Genomic DNA encoding a class IV chitinase was cloned from yam (*Dioscorea opposita* Thunb) leaves in previous research (*Biosci. Biotechnol. Biochem.*, 68, 1508–1517 (2004)). But this chitinase had an additional sequence composed of eight amino acids (a C-terminal extension) at the C-terminal, compared with class IV chitinases from other plants. In order to clarify the role of this C-terminal extension in cellular localization, plants and suspension-cultured cells of *Nicotiana tabacum* were transformed with either the cloned yam class IV chitinase gene carrying the C-terminal extension or its truncated gene by the *Agrobacterium*-mediated method, and then their localization was investigated. The results suggest that the C-terminal extension of yam class IV chitinase plays a role as a targeting signal for plant vacuoles. This is the first report presenting the existence of vacuolar type class IV chitinase.

**Key words:** yam (*Dioscorea opposita* Thunb); class IV chitinase; C-terminal extension; transgenic tobacco; vacuolar targeting signal

Plant chitinases (EC 3.2.1.14) occur in higher plants.1,2 These enzymes catalyze the hydrolysis of chitin, a major component of the cell walls of many fungi, including plant pathogens,3,4 whereas no endogenous substrate has so far been found in higher plants. In addition, some purified plant chitinases inhibited fungal growth in test tube5 and in field.6 Hence it appears that plant chitinases play an important role in self-defense against fungal pathogens. Chitinases are largely classified into 2 families, namely families 18 and 19 of glycosyl hydrolases,7 and at least 4 classes of plant chitinases have been proposed on the basis of their primary structures.2 Class I, II, and IV chitinases belong to family 19, while class III chitinases belong to family 18. We previously purified and characterized 5 chitinase isoforms designated A, E, F, H1, and G from yam tuber (*Dioscorea opposita* Thunb).8 Chitinases E, F, and H1 were demonstrated to have higher lytic activity against the pathogen *Fusarium oxysporum* among these isoymes. In particular, chitinase E, which belongs to class IV, was shown to be a possible biocontrol agent against strawberry powdery mildew (*Spherotheca humuli*).9 In order to investigate the structure of chitinase E, therefore, its genomic DNA was cloned from yam leaves in the previous study.9 The nucleotide sequence (*YC4-v*) has been deposited in the GenBank data base (under accession no AB102714). The deduced amino acid sequence showed 50 to 59% identity to class IV chitinases from other plants. But this chitinase also had an additional sequence composed of 8 amino acids (the C-terminal extension) following the cysteine, which was reported as the last amino acid for other class IV chitinases. This C-terminal extension is composed mainly of hydrophobic amino acids.

Class I chitinases carrying a short C-terminal extension are localized in vacuoles.2 Neuhaus et al.10 have demonstrated that a short C-terminal extension of about 6 amino acids present in a tobacco basic class I chitinase is both necessary and sufficient for vacuolar localization. Vacuolar chitinases have been reported only from class I chitinases such as bean11,12 and tobacco.10 It has not been reported that class IV chitinases carry a C-terminal extension and are localized in the vacuole.

We hypothesized that this C-terminal extension of yam class IV chitinase may be a targeting signal for the plant vacuole. In order to clarify the role of this C-terminal extension in cellular localization, transgenic tobacco was developed by introducing the cloned yam class IV chitinase gene carrying the C-terminal extension and its truncated gene into tobacco plants and suspension-cultured cells using the agrobacterium system.

We report here the localization of this class IV chitinase and the role of the C-terminal extension in intracellular localization.

**Materials and Methods**

Preparation of total DNA and oligonucleotide primers. Total DNA was extracted from frozen leaves with a DNeasy plant kit (Qiagen K.K., Japan) according to the supplier’s protocols. Gene-specific primers were designed based on the genomic DNA encoding yam class IV chitinase *YC4-v* and the construction vector.

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Abbreviations: PCR, polymerase chain reaction; GSP, gene-specific primer; PAGE, polyacrylamide gel electrophoresis
pMLH7133-GUS. Their melting temperatures (°C) were calculated according to the formula 69.3 + 0.41(%GC) – 650/L, where L is the primer length (Table 1).

**Construction of binary vector.** To investigate the role of the C-terminal extension in cellular localization, **YChi4-v** and **YChi4-v-Cdel**, which encode yam class IV chitinase carrying and lacking the C-terminal extension, and contain the **BamHI** site, as shown in Table 1. **GSP-R1KnI** and **GSP-R2KnI** are reverse primers to amplify **YChi4-v** and **YChi4-v-Cdel** respectively, which contain the **KpnI** site as shown in Table 1.

The constructions were designated pMLH-YChi4-v and pMLH-YChi4-v-Cdel, and introduced into **Agrobacterium tumefaciens** strain EHA101 by freeze-thaw methods. A. tumefaciens carrying pMLH-YChi4-v (EHA101-YChi4-v) or pMLH-YChi4-v-Cdel (EHA101-YChi4-v-Cdel) was grown for 3 days in luria broth medium containing 50 mg/l kanamycin, 50 mg/l hygromycin, 20 mg/l rifampicin, and 20 mg/l Chloramphenicol at 28 °C with shaking at 150 rpm, and used for transformation.

**Plant and cell transformation.** Tobacco (Nicotiana tabacum cv. Xanthi) plants were transformed with EHA101-YChi4-v or EHA101-YChi4-v-Cdel by the method of Horsch et al., with a modification as follows: The turbidity of A. tumefaciens was adjusted to an absorbance of 0.001 at 600 nm for the infection. The selection medium contained 0.2 mg/l kanamycin, 50 mg/l hygromycin, 100 mg/l carbenicillin. The shoot elongation/rooting medium contained 50 mg/l kanamycin, 12.5 mg/l hygromycin, and 100 mg/l carbenicillin.

Tobacco (Nicotiana tabacum cv. Bright yellow 2) suspension-cultured cells were also transformed with EHA101-YChi4-v or EHA101-YChi4-v-Cdel by the method of An, with a modification as follows: The

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**Table 1.** Oligonucleotide Primer Sequences Used in PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Melting temperature° C</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSP-F1BmI</td>
<td>5'-GCGGGATCCATGCATTTAGTAGATTTCCCTTG-3'</td>
<td>68.4</td>
</tr>
<tr>
<td>GSP-F2</td>
<td>5'-CGACTATTGGAAGCCGGG-3'</td>
<td>58.2</td>
</tr>
<tr>
<td>GSP-F3</td>
<td>5'-AATGCCATTCCTCCCTCG-3'</td>
<td>54.5</td>
</tr>
<tr>
<td>GSP-R1KnI</td>
<td>5'-GGGCTACCTAATAACTAAGGCGAGTTAGTGTG-3'</td>
<td>71.7</td>
</tr>
<tr>
<td>GSP-R2KnI</td>
<td>5'-GGCCACGGTACCTAATAACAGTGAGATCATTCCCGAGA-3'</td>
<td>72.6</td>
</tr>
<tr>
<td>GSP-R3</td>
<td>5'-GGTCAGGTCGAAGTAC-3'</td>
<td>58.2</td>
</tr>
<tr>
<td>GSP-R4</td>
<td>5'-AAGGCTACCAGTTACTAGGAC-3'</td>
<td>57.9</td>
</tr>
</tbody>
</table>

°Melting temperatures (°C) were calculated according to the formula 69.3 + 0.41(%GC) – 650/L, where L is the primer length.  

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**Fig. 1.** The Primer Positions on Yam Chitinase Gene **YC4-v**.  
GSP-F1BmI is a forward primer to amplify **YChi4-v** and **YChi4-v-Cdel**, which encode yam class IV chitinase carrying and lacking the C-terminal extension, and **BamHI** site, as shown in Table 1. **GSP-R1KnI** and **GSP-R2KnI** are reverse primers to amplify **YChi4-v** and **YChi4-v-Cdel** respectively, which contain the **KpnI** site as shown in Table 1.
turbidity of *A. tumefaciens* was adjusted to an absorbance of 0.001 at 600 nm for the infection. The selection medium contained 100 mg/l kanamycin, 25 mg/l hygromycin, and 500 mg/l carbenicillin.

**Selection of transformants.** To detect the introduced gene of *YChi4-v* or *YChi4-v-Cdel* in the transformed tobacco plants and cells, PCR was carried out with a primer combination of GSP-F2 and GSP-R3 designed on the basis of the common region for both transgenes, and with a primer combination of GSP-F3 and GSP-R4, which was designed on the basis of the C-terminal extension (Table 1, Fig. 2A) in a 50-μl reaction mixture containing 50 ng of total DNA, 0.4 μM each gene-specific primer, 200 μM each of the dNTPs, 1 U Ex Taq polymerase, and 1× Ex Taq™ buffer supplied with the enzyme (Takara Co., Ltd.). Thermal cycling conditions were set as follows: preheating at 94°C for 2 min and 25 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min for a primer combination of GSP-F2 and GSP-R3, and preheating at 94°C for 2 min and 25 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 2 min for a primer combination of GSP-F3 and GSP-R4.

**Fig. 2.** Detection of Transgene in Transgenic Tobacco.

Panel A: The binary vector (pMLH-YChi4-v) used for transformation and the relative position of primers used for detection of the transgenes. CTE, C-terminal extension; E7-P3SSS-Ω-I, expression promoter; Tnos, terminator of nopaline synthase gene; Km^R^, kanamycin-resistance gene; Hyg^R^, hygromycin-resistance gene; RB and LB, right and left borders of T-DNA of *Agrobacterium tumefaciens* Ti plasmid respectively. Panels B and C: PCR amplification of the transgenes in R_0 regenerated plants or suspension-cultured cells using a primer combination of GSP-F2 and GSP-R3 (B) and a primer combination of GSP-F3 and GSP-R4 (C). Symbols: M, DNA molecular weight markers of φX174/Hind III digest (B) or φX174/Hinc II digest (C); pMLH-YChi4-v and pMLH-YChi4-v-Cdel, transformation vectors used; Leaf, leaves from R_0 regenerated plants; Cell, suspension-cultured cells; NT, non-transformant; YChi4-v and YChi4-v-Cdel, transformant carrying YChi4-v and YChi4-v-Cdel respectively.
Chitinase activity staining and α-mannosidase activity assay. Chitinase activity on native PAGE was carried out by the method of Koga et al. In the case of the transformed tobacco suspension-cultured cells, the cultured cells and medium were separated by filtration with a filter paper/type 2 (Toyo Roshi Co., Ltd.) 7 days after subculture. The cells were homogenized in equal volumes of the extraction buffer and centrifuged at 4 °C for 10 min at 15,000 rpm. The protein concentration of the supernatants was determined using Bio-Rad DC Protein Assay kit II (Bio-Rad Laboratories). Fifty micrograms of total protein from cells was loaded to each gel lane. The medium was diazylated toward the extraction buffer and concentrated with an Ultra filter unit/USY-1 (Toyo Roshi Co., Ltd.). Then the medium corresponding to 200 μl was loaded to each gel lane.

In the case of the transformed tobacco plants, fresh leaves were ground with a mortar and pestle, homogenized in 3 volumes of the extraction buffer (50 mM phosphate buffer, pH 8.0) and centrifuged at 4 °C for 10 min at 15,000 rpm. The protoplasts were isolated from the leaves by digestion with 0.2% Macerozyme R10 (Yakult Honsya Co., Ltd.) and 2% Cellulase Onozuka R-10 (Yakult Honsya) in the presence of 10 mM CaCl₂ and 0.7 M mannitol (pH 5.6) for 5 hours at 30 °C. The protoplasts were filtered through a miracloth (Calbiochem-Novabiochem Corp.) and collected as precipitate by centrifugation at 500 rpm for 3 min. For chitinase activity staining and α-mannosidase activity assay, the protoplasts were homogenized in equal volumes of the extraction buffer. These enzyme solutions were subjected to α-mannosidase activity assay carried out by the method of Boller and Kende using PNP-α-mannopyranoside as the substrate. The reaction mixture consisted of 250 ml of 100 μM sodium succinate buffer (pH 5.0) and 250 ml of 0.6 mM PNP-α-mannopyranoside. The reaction was performed with 10 μl of enzyme solution for 5 h at 27 °C and stopped by 0.8 ml of 1 mM Na₂CO₃. Absorbance was determined at 405 nm. One unit was defined as 1 nmol product/min. Chitinase activity on native PAGE was done as described above. The enzyme solution containing 40 to 50 units of α-mannosidase activity was loaded to each gel lane.

Results

Localization of chitinase in transgenic plants and cells

To investigate the role of the C-terminal extension in cellular localization, plants and suspension-cultured cells of Nicotiana tabacum were transformed with this yam class IV chitinase gene carrying or lacking the C-terminal extension by the Agrobacterium-mediated method. To examine whether the transgenic plants and cells carry the introduced chitinase gene, PCR was performed using the gene-specific primers shown in Table 1 and Fig. 2A. PCR products were obtained in the transformants with YChi4-v and YChi4-v-Cdel in the expected size when using a primer combination of GSP-F2 and GSP-R3 designed on the basis of the common region for both transgenes (Fig. 2B), while PCR products were obtained only in the transformants with YChi4-v in the expected size when using a primer combination of GSP-F3 and GSP-R4, which was designed on the basis of the C-terminal extension (Fig. 2C). These results suggest that the transgenic plants and cells carrying YChi4-v or YChi4-v-Cdel were developed successfully.

To investigate the localization of the yam chitinase in the transgenic tobacco plants and cells, activity staining of chitinase was performed on native PAGE. In order to investigate whether the yam chitinase is accumulated in the cell or secreted out of the cell, we used the suspension culture of transgenic cells. Although it is difficult to compare quantitatively the expression of chitinase activity as between in the cell and in the medium, we compared them relatively. Chitinase active bands were observed strongly in the cells of the transformant with the yam chitinase gene carrying the C-terminal extension (YChi4-v) rather than in the medium. On the other hand, active bands were observed in the medium of the transformant with the yam chitinase gene lacking the C-terminal extension (YChi4-v-Cdel) rather than in the cells. In the non-transformant, a strong active band was not observed (Fig. 3A). These results indicate that the yam chitinase is localized in the intracellular space, suggesting that this C-terminal extension plays a role in targeting the chitinase to the intracellular space. Furthermore, the result that the yam chitinase lacking the C-terminal extension was secreted out of the cell confirmed that the N-terminal signal sequence correctly functioned in the transgenic tobacco to target the chitinase to the endoplasmic reticulum, probably followed by the region outside the cells, the plasma membrane, or the vacuoles. Then, in order to investigate the role of the C-terminal extension in intracellular localization in further detail, we isolated protoplasts from the leaves of the transgenic tobacco plants and measured a vacuolar enzyme, α-mannosidase in the expected size when using a primer combination of GSP-F3 and GSP-R4, which was designed on the basis of the common region for both transgenes (Fig. 3B). Both the leaves and protoplasts of the transgenic plants transformed with YChi4-v carrying the C-terminal extension, which have the same α-mannosidase activities as unit 45 and unit 40 respectively, had similar chitinase-activity in amount and number of the active band. On the other hand, in the case of the transgenic plants transformed with YChi4-v-Cdel lacking the C-terminal extension, the active bands were observed mostly in the leaves and not in the protoplasts, even though α-mannosidase activities were almost the same as in unit 50 and unit 40 respectively, meaning that the chitinase was targeted to the region outside the cells, but not to the vacuole or the plasma membrane. As for the control, both the leaves and the protoplasts of the non-transformant did not show any
chitinase-active band, even though their α-mannosidase activities were unit 45 and unit 44 respectively (Fig. 3B), suggesting that the chitinase was not expressed in the non-transformant. All these results suggest that the C-terminal extension is a vacuolar targeting signal.

Discussion

The yam class IV chitinase, which was cloned in the previous study, has a signal sequence at the N-terminal.9) Hence this chitinase is secreted out of the cell or targeted into the plasma membrane or the vacuole. Furthermore, this yam chitinase has an additional 8 amino acids (the C-terminal extension) following the last cysteine at the C-terminal, compared with other class IV chitinases.9) With respect to the C-terminal extension, some class I chitinases have been found in bean,23) potato,24) tobacco,25) rice26,27) and grape28) to have a C-terminal extension like this yam class IV chitinase. As a general rule, chitinases carrying such a C-terminal extension are localized in the vacuole and those lacking it are secreted out of the cell.29)

In this study, to investigate the role of the C-terminal extension of the yam class IV chitinase in cellular localization, transgenic tobacco plants and cells were developed by introducing yam chitinase gene (YChi4-v and YChi4-v-Cdel) driven by a constitutive strong promoter. These transgenic plants and cells were analyzed on the localization. Chitinase activity was detected mainly in the protoplasts, including vacuoles in the transgenic plants carrying a transgene encoding the unmodified yam chitinase, whereas in the case of the transgenic plants and cultured cells carrying mutant yam chitinase lacking this C-terminal extension, chitinase activity was detected mainly in the extracellular space, such as the medium of the suspension culture. These results suggest that this C-terminal extension works as an intracellular targeting signal, probably a vacuolar targeting signal like that of a tobacco basic class I chitinase.10) Therefore, the yam class IV chitinase carrying this C-terminal extension is localized in the vacuole. In order to confirm this, we are now working to isolate the vacuole.

In chitinase activity staining of the transgenic plants and cells, a few active bands were detected. Lange et

![Fig. 3. Chitinase and α-Mannosidase Activities in Transgenic Tobacco.](image-url)
understand the plant self-defense mechanism. Class IV chitinase is significant to the attempt to produce from these transgenic tobacco plants and cells. The genes, YChi4-v and YChi4-v-Cdel, to be introduced into the transgenic tobacco were cloned on the basis of the partially known amino acid sequence of yam chitinase E. But the deduced amino acid sequence of the cloned chitinase gene showed about 90% sequence identity to yam chitinase E. Hence it is reasonable to suppose that the mobility of the chitinases produced from these transgenic tobacco plants and cells is not the same as that of chitinase E.

As far as we know, this is the first report to the finding that a vacuolar type class IV chitinase occurs in higher plants such as the yam. The existence of such a novel class IV chitinase is significant to the attempt to understand the plant self-defense mechanism.

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