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DO ARBUSCULAR MYCORRHIZAL FUNGI AFFECT METALLOTHIONEIN *MT2* EXPRESSION IN *BRASSICA NAPUS* L. ROOTS?

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Arbuscular mycorrhizal fungi are the most widespread root fungal symbionts, forming associations with the vast majority of plant species. Ectomycorrhizal development alters gene expression in plant symbionts. In this work we examined the effect of arbuscular mycorrhizal fungi spores on the growth and development of *Brassica* and on the expression of *BnMT2* in winter rape. In a pot experiment, rape seedlings growing on different types of sterile and nonsterile soils were inoculated simultaneously with mycorrhizal fungi spores of *Acaulospora longula, Glomus geosporum, Glomus mosseae* and *Scutellospora calospora*. As compared with control plants growing in the absence of spores, ten-week-old seedlings of *Brassica napus* L. in sterile soil inoculated with arbuscular spores had longer shoots and higher fresh biomass of above-ground plant parts. In other types of substrates enriched with mycorrhizal fungi spores, the plants were smaller than non-inoculated plants. The presence of AMF spores stimulated the elongation growth of hypocotyls in both analyzed substrates. *BnMT2* expression was highest in plants growing on the sterile substrate. Generally, the presence of mycorrhizal fungi spores appeared to have an adverse effect on the growth of rape plants.

Key words: Oilseed rape, mycorrhiza, gene expression, metallothionein.

INTRODUCTION

Many plants, including crops, are exposed to different unfavorable biotic and abiotic factors of the soil environment (e.g., the presence of antagonistic microorganisms, nutrient deficiency) which can induce stress in their cells. Stress genes can be activated in plant cells in response to those conditions. The end-products encoded by these genes include peroxidases (POD) (Dąbrowska et al., 2007), catalases (CAT), dismutases (SOD) (Scandalios, 1993; Zhao et al., 2010) and metallothioneins (MT) (Koszucka and Dąbrowska, 2006). Plant metallothioneins encoding low-molecular weight proteins have cysteine residues (Cys) gathered in two terminal domains separated by the central region 40 amino acids long. Four different types of plant MTs are distinguished according to the quantity and arrangement of Cys residues: type 1 (12 cysteine residues in Cys-X-Cys motifs), type 2 (14 cysteine residues in Cys-X-X-Cys and Cys-Cys motifs), type 3 (10 cysteine residues in Cys-X-Cys motifs) and type 4 (three domains rich in cysteine residues) (Cobbett and Goldsbrough, 2000; Robinson et al., 1993).

The expression of genes encoding plant metallothioneins is induced under the influence of both endo- and exogenous factors, such as heavy metal ions (Ma et al., 2003), high/low temperature, strong light (Dunaeva and Adamska, 2001), darkness (Chen et al., 2003), oxidative stress (Mir et al., 2004), pathogen attack (Choi et al., 1996) and the presence of rhizosphere microorganisms (Hrynkiewicz et al., 2011).

Most terrestrial plant species, including several crops, are colonized by mycorrhizal fungi (Smith and Read, 1997; Jansa et al., 2003). Arbuscular mycorrhizal (AM) symbiosis is a mutually beneficial interaction and is an integral component of the natural ecosystem (Gard and Chandel, 2010). AM fungi form structures inside root cells (arbuscules), which participate in nutrient exchange between the fungal symbiont and the host plant (Read, 1999). AM fungi increase nutrient uptake and the growth of the host plant (Miller, 2000). The positive influence of inocu-

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lation with mycorrhizal fungi spores on agricultural plant growth has been confirmed in *Lycopersicon esculentum* Mill. and *Capsicuum annuum* L. (Olsen et al., 1999), *Zea mays* L. (Jansa et al., 2003) and *Avena sativa* (Khan et al., 2003). Members of the families Brassicaceae, Chenopodiaceae and Amaranthaceae are exceptions, and form only sparse or no mycorrhizae (Varma et al., 1999; Singh et al., 2003). Rape (*Brassica napus* L.) is one of the plants that form no mycorrhizae at all (Smith and Read, 1997). Thus, AM fungi might be expected to affect *B. napus* negatively. The effect of AM fungi on the growth of non-mycorrhizal plants and mechanisms of stress tolerance of such plants is poorly known at present.

Brassica napus is the world's third most commonly grown vegetable oil crop. It is cultivated in Northern Europe, including Poland. Canola is cultivated for edible oil and for biofuel production, and can be used for phytoextraction of heavy metals (Turan and Esringü, 2007). Since rape is cultivated very often in soils where mycorrhizal plants grew previously (e.g., maize, wheat), the fungal spores might affect the non-mycorrhizal plants. In this study we examined (i) the effect of AM fungal spores in substrate on the growth and development of winter rape (*Brassica napus*), and (ii) the expression of the stress protein metallothionein MT2 in their cells.

MATERIAL AND METHODS

GROWING CONDITIONS

Seeds of *Brassica napus* L., winter variety Kronos, were rinsed with 20% sodium hypochlorite (5 min) and with sterile distilled water (30 min) to sterilize them and to remove the fungicide. Thirty seeds each were sown in pots (diam. 15 cm) filled with soil (K: 250–350 mg l^{-1}, N: 150–250 mg l^{-1}, P: 100–200 mg l^{-1}, pH 6.0–6.5) or a 2:1:1 mixture of peat, sand and vermiculite. Two variants of the same substrate, sterile and nonsterile, were used in the pot experiment to investigate the impact of autochthonous microorganisms. The soil was sterilized at 0.75 atm (15 min).

Three days later, spores of AM fungi *Acaulospora longula, Glomus geosporum, Glomus mosseae* and *Scutellospora calospora* (INVAM, International Culture Collection of Arbuscular Mycorrhizal Fungi; ~20 spores of each fungus) were introduced to the pots with growing *B. napus* seedlings. Inoculation with AM spores was repeated during the next 3 days. The control consisted of non-inoculated plants. Three replicates (pots) were prepared for each variant of the experiment (90 plants per variant, 360 plants total). The plants were watered with sterile distilled water. Growth conditions were 25±1°C and a 16 h photoperiod. After 10 weeks the growth parameters of the plants were measured: shoot weight (g), hypocotyl length (cm) and number of nodes.

ISOLATION AND QUANTIFICATION OF NUCLEIC ACIDS

Total RNA was isolated from 100 mg root tissue of *B. napus* according to Chomczyński and Sacchi (1987).

Plasmid DNA containing the *Brassica* metallothionein gene *BnMT2* fragment was isolated on columns in the Gene Matrix Plasmid Miniprep DNA Purification Kit (Eurx, Gdańsk, Poland). The quantity and quality of the isolated nucleic acids were checked spectrophotometrically with a GeneQuant DNA/RNA Calculator (Pharmacia, U.S.A.).

PREPARATION OF THE MOLECULAR PROBE

PCR-based amplification of the *BnMT2* gene sequence was performed using the PCR DIG Probe Synthesis Kit (Roche, Switzerland). Besides the components included in the kit, the mixture for the PCR reaction contained 100 ng plasmid DNA and starters BnMet2-for (5'-tcaatttgattaacattctctgct-3') and BnMet2-rev (5'-aagcctgcagccattattaca-3') (0.2 μM). PCR conditions: 95°C – 5 min, 30 cycles (95°C – 30 s, 53°C – 30 s, 72°C – 45 s), 72°C – 10 min. The digoxigenin-labeled *BnMT2* DNA sequence constituted the molecular probe for hybridization.

NORTHERN HYBRIDIZATION

Total RNA (10 μg) was separated in 1.2% denaturing agarose gel with 6% formaldehyde at 13°C. After electrophoresis, RNA was transferred to a Hybond-N+ nylon membrane (Sigma-Aldrich, Germany) by the standard capillary method according to the protocol of Sambrook et al. (1989). The membrane was exposed to UV radiation to bind RNA (GS Gene Linker, BioRad). Prehybridization and hybridization were conducted in DIG Easy Hyb buffer (Roche, Switzerland) at 50°C. Total products of the 400 bp PCR reaction, labeled with digoxigenin, were denatured at 100°C for 5 min and used as molecular probe for hybridization conducted over 16 h. Unbound molecular probe was removed by washing the membrane in Low Stringency Buffer (5 min at 25°C) and in High Stringency Buffer (Roche, Switzerland) (15 min at 50°C). Washing was done twice. Detection was done according to the manufacturer's protocol for the DIG Nucleic Acid Detection Kit (Roche, Switzerland). The membrane was exposed to a plate for 12 h at -80°C. The hybridization reaction was run twice. The hybridization signals were digitized and quantified with Image GaugeTM ver. 3.46 (Science Lab Image Analysis Software, Fuji Science, Tokyo, Japan).

TABLE 1. Effect of interaction between soil type (substrate) used for plant growth (NST – nonsterile; ST – sterile) and inoculation with AM spores (Ctr – non-inoculated; AM – inoculated with AM spores) on fresh biomass (g), hypocotyl length (mm) and number of nodes of *Brassica napus* (ANOVA2).

Factor	Fresh biomass (g)			Length of hypocotyl (mm)			No. of nodes		
	ST Effect	F	p-level	ST Effect	F	p-level	ST Effect	F	p-level
(1) Soil	34.36	46.61	0.0000*	262.81	4.01	0.0488*	9.11	34.37	0.0000*
(2) Inoculation	9.56	12.96	0.0006*	4697.11	71.65	0.0000*	2.11	7.97	0.0061*
(1) × (2)	0.00	0.00	0.9803	262.81	4.01	0.0488*	1.01	3.82	0.0543
Error									
Newman–Keuls test									
(1) Soil	NST	3.6590 b		NST	57.8750 b		NST	6.05 b	
	ST	2.3483 a		ST	54.2500 a		ST	5.38 a	
(2) Inoculation	Ctr	3.3493 b		Ctr	48.4000 a		Ctr	5.88 b	
	AM	2.6580 a		AM	63.7250 b		AM	5.55 a	

* $p \leq 0.05$, F – ratio of MS (effect) to MS (error), MS – mean square, n=90.
Values with different letters differ significantly by the Newman-Keuls test.

RESULTS AND DISCUSSION

Brassica napus is a non-mycorrhizal plant, so the presence of AM mycorrhizal spores in the soil might be expected to affect plant growth and the harvest. In our pot experiments the presence of AM spores in the soil had a significant effect on the growth parameters of *Brassica napus*. Inoculation of plants with AM spores significantly decreased fresh biomass production and the number of nodes (Tab. 1, Fig. 1a,b). The only parameter that was increased by inoculation was plant length, but together with decreased biomass this result also suggests a negative effect of AM spore inoculation: plant elongation in response to stress (as occurs, for example, in response to light deficit) (Tab. 1, Fig. 1c). We also note a positive effect of other microorganisms naturally occurring in the substrate in this pot experiment. In nonsterile substrate all the investigated plant parameters (fresh biomass, hypocotyl length, number of the nodes) were significantly higher than in sterile soil.

The inability of some plants to establish associations with AM has not yet been satisfactorily explained, but there has been some work in that direction. *Brassica*ceae roots have been found to release secondary plant compounds or glucosinolates which reduce the germinability of AMF spores (Schreiner and Koide, 1992; Kabir et al. 1996). Metabolic processes of rape connected with secretion of substances that inhibit AMF growth decrease their growth in general. Watrud et al. (2011) suggest that increased representation of *Brassica* within plant communities may indirectly harm beneficial ecosystem dependency associated with arbuscular mycorrhiza via glyphosate (herbicyde-resistant) drift which leads to the expression of the *CP4 EPSPS* transgene. Incorporation of *B. napus* residues in soil had no negative effect on the colonization of maize roots by mycorrhizal fungi (Pellerin et al., 2007). To our knowledge, however, the direct impact of mycorrhizal fungi on biomass production and stress response in *B. napus* has not been investigated previously.

Expression of MTs in plants can be altered by the formation of ectomycorrhizae. The metallothionein gene was downregulated in the *Eucalyptus globulus-Pisolithus tinctorius* ectomycorrhizal association (Voiblet et al., 2001). In our experiments the molecular probe labeled with the digoxygenin detected a transcript 400 bp long, the size of rape *BnMT2* (Fig. 2b). *BnMT2* expression in winter rape was highest in plants growing in sterile soil (ST); in nonsterile soil (NST) no or very low (<1.0) expression was detected. mRNA amounts of *BnMT2* were at similar levels in plants growing in the sterile soil with or without AM spores (Fig. 2a–c); there was no significant correlation with the presence of arbuscular mycorrhizal fungi in the soil. *PaMT1-3* expression in *Populus alba* L. leaves has been found to depend on inoculation with AMF (*Glomus mosseae, G. intraradices*) and on substrate type (unpolluted vs. heavy metal contaminated) (Cicatelli et al., 2010). In our earlier studies, *SvMT1* molecular probes of *Salix viminalis* did not detect transcripts of the metallothionein in plants growing in soil without heavy metals. In leaves of *S. viminalis* from soil with heavy metals and containing spores of *Hebeloma mesopheum* there was no *SvMT1* expression (Hrynkiewicz et al., 2012). Transcripts of metalloth-

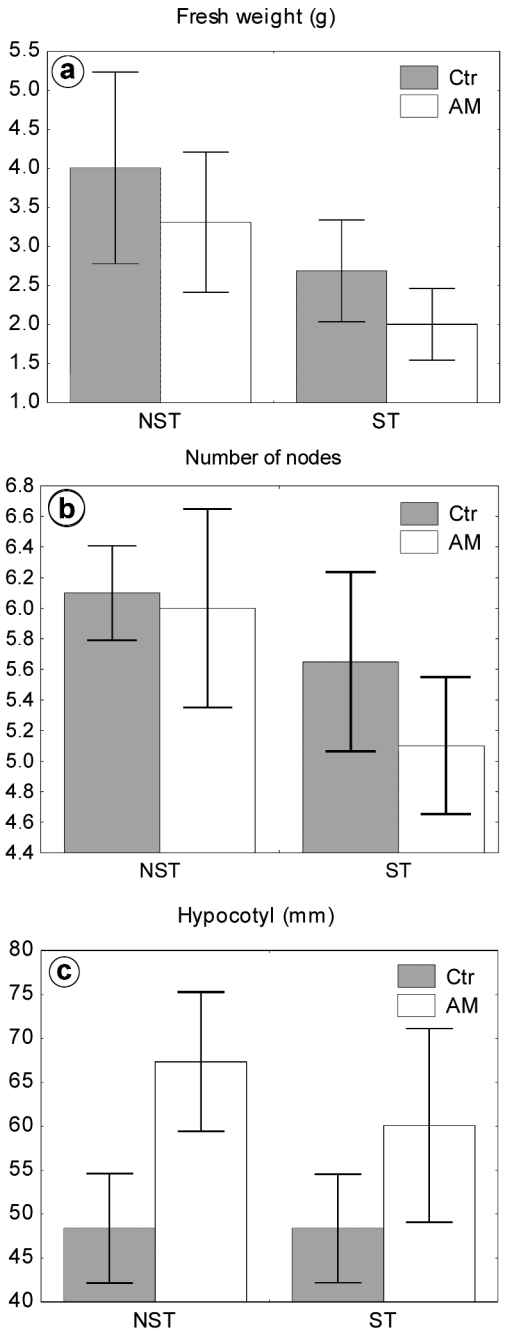

Fig 1. Effect of AM spores on growth parameters of 10-week-old *Brassica napus* plants in pot experiment. NST – nonsterile soil; ST – sterile soil; Ctr – soil without AM spores; AM – soil with AM spores; mean ± SE, n=90

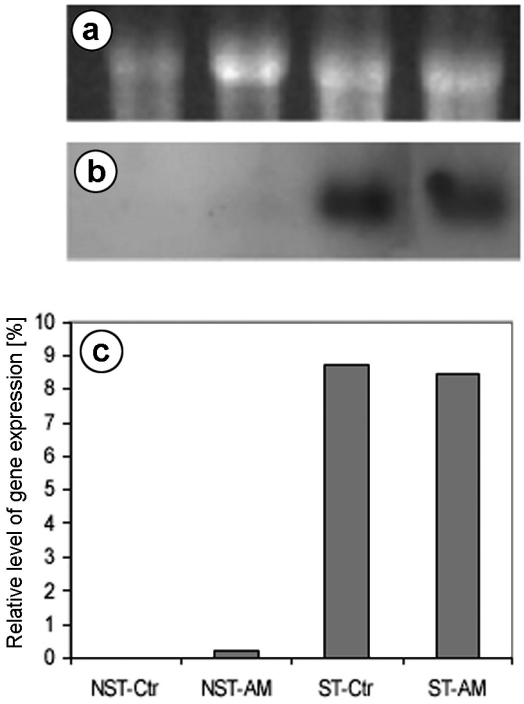

Fig 2. (**a**) Electrophoretic separation of total RNA in agarose gel stained with ethidium bromide. Lanes show RNA isolated from roots of control plants and growing in the presence of AM spores cultivated in nonsterile soil (NST-Ctr, NST-AM) and sterile soil (ST-Ctr, ST-AM), (**b**) Results of molecular probe hybridization for type-2 metallothionein in *Brassica napus* (*BnMT2*) for transcripts from experimental variants, (**c**) Relative level of *BnMT2* gene expression in roots of winter rape for each sample as assessed from differences in amounts of RNA separated on gel.

ionein *PsMTA* increased in roots of *Pisum sativum* cv. Frisson growing in the presence of *G. intraradices* as compared with plants growing in the absence of AMF (Rivera-Becerril 2005). On the other hand, Ouziad et al. (2005) found no significant differences in expression levels of metallothionein *Lemt1*, *Lemt3* and *Lemt4* in roots of tomatoes grown in nonsterile, heavy metal-polluted soil with or without the presence of AM fungi. However, *Lemt2* was strongly expressed only in non-AMF-colonized roots of plants growing in the presence of heavy metals. AMF colonization distinctly reduced the level of *Lemt2* transcripts.

Here we confirmed that the level of *BnMT2* transcripts depends significantly on substrate (whether sterile or nonsterile). We suggest that the absence/presence of microorganisms other than AMF in the NST/ST variants, such as rhizosphere bacteria, may be a key factor in *BnMT2* expression in *B. napus* roots. Further experiments should verify this suggestion.

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