Genetic Evidence That Two Types of Retroelements Evolved through Different Pathways in Ectomycorrhizal Homobasidiomycetes Tricholoma spp.

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We designed a polymerase chain reaction that allows us to clone two types of putative reverse transcriptase genes from 11 species of ectomycorrhizal basidiomycetes that belong to the genus Tricholoma. One corresponds to the putative gene of marY1, a long terminal repeat (LTR) retroelement from Tricholoma matsutake, and the other, marY2N, a LINE-like non-LTR (L1-like) retroelement from this fungus. Putative protein products predicted from nucleotide sequencing of cloned fragments were phylogenetically analyzed. marY1-like elements had a parallel phylogenetic relationship with no apparent correlation to a current taxonomic profile, while marY2N-like elements showed a vertical one in relation to host-plant species. Data suggest that marY1-like elements and marY2N-like elements have evolved independently, and the evolution of marY1-like elements could have occurred later than the evolution of marY2N-like elements in the species of Tricholoma.

Key words: basidiomycetes; ectomycorrhizal fungi; molecular evolution; reverse transcriptase; Tricholoma matsutake

Retroelements are retrovirus-like DNA elements that may replicate through an RNA intermediate by reverse transcriptase (RT) activity.1-3 Because of their major influence on the evolution of eukaryotic genomes, retroelements are useful as markers for genome analysis, and in some cases as mutators for genetic analysis of their host organisms.4-11 Tricholoma, a genus of homobasidiomycetes, consists of many ectomycorrhizal fungi, most of which have symbiotic profiles specific to certain plant species.12-14 We have cloned two types of retroelements designated marY1 and marY2N from Tricholoma matsutake, a symbiont that produces economically important “matsutake” mushrooms.15-16 marY1 is a member of the gypsy-type long terminal repeat (LTR)-retroelements, which are closely related to mammalian retroviruses and have also been found in various plant-associated ascomycetes.5,16-19 marY2N is a member of LINE-like non-LTR retroelements, which are closely related to L1-elements present in human genomes, and have been found in only a few cases of plant-associating fungi.7,8,11,15,20,21 So far, the recognition of both LTR and L1-like retroelements residing in the same genome of plant-associating fungi was reported in the rice blast pathogen Magnaporthe grisea, which belongs to filamentous ascomycetes, and in T. matsutake.11,15,16,18,21,22 In fact, marY1 and marY2N are the first retroelements of each class reported in basidiomycetes.15,16

In this study, we attempted to clone putative rt genes that correspond to those of either marY1 or marY2N from 11 species of ectomycorrhizal Tricholoma and to explore their phylogenetic relationships. The analysis will provide us with information on how retroelements evolved in the genus Tricholoma. This information may show us a way of using these DNA elements for genetic and genome analyses in the species of Tricholoma.

Materials and Methods

Fungal strains and media. Strains of 11 Tricholoma species used in this study are listed in Table 1. Fungal mycelia were cultured in MMN liquid medium modified by the addition of V8 juice (Campbell Soup Co., Camden, NJ) to the final concentration of 1.5% instead of NaCl at 23°C for 24 days. For long-term storage, the fungi were grown on the medium

Nucleotide sequences from species of Tricholoma used in this study have been deposited in the DDBJ database under accession numbers AB028236, AB047280, and AB078743-AB078764.

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Abbreviations: LINE, long interspersed nuclear element; LTR, long terminal repeat; ORF, open reading frame; RT, reverse transcriptase; rt, reverse transcriptase gene
solidified by 2.0% agar (Wako Pure Chemicals, Osaka, Japan).

PCR and TA-cloning. Genomic DNA was extracted from fungal mycelia using a lysis buffer containing hexadecltrimethylammonium bromide. A high-fidelity PCR was done with LA-Taq (Takara Shuzo, Japan) (Table 1). The products were isolated from 1X 

Nucleotide sequencing. Plasmids containing PCR products were isolated from E. coli by using Wizard SV Plus mini-prep system (Promega, Madison, WI). The nucleotide sequence of the insert was analyzed with a Big Dye terminator FS core reaction kit ver 3 and ABI prism 377 sequencer (Applied Biosystems). Data were analyzed using the computer software GENETIX-Mac ver 9.0 (Software Development Co., Tokyo, Japan). A homology search was done using the BLAST program (http://blast.genome.ad.jp/). A neighbor-joining phylogenetic tree was constructed after a multiple sequence alignment and a bootstrap analysis based on 1000 replications with the CLUSTAL X program.23) Sequences used in designing primers and gaps detected in the alignment were excluded during the bootstrap analysis.23)

Results and Discussions

Cloning of marY1-like rt genes

We had previously cloned marY1-like rt genes by PCR using primers designed based on nucleotide sequences that could encode the RT domains II and RH (=-RNase H) of marY1 1.0 kb DNA segments from T. matsutake Y1, T. magnivelare Tp-C3, T. portentosum 615, T. fulvocastaneum WK-N1, and T. sectaunum NA12.24) The fragments were predicted to encode RT domains II, III, IV, V, VI, VII, and RH, which corresponded to those of marY1.24) In the same attempt, however, we could not obtain any such products from the samples of six other Tricholoma species tested.24) Therefore, we redesigned primers for PCR using nucleotide sequences that correspond to the amino acid sequences conserved in the former five species of Tricholoma to clone the segment from all the 11 species of Tricholoma. The primers 5'-pMM [5'-ACCTTTTACAGACATGATGAAGCAGACTCTCT-3'], Tm(°C) = 61.9] and 3'pKK [5'-TTTCTTGGCGCTCAGAACTCAGAG-3'], Tm(°C) = 63.3] were designed based on the amino acid sequence TFQTMMNDIF at the RT domain IV and the sequence NLEYMTAKKL at the domain RH of marY1, respectively. Using these primers, we obtained 0.76-kb DNA segments from all 11 species of Tricholoma (Fig. 1(A)). Although the fragments were barely detected in the samples of T. saponaceum 616 and T. japonicum FK-J1 under the assay conditions described above (Fig. 1(A)), some fragments were recovered from these samples by reducing the annealing temperature to 50°C during PCR. All of these fragments were cloned into the vector pCR2.1 for nucleotide sequencing.

Cloning of marY2N-like rt genes

We designed primers for PCR to clone marY2N-like rt genes from species of Tricholoma using nucleotide sequences of marY2N that correspond to amino acid sequences conserved to some extent among marY2N and other L1-like retroelements, such as CgT1 from Colletotrichum gloeosporioides, jockey from Drosophila melanogaster, and Tad1 from Neurospora crassa.20,25,26) The primers 5'pSF [5'-TCATTCAGGCCCTTGTGCTCTCG-3'], Tm(°C) = 62.2] and 3'pLG [5'-GAGTTTTCGCGTCAAGAGAATACTCGAG-3'], Tm(°C) = 62.0] were designed on the basis of the amino acid sequence SFRPIV at the RT domain II and the

Table 1. Fungal Strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Sampling site</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricholoma matsutake</td>
<td>Y1</td>
<td>Pinus densiflora forest, Ibaraki, Japan</td>
<td>1993²⁴</td>
</tr>
<tr>
<td>Tricholoma bakamatsutake</td>
<td>B1</td>
<td>Quercus serrata forest, Ibaraki, Japan</td>
<td>1993²⁴</td>
</tr>
<tr>
<td>Tricholoma magnivelare</td>
<td>Tp-C3</td>
<td>Canada (A fruiting body was obtained from a local grocery)</td>
<td>1993²⁴</td>
</tr>
<tr>
<td>Tricholoma fulvocastaneum</td>
<td>WK-N1</td>
<td>Quercus phillyreoides forest, Wakayama, Japan</td>
<td>1988²⁴</td>
</tr>
<tr>
<td>Tricholoma robustum</td>
<td>KB1</td>
<td>P. densiflora forest, Nagano, Japan</td>
<td>2000</td>
</tr>
<tr>
<td>Tricholoma ustula</td>
<td>611</td>
<td>P. densiflora forest, Ibaraki, Japan</td>
<td>1997²⁴</td>
</tr>
<tr>
<td>Tricholoma flavivirens</td>
<td>613</td>
<td>P. densiflora forest, Ibaraki, Japan</td>
<td>1997²⁴</td>
</tr>
<tr>
<td>Tricholoma portentosum</td>
<td>615</td>
<td>P. densiflora forest, Ibaraki, Japan</td>
<td>1997²⁴</td>
</tr>
<tr>
<td>Tricholoma saponaceum</td>
<td>616</td>
<td>P. densiflora forest, Ibaraki, Japan</td>
<td>1997²⁴</td>
</tr>
<tr>
<td>Tricholoma japonicum</td>
<td>FK-J1</td>
<td>P. densiflora forest, Fukui, Japan</td>
<td>1988²⁴</td>
</tr>
<tr>
<td>Tricholoma sectaunum</td>
<td>NA12</td>
<td>P. densiflora/Q. serrata forest Nagano, Japan</td>
<td>1993²⁴</td>
</tr>
</tbody>
</table>

* ( ) = IFO strain numbers (Institute of Fermentation, Osaka, Japan).
sequence LGFFFRDKL at the RT domain VIII of *marY2N*, respectively. Using these primers, we obtained 0.76-kb DNA segments from *T. matsutake* Y1, *T. magnivelare* Tg-C3, *T. robustum* KB1, *T. bakamatsutake* B1, *T. fulvocastaneum* MR26, *T. sejunctum* NA12, *T. japonicum* MR27, *T. flavovirens* 613, and *T. portentosum* 615 (Fig. 1(B)). These DNA segments were cloned into the vector pCR2.1 for nucleotide sequencing.

We could not, however, obtain any such DNA segments from *T. ustale* 611 and *T. saponaceum* 616 even if the annealing temperature was decreased to 50°C. Also, subsequent nucleotide sequencing showed that the 0.76-kb DNA segment from *T. fulvocastaneum* WK-N1 resulted from the extension of a set of the single primer 5′pSF and did not contain any rt genes. Therefore, we designed primers based on the amino acid sequences predicted to be encoded in common in the inserts of all clones from species of *Tricholoma*. 5′pIV [5′-ATTGTGCT-CCTGAATACCTTGGCG-3′, \( Tm^\circ = 60.5 \)] was designed based on the amino acid sequence IYLLNLTG at the RT domain II of *marY2N*, and 3′pWR [5′-GTCGAAGAAAAATCCGAGGTACC-TCCA-3′, \( Tm^\circ = 63.0 \)] based on the sequence WRYLGFFFD at the RT domain VIII of *marY2N*. Using these primers, we obtained 0.75-kb DNA segments from *T. ustale* 611, *T. saponaceum* 616, and *T. fulvocastaneum* WK-N1 (Fig. 1(C)), and cloned them into the vector pCR2.1 for nucleotide sequencing.

**Phylogenetic analysis of deduced RT**

The neighbor-joining analysis was done to examine phylogenetic relationships among the deduced amino acid sequences of *marY1*-like and *marY2N*-like proteins from *Tricholoma* spp. Corresponding sequences of retroelements from various ascomycetes were included in the analysis as out-group controls: e.g., Cft-1 from *Cladosporium fulvum*,29) skippy from *Fusarium oxysporum*,27) MAGGY and MGR583 from *M. grisea*,21–22) REAL from *Alternaria alternata*,27) Ty1-H3 from *Saccharomyces cerevisiae*,28) Zorro from *Candida albicans*,29) Tad1 from *N. crassa*,26) and CgT1 from *C. gloeosporioides* (Fig. 3). The analysis supports the idea that all the putative rt genes segmentally cloned from *Tricholoma* spp. are part of either gypsy-type LTR or L1-like non-LTR retroelements, appropriately (Fig. 3).

**Phylogenetics of marY2N-like elements**

The phylogenetic relatedness is more pronounced in RTs of *marY2N*-like elements than those of *marY1*-like elements (Fig. 3). It is interesting to note that *marY2N*-like elements from *T. matsutake*, *T.
magnivelare, T. bakamatsutake, T. fulvacastaneum, and T. robustum, which are fungi closely related by producing fruiting bodies named “matsutake” or “matsutake-like mushrooms”, show phylogenetic relationships reflecting their host-plant specificity (Fig. 3). For example, T. fulvacastaneum and T. bakamatsutake are known to associate with Fagaceae plants, while T. matsutake, T. magnivelare and T. robustum with Pinaceae like T. japonicum. In contrast to these rather host-specific fungi, T. portentosum, T. sejunctum, T. flavovirens, T. saponaceum, and T. ustale are known to have symbiosis with both Pinaceae and Fagaceae. On the basis of the data, we hypothesize that marY2N-like retroelements, like L1 and other L1-like elements, might be an old component of the genome acquired by an ancestor of these fungi, which could have evolved along with the evolution of the host fungi through a vertical transmission.

If the evolution of marY2N-like elements reflects the fungus-plant interaction, the ancestor of the genus Tricholoma should have been associated with broad-leaved trees, from which symbionts of both broad-leaved trees and conifers, and those of coniferous plants could have evolved. This notion contradicts a general concept of the evolution of tree species currently accepted, in which broad-leaved trees are considered to have appeared after the occurrence of conifers. It looks somewhat strange that RTs of marY2N-like elements from T. portentosum, T. sejunctum, or T. flavovirens (Fig. 3). In fact, our unpublished rRNA gene analysis also showed that the nucleotide sequence of the spacer-5s rRNA region from T. robustum was identified in the phylogenetic cluster of those of T. ustale.
and *T. saponaceum* rather than the the cluster of *T. matsutake*, *T. magnivelure*, *T. bakamatsutake*, and *T. fulvocastaneum*. We may interpret the observation as being the consequence of *Tricholoma* that has diverged into *T. robustum*, *T. ustale*, and *T. saponaceum* by broadening its host specificity unlike other *Tricholoma*. Alternatively, we may hypothesize that *T. ustale* and *T. saponaceum* are the species composed of strains specific either to conifers or to broad-leaved trees, rather than the species without host specificity. If this is the case, our observation may be more plausible since the *T. ustale* and *T. saponaceum* strains used in this study were isolated from *Pinus densiflora* (Table 1). It may be interesting to assay *T. ustale* and *T. saponaceum* isolated from Fagaceae. Because of such complexities, it still remains open to be explored how a certain genus of ectomycorrhizal fungi, especially *Tricholoma*, which consists of over 90 symbiotic species, could have evolved.\(^{13}\) In this respect, further phylogenetic investigations based on the retroelement evolution along with phylogenetics based on the rRNA gene may help in the elucidation of the evolution of symbiotic fungi.

**Phylogenetics of *marY1*-like elements**

In contrast to *marY2N*-like elements which showed a tight phylogenetic relationship within the group suggesting a vertical process of genetic differentiation, *marY1*-like elements showed rather parallel relationships leading eventually to three major clusters, which suggests the involvement of a lateral transfer during the evolution of *marY1*-like elements (Fig. 3). The first group consists of RTs from *T. japonicum* FK-J1, *T. bakamatsutake* B1, *T. matsutake* Y1, and *T. magnivelare* Tp-C3, the second, *T. portentosum* 615, *T. robustum* KB1, *T. fulvocastaneum* WK-N1, *T. flavovirens* 613, *T. ustale* 611, and *T. sequentum* NA12, and the third, *T. saponaceum* 616 (Fig. 3). Multiple sequence alignment analysis showed that RTs of the former group shared sequences with 79.9% similarity and the latter with 79.5%. RT from *T. saponaceum* 616 apparently does not belong to these groups, though it had 75–77% similarity to the rest of RTs of *marY1*-like elements. RTs from *T. bakamatsutake*, *T. matsutake*, and *T. magnivelare* are in the same cluster, but those from two other “matsutake-like mushrooms” are in clusters with no clear relationship with currently known taxonomy of fungi nor with a host-plant specificity (Fig. 3). The fact that the sequences from *T. bakamatsutake*, *T. matsutake*, and *T. magnivelare* form a cluster is interesting in that these organisms are rare carbonizing mycorrhizal fungi symbiotic to arboreal plants unlike many other ectomycorrhizal fungi (Fig. 3).\(^{34,35}\) The characteristics regarding this trait of *T. japonicum* have to be clarified to figure out whether this feature is shared in this group of fungi (Fig. 3). Based on the data, we hypothesize that the evolution of *marY1*-like elements could have occurred in a rather later evolutionary stage of host fungi and might be more influenced by a lateral gene transfer as compared with *marY2N*, as is the case with other LTR retroelements.\(^{30}\)

**Concluding remarks**

We demonstrated that a *gypsy*-type LTR retroelement and an L1-like retroelement have evolved through different phylogenetic pathways in the genus *Tricholoma*. To our knowledge, this is the first to report this phenomenon in both basidiomycetes and ectomycorrhizal fungi. These data suggest that *marY2N*-like elements are components of the genome apparently older than *marY1*-like elements, and that the evolution of *marY2N*-like elements could have been greatly influenced by a vertical transmission, but that of *marY1*-like elements by a lateral transfer. This interpretation may be plausible in view of the fact that *gypsy*-type LTR retroelements are closely related to mammalian retroviruses, and also be consistent with a general hypothesis currently accepted that extensive lateral transfer has maintained the distinctive character of *gypsy*-type and *copia*-type LTR retroelements.\(^{30}\) On the contrary, L1-like elements are considered to be ancient components, which could have required host DNA repair systems for the integration process.\(^{7–10,30–32}\)

We previously reported that parts of the LTR of *marY1* have been distributed to a wide range of higher fungi.\(^{36}\) In fact, the 5′-LTR region is expressed in the budding yeast at the transcriptional level, which indicates that *marY1* promoters are recognized by *trans*-acting factors common to higher fungi.\(^{17}\) This notion is apparently consistent with the general view on the evolution of retroviruses in that the viruses could have evolved from *rt* flanked by IS-elements, *i.e.*, LTR, and could have been transferred horizontally among related eukaryotes.\(^{30}\)

Unlike LTR-retroelements, L1-like retroelements lack LTR, but carry a poly (A) tail at the 3′ end instead, and are considered to be old genetic components involved in early evolution of eukaryotic genomes.\(^{4,7–10,30–32}\) In the course of cloning of *marY2N* from *T. matsutake*, we observed that a copy of the element is located within an ORF predicted to encode a protein similar to CcZA, which is involved in a divergent cation efflux system in a soil bacterium.\(^{15}\) Coincidentally, ectomycorrhizal basidiomycetes are known to accumulate divalent cation metals.\(^{14}\) In the rice blast filamentous ascomycete *M. grisea*, several retroelements have been identified and used effectively as genetic markers as well as a gene-manipulating tool for the genetic analysis of this host-specific phytopathogen.\(^{5,11,18,21,22,39}\)

The analysis presented here, along with previous observations, strongly suggests that *marY1*-like retroelements may be useful as genetic markers in
analyzing the late evolution of ectomycorrhizal basidiomycetes and as a tool in developing the molecular identification system of mycorrhizae. Should they be demonstrated as functional, they may also provide us with new means to construct gene transfer systems in this fungal group. In contrast, \textit{maryYN}-like elements may be useful as genetic markers in the analysis of phylogenetics of ectomycorrhizal basidiomycetes with a scope different from the conventional rRNA gene-related analysis. The fact that the two-types of retroelements are shared in common in the genus \textit{Tricholoma} may encourage further search for their ubiquitousness in ectomycorrhizal basidiomycetes to explore new means to elucidate genome-related characteristics of this fugal group.

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