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Cross-protection against anthracnose with heat stress, antioxidative changes and proteomic analysis in mycorrhizal cyclamen

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Abstract: Cross-protection against anthracnose with shock heat stress, antioxidative changes and proteomic analysis in mycorrhizal cyclamen were investigated. Eleven weeks after arbuscular mycorrhizal fungus (AMF, *Glomus fasciculatum*) inoculation, cyclamen (*Cyclamen persicum*, cv. Pastel) plants were acclimated under 20°C for 4 days, followed to 35°C (shock heat stress, SHS) for 7 days, and inoculated with *Colletrichum gloeosporioides* (CG) as anthracnose pathogen. Seven days after SHS treatment, dry weights of leaves, bulbs and roots increased in mycorrhizal plants, thus, growth promotion appeared. In addition, mycorrhizal plants showed higher resistance to anthracnose compared to non-mycorrhizal control plants. Regarding antioxidative activity, superoxide dismutase (SOD) activity increased in roots of mycorrhizal plants under 7d after SHS. DPPH radical scavenging activity increased in some parts of the mycorrhizal plants under SHS and CG-inoculated conditions. As for proteomic analysis, totally 29 spots changed in mycorrhizal plants through SHS and CG-inoculated conditions. In this case, the spot of 20.4kDa was detected only in mycorrhizal plots, furthermore, 4 spots intensively appeared in mycorrhizal plots through SHS and CG conditions. From these findings, AMF could alleviate heat shock stress with promoting host plant growth and induce resistance to anthracnose under heat stress. In addition, it supposed that antioxidative modification would have cross association with the resistance to heat shock and anthracnose, and the symbiosis-specific changes in some proteins might have concern with the cross protection.

1. Introduction

Cyclamen is an herbaceous perennial used as a flowering potted plant (Karlsson and Werner, 2001; Elmer and McGovern, 2004). In the genus *Cyclamen*, *Cyclamen persicum* is the major species used for commercial cultivation (Ishizaka *et al.*, 2002). It has a longer growing season and temperature is one of the most important environmental factors that affect the growth, development and distribution of this plant (Yesson and Culham, 2006). Cyclamen is highly susceptible to various temperature conditions and the recommended temperature for cyclamen production from seeding to appearance of flower bud is 20°C (Ball, 1991). Currently, due to global warming, summer temperatures are increasingly elevated. However, garden type cyclamen is intolerant to heat stress, which severely affects growth and development of this plant in southwest Japan during summer (Goto *et al.*, 2011). On the other hand, in summer, heat is not the only stress factor, but pathogens also pose a threat. Pathogenesis infection impacts commercial cyclamen production. Although various diseases are reported, anthracnose is especially destructive disease worldwide. Anthracnose is responsible for *Colletotricum gloeosporioides* (CG) and it causes extensive lesions on aerial plant parts.

Today, cost effective and eco-friendly control strategies against heat and anthracnose are needed. Arbuscular mycorrhizal fungus (AMF) has drawn attention by crop researchers for its benefits to host plants. Improving nutrient uptake, especially of phosphate, of the host plant is well known as one of the benefits of arbuscular mycorrhizal symbiosis (Marschner and Dell, 1994). Additionally, several investigators have reported a higher resistance of mycorrhizal plants to biotic and abiotic stresses (Garmendia *et al.*, 2006 ; Wu *et al.*, 2006; Li *et al.*, 2010). As for cyclamen, mycorrhizal plants showed growth promotion under heat stress (30°C) (Maya and Matsubara, 2013 a) and higher tolerance to anthracnose caused by CG (Maya and Matsubara, 2013 b). However, growth improvement under extreme shock heat stress, resistance to anthracnose under such heat stress and proteomic changes through heat stress and anthracnose in mycorrhizal plants are yet to be revealed.

When plants suffer to heat stress and pathogenesis infection, reactive oxygen species (ROS) generate in cells and the oxidative stress to intercellular structures they cause are major damaging factors in plants (Wahid *et*

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al., 2007). In order to cope with these toxic ROS, plants have antioxidants as detoxification factors (Kuzniak and Sklodowska, 2004; Wu *et al.*, 2006). Antioxidative defense mechanisms consist of antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) and nonenzymatic antioxidants such as polyphenols and ascorbic acid (Asada, 1999). Enhancement of antioxidative activity in mycorrhizal plants has been already reported (Lambais *et al.*, 2003).

Physiological changes in plants due to heat stress have not yet been revealed. However, plants have shown different responses under heat acclimation and heat shock (Durand *et al.*, 2012). According to Li *et al.* (1984), heat shock caused stronger growth inhibition than heat acclimation. Although heat shock is thought to be a stronger heat stress treatment, some researchers reported that heat shock (around 45°C) induced tolerance against aboveground diseases such as anthracnose to host plants (Teruya *et al.*, 2012; Yoshino *et al.*, 2012). Therefore, heat shock could be considered as a trigger defense system toward pathogens. On the other hand, AMF symbiosis also causes alteration of gene expression in leaves and roots (Liu *et al.*, 2007). Campos *et al.* (2012) reported that expression of some defense related genes such as transcriptional factors, regulators of Ca^{2+} signaling pathway, MAPKs and NPR1 (nonexpressor of PR genes) etc. had been up-regulated in mycorrhizal rice leaves. Yet, the influence of AMF on protein expression in cyclamen under heat stress and CG inoculation have not been revealed.

The aim of this study was to determine cross-protection against heat stress and anthracnose in mycorrhizal cyclamen in association with antioxidative changes and proteomic analysis.

2. Materials and Methods

Plant material and mycorrhizal inoculation

Cyclamen (*Cyclamen persicum*, cv. Pastel) seedlings (three months of age) were replanted in plastic pots containing autoclaved (121°C, 1.2 kg/cm^2, 15 min) commercial potting media (Sogemix SM-2). In the meantime, plants were inoculated with AMF (*Glomus fasciculatum*) inoculum for mycorrhizal (AMF) plants and autoclaved inoculum for non-mycorrhizal (control) plants. In both cases, inocula (3 g/plant) were placed 4 cm below the bulbs. Commercial AMF inoculum supplied by Idemitsuagri Co. Ltd, Tokyo, Japan was used in this study: the spore density was unknown. Plants were transferred into poly silver pot (9 cm diameter) after 11 weeks of AMF inoculation. Plants were fertilized with slow release granular fertilizer (N:P:K= 5:10:15, Ube Industries Ltd.) once a month and raised in a greenhouse.

Treatment with shock heat stress (SHS)

Eleven weeks after AMF inoculation, the plants are transferred to a growth chamber (20°C constant, 12 hr daylength, RH 60%) for 4 days (d) as acclimation under optimum environmental conditions. After 4 days at 20°C, the temperature was suddenly increased to 35°C (constant) as shock heat stress treatment (SHS). The plants were grown for 7 d under SHS.

Inoculation of Colletrichum gloeosporioides

The isolate (MAFF744024) of *Colletrichum gloeosporioides* (CG) was collected from the Ministry of Agriculture, Forestry and Fisheries, Japan. It was grown in potato dextrose agar (PDA) medium and was incubated at 25°C for two weeks in dark conditions to prompt sporulation. The spore concentration was adjusted to 10^5 cfu using sterile distilled water. The shoots of each plant were sprayed at 7 d after SHS with 10 ml of CG suspension. Symptoms of anthracnose were evaluated 9 d after CG inoculation. A completely randomized design with five replicates was used.

Disease incidence and index of anthracnose

Disease symptoms were checked 9d after CG inoculation. The disease severity in individual plants was rated visually on a scale of 0-5.

0= no symptoms (healthy plants);
1= <20% disease symptoms (small discolored leaf lesions covering less than 20% of total leaves of a plant);
2= 20-40% disease symptoms (minor small discolored lesions covering 20-40% of leaves);
3= 40-60% disease symptoms (moderate brown lesions in 40-60% of leaves and 15% defoliation);
4= 60-80% disease symptoms (mild wilt discolouration covering 60-80% of leaves and more than 50% leaf defoliation in case of *Fusarium* wilt);
5= 80-100% disease symptoms (stems and leaves severely affected).

The disease index for CG was calculated using the following formula:

Disease index= [Σ (number of diseased plants x severity level)] x 100/ (total number of plants x maximum level of disease severity)

Plant growth and mycorrhizal colonization

Dry weight of leaves, bulbs and roots of five plants which were grown at 20°C for 4 d, under SHS for 2 d, 7 d and for 9 d after CG inoculation was determined after plant material was dried at 100°C for 24 h. The lateral roots of each plant were sampled and used to check the level of AMF colonization according to Phillips and Hayman (1970). Lateral roots were sampled in 70% ethanol and later washed with distilled water. They were then ground in 10% NaOH and autoclaved (121°C, 1.2 kg/cm^2, 10 min) and subsequently washed with distilled water and stained with trypan blue. The ratio of AM fungal colonization was checked in 1-cm segments of lateral roots and approximately 60 samples of 1-cm segments were checked per plant. The average was calculated from the values of five plants for each time.

Antioxidative analysis

Superoxide dismutase (SOD) as enzymatic antioxidative activity and DPPH radical scavenging activity as non-enzymatic antioxidative activity were analyzed. Plants which were sampled 4 d after 20°C, 2 d and 7 d after SHS, and 9 d after CG inoculation preserved with liquid nitrogen were used for each analysis. Total SOD activity was measured according to Beauchamp and Fridovich (1971). DPPH radical scavenging activity test was carried out as described by Burits and Bucar (2000). All experiments were replicated three times.

Proteomic analysis

Protein extraction. One g of frozen extended leaves was homogenized in liquid nitrogen with mortar and pestle. Powdered leaf sample was mixed with 2 ml of extraction buffer containing 7 M urea, 2 M thiourea, 4% CHAPS and 50 mM Tris (pH 7). The mixture was then centrifuged (14,000 rpm, 4°C, 60 min), and the collected supernatant was mixed with 400 ml of 50% TCA. This mixture was centrifuged (14,000 rpm, 4°C, 10 min) and supernatant was discarded. One ml of 100% acetone was added to the pellet and this mixture was centrifuged (14,000 rpm, 4°C, 10 min); the supernatant was discarded. These steps were repeated three times. After acetone was dried at RT, 200 µl of dissolution buffer (8 M urea, 2% nonidet p-40, 1% dithioslatol) was added to the pellet and centrifuged (14,000 rpm, 4°C, 5 min). The supernatant was sampled and mixed with 20 µl of 1M iodoacetamide.

Electrophoresis. The first dimension IEF was run using WSE 1500 (ATTO Corp.) according to the manufacture's instruction manual with some modifications (300V, 210 min). After electrophoresis, the gel was soaked in 100 ml of 2.5% TCA for 5 min. Then, it was washed with distilled water for 2 h. For the second dimension (SDS-PAGE), it was soaked in 100 ml of SDS equilibration buffer A (50 mM Tris-HCl, 2% SDS, 0.0001% BPB, 0.001% DTT), shaken for 10 min and subsequently shaken in SDS equilibration buffer B (50 mM Tris-HCl, 2% SDS, 0.0001% BPB, 0.001% iodoacetamide) for 10 min. SDS-PAGE was run on AE-6500 (ATTO Corp.) using 12.5% polyacrylamide gel (ePAGEL E-D12.5L, ATTO Corp.) according to the manufacture's instructions (100V, 20 mA, 150 min). Finally, proteins were visualized by CBB staining.

3. Results

Seven days after SHS treatment, dry weights of leaves, bulbs and roots increased in mycorrhizal plants (Fig. 1 and 2). In addition, leaf yellowing and browning caused by shock heat stress were alleviated in mycorrhizal plants compared to non-mycorrhizal control plants. The AMF colonization level reached around 50% through all the treatments and did not differ among the treatments (data not shown). With regard to anthracnose symptoms, greater

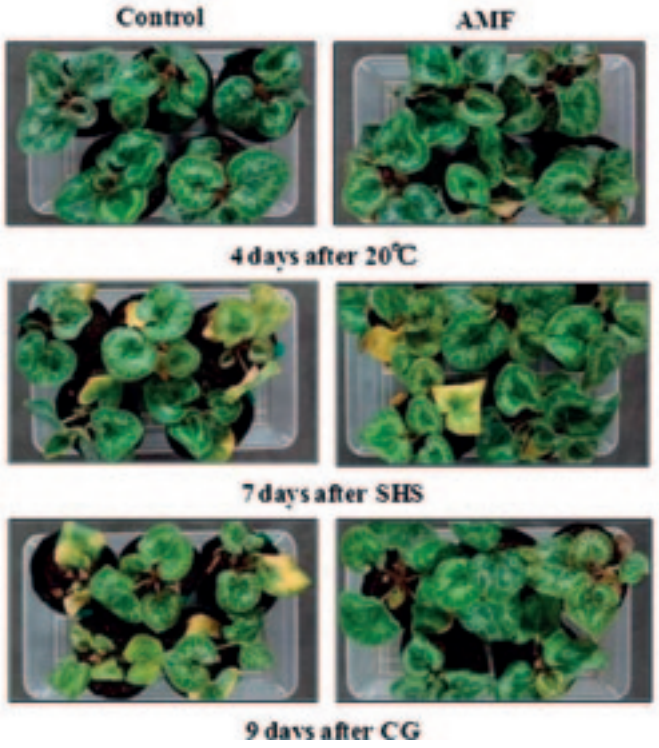

Fig. 1 - Growth of mycorrhizal cyclamen under shock heat stress (SHS) and *Colletotricum gloeosporioides* (CG) inoculation.

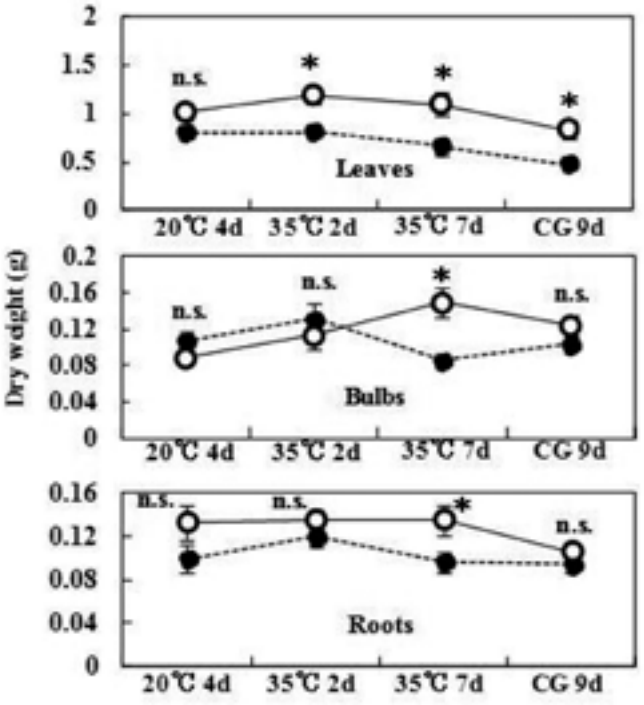

Fig. 2 - Dry weight of mycorrhizal cyclamen before and after SHS and CG inoculation. ● Control; O AMF (*Glomus fasciculatum*). SHS, CG, see Figure 1. 20°C 4d, 4 days after 20°C; 35°C 2d, 2 days after SHS; 35°C 7d, 7 days after SHS; CG 9d, 9 days after CG inoculation. Bars represent standard errors (n=5). NS, non-significant. *, significantly different between C and AMF by *t*-test (P<0.05).

severity appeared in non-mycorrizal plants and mycorrhizal plants showed less severity and lower indices (Fig. 3).

Differences in antioxidative activity occurred between AMF and control plants during the experimental period. As for SOD activity, significant change was not detected in leaves and bulbs but SOD activity was enhanced in roots at 20°C, 2 d and 7 d after SHS (Fig. 4A). However, no difference in SOD activity appeared under CG inoculation. DPPH radical scavenging activity as non-enzymatic antioxidative activity increased roots of mycorrhizal plants through the experimental period, and some parts of mycorrhizal plants also showed higher DPPH levels compared to control (Fig. 4B).

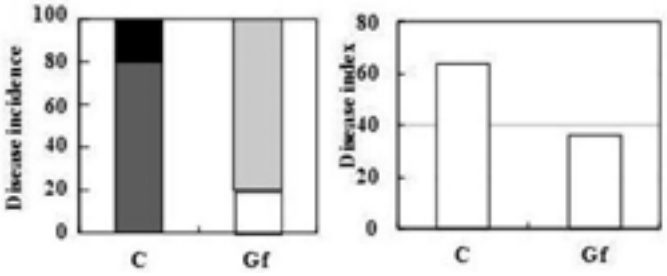

Fig. 3 - Disease incidence and index of anthracnose in mycorrhizal cyclamen 9 days after CG inoculation under SHS.
SHS, CG, C, AMF, see figure 2.
Ratio of diseased leaves to the total in each plant.
□ -20% ; ▒ 20-40% ; ▓ 40-60% ; ■ -80%.

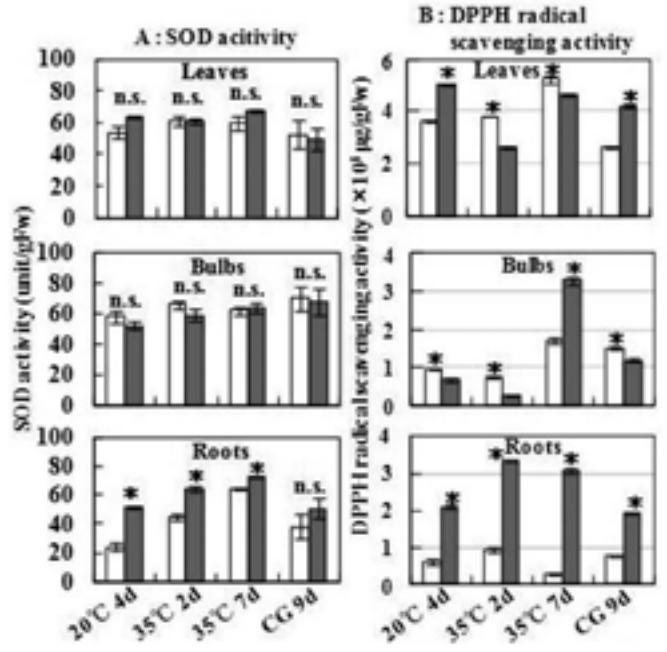

Fig. 4 - SOD activity, DPPH radical scavenging activity in mycorrhizal cyclamen before and after SHS and CG inoculation.
□ Control ; ▓ AMF. SHS, CG, see figure 2. Bars represent standard errors (n=3). NS, non-significant. *, significantly different between C and AMF by *t*-test (P<0.05).

As a result of proteomic analysis, overall the expressions of 29 spots were detected as different spots in mycorrhizal plants compared to control through SHS and CG inoculation (Table 1). Four days after 20°C, differences in expression of 11 spots between mycorrhizal and non-mycorrhizal plants appeared. In this case, although six increased spots and three newly appeared spots were detected in mycorrhizal plants, two spots slightly decreased in contrast to

Table 1 - Expression levels in 29 proteins in leaves of mycorrhizal cyclamen under SHS and CG inoculation

Spot ID	Estimated MW (kDa)	20°C 4 d		35°C 7 d		CG 9 d	
		C	AMF	C	AMF	C	AMF
A	68.0		+	+		- -	
B	68.0		+		+		+
C	87.1		++	++	++		+
D	33.4		+	++	+		++
E	29.9		-	+			- -
F	26.9		-		-		++
G	20.4		N		N		N
H	19.4		++	+	+		++
I	37.5		N	N	-	D	
J	36.7		N	N	-	D	
K	24.3		+				
L	27.3			+	+	+	
M	27.7				+		
N	19.7			+	+		++
O	19.5			+	+	+	
P	32.2			+	++		
Q	36.0			-		-	-
R	36.0			-		-	-
S	36.0			-		-	-
T	21.0				+		
U	27.0				++		
V	16.9			+	++	+	++
W	47.4			++	-	- -	-
X	20.7					- -	++
Y	36.3			+	++	+	++
Z	28.5					++	++
α	26.9					+	++
β	19.1					++	++
γ	19.1					++	++

SHS, CG, C, AMF, see Figure 2.
+ = slightly increased spot in expression;
++ = greatly increased;
- = slightly decreased;
-- = greatly decreased;
N= new spot;
D= disappeared spot.
In C plots, marks represent changes in expression level compared to former treatment.
In AMF plots, marks mean changes in comparison to C under same treatment.

control. Seven days after SHS, expression in 17 spots changed compared to 4 d after 20°C in control (Fig. 5A). As for mycorrhizal plants, 14 spots showed higher expression levels than non-mycorrrhizal plants at 7 d after SHS (Fig. 5B). After CG inoculation, expression levels of a total of 23 spots changed compared to 7 d SHS. In the control, eights spots increased in expression but six spots decreased and two spots disappeared compared to 7 d SHS (Fig. 5C). Regarding AMF, 13 spots increased in expression and two new spots appeared compared to control (Fig. 5D). Spot G (20.4kDa) was mycorrhizal-specific expression through SHS and CG treatments. The spots increased in expression by both SHS and CG were L (27.3kDa), O (19.5kDa), V (16.9kDa), Y (36.3kDa). In this case, especially, V and Y increased in mycorrhizal plants compared to the control.

4. Discussion and Conclusions

Mycorrhiza-induced growth promotion in cyclamen under heat stress (30°C) and anthracnose were reported in previous study (Maya and Matsubara, 2013 a, b). However, growth improvement under shock heat stress (35°C), resistance to anthracnose in light of cross-protection under heat stress, and proteomic changes through heat stress and

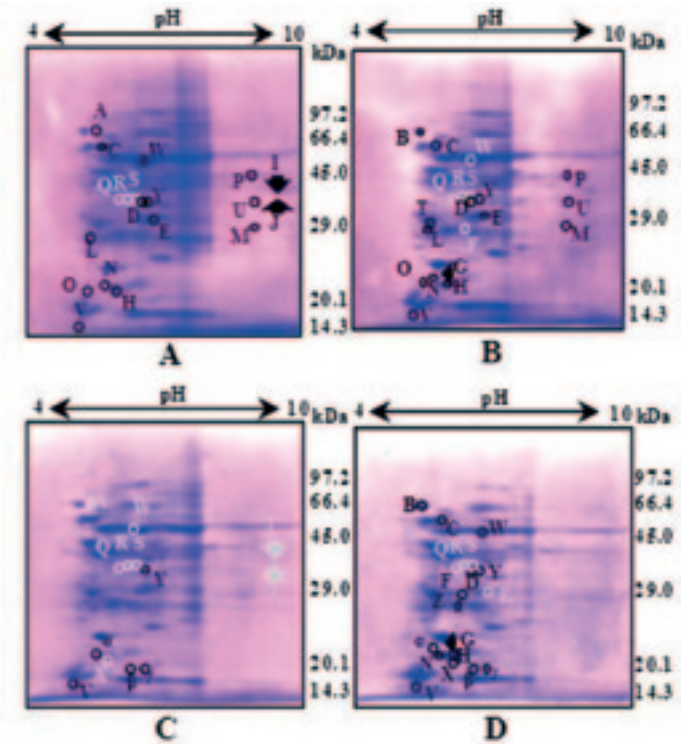

Fig. 5 - 2-D electrophoresis gel with leaf protein in mycorrhizal cyclamen 7 days after SHS and 9 days after CG inoculation. A (control), B (AMF) in 7 days after SHS. C (control), D (AMF) in 9 days after CG inoculation. SHS, CG, see figure 2. Letters show spot ID in Table 1. In A and C, black circle, expression-increased spots compared to former treatment; white circle, decreased spots; black arrows, newly appeared spots; white arrows, disappeared spots. In B and D, black circle, expression-increased spots compared to control under same treatment; white circle, decreased spots; black arrow, newly appeared spot.

disease in mycorrhizal plants have not been clarified. In the present study, growth of mycorrhizal cyclamen under SHS was greater than control, and AMF plants showed greater increases in dry weight of leaves than other parts. This phenomenon might be caused by an allocation of carbohydrate by AMF in shoots that was proportionally greater than tuberous tissues and below ground tissues (Shokri and Maddi, 2009). Generally, temperature influences mycorrhizal symbiosis and growth promotion (Zhu *et al.*, 2011) i.e. high temperature caused negative effect or no effect on mycorrhizal colonization (Li *et al.*, 2008; Compant *et al.*, 2010). In the present study, no significant differences occurred in AMF colonization levels, meaning heat stress might affect mycorrhizal symbiosis less.

Although many reports suggest that AMF had induced disease resistance to host plants, most of them refer to soil-borne disease interactions (Whipps, 2004; Li *et al.*, 2006). In contrast to soil-borne diseases, shoot disease resistance induced by AMF is rarely reported. However, some study have shown AMF alleviated shoot disease and induced systemic acquired resistance (SAR) (Campos *et al.*, 2012). As for anthracnose caused by *Colletotricum gloeosporioides*, one study demonstrated that AMF symbiosis suppressed anthracnose in cyclamen (Maya and Matsubara, 2013 b). In this experiment, mycorrhizal cyclamen plants also showed resistance to anthracnose even under the shock heat stress condition. Hence, it is supposed that AMF induced cross protection to heat stress and anthracnose in the cyclamen examined in this study.

Biotic (pathogen, nematode etc.) and abiotic stresses (heavy metal, drought, high temperature etc.) cause reactive oxygen species (ROS) production in plant tissues. Although ROS have a positive role as a signal which triggers stress responses, it causes destructive damage to organs in cells and a loss of homeostasis. In order to limit oxidative damage and remove ROS, plants have developed a detoxification system through changing antioxidative activity. Antioxidative activity includes enzymatic and non-enzymatic antioxidants where SOD is a key enzyme in the enzymatic antioxidation system, and non-enzymatic antioxidation is also an effective defense system, because it efficiently prevents accumulation of ROS under stress conditions. There are reports suggesting that antioxidative modifications in mycorrhizal plants refer to AMF-induced stress resistance (Nahiyan and Matsubara, 2012; Lambais *et al.*, 2003). However, as for antioxidative changes in host plants, both increases and decreases in activity has been reported (Roldan *et al.*, 2008). Generally, ROS is produced in roots under heat stress (Kolupaev *et al.*, 2013). Increases in SOD activity in roots of mycorrhizal cyclamen under heat stress have also observed (Maya and Matsubara, 2013 a). In this study, the same phenomenon occurred in mycorrhizal cyclamen. In addition, as for DPPH radical scavenging activity that means total activity of non-enzymatic antioxidants: ascorbic acid, polyphenols, flavonoid etc., DPPH radical scavenging activity increased in roots of mycorrhizal plants in this experiment. Some investiga-

tions have shown that AMF-induced increases in DPPH radical scavenging activity under salinity stress and pathogen infection (Hichem *et al.*, 2009; Nahiyan and Matsubara, 2012). Although decreases and increases occurred in leaves and bulbs in this experiment, the decrease might be caused by the resolution of ROS by such increased antioxidants.

Some investigators have reported that AMF has enhanced activity of various defense mechanisms as well as antioxidants. Campos *et al.* (2012) reported that various genes which regulate stress response in plants had were up-regulated in mycorrhizal rice (*Oryza Sativa* L.), and suggested that salicylic acid (SA)-dependent and jasmonic acid (JA)-dependent mechanisms associate with AMF-induced disease resistance. They also implied that systemic acquired resistance (SAR) is caused in mycorrhizal plants. In the present experiment, expression of many protein spots changed following heat stress and CG inoculation. Moreover, expression levels of some spots also changed by AMF. In this case, 4 d after 20°C, an intensive increase in six spots and three newly appeared spots (G, H and I) were recognized in AMF plants. These spots may supposed to be associated with growth promotion through symbiosis. Although these changes might be defense response to AMF colonization itself, which is caused temporary in initial phase of AMF colonization (Campos *et al.*, 2010). At 7 d after SHS, an intensive increase in 12 spots and decrease in three spots (36.0 kDa) was visible in control plants compared to 20°C. Spots I and J, which were visible in the AMF treatment at 20°C, were expressed by SHS treatment. In mycorrhizal plants, 13 spots increased and especially six spots showed greater increase; spot G (20.4 kDa) was still visible at SHS treatment. After CG inoculation, eight spots increased intensively in non-mycorrhizal plants. Compared to control plants, mycorrhizal plants showed considerable increase in 13 spots and spot G was still detected. In this study, proteins which were up-regulated under both SHS treatment and CG inoculation were L (27.3 kDa), O (19.5 kDa), V (16.9 kDa) and Y (36.3 kDa). Especially, V and W showed greater expression levels in mycorrhizal plants than in the control under both stress conditions. Yoshino *et al.* (2012) reported that heat shock treatment induced tolerance to disease and increased free SA. Hence, it is supposed that these four spots are related with both heat stress tolerance and anthracnose resistance induced by AMF.

In conclusion, AMF induced cross-protection to shock heat stress and anthracnose in this study, and the defense systems could include antioxidative modification and changes in some protein expressions associated with such stress tolerances.

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