Analysis and utilization of proteins related to mycorrhizal symbiosis

（菌根共生に関与するタンパク質の分析および応用）

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General introduction

Mycorrhizal fungi are beneficial fungi that colonize plant roots, and help host plants to uptake water and inorganic nutrients, especially phosphate, and to provide the resistance against soilborne pathogens and nematodes, and the tolerance against environmental stresses. Mycorrhizal fungi are mainly classified as arbuscular mycorrhizal fungi (AMF), ectomycorrhizal fungi (ECMF), ericoid mycorrhizal fungi (ERMF) and orchid mycorrhizal fungi (OMF).

In particular, AMF can colonize nearly all of the terrestrial plants symbiotically and are effective in the production of safe, secure and sustainable foods. To develop useful methods for AMF applications in food production, it is necessary to understand the mechanism of AMF symbiosis, but the mechanisms is not adequately clear yet. For the reasons, it is very difficult to analyze genetically by genome sequencing, since the nuclei of AMF are multiform, and the diversity of the nuclei is still increasing by taking away nuclei from host plants. Therefore, the author has focused on proteomic approaches, because proteins are genome products as gene expression. Analysis of proteins related to AMF symbiosis, however, requires new techniques for mass-separation and mass-purification of the proteins.

Mycorrhizal colonization of plant roots is an effective indicator of soil productivity. Mycorrhizal observations, however, are cumbersome and require various techniques to discriminate between AMF and root tissues. Thus, the author was attempted to develop a new reagent to detect mycorrhizal fungi, by using the antigen-antibody reaction.

Using the proteomic techniques developed, the author investigated the discovery of new types of mycorrhizal fungi. Furthermore, genetically modified plants (GMP) are recently cultivated in over 25 countries, and their harmful effects on ecology and human health have been clarified, but few studies have examined their impacts on AMF has been obtained. Thus, qualitative and quantitative comparisons between proteins in both GM soybean and wild-type roots inoculated with AMF were analyzed, by using a new type of preparative chromatograph.

Chapter 1

Development of preparative chromatography for proteomic approach of mycorrhizal symbiosis

Abstract

Although mechanism of symbiosis between arbuscular mycorrhizal fungi (AMF) and host plants has been investigated by genetic analysis, very little knowledge has been obtained because genome analysis of AMF is not perfect yet. Thus, we tried to develop mass purification of proteins using preparative chromatography in order to progress proteomic analysis of proteins related to mycorrhizal symbiosis, such as 24 and 53 kDa proteins. In particular, our data showed that 53 kDa proteins would be restrictively expressed when mycorrhizal fungi and host plants were stressed. However, 24 kDa proteins, which appear to be an usable indicator for the existence of various mycorrhizal fungi, were habitually detected in not only AMF but also other mycorrhizal fungi such as ectomycorrhizal fungi (ECMF). Moreover, we discovered new preparative chromatographical techniques for isolation and mass purification of those proteins. We are convinced that these chromatographical techniques will greatly contribute to proteomic approach of mycorrhizal symbiosis.

Introduction

Although importance of AMF for sustainable food production is widely known, the mechanism of AMF symbiosis is not so clear. Since AMF have been known to be multinuclear and multiform (Tisserant et al. 2013) and to take away cell nuclei from plant hosts (Ishii et al. 2011), it is difficult to approach from genome sequence and often causes confusion in classification of species (Krüger et al. 2012). Therefore, we focused on proteomic approach for identification of proteins related to mycorrhizal symbiosis, which would indicate the expression of genes in AMF. Our previous reports show that AMF (Ishii et al. 1999) and orchid mycorrhizal fungi (OMF) (Matsubara et al. 2012) have proteins of 24 and 53 kDa in molecular weight that are related to mycorrhizal symbiosis. These facts indicate that various kinds of mycorrhizal fungi will have similar proteins for symbiotic relationship with plants. However, there is now no useful techniques for mass purification of the proteins, which contribute to proteomic approach of mycorrhizal symbiosis.

Therefore, we examined not only separation of proteins related to mycorrhizal symbiosis in AMF and ECMF using SDS-PAGE techniques, but development of new preparative chromatography for isolation and mass purification of the proteins on proteomic approach of the symbiosis.

Materials and Methods

1. SDS-PAGE of proteins

In this experiment, we used pot-cultured bahiagrass (*Paspalum notatum*) roots inoculated with and without *Glomus clarum*, AMF spores, such as *Gigaspora albida*, *Gigaspora margarita* and *G. clarum*, stocked in a refrigerator at 4ºC, and ECMF hyphae, such as *Tricholoma matsutake* (NBRC 6933) at 4, 25 and 40ºC and *Rhizopogon roseolus* at 25ºC in Ebios media.

Crude proteins were extracted from 5 g of bahiagrass roots inoculated with and without *G. clarum*, approximately 5000 of AMF spores, and approximately 0.1 g of ECMF hyphae by modified methods of Shannon et al. (1966). That is, these samples were homogenized with 0.05 M Tris buffer (pH 7.5) containing 0.05 M NaCl, and then the homogenates were filtered through a cheese cloth. After ammonium sulfate were added into the liquid to 35% saturation, they were stood overnight at 4ºC. They were centrifuged at 8000 g for 30 min and then added ammonium sulfate into the supernatant to 95% saturation. After standing overnight at 4ºC, they were centrifuged at 8000 g for 30 min and then the residues were dissolved in 2 ml of 0.005 M Tris buffer (pH 7.0). Dialyzation was done against 0.005 M Tris buffer (pH 8.0) containing 0.1 M KCl for overnight. After centrifugation at 8000 g for 30 min, we obtained the supernatant with crude proteins.

To detect 24 and 53 kDa proteins in the crude proteins, the SDS-PAGE was carried out by the methods of Laemmli (1970). Each of the crude proteins obtained from bahiagrass roots, *Gi. albida*, *Gi. margarita*, *G. clarum*, *T. matsutake* and *R. roseolus* were used, and the protein bands on the SDS-PAGE gel plates were analyzed by the iMeasure Scan software (iMeasure Inc.).

2. Isolation and mass purification of proteins by chromatography

As shown in Figure 1-1, we developed a new method for isolating and purifying 24 kDa protein related to mycorrhizal symbiosis from crude proteins of soybean (*Glycine max*) roots inoculated with *Glomus intraradices* and sod-cultured peach orchard soil. That is, crude proteins were extracted by modified procedures of Shannon et al. (1966), and then were

separated by medium pressure liquid chromatography (MPLC) with a Diol column to remove ammonium sulfate and to obtain the fractions including 24 and 53 kDa proteins. The solvent was 0.001 M Tris- HCl + 0.001 M NaCl (pH=7.5) and the flow rate was 3 ml/min. A detector was used at 280 nm. Moreover, we purified these proteins on gradient methods using a preparative high pressure liquid chromatograph (HPLC) with a Diol column.

3. Properties of 24 kDa protein

To analyze the properties of pure 24 kDa protein related to mycorrhizal symbiosis, the protein solution was spotted on thin-layer chromatographic plates, and then various stains, such as ninhydrin, Rhodamine 6G, fluorescein, Dittmer reagent, $FeCl_2$ and hydroxylamine chloride, were sprayed on protein spots. Then, color changes of the spots were observed.

Results

Although non-mycorrhizal bahiagrass roots have no 24 and 53 kDa proteins (Figure 1-2), 24 kDa proteins were detected in all of crude proteins extracted from mycorrhizal samples (Figures 1-2, 1-3 and 1-4), but 53 kDa proteins were not detected in crude proteins of *T. matsutake* cultured at 25ºC (Figure 1-3). The 53 kDa proteins were appeared when *T. matsutake* was cultured under low or high temperature stressed conditions such as 4ºC and 40ºC (Figure 1-3). The 53 kDa proteins were also detected in pot-cultured bahiagrass roots inoculated with AMF, all AMF spores stored at 4ºC and *R. roseolus* cultured at 25ºC (Figures 1-2 and 1-4).

On mass purification of proteins, both of preparative chromatographs, MPLC and HPLC, with a Diol column were very useful for isolating and purifying great amounts of crude proteins extracted by ammonium sulfate precipitation. Moreover, a gradient method on preparative HPLC using a Diol column separated each protein in 24 kDa protein fraction. As shown in Figure 1-5-A, the chromatogram of soil proteins fractionated by an isochromatic method on a preparative HPLC with a Diol column shows existence of 24 kDa proteins as well as mycorrhizal fungi and plant roots, but it was impossible to isolate pure 24 kDa protein (Peak 5) related to mycorrhizal symbiosis from the 24 kDa protein fraction. The development of a gradient method on preparative HPLC, however, solved the isolation of the same or almost the same as molecular weight of proteins. Thus, it became possible to isolate the pure 24 kDa protein (Figures 1-5-B and 1-6).

The properties of the 24 kDa protein purified were analyzed using some kinds of stains (Table 1-1). That is, the detection by Rhodamine 6G showed that the 24 kDa protein is

bound with lipids. Other stains, such as fluorescein, Dittmer reagent, FeCl2 and hydroxylamine chloride, showed that the lipids were simple ones such as ester lipids, sterol lipids and non-phospholipids. The pink spot made by ninhydrin indicated the existence of peptides. Therefore, the pure 24 kDa protein was lipoproteins with ester and sterol lipids (Table 1-1).

Discussion

The results of SDS-PAGE indicated that 24 kDa proteins were habitually detected in not only AMF (Ishii et al., 1999), OMF (Matsubara et al., 2012) and ericoid mycorrhizal fungi (unpublished), but AMF and ECMF used. Therefore, the proteins would be related to symbiosis between plants and mycorrhizal fungi. Furthermore, pure 24 kDa protein, which isolated from crude 24 kDa proteins by preparative chromatography, was revealed to be lipoproteins. It suggests that the protein is similar to non-specific lipid transporter proteins with anti-fungal activity reported by Bazghaleh et al. (2012). The 24 kDa protein can be related to formation or maintenance of membrane structure of mycorrhizal fungi at inside or around plant roots and protects their hosts from pathogenic fungi.

On the other hand, 53 kDa proteins seem to be expressed only when mycorrhizal fungi and host plants were stressed, because the proteins in *T. matsutake* had appeared at 4°C and 40°C. Further, the expression of the proteins in pot-cultured bahiagrass roots may result from water stress in summer. These results suggest that the proteins will be glomalin-like proteins which relieve cells from stresses. Glomalin has been already known as glycoprotein weighs approximately 60 kDa (Wright and Upadhyaya, 1996), which has similarity to heat shock protein 60 (Gadkar and Rillig, 2006).

New creative technique of chromatography developed is very useful for isolation and mass purification of pure 24 kDa protein related to mycorrhizal symbiosis. The protein would contribute to the development of reagents which can detect mycorrhizal fungi by the immunologic analysis. Therefore, we are much in need of sufficient amounts of pure objective protein for building up antibodies.

The Diol column has the ability of gel filtration. Thus, our results indicate that MPLC with the Diol column can easily remove ammonium sulfate from large amounts of proteins without conventional dialysis. Moreover, the Diol column in preparative HPLC is effective in isolating single protein from proteins in similar molecular weight under pH gradient conditions. Except for gel filtration of the Diol column, the column would show positive electric charge of silica residue, since this has small amounts of silica without a Diol group. That is, the pH gradient method in range of pH 7.5-6.5 causes that silica without the

Diol group becomes positively charged, so that retention time of each protein is slightly changed. Although the idea of preparative HPLC in gradient methods for proteins had been already reported by Unger et al. (1987), the methods have been not utilized. One of the possible reason would be that the pH range used in their experiments was pH 2.0-5.0, so that their methods were usable only for proteins stable at low pH. The fact that we had the pure protein at pH near 7.0 was an advantage of our methods. Further experiments are needed to develop the utility of the Diol column for protein analysis,

Couto et al. (2013) have reported that it is difficult to purify proteins from AMF and plants. One of their insistent reasons was difficulty of removing non-protein compounds in plants or fungal tissues. In this investigation, however, these compounds were easily removed from crude proteins by MPLC with the Diol column, and large amounts of the objective proteins were isolated and purified from the crude proteins by gradient preparative HPLC with the Diol column. This new chromatographical techniques will greatly contribute to proteomic approach of mycorrhizal symbiosis. In particular, this preparative chromatography would create a new path to analyze proteomically mycorrhizal symbiosis in vitro using the axenic culture of AMF (Ishii and Horii, 2007; Ishii, 2014).

Roots 40 g

↓

Homogenized with 80 ml of 0.001 M Tris-HCl buffer (pH 7.5) containing 0.001 M NaCl

↓

Soils 100 g

↓

Shook with 200 ml of 0.1 M phosphate buffer (pH 7) containing 0.01 M EDTA and 0.3 M KCl

↓

Extracted crude proteins by modified methods of Shannon *et al.* (1966)

Filtered the homogenates through a cheese cloth.

Added ammonium sulfate into the liquid to 35% saturation.

Stood overnight at 4ºC and then centrifuged at 8000 g for 30 min.

Added ammonium sulfate into the supernatant to 95% saturation.

Stood overnight at 4ºC and then centrifuged at 8000 g for 30 min.

Residues were dissolved in 2 ml of 0.005 M Tris buffer (pH 7.0).

↓

Removed ammonium sulfate and fractionated crude proteins using a MPLC

Column: Diol (75-200 μm) (Fuji Silysia Chemical Ltd.), φ35×400 mm

Solvent: 0.001 M Tris-HCl + 0.001 M NaCl (pH=7.5)

↓

Isolated 24 kDa proteins using a preparative HPLC with a Diol column by gradient methods

Column: Develosil 300 Diol (Nomura Chemical Co., Ltd.), φ10×250 mm

Solvent: 0-10 min 0.001 M Tris-HCl + 0.001 M NaCl (pH=7.5)

10-50 min 0.001 M Tris-HCl + 0.001 M NaCl (pH=6.5)

50-60 min 0.001 M Tris-HCl + 0.001 M NaCl (pH=7.5)

Flow rate: 0.5 ml/min, Temperature: 20°C, UV: 280 nm

Figure 1-1. Procedures for the extraction and purification of proteins in roots and soils.

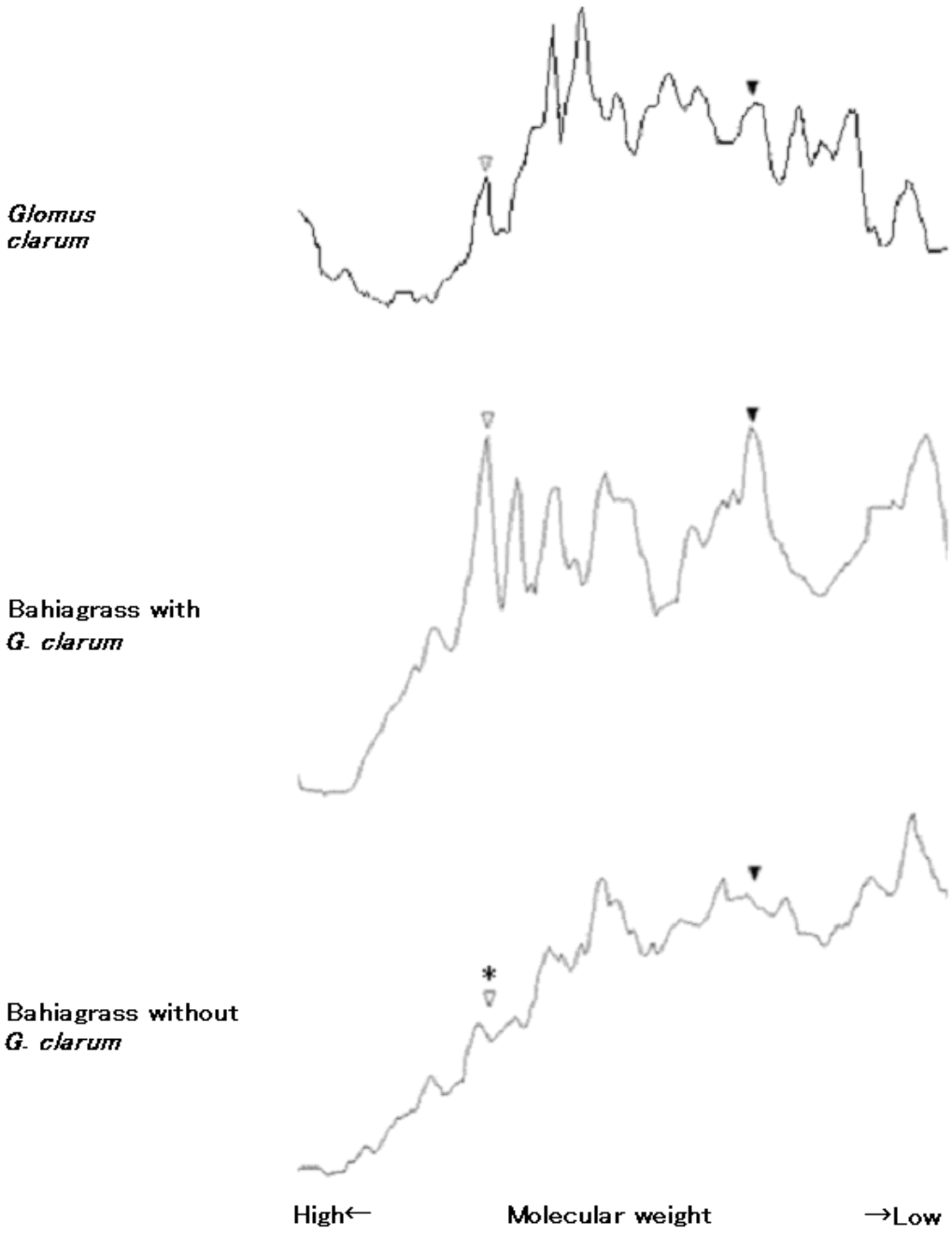

Figure 1-2. Densitograms of crude proteins extracted from *Glomus clarum* spores and inoculated bahiagrass roots.

Bahiagrass roots were harvested on September 6th.

White and black arrows show 53 and 24 kDa proteins, respectively.

An asterisk shows no significant peak for 53 kDa proteins in bahiagrass without AMF.

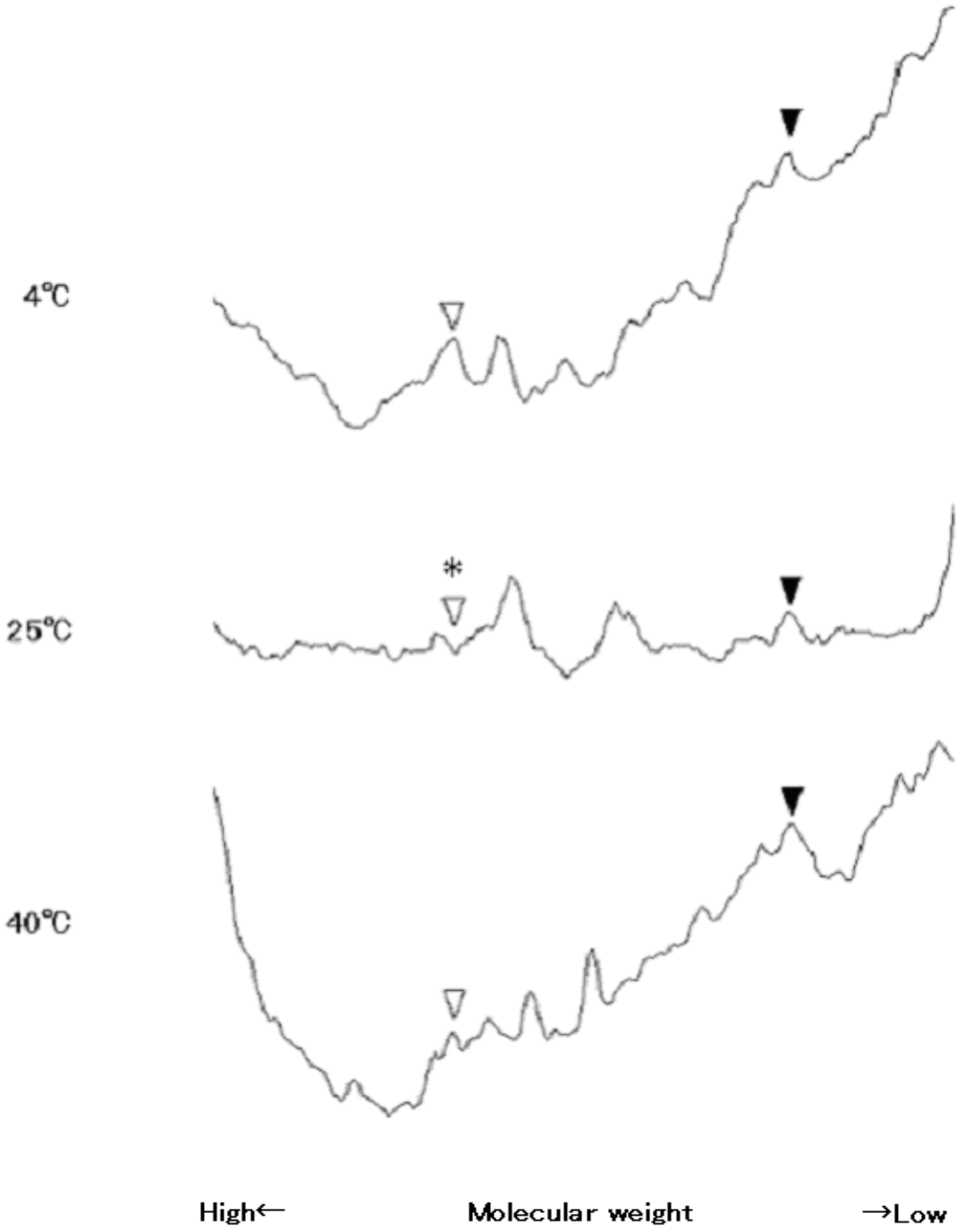

Figure 1-3. Densitograms of crude proteins *T. matsutake* at 4, 25 and 40°C for 3 days.

White and black arrows show 53 and 24 kDa proteins, respectively.

An asterisk shows no significant peak for 53 kDa proteins at 25°C .

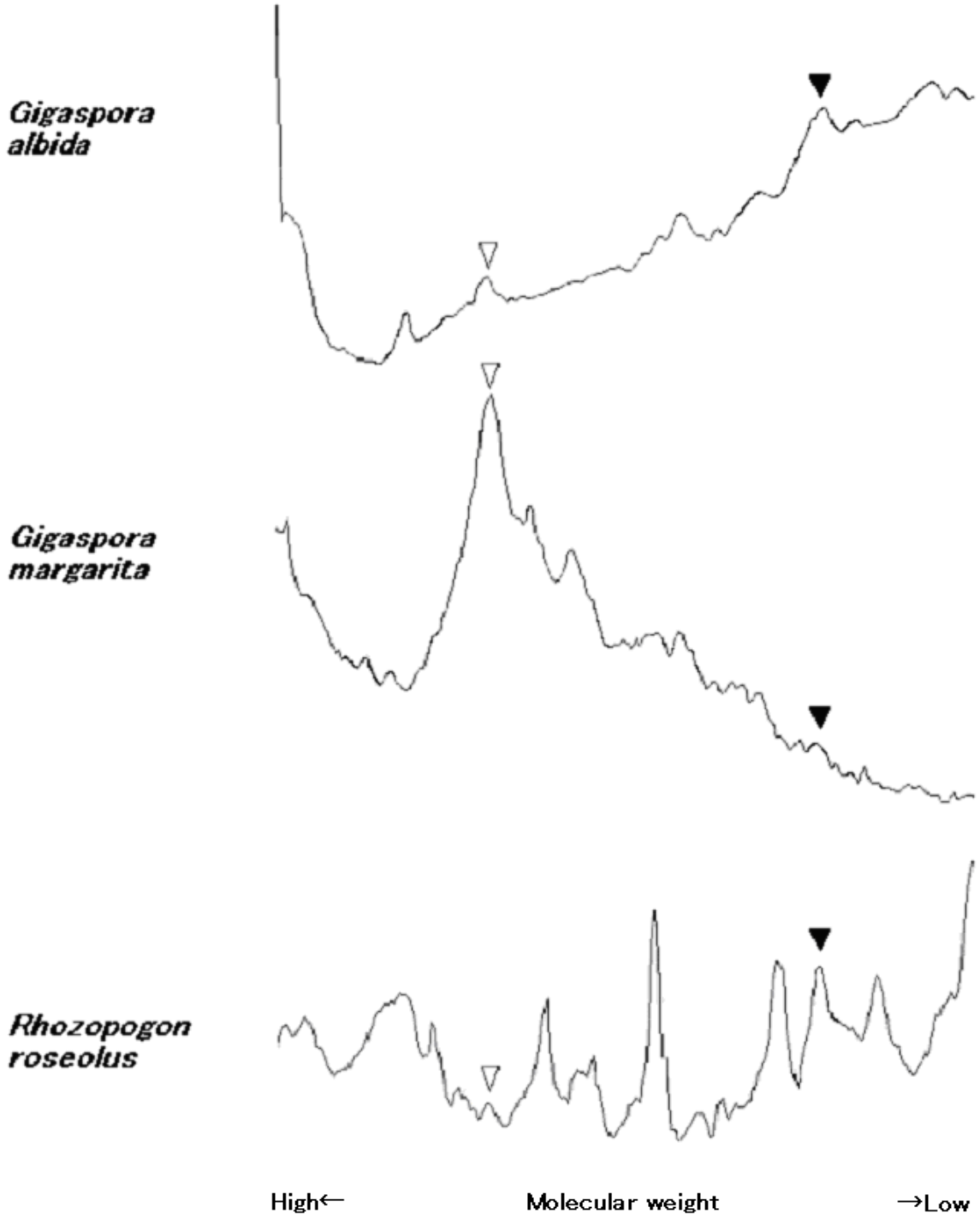

Figure 1-4. Densitograms of crude proteins extracted from other AMF and ectomycorrhizal fungi.

White and black arrows show 53 and 24 kDa proteins, respectively.

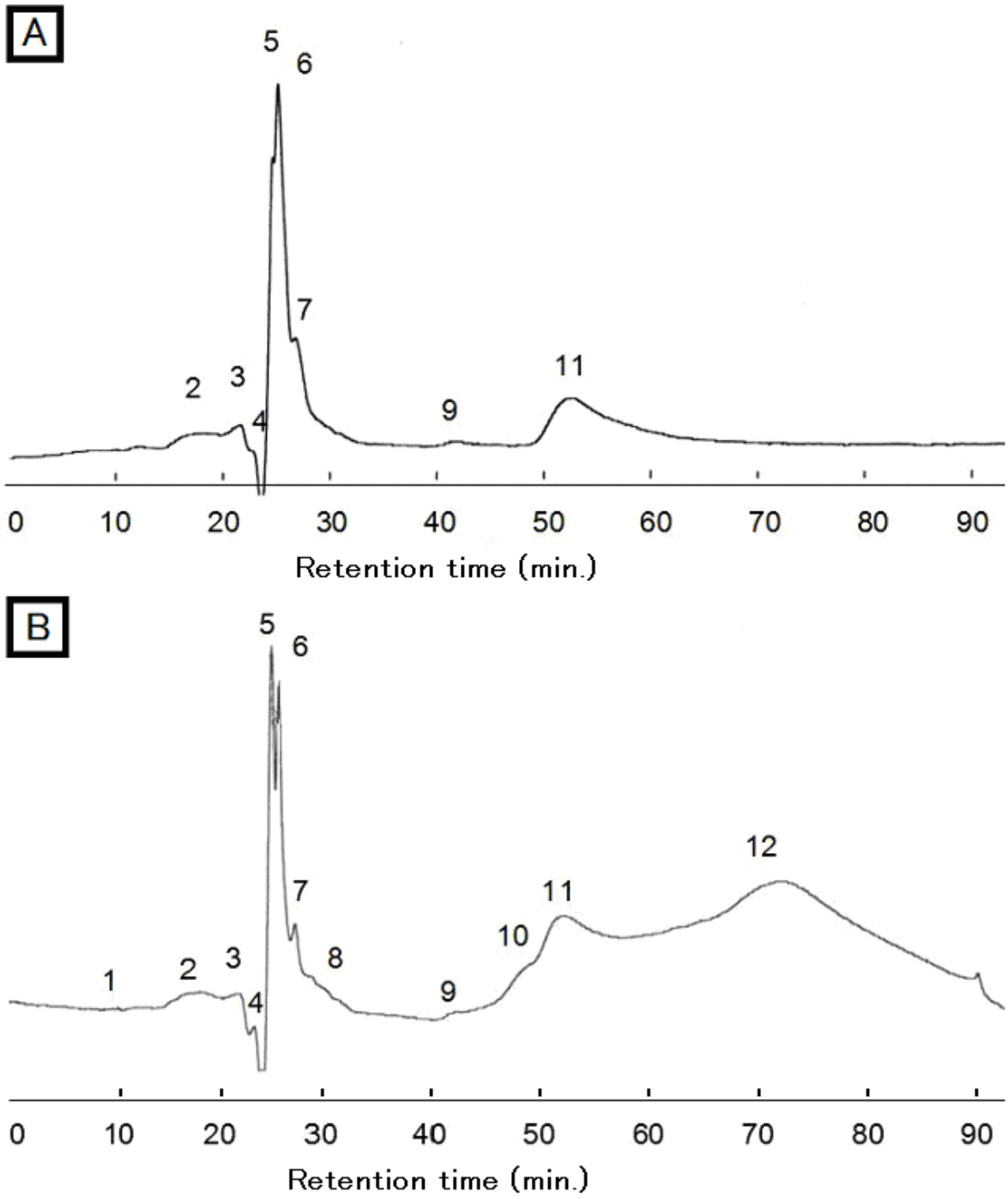

Figure 1-5. Chromatogram of soil proteins fractionated by HPLC.

A: Isochromatic methods

Column: Develosil 300 Diol, φ10×250 mm,

Solvent: 0.001 M Tris-HCl + 0.001 M NaCl (pH=7.5),

Flow rate: 0.5 ml/min, Temperature: 20°C, UV: 280 nm

B: Gradient methods

See in Figure 1.

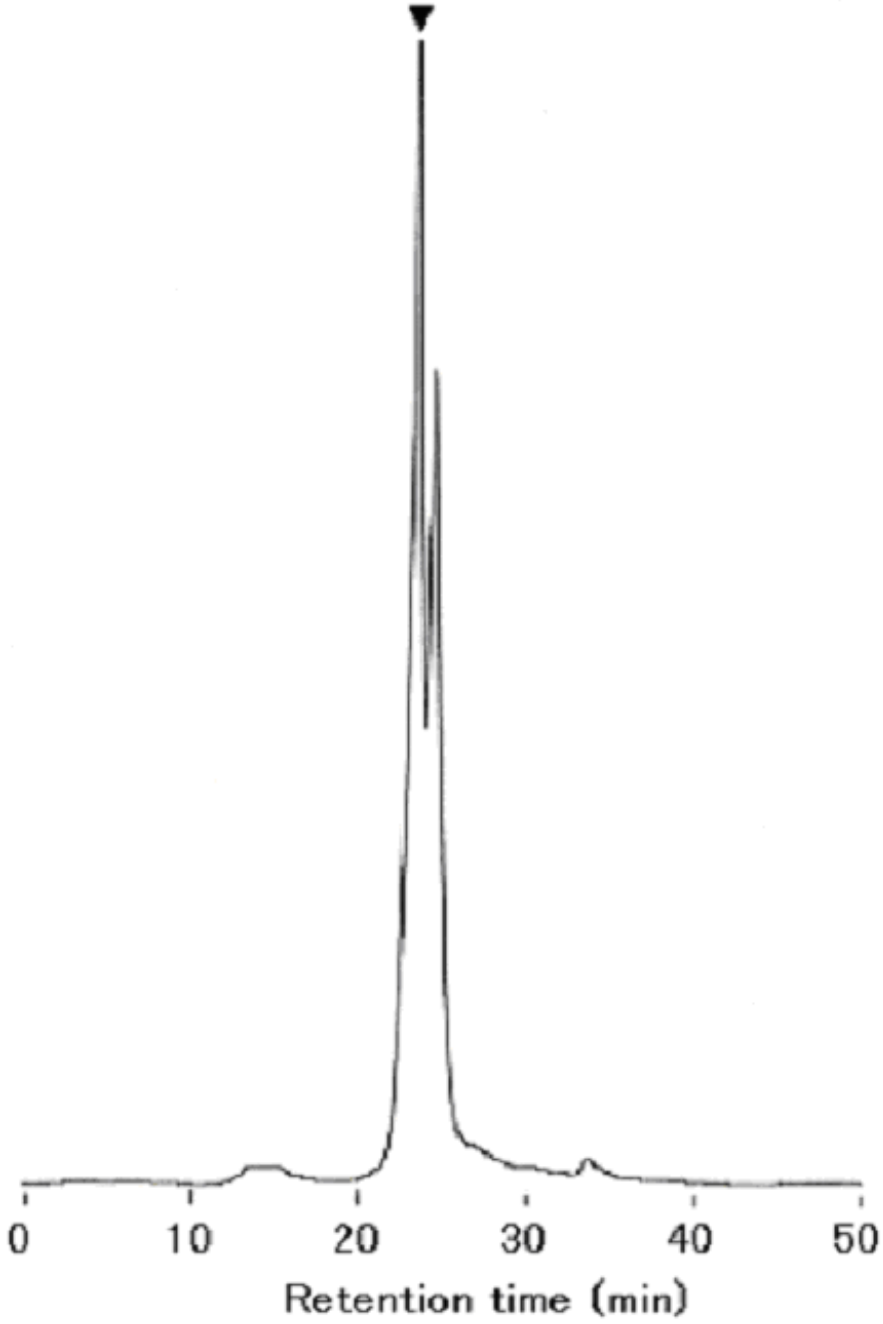

Figure 1-6. A chromatogram of 24 kDa proteins of *Glomus intraradices* inoculated soybean roots fractionated by gradient HPLC.

Gradient methods: See in Figure 1-1.

The black arrow shows 24 kDa proteins in the electrophoresis with SDS plates.

Table 1-1. The properties of a 24 kDa protein using some kinds of stains.

Stains	Spot color	Decision
ninhydrin	slight pink	amino groups
Rhodamine 6G	orange	lipids
fluorescein	yellow	simple lipids
Dittmer reagent	-	non phospholipids
FeCl2	purple	sterol lipids
hydroxylamine chloride	slight purple	ester lipids

Chapter 2

A new reagent to detect mycorrhizal fungi

Abstract

The existence of arbuscular mycorrhizal fungi (AMF) in plant roots indicates the productivity of a field. The procedure to measure AMF root colonization rates, however, is cumbersome, and various light microscopical techniques are required to clearly distinguish between AMF and root tissues. In this study, we developed a reagent to detect a specific 24 kDa protein related to mycorrhizal symbiosis via antigen-antibody reaction. We built up antibodies against the protein and added fluorescein. Using the reagent, it was easy to observe not only AMF, but also ericoid mycorrhizal fungi (ERMF), ectomycorrhizal fungi (ECMF) and orchid mycorrhizal fungi (OMF) on fluorescence microscopy. We also developed a technique to measure the mycorrhizal root colonization rate by fluorometric analysis of plant root extracts containing the 24 kDa protein. The new reagent facilitates observation of root colonization rates, and will greatly contribute to the establishment of sustainable agriculture by using beneficial microorganisms such as mycorrhizal fungi.

Introduction

AMF form symbiotic relationships with plant roots, and are therefore important for sustainable food production. Thus, analysis of AMF root colonization is often used as an indicator of soil productivity. The measurement of the root colonization rates, however, is cumbersome. Conventional methods introduced by Phillips and Hayman (1970) require preparations before using light microscopical observation. Additionally, it is difficult to discriminate between AMF and root tissues. For these reasons, a simple and facile method to observe mycorrhizal root colonization is needed.

In this study, we developed a new reagent to detect mycorrhizal fungi via the antigen-antibody reaction. Our previous reports indicate that a 24 kDa protein is closely related to various mycorrhizal symbioses, including AMF (Ishii et al., 1999), OMF (Matsubara et al., 2012), ECMF (Matsubara and Ishii, 2014) and ERMF (unpublished); therefore we used the protein to develop the new reagent. We also examined root colonization rates by fluorometric analysis with the reagent.

Materials and methods

1. Building up antibodies to the 24 kDa protein and the addition of fluorescein

Using a specific 24 kDa protein related to mycorrhizal symbiosis isolated from crude proteins of mycorrhizal roots by the methods such as SDS-PAGE techniques (Ishii et al., 1999; Matsubara and Ishii, 2014) and preparative chromatography with a Diol column (Matsubara et al., 2011), we accumulated antibodies to the protein using rabbits, and purified them using the Protein G HP SpinTrap (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's directions. After purification of the antibodies, fluorescein was added using the Fluorescein Labeling Kit-NH2 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's directions. Then, the antibody solution was diluted 50-fold with 0.02 M phosphate buffer (pH 7) and used as a detection reagent in the following experiments.

2. Observation of mycorrhizal fungi by fluorescence microscopy

Rootlets of various plants such as bahiagrass (*Paspalum notatum*), soybean (*Glycine max*), *Rhododendron* × *pulchrum,* Japanese red pine (*Pinus densiflora*) and *Dendrobium* sp. were cut in 1-2 cm from the apex. Then, 10 μl of the detection reagent was dropped onto each root and observed using a fluorescence microscope (CyScope plus CY-S-4005, Partec GmbH, Gorlitz, Germany) at wave length of 472 nm. In addition, pathogenic and saprobic fungi such as *Rhizoctonia solani* (KB and MAFF 305238), *Fusarium oxysporum* (EI and f.sp. *fragariae*), *Rosellinia necatrix* (KI-D-10-4 and KI-D-38-17), *Pythium rostratum* UOP383, *Pythium ultimum* CPU407, *Aspergillus niger* and *Penicillium expansum* were also observed.

3. Measurement of mycorrhizal root colonization rates by fluorometric analysis

Mycorrhizal root colonization rates of plants such as apple mint (*Mentha suaveolens*), bahiagrass, flatsedge (*Cyperus microiria*), paprika (*Capsicum annuum* 'grossum'), strawberry (*Fragaria* × *ananassa*), lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), blueberry (*Vaccinium corymbosum*), raspberry (*Rubus idaeus*), *Rhododendron indicum*, *Rhododendron* × *pulchrum*, apple (*Malus pumila* 'Fuji' with JM7 rootstock), *Citrus natsudaidai*, grape (*Vitis* × *labruscana* 'Pione' with hybrid Franc rootstock), Japanese apricot (*Prunus mume* 'Nanko' with free stock), peach (*Prunus persica* 'Akatsuki' with with free stock), plum (*Prunus salicina* 'Akihime' with with free stock), trifoliate orange (*Poncirus trifoliata*), Japanese black pine (*Pinus thunbergii*) and Japanese red pine were stained following the methods of Phillips

and Hayman (1970), and then mycorrhizal root colonization rates were measured according to the methods of Ishii et al. (1996).

Rootlets (0.5 g) of the plants described above were homogenized using 1.5 ml of 0.02 M phosphate buffer (pH 7). Then, these homogenates were centrifuged at 8000 x g for 15 min, and the supernatants were purified using the syringe column (φ16 x 30 mm) with a Diol filler (Fuji Silysia Chemical Ltd., Aichi, Japan) to obtain the pure 24 kDa protein and remove non-protein compounds such as pigments, lipids and phenolic compounds using 0.02 M phosphate buffer (pH 7). Each sample was divided into 2 equal volumes; one received 20 µl of detection reagents; the other received 20 µl of 0.02 M phosphate buffer (pH 7) and was used as the control. The fluorescence density at 494 nm for each pair of samples was measured using a fluorescence spectrophotometer (F-2500 FL Solutions 2.0, Hitachi Ltd., Tokyo, Japan). The correlation between fluorescence density (i.e. the value of samples with the detection reagents reduced by the control value) and the root colonization rates using the methods of Phillips and Hayman (1970) and Ishii et al. (1996) was determined by statistical analyses.

Results

1. Observation of mycorrhizal fungi, and pathogenic and saprobic fungi by fluorescence microscopy

Using the newly developed reagent, AMF in bahiagrass and soybean roots were clearly observed by fluorescence microscopy (Figure 2-1-A and 2-1-B). Similarly, ERMF and AMF in *Rhododendron* × *pulchrum* roots (Figure 2-1-C), ECMF in Japanese red pine roots (Figure 2- 1-D) and OMF in *Dendrobium* sp. roots (Figure 2-1-E) were detected. On the other hand, pathogenic and saprobic fungi showed no yellow-green fluorescence except for *Rhizoctonia solani* (Table 2- 1 and Figure 2- 2).

2. Measurement of mycorrhizal root colonization rates by fluorometric analysis

Protein samples extracted from various plant roots were purified by simple Diol column chromatography. When the purified protein samples were used, a significant correlation between the root colonization rate and the value of fluorescence density was found (regression, $y = 0.1902 x$; R^2=0.3516) (Figure 2-3).

Discussion

The new reagent using the 24 kDa protein-antibody was successfully used to detect various kinds of mycorrhizal fungi by fluorescence microscopy. This method was simple; the only operation required before observation is the addition of 10 µl of the detection reagent. Compared with the methods of Wright and Upadhyaya (1996), which are used to detect glomalin by antigen-antibody reaction, our method has an advantage. Glomalin is known to have similarity with heat shock protein 60 (Gadkar and Rillig, 2006), which seems to be expressed only when mycorrhizal fungi and host plants are stressed (Matsubara and Ishii, 2014). Moreover, since the 24 kDa protein is habitually detected in mycorrhizal fungi (Matsubara and Ishii, 2014), it is a useful indicator.

Before fluorometric analysis, we used the simple Diol column to remove non-protein compounds from each crude protein sample. This technique is very effective to purify the 24 kDa protein from crude root protein samples.

Various kinds of mycorrhizal fungi were observed by fluorescence microscopy with our reagent, but pathogenic *Rhizoctonia solani* also showed fluorescence. This fact indicates that 24 kDa protein may be essential for the hyphae of pathogenic *Rhizoctonia* to invade root tissues as well as mycorrhizal *Rhizoctonia.* Since *R, solani* is a known pathogen of Solanaceae, such as eggplant (*Solanum melongena*), paprika and tomato, the healthiness of their rootlets should be checked when the present reagent developed is utilized for the observation of mycorrhizal fungi in the genera of Solanaceae. Now, we are continuing to assess the difference in proteins between OMF and pathogenic *Rhizoctonia* in further studies to develop the reagents with different fluorescence colors to detect pathogenic *Rhizoctonia*. Furthermore, differences in the genome between mycorrhizal and pathogenic *Rhizoctonia* present new opportunities to indirectly analyze the mechanism of mycorrhizal symbiosis.

Further investigations are needed to develop simple and reasonable instruments to detect mycorrhizal fungi using the new reagent for wide implementation on farms.

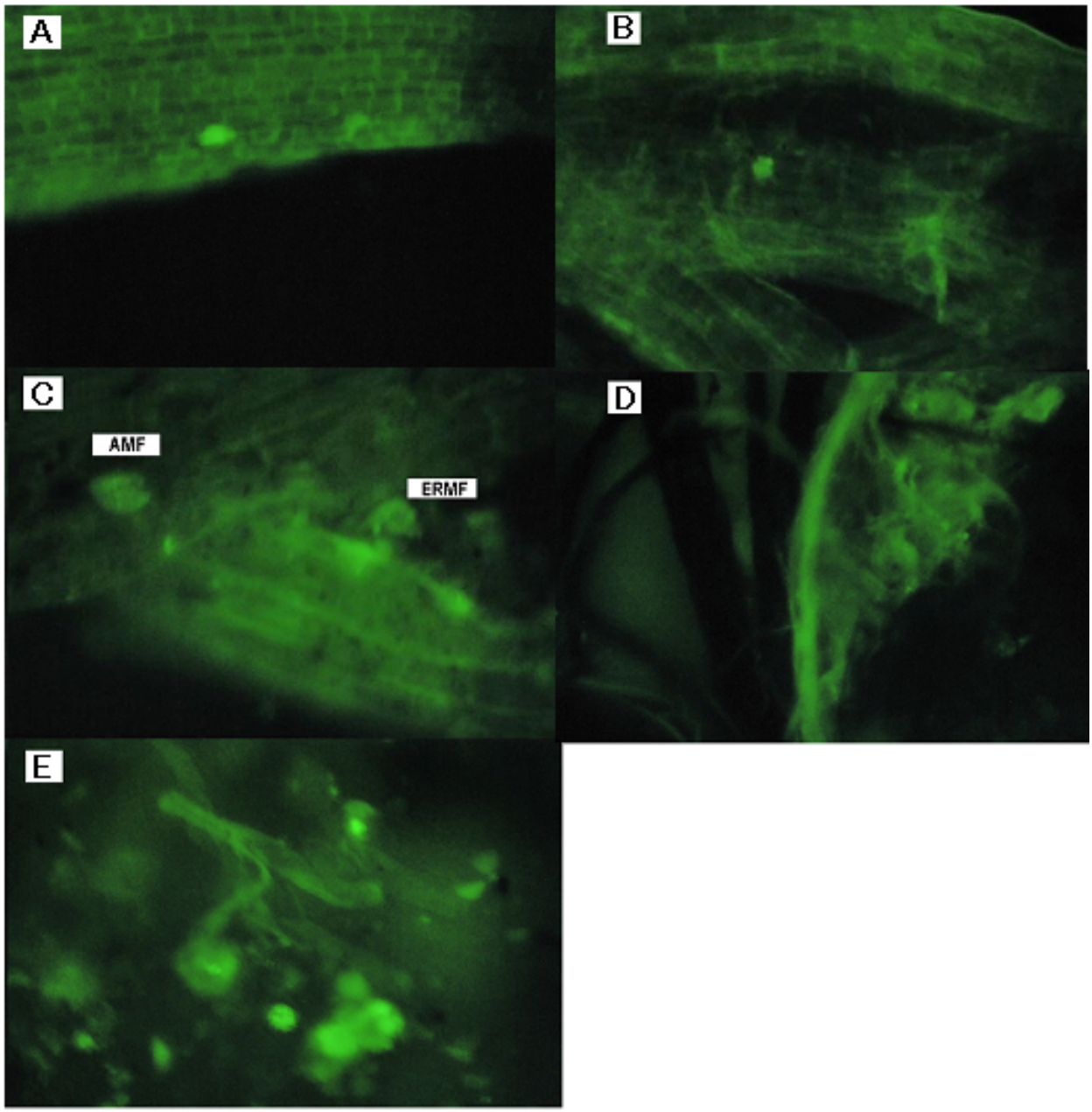

Figure 2-1. Various mycorrhizal fungi in plant roots observed by fluorescence microscopy.

A: AMF in bahiagrass (x200), B: AMF in soybean roots (x200),

C: AMF and ERMF in *Rhododendron* x *pulchrum* roots (x400),

D: ECMF in Japanese red pine roots (x400),

E: OMF in *Dendrobium* sp. roots (x400).

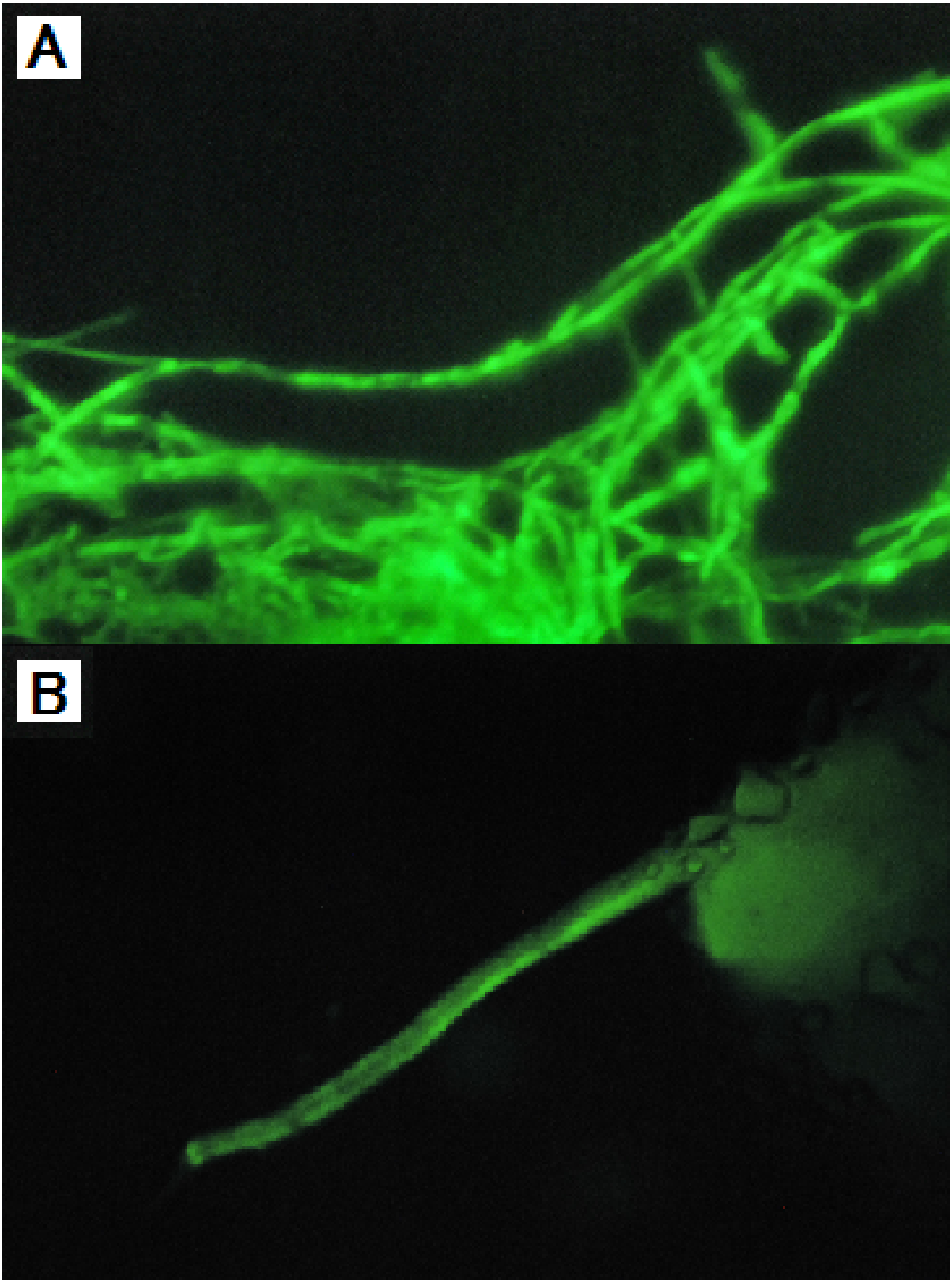

Figure 2-2. Pathogenic *Rhizoctonia solani* KB (A) and MAFF 305238 (B) observed by fluorescence microscopy (x400).

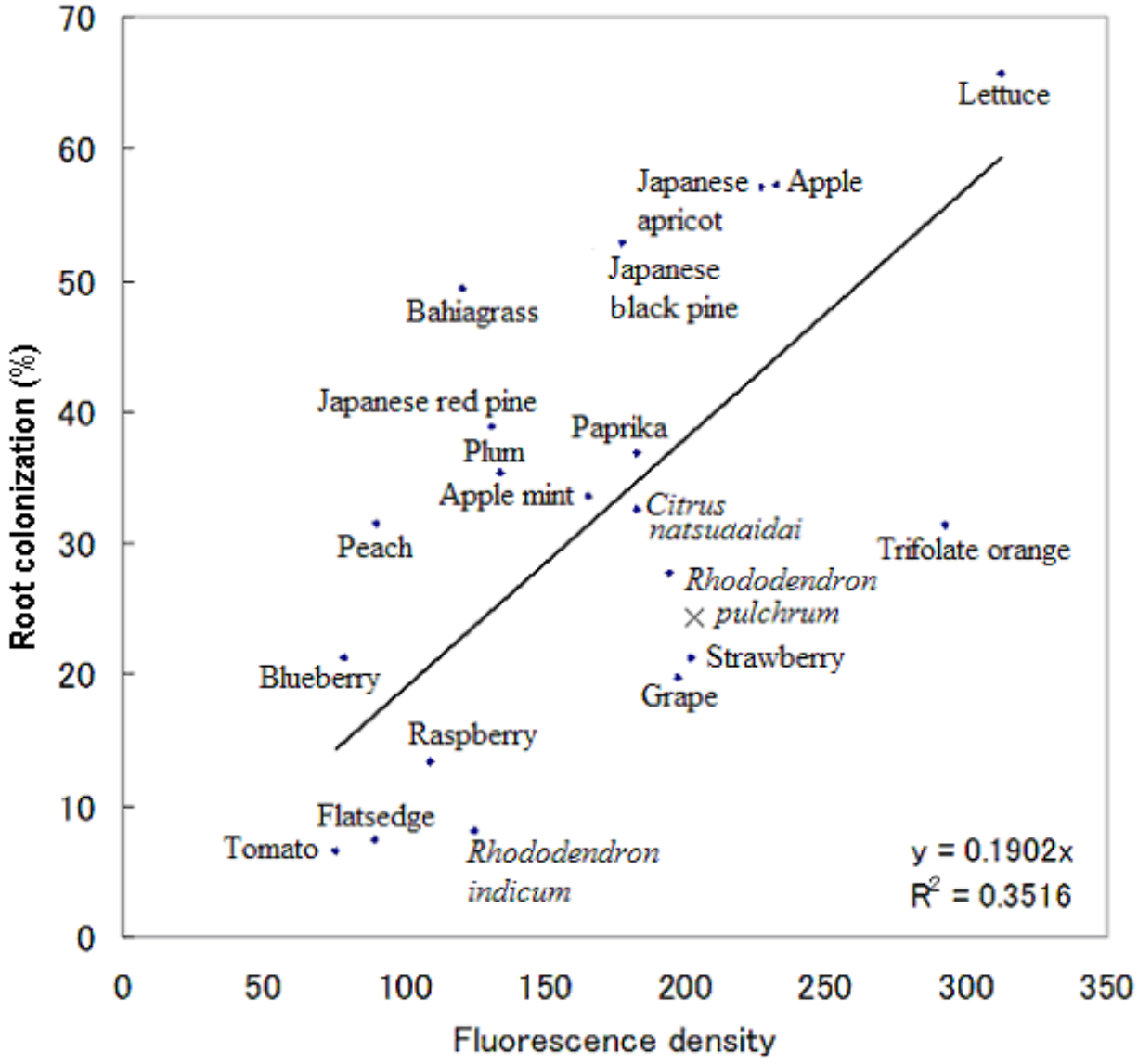

Figure 2-3. Correlation between mycorrhizal infection rate and fluorescence density.

Table 2-1. Pathogenic and saprobic fungi observed by fluorescence microscopy.

Fungus	**FM**	**Fungus**	**FM**
Rhizoctonia solani KB	+	*Rosellinia necatrix* KI-D-10-4	-
Rhizoctonia solani MAFF 305238	+	*Rosellinia necatrix* KI-D-38-17	-
Fusarium oxysporum EI	-	*Pythium rostratum* UOP383	-
Fusarium oxysporum f.sp. *fragariae*	-	*Pythium ultimum* CPU407	-
Aspergillus niger	-	*Penicillium expansum*	-

FM: fluorescence microscopy at 472 nm, +: detection, -: no detection.

The newly developed reagent was used.

Chapter 3

Fungal isolate 'KMI' is a new type of orchid mycorrhizal fungus

Abstract

Fungi which help orchid seeds to germinate, and colonize the root tissues near from the surface of adult orchid plant roots are known as orchid mycorrhizal fungi. We analyzed an isolate of fungus that seems to be a new type orchid mycorrhizal fungus 'KMI' obtained from the roots of *Paphiopedilum thailandense*. KMI has hyphal branching into right angle similar to *Rhizoctonia*-like fungi, but its spore formation was completely different from the fungi. Its ribosomal DNA sequences of 18S and ITS have no similarity with any known fungal species. Proteins in molecular weight of 53 and 24 kDa, which are common to mycorrhizal fungi, were detected in KMI. When KMI was inoculated onto orchids, the formation of mycorrhizae was observed. No significant symptoms of *Rhizoctonia* or *Fusarium* disease were developed on tomato and cucumber plants. From the evidence obtained, KMI might be a new type of orchid mycorrhizal fungus.

Introduction

It is known that orchid mycorrhizal fungi help orchid seeds to germinate, and they generally colonize the root tissues near from the surface of adult orchid plant roots. In 1904, N. Bernard observed *Rhizoctonia repens* (teleomorph: *Tulasnella deliquescens*) in roots of *Goodyera repens*. *Rhizoctonia*-like orchid mycorrhizal fungi can be classified into *Thanatephorus* (=*Corticium*), *Ceratobasidium*, *Ypsilonidium*, *Sebacina*, *Tulasnella* and other groups based on the morphology of their of teleomorph (Currah and Zelmer, 1992). They were distinguished from other fungi by their of hyphae branching at right angle and the septa forming near the branching point.

Fungal species, which can make orchid mycorrhiza, are not only *Rhizoctonia*-like species. Kusano (1911) discovered that *Armillaria mellea* can have symbiotic relation with *Gastrodia elata*. It turned out that not only *G. elata* but *Galeola septentrionalis* (Hamada, 1940) and *Gastrodia cunninghamii* (Campbell, 1962) are *A. mellea*'s host. Not having chlorophyll, these 3 species of orchids are all depending on the fungi for nutrients.

In this study, we analyzed an isolate of fungus that seems to be a new type orchid mycorrhizal fungus obtained from *Paphiopedilum thailandense* roots.

Materials and Methods

1. Isolation of KMI

Roots of *Paphiopedilum thailandense* (from Kyoto Prefectural Botanical Garden, 3 cm from the apex) were surface-sterilized for 12 minutes in solution of 700 ppm chloramines-T, 5.6 ppm streptomycin and 2.1 ppm chloramphenicol. Then, they were homogenated by a glass mortar and pestle in a clean bench and placed on potato dextrose agar media (PDA; Eiken Chemical Co., LTD.), and incubated at 25 ºC for 3 days.

2. Microscopic examination of KMI

In a clean bench, a small volume of 20% potato dextrose media was dropped on glass slide with two holes. The slide was placed in a 9 cm plastic Petri dish, then hyphae of KMI were added. The slide was covered with a cover glass before observing fungal growth under the optical microscopy (x 400) after incubation at 25 ºC for 3 days.

3. DNA sequencing

KMI was inoculated on 20 ml of 20% potato dextrose media and incubated in 25 ºC for 3 days.

A small amount of mycelium was crushed in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM EDTA, 0.1% n-lauroylsarcosine sodium salt, 500 μg/ml actinase E) under liquid nitrogen, incubated at 65ºC for 20 min, then centrifuged at 3,000 g for 10 min. The supernatant was mixed gently with phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v), and centrifuged at 3000 g for 10 min. The supernatant was collected and DNA was precipitated with an equal volume of ethanol, and then centrifuged at 10,000 g for 1 min. The supernatant was removed and the precipitate (DNA) was washed with 70% ethanol, and suspended in sterile distilled water. This solution was used as the template for PCR.

The oligonucleotide primers for 18S rDNA partial sequence specific PCR, NS1: 5'-GTAGTCATATGCTTGTCTC-3', NS4: 5'-CTTCCGTCAATTCCTTTAAG-3' and ITS rDNA specific PCR, ITS4: TCCTCCGCTTATTGATATGC-3', ITS5:5'-GGAAGTAAAAGTCGTAACAAGG-3' (White et al., 1990), were made by Proligo (Japan). The reaction mixture consisted of 1 μl of template solution, 2.5 μl of each primer (2 pmol), KOD Dash (2.5 U/μl, TOYOBO) as taq polymerase, 5 μl of dNTPs (2mM dATP, dGTP, dCTP, dTTP each, TOYOBO) and 5 μl of 10 × reaction buffer (TOYOBO). Sterilized distilled water

was added to increase the volume to 50 µl.

The PCR cycling conditions were one cycle of 94ºC for 3 min, 30 cycles of 94ºC for 30 sec, 50ºC for 2 sec, and 74ºC and 30 sec, and final extension step at 20ºC for 3 min.

The PCR products were purified by GENECLEAN Kit (Q-BIOgene) according to the manufacture's instruction. Purified PCR products were used in sequencing reactions with the same primers using a BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed on Genetic Analyzer 310 (Applied Biosystems).

Sequences were searched on the nucleotide sequence database using BLAST program (http://www.ebi.ac.uk/blastall/). Then, phylogenic analysis was conducted by using ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html) and TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

4. Extraction and purification of proteins

It is known that arbuscular mycorrhizal fungi have proteins that their molecular weights are 53 and 24 kDa, respectively (Ishii et al., 1999). To check whether identified orchid mycorrhizal fungi and KMI have those proteins, a modified procedure of Shannon et al. (1966) was used to extract and partially purify proteins in KMI, arbuscular mycorrhizal fungi (AMF) such as *Glomus clarum*, identified orchid mycorrhizal fungi such as *Screrotium tulipalum* (NBRC 6168), *Thanatephorus cucumeris* (NBRC 101554) and *Rhizoctonia candida* (NBRC 7033). Each fungus were homogenized with 0.05 M Tris buffer, pH 7.5, containing 0.05 M NaCl by a mortar and pestle. The homogenates were filtered through cheesecloth, and ammonium sulfate was added to 35% saturation.

After standing overnight at 4 ºC, the liquid was centrifuged at 8000 g for 30 min. The supernatant liquid was made up to 95% ammonium sulfate saturation. After standing overnight at 4 ºC, the liquid was centrifuged. The residues were dissolved in 2 ml of 0.005 M Tris buffer, pH 7.0. After centrifugation, the supernatant liquid was dialyzed against 0.005 M Tris buffer, pH 8.0, containing 0.1 M KCl. The dialyzate was centrifuged. The supernatant liquid was used as protein samples.

5.SDS-PAGE of protein

SDS-PAGE in method of Laemmli (1970) was used to check whether known orchid mycorrhizal fungi and KMI have proteins that their molecular weights are 53 and 24 kDa.

Each protein samples from KMI, *G. clarum*, *S. tulipalum*, *T. cucumeris* and *R. candida* were analyzed. After the SDS-PAGE, bands on the gel were analyzed by the iMeasureScan software (iMeasure Inc.).

6. Inoculating on plants

In order to confirm that KMI is a mycorrhizal fungus, it was inoculated on several kinds of orchids. Because *P. thailandense*, the host of KMI is one of rare species which can not be propagated easily and both of its seeds and plants were unavailable, axenic cultured plants of two kinds of *Phalaenopsis* (code: PATO79 'Memorial Day' and code: PATOB1 'Ice Lady'), which have resemblance of morphologic characteristics and *Miltonia* (code: M203 ENZAN LADY 'Stork Feather') were used for the experiment. (These orchids were from Mukoyama Orchids Co., Ltd.)

Eighty ml of IH base media (Ishii and Horii, 2007) were poured into 800 ml jars. After the media solidified, one plant was transplanted in each jar. Half of them were inoculated with 5 ml of KMI culture (cultured by 20% potato dextrose liquid media for 3 days in 25 ºC). After incubated at 28 ºc for a week, their roots were stained using the method of Phillips and Hayman (1970) and observed by using a light microscope.

To check whether KMI is pathogenic or not to vegetables which are sensitive to *Rhizoctonia* and *Fusarium*, 5 plants each of tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*) were inoculated with KMI. Each plant received 5 ml of KMI culture.

Results

On the PDA plate, KMI formed white colonies. Diameter of its hyphae was approximately 1.5-2.5 μm and branched into right angle (Figure 3-1-A). It seldom made septa. Its spores were oval shape in size of 2 μm x 4 μm and had two nuclei each (Fig. 3-1-B).

KMI had no significant similarity to known species in both of 18S rDNA partial sequence:

5'-

CTCATTATATAAGTTATCGTTTATTTGATAGTACCTTACTACTTGGATAACCGTGGTAA
TTCTAGAGCTAATACATGCTAAAAATCCCGACTTTGGAAGGGATGTATTTATTAGAT
TAAAAACCAATGCCCTTCGGGGCTCCTTGGTGATTCATGATAACTCCTCGAATCGCA
TGGCCTTGTGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTTCGATGTTT
GGGTATTGGCCAAACATGGTTGCAACGGGTAACGGAGGGTTAGGGCTCGACCCCG
GAGAAGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAN
ATTACCCAAATCCTAACACGGGGAAGGTAGTGACAATAAATAAACAATACCGGGCT
CATTGAAGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGATCCATT
GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATT

AAAGTTGTTTGCAGTTAAAAGCTCGTAGTTGGACCTTGGGNTGGGTCGATCGGTCC
GCCTCTGGGTGTGCACCGGTCGGCTCGTCCCTTCTGC-3' and ITS rDNA partial sequence:

5'-
CCCCTGTGAACTATACCGATTTGTTGCTTCGGCGGTGCCCCTCGCTTTCGCGGCCA
GGGCCCGCCAGAGGACCCAAATTCTTTGTTTTTTTGTATTCGTTTTATATTCTTCTGA
GTGGAATTTTTTAAATAAAAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCT
GTTCGAGCGTCATTTCAACCCTCAAGCCCCCGGGCTTGGTGTTGGGGATCGGCAC
AAGGCCCCTCGTGGGCCCGCCGTCCCCCAAATGCAGTGGCGGTCACGTCGCAGCC
TTCTATGCGTAGTAGCAACACCTCGCACTGGAGCGCGACGCGGCCACGCCGTAAA
ACCCCCGACTTTTTTCTGGTTGACCTCGAATCAGGTAGGACTACCCGCTGAACTTA
AGCATATC-3' (Figure 3-2 and 3-3).

Proteins in molecular weights of 53 and 24 kDa were detected from *G. clarum*, KMI and all of three orchid mycorrhizal fungi (Figure 3-4). The analysis of iMeasureScan software showed the fact that KMI have both molecular weights of proteins.

KMI infected all three types of orchids (Figure 3-5). Hyphae were observed in tissues near from surface of the roots and colonized cells were not decayed. Not only in potato dextrose media but also when inoculated on orchid roots, the morphological characteristics of orchid mycorrhizal fungi i.e. branching into right angle and seldom makes septa, were observed (Fig. 3-5-C).

No significant symptoms of *Rhizoctonia* or *Fusarium* disease were developed on both plants of tomato and cucumber after two weeks from inoculation of KMI (Table 3-1).

Discussion

KMI has hyphal branching into right angle and can infect to surface tissues of orchid roots. Even when the hyphae penetrated into the root tissues, the root cells were not decayed. From the evidence, KMI might be an orchid mycorrhizal fungus. The morphological characteristics of spores, however, are different from those of *Rhizoctonia* (Uetake et al., 1999). It seems to resemble *Nectoria*, which is known as a teleomorph of *Fusarium* rather than *Rhizoctonia*.

Since Vujanovic et al. (2000) have reported that an isolate of *Fusarium* stimulated seed germination and protocorm formation in *Cypripedium reginae*, some of *Fusarium* species also can be orchid mycorrhizal fungi. But KMI rDNA shows low similarity with *Fusarium* species both in 18S (<94%) and ITS (<92%). It suggests that KMI is not belonging

to group of *Fusarium*. Actually, when the endophyte of tropical tree *Cecropia insignis* of KMI was analyzed, it showed low similarity (<94%) to known species so that analyze of ITS sequence was conducted. Interestingly, when the similarity of 18S rDNA between KMI and *Rhizoctonia* species were analyzed, one of the *Rhizoctonia* species *Sclerotium rolfsii* and KMI were in a same cluster. But the similarity between them were only 88%. Similarity of their ITS rDNA were even low. Fungal sp. ARIZ B394, which had highest similarity of the ITS sequence of KMI is reported as an endophyte of tropical tree *Cecropia insignis* (U'Ren et al., 2009) and may help germination of their seeds. Thus, it seemed to be a new type of mycorrhizal fungus that can have symbiotic relation with orchid plants. In addition, the fact that the inoculation of KMI on tomatoes and cucumbers did not cause the symptom such as wilt suggested that KMI is not seemed to be a pathogenic fungus.

Proteins in molecular weight of 53 and 24 kDa were common to AMF and orchid mycorrhizal fungi. Because KMI also has proteins in same molecular weights, those can be used for symbiosis. It is necessary to search the mechanism of symbiosis between orchid plants and KMI.

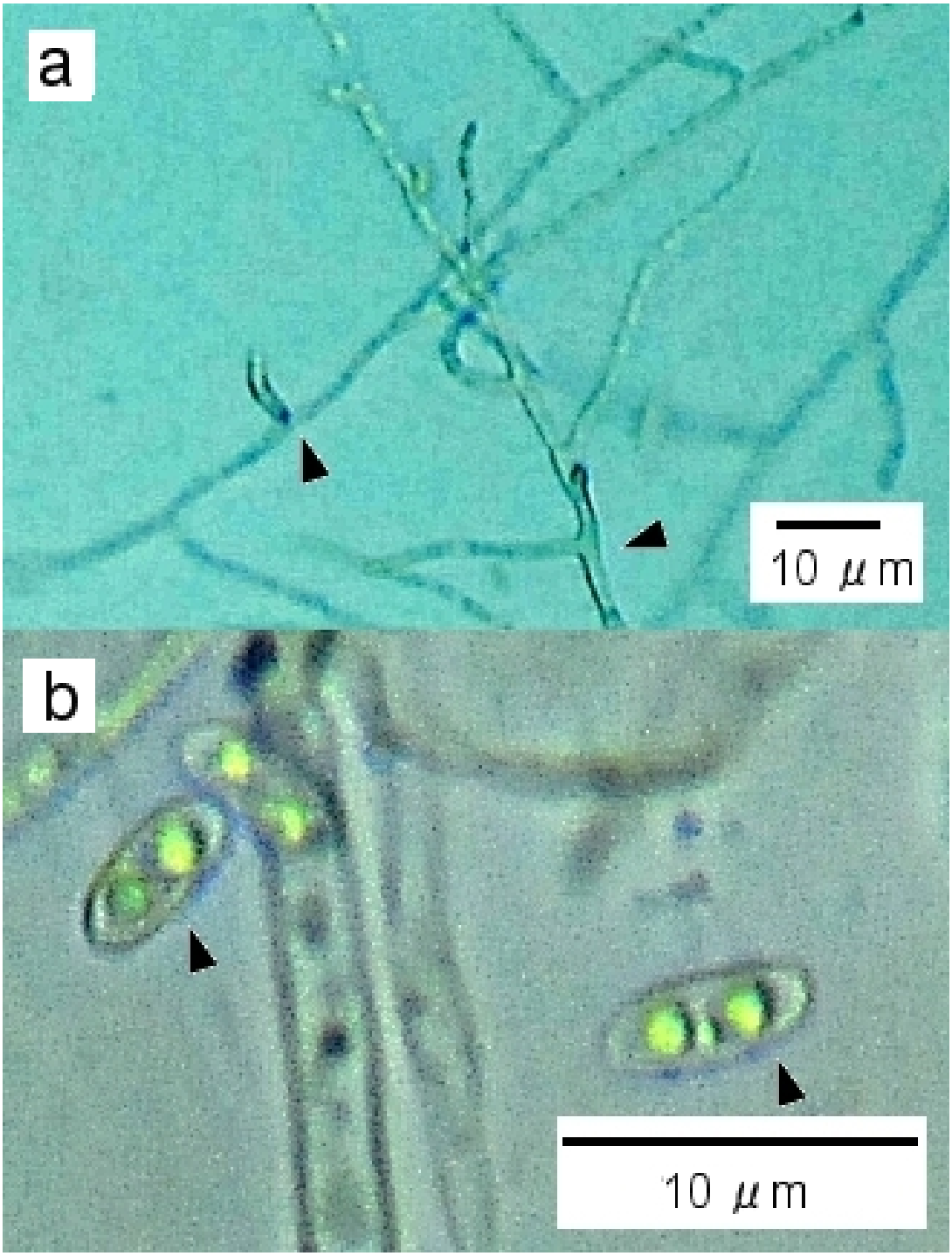

Figure 3-1. Morphological characteristics of KMI.

a: The arrows show the right-angle branching points.

b: KMI spores have two nuclei.

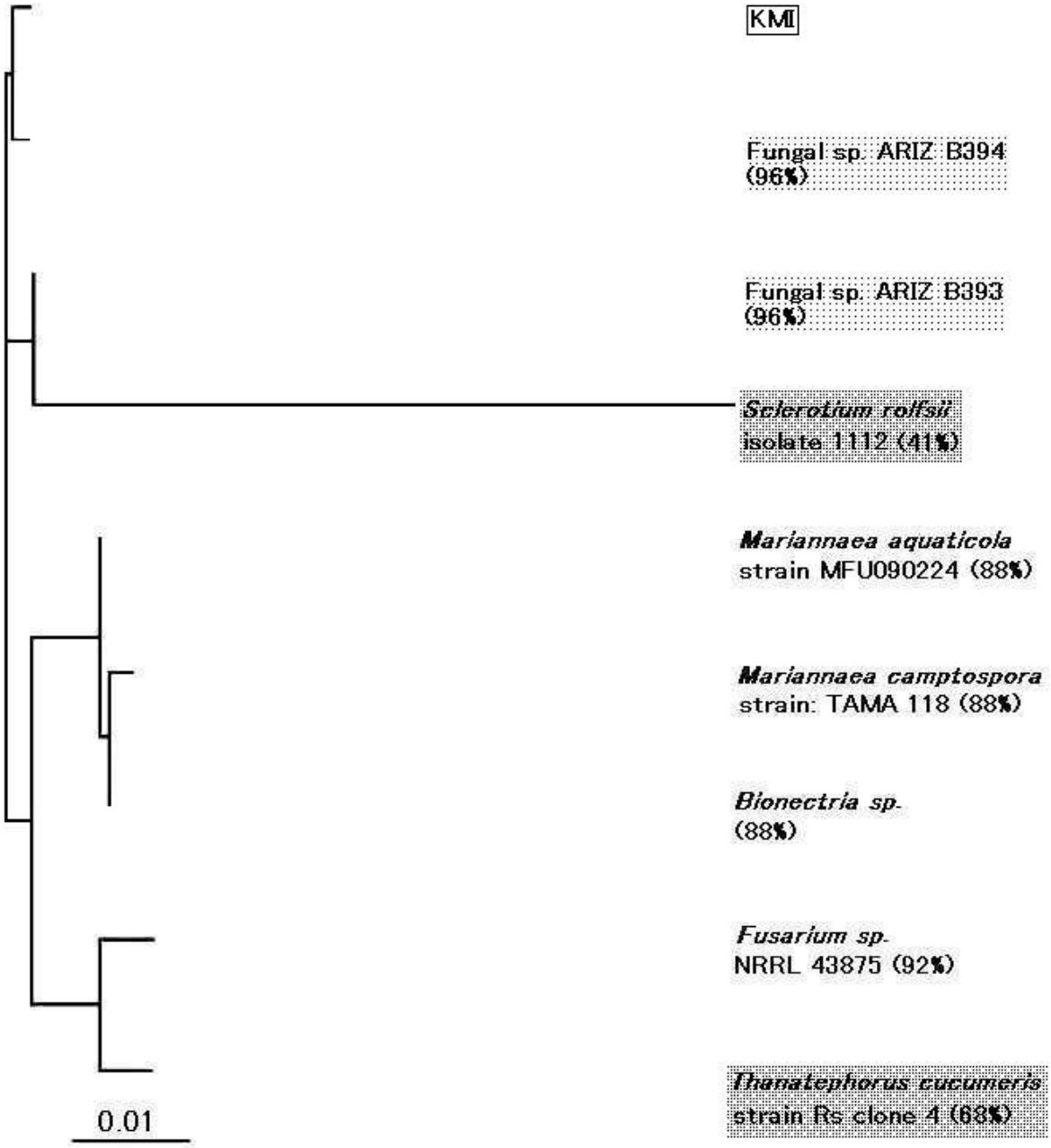

Figure 3-2. Phylogenetic analysis of KMI by the 18S rDNA sequence.

The white and gray box shows KMI and species which belongs to *Rhizoctonia*, respectively.

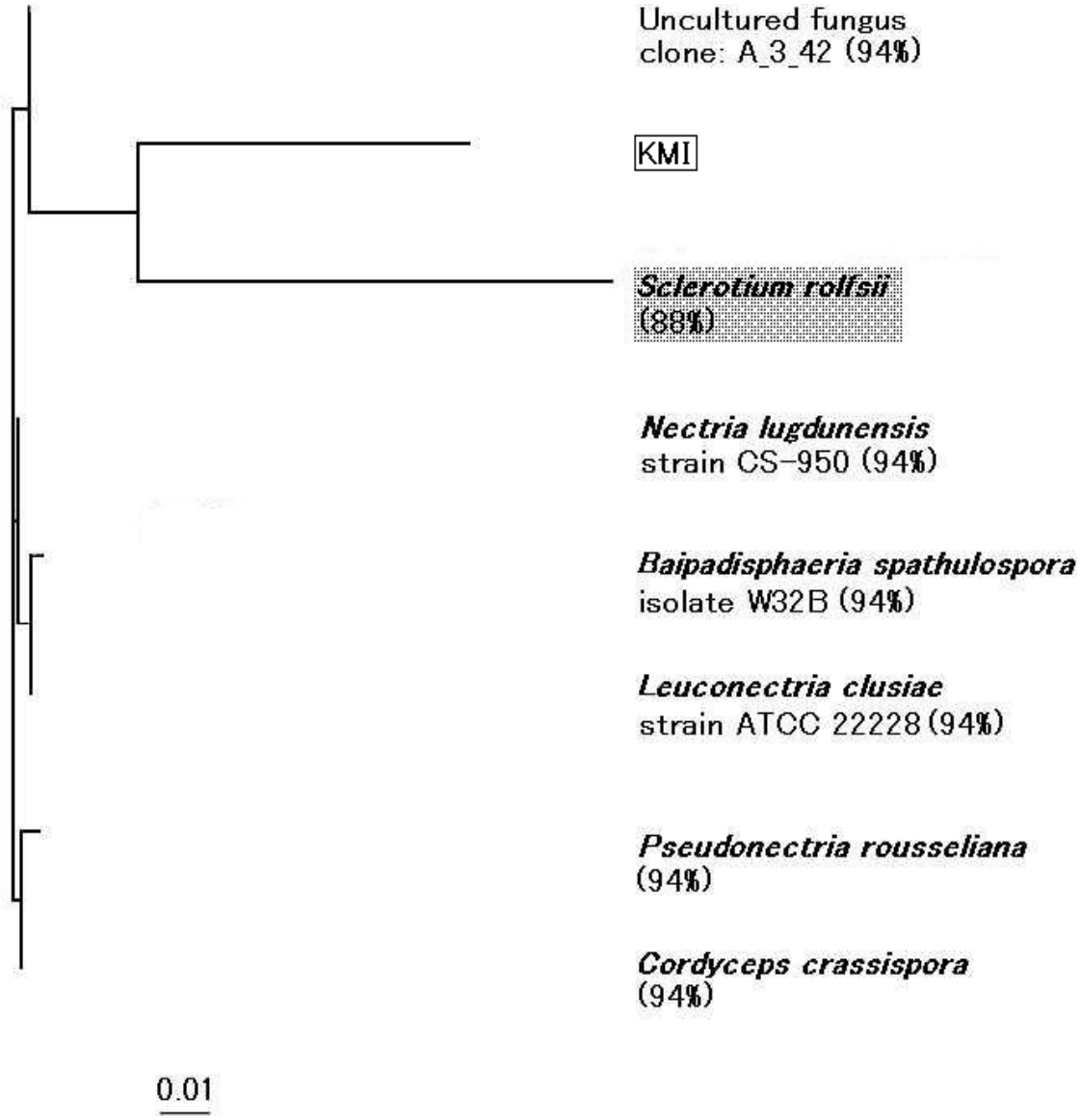

Figure 3-3. Phylogenetic analysis of KMI by the ITS rDNA sequence.

The white box shows KMI.

The Dark gray boxes show species which belong to *Rhizoctonia*.

The light gray boxes show species which might help seeds of tropical tree *Cecropia insignis* to germinate.

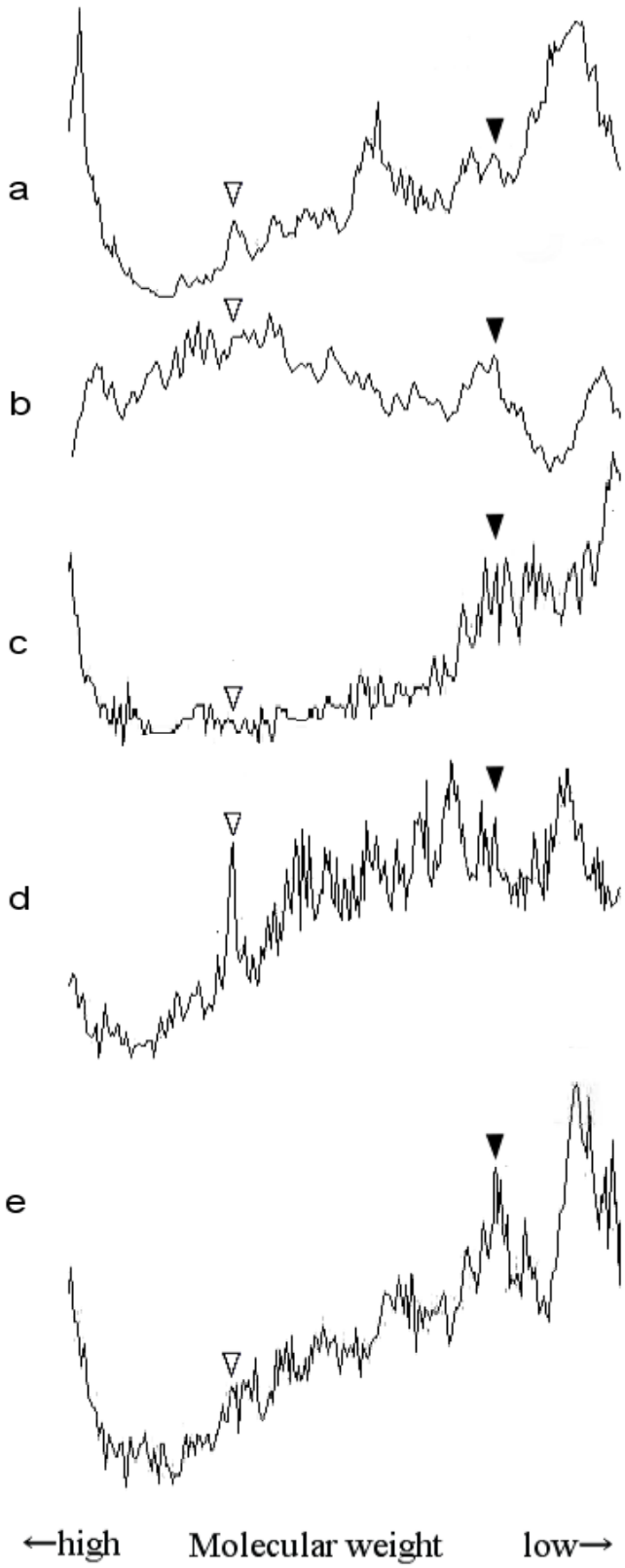

Figure 3-4. The digitize image of proteins extracted from some kinds of mycorrhizal fungi.
a: KMI, b: *Glomus clarum*, c: *Sclerotium tulipalum* (NBRC 6168),
d: *Thanatephorus cucumeris* (NBRC 101554), e: *Rhizoctonia candida* (NBRC 7033) .
White and black arrow show 53 and 24 kDa proteins, respectively.

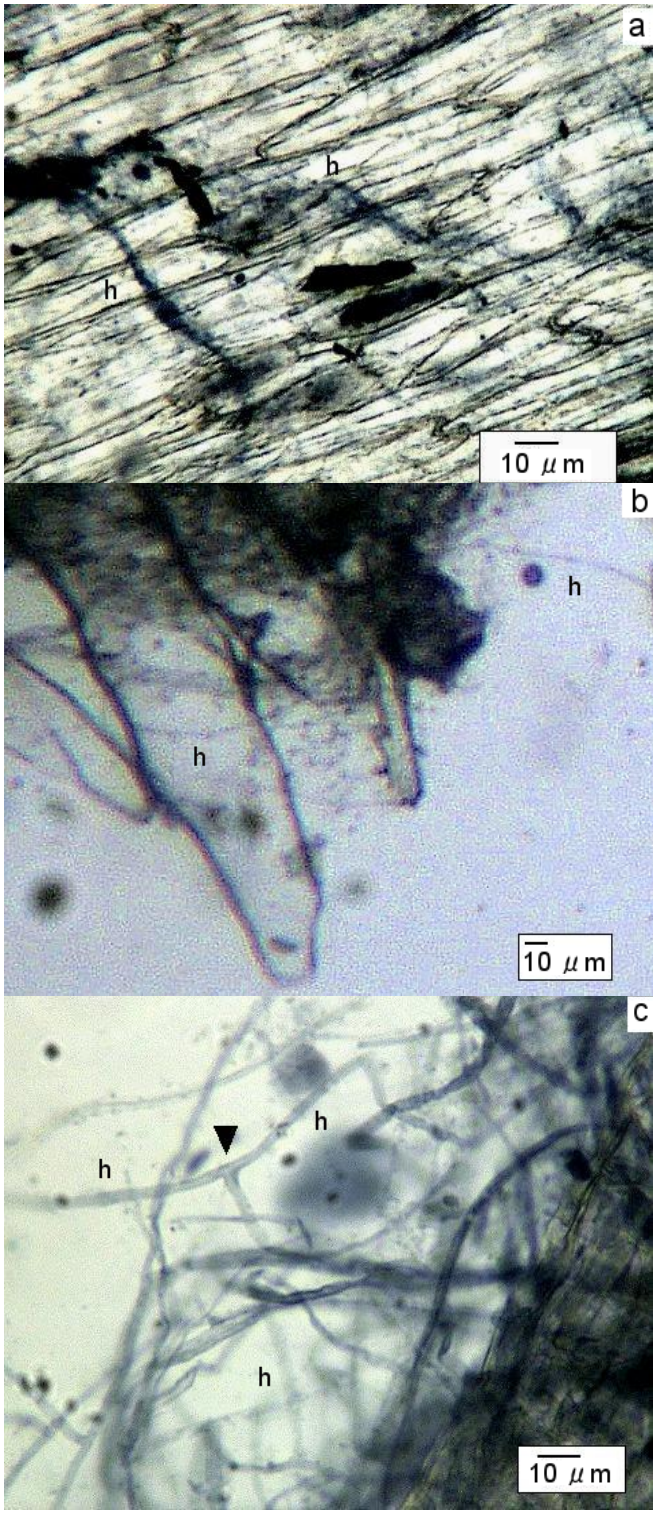

Figure 3-5. Orchid roots inoculated by KMI, one week after the inoculation.

a: *Phalaenopsis* 'Memorial Day' roots,

b: *Phalaenopsis* 'Ice Lady' roots.

c: *Miltonia* 'Stork Feather' roots.

The arrow shows the right-angle branching point.

The 'h' in each figure refers to 'hypha'.

Table 3-1. Effect of the inoculation of KMI on the growth of tomato and cucumber plants.

	symptoms [Z]	height (cm)	symptoms	height (cm)
KMI-inoculated	-	19.4±0.9 [Y]	-	13.4±0.8
Control	-	28.2±0.9	-	20.6±2.3
t-test		*		ns

[Z]: Symptoms of disease: - no symptoms.

[Y]: Mean±standard error (n=5). ns= no significant,

*= significant difference at 95% level.

Chapter 4

Protein changes in genetically modified plants

Abstract

Although harmful effects on ecology and human health by genetically modified plants (GMP) have been clarified, little is known about the effect of GMP on AMF. Therefore, soybean plants with genetically introduced oxalate oxidase (OXO) to protect from a plant pathogen, *Sclerotinia minor*, were examined. The qualitative and quantitative changes in proteins in the genetically modified (GM) soybean roots inoculated with AMF were analyzed by a new technique of preparative chromatography. The concentration of toxic proteins derived from OXO around 24 kDa in *OXO* soybeans was higher than that of wild type soybeans, and mycorrhizal 24 kDa protein were separated from these proteins around the 24 kDa, by using a preparative HPLC with a Diol column in a gradient method. This new chromatographical techniques will greatly contribute to the separation and purification of toxic proteins derived from GMP.

Introduction

GMP, such as *Bacillus thuringiensis* toxin and glyphosate tolerant plants, are cultivated in over 25 countries as of 2008 (Liu, 2010). Since a report of Chakravarty and Chatarpaul (1990) has shown that the growth of all five ectomycorrhizal fungi were significantly reduced by very low concentrations of glyphosate, such as the concentrations above 50 μl formulation liter^{-1}; therefore, it is necessary to understand the impact of GMP on mycorrhizal fungi for ecological conservation and safe, secure and sustainable food production.

Liu (2010) has reported that most of previous studies have focused on colonization rate, spore formation and morphology. However, the qualitative and quantitative changes in proteins in GMP have not yet been investigated. Therefore, the impact of *OXO* soybeans on AMF was analyzed by a proteomic approach using chromatographical techniques.

Materials and Methods

GM and wild type soybean (*Glycine max* 'Westag 97', 'AC Colibri' and 'AC Orford' with and without *OXO*) plants were inoculated with *Glomus intraradices*. One month after the inoculation, crude proteins of each soybean root were extracted by modified methods of

Shannon et al. (1966). Cultivation of the soybean plants and the extraction of proteins in their roots were done in Semiarid Prairie Agricultural Research Centre with permission of Agriculture and Agri-Food Canada. Then, fractions with 24 kDa proteins were obtained by MPLC and isocratic HPLC as mentioned in Chapter 1. Furthermore, the proteins that were around 24 kDa were purified by HPLC in a gradient method as described in Chapter 1. Then, the qualitative and quantitative differences in these proteins between GM and wild type soybean roots were analyzed. The peak area of the mycorrhizal 24 kDa protein per gram of roots in the GM and wild type soybean roots were also measured.

Results

The chromatograms in Figure 4-1 indicated that proteins around 24 kDa in every GM soybean were higher than those in the wild type one, and that these proteins showed single peaks in isocratic HPLC. Using a gradient HPLC, however, the single peaks of proteins around 24 kDa were separated into two peaks (Figure 4-2). The black arrows indicate mycorrhizal 24kDa proteins and asterisks show the proteins including OXO. The mycorrhizal proteins and the proteins including OXO in wild type soybeans used were increased by gene recombination events (Figure 4-2 and Table 4-1) .

Discussion

Originally, OXO was identified as a Germin-like protein of *Poaceae* with antifungal activity (Zhang et al., 1995). Even in every wild type soybean root infected by AMF, the proteins around 24 kDa were detected. These proteins have been reported to have antifungal activity (Bazghaleh et al., 2012). Furthermore, 75% of rabbits died off through the administration of the proteins around 24 kDa, while antibodies against mycorrhizal 24 kDa protein were being built up in Chapter 2. This fact suggests that the proteins around 24 kDa have strong cytotoxicity (unpublished data). Proteins around 24 kDa increased in GM soybean roots seem to be isozymes of OXO in molecular weight of 20-25 kDa (Zhang et al., 1995). According to a report by Donegan et al. (1995), unexpected changes in plant characteristics, such as the regulation of *OXO* expression, occur in GM soybeans by gene recombination or tissue culture. As shown in Chapters 1 and 2, the mycorrhizal 24 kDa protein may be masked by the OXO isozymes. Thus, an increase in the 24 kDa proteins in GM soybean roots results from OXO isozymes and not the mycorrhizal protein, since mycorrhizal colonization rate of GM soybean roots was slightly lower than that of wild type soybean roots. The *OXO* soybeans produced high concentrations of toxic substances encoded by proteins around 24 kDa (isozymes of

OXO). In this chapter, however, the preparative HPLC with a Diol column in a gradient method was very useful for the separation and purification of the mycorrhizal 24 kDa protein and other proteins around 24 kDa.

Conventionally, the effect of the direct products of a modified gene has been examined (Liu, 2010), but further studies examining unexpected products of GMP which negatively affect the safe, secure and sustainable food production are needed. In this respect, the new chromatographical techniques developed will greatly contribute to the separation and purification of toxic proteins derived from GMP.

Table 4-1. The peak area of mycorrhizal 24 kDa protein /g roots.

	Westag 97	AC Colibri	AC Orford
WT	329086	427139	155298
GM	805264	636264	206721

WT: wild type, GM: genetically modified

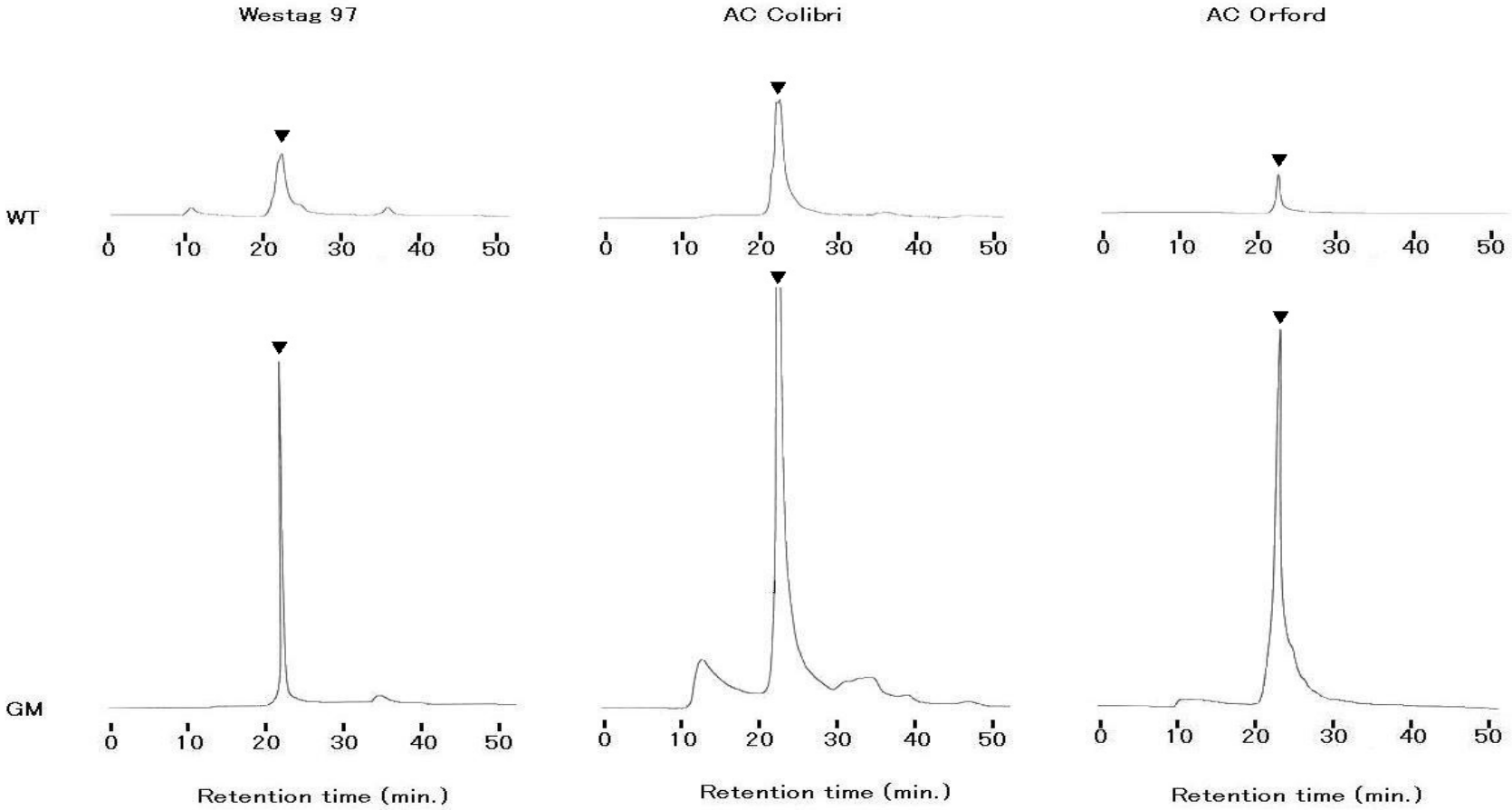

Figure 4-1. A comparison between chromatograms of 24 kDa protein fractions extracted from wild type and genetically modified soybeans in an isocratic method.

WT: Wild type, GM: Genetically modified.

Protein fractions from 2.5 g roots were analyzed.

The black arrows show 24 kDa proteins.

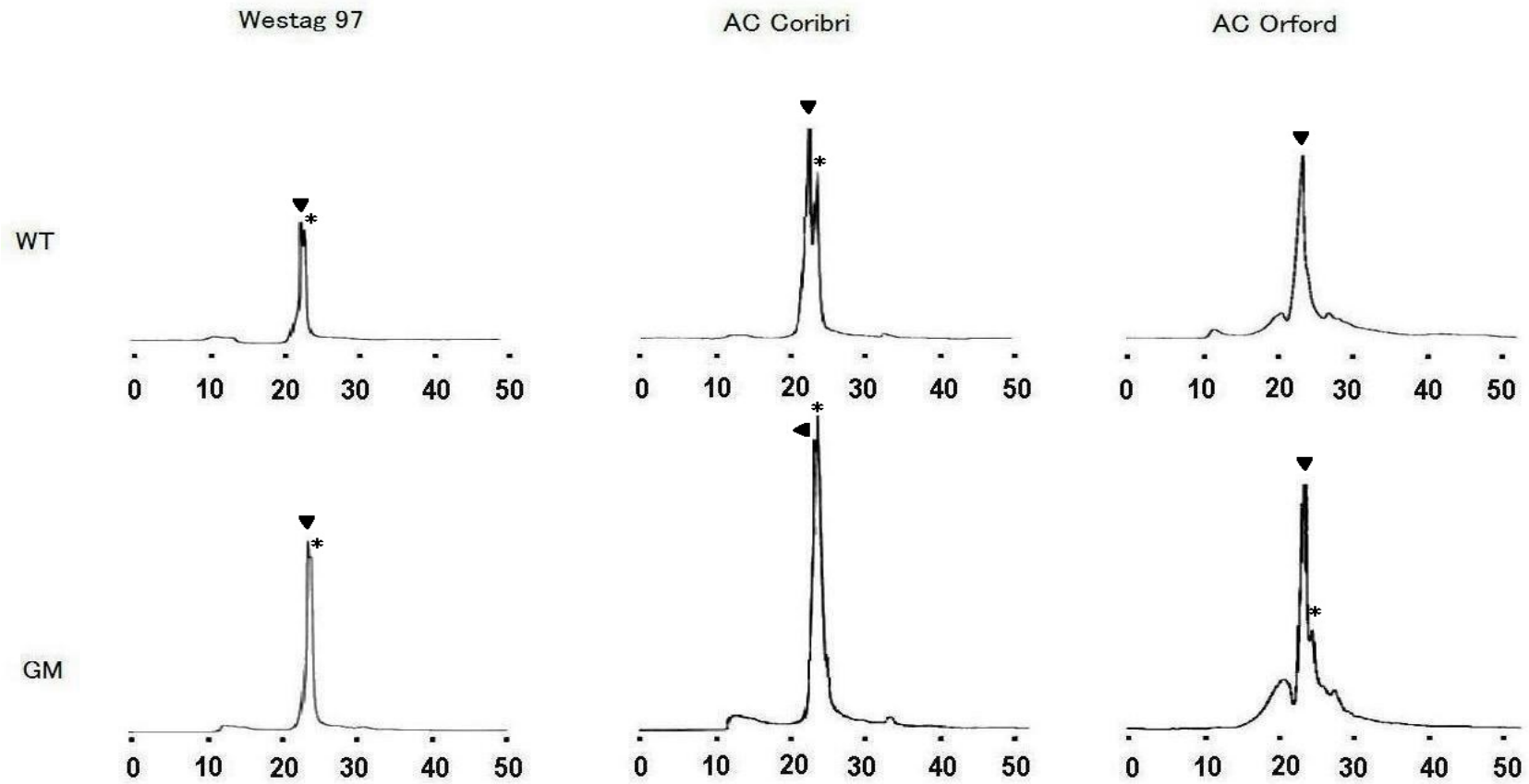

Figure 4-2. A comparison between chromatograms of 24 kDa proteins extracted from wild type and genetically modified soybeans in a gradient method.

WT: Wild type, GM: Genetically modified.

24 kDa proteins from 2 g roots were analyzed.

The black arrows show 24 kDa proteins.

General Conclusions

This research focused on proteins because AMF are multinuclear and multiform, so genome sequence analyses are impossible. Both 24 and 53 kDa proteins related to mycorrhizal symbiosis were detected from not only AMF, but ERMF, ECMF and OMF. This suggests that all kinds of mycorrhizal fungi act via the same mechanism to form symbiotic relationship with plants. The characteristics of the 24 kDa protein, which is habitually expressed as a non-specific lipid transporter, indicate its involvement in the growth of host plants. However, 53 kDa protein is apparently expressed only when the mycorrhizal fungi and their host plants are stressed. Therefore, the author focused on the 24 kDa protein and attempted to develop new chromatographical techniques. The techniques will greatly contribute to further proteomic studies of the mechanism of mycorrhizal symbiosis.

The 24 kDa protein is a very effective indicator of mycorrhizal fungi. Thus, the author first succeeded to develop a new reagent to detect mycorrhizal fungi under fluorescence microscopy, by using antigen-antibody reactions for this protein. To be utilized widely on farms, simple and reasonable instruments are required.

Interestingly, the 24 kDa protein was detected in not only mycorrhizal fungi but also pathogenic *Rhizoctonia.* It is possible that the 24 kDa protein is essential for both of mycorrhizal and pathogenic *Rhizoctonia* to invade into root tissues. Structural elucidation of the protein in both fungi is needed. Furthermore, a new type of OMF was isolated using morphological observation, DNA sequencing and the mycorrhizal 24 and 53 kDa proteins.

Since the impact of GMP on AMF is concerned, the author used chromatographical techniques to analyze proteins in *OXO* and wild type soybean roots inoculated with AMF. The concentration of proteins around 24 kDa (isozymes of OXO) with toxicity was higher in GMP than in wild type plants. Although it was difficult to separate the mycorrhizal 24 kDa protein from the OXO isozymes including toxic proteins, the author successfully obtained pure mycorrhizal 24 kDa protein by using the preparative HPLC with a Diol column in a gradient method. This new chromatographical techniques will greatly contribute to mass-separation and mass-purification of these proteins, and the protein quantity obtained was sufficient to analyze precisely their characteristics in future.

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