed from \((\text{GlcNAC})_6\) during the first 5 min, was degraded into two molecules of \((\text{GlcNAC})_2\) during further incubation. The product \((\text{GlcNAC})_3\), but not \((\text{GlcNAC})_2\), was slowly hydrolyzed into \(\text{GlcNAc}\) and \((\text{GlcNAC})_2\) by further incubation. The relative activities of OsChia2b to \((\text{GlcNAC})_2\) and \((\text{GlcNAC})_3\) were roughly 20- and 5-fold those of OsChia1c.

**Discussion**

These and other studies revealed that \(O.\ sativa\) cv. Nipponbare has at least 7 family 19 chitinases (Fig. 1A and Table 1). The formation of these enzymes appears to be regulated by different mechanisms. OsChia1a and OsChia1c (84% identity each other) are expressed at low levels in roots and leaves and are induced to high levels by ethylene, salicylic acid, and \(N\)-acetylchitooligosaccharide elicitors. OsChia1a(94% identity) and CH6 (91% identity) is likely the OsChia1c counterpart in other cultivars (Table 1). Like the Nipponbare class I enzyme, synthesis of these enzymes is induced by elicitors. Vacuole OsChia1b is constitutively expressed at relatively high levels in roots but not in leaves, and it is not induced by elicitors. OsChia1d, as well as its counterpart (100% identity) OsChia1;175 of indica cv. IR24, is rather distantly related to other class I enzymes (Fig. 1B). Consistent with the high-level of OsChia1;175 expression in floral organs, the EST clones of OsChia1d accounted for 0.35% of the total number of clones obtained from flowering panicles.

Class II enzymes (OsChia2a and OsChia2b) are also differently regulated. OsChia2a is the probable counterpart of RCHT2 in the *indica* cv. Cheongcheongbyeo, as they share 95% sequence identity. Rcht2 is formed in response to glycol chitin or fungal elicitors. Another class II enzyme (OsChia2b) with a class IV-type catalytic domain (Fig. 1A) has a sequence that is identical to that of the partially deduced Chi-4, which has been identified as a husk protein expressed under high temperatures that induces seed dormancy. The antifungal property of the class II enzyme (Fig. 4) implies that it helps to protect rice seeds from fungal infection together with molilactones (phytoalexins), which are also formed under the same conditions.

OsChia4a, is a class IV chitinase that has similarity (about 70% identity) to both maize ChiA and Arabidopsis ChIV of class IV chitinases. This notion is in accordance with the hypothesis that the class I and IV genes diverged before the separation of dicots and monocots. Class II genes might have arisen from the relevant class I genes by the deletion of CBD or perhaps class I genes were created by the transposition of a CBD sequence into a class II
gene. The high sequence conservation (95% identity) between the catalytic domains of OsChia4a (class IV) and OsChia2b (class II) provides an example of the direct and recent diversion of their genes via either deletion or insertion mechanisms within rice.

The class IV catalytic domains have three deletions (Fig. 1A) that correspond to the loops between $\alpha$-helices C and D, F and G, and G and H. These loops do not appear to significantly contribute to stability and activity towards chitin polymers (Table 2). However, the hydrolytic activities of OsChia2b towards N-acetylchitooligosaccharides were significantly distinct from those of OsChia1c and OsChia1aCBD. As proposed by X-ray crystallography, molecular dynamics simulations, and kinetic studies, barley class II chitinase with the class I-type catalytic domain would bind to a substrate at subsites $-3$ through $+3$ (or A through F) and cleave the substrate between $-1$ and $+1$ sites by a single-displacement inverting mechanism. OsChia1c appears to share the similar binding-subsite structure with the barley enzyme. According to this binding model, OsChia2b may lack the loop between $\alpha$-helices F and G that contains the substrate-binding residue K188 of sites $-3$ and $-2$. The protein also has a substitution of T69 at the site $+2$ to G. Regardless of these altered binding-subsite structures, OsChia2b efficiently hydrolyzes (GlcNAC)$_4$ and (GlcNAC)$_5$, while OsChia1c, in which the proposed binding residues are perfectly conserved, does so very poorly (Figs. 6A and B). At this stage the structures responsible for the different hydrolytic activities are unknown. The activities of plant chitinases towards N-acetylchitooligosaccharides are quite important with respect to their roles in elicitor formation (see below). The P. pastoris expression system allows mutant enzymes to be produced that have a specific amino acid substitution(s) at a position(s) of interest for elucidation of its role in substrate binding and catalysis.

The antifungal properties of OsChia1c and OsChia2b (Fig. 4) support the notion that they participate in defense against fungal pathogens. OsChia1c has high antifungal activity, but low activity towards (GlcNAC)$_4$, which is less than hexameric (Figs. 6A and B), and which can elicit chitinase synthesis, thus making it (and its isoform OsChia1a) a likely generator of N-acetylchitooligosaccharide elicitors from the cell walls of infecting fungi. OsChia2b and OsChia4b may not significantly contribute to elicitor formation. Rather they would degrade elicitors due to their powerful hydrolytic activity on N-acetylchitooligosaccharides (Figs. 6A and B).

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References


