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Medicago truncatula Rop GTPases expression in young nodules

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ABSTRACT

Small GTPases are monomeric guanine nucleotide binding proteins with molecular weight of 21 to 30 kD. Plants have plant-specific small GTPases, termed Rop (Rho GTPases from plants) which serve as signaling proteins in plant signal transduction. To have better understanding on nodulation process in legume, which Rop proteins that play a dominant role in nodule formation would be determined. Therefore, the expression of five ***Medicago truncatula*** *Rop GTPase genes (MtRops) in young nodules were studied in this experiment. RT-PCR method was used to examine the expression of MtRops. The MtRops expression in inoculated* ***M. truncatula*** *plants were compared with those in uninoculated plants. Results of this studies showed that the expression of MtRop5 and MtRop6 were significantly induced in young nodules, it was suggesting that these Rops may have important role during nodule formation.*

Key words: *nodule, Rop, small GTPase*

INTRODUCTION

The symbiotic interaction between nodule bacteria (collectively called rhizobia) and legume results in mature nitrogen fixing nodules. Nodulation process involves differentiation, cell division, gene expression and organ development. Those processes are resulted from a variety of signaling processes.

In Arabidopsis, Rop GTPases, a group of signaling proteins, participates in the signaling of multiple distinct developmental processes, such as embryo development, seedling development and morphogenesis. Further, the expression of several *Rop* genes in all part of Arabidopsis plants indicates that Rop has a broad role in the signaling of various plant processes (Li *et al.,* 2001). Thus, it is suggested that Rop GTPases also play a role in nodulation.

Before investigate the role of Rop GTPase in nodulation process, it is necessary to know which Rop GTPase is expressed in nodules, especially in young nodules which are still undergone developmental stages to form mature nodules. The expression of a specific Rop GTPase indicates its role during nodulation. Therefore, the expression of Rop GTPases in young nodules on *Medicago truncatula* was studied here.

MATERIAL AND METHODS

Material

Materials used in this research were *Medicago truncatula* ecotype Sickle, *Agrobacterium rhizogenes* MSU440 and *Rhizobium meliloti* 2011.

Agrobacterium rhizogenes MSU440 were grown in TY medium containing NaCl 8 g/l, trypton 10 g/l, yeast extract 5 g/l, antibiotic used was kanamycin 50 µl/l.
Rhizobium meliloti 2011 were grown at 30° C in YEM medium containing mannitol 5 g/l, sodium gluconate 5 g/l, yeast extract 0.5 g/l, $MgSO_4.7H_2O$ 0.2 g/l, K_2HPO_4 0.5 g/l and supplemented with 16.6% $CaCl_2$ 1 ml/l.

Plant Growth

The *M. truncatula* seeds were surface sterilized by incubating for 10 minutes in concentrated sulphuric acid and 6× washed in sterile water and 10 minutes in 4% chlorite bleach and 7× washed in sterile water, and then seeds were plated on Färhaeus medium. Seeds were cold shocked overnight at 4° C and placed in dark for one day at 25° C to germinate. The seedlings were put in Färhaeus plates and the plates were partially sealed with parafilm to allow gas exchange. The plates were incubated in growth chamber in a vertical position with 16-h daylight period at 21° C and 70–80% relative humidity. On the 8th day, the seedling were transferred into the gravel system. The plants were divided into two groups, i.e., inoculated and uninoculated group. The inoculated group was inoculated with *R. meliloti* after the 3rd leaf were grown. On the 5th day of postinoculation, young nodules were harvested from inoculated plants and the same part of root were taken from the uninoculated plants. The total RNA was extracted from the nodules.

RNA Extraction

The RNA extraction was done by preparing a 1:1 mixture of RNA extraction buffer (100 mM LiCl, 1% SDS,

100 mM Tris-HCl pH 9.0, 10 mM EDTA) and phenol, then heated at 90° C in a waterbath in a fume hood. The harvested material was grinded in liquid N_2 in a pre cooled mortar and was pestled until a fine homogenous powder was obtained. The frozen powder was transferred to the plastic tube with the aid of a metal or plastic spatula cooled in liquid N_2. Two ml of well mixed phenol/extraction buffer was added per g fresh weight of plant material. The mixture was vortexed for 5 minutes and centrifuged at 4000 rpm for 30 minutes at room temperature. The aqueous layer was transferred to a fresh tube. Then, chloroform with equal volume was added, the mixture was vortexed 10–30 seconds, centrifuged at 3000–40000 rpm for 10 minutes. The aqueous layer was transferred to new tubes. This was done twice. 8 M LiCl (cold, kept in –20° C or 4° C) was added to final concentration 2 M and the mixture was incubated at 4° C for 4–16 hours. The mixture was spinned at 12000 rpm for 20 minutes at 4° C. The supernatant was discarded, all LiCl was removed (an extra, short centrifugation might needed). The resulting pellet was washed with 1 ml 70% ethanol (–20° C) and was vortexed for few seconds, centrifuged at 13000 rpm for 10 minutes. This was done twice. Then the pellet was vacuum dried. The resulting RNA was dissolve in 50 ml H_2O (double distilled water), 2 ml DNase and 10 ml DNase buffer were added. The mixture was incubated for 45 minutes at 37° C. 3 M sodium acetate pH 5.2 was added to final concentration 0.3 M. Chloroform with equal volume was added, the mixture was vortexed for 1 minutes, then centrifuged at 4000 rpm for 10 minutes. The resulted aqueous layer was transferred to new tubes, and 2.5 volume 100% ethanol (–20° C) was added. The mixture was vortexed and incubated at –20° C for 30 minutes. Then the mixture was centrifuged at 14000 rpm for 25 minutes at 4° C. All supernatant were removed (an extra, short centrifugation could be needed). The pellet was washed with 70% cold ethanol, centrifuged at 14000 rpm for 5 minutes at 4 ° C. All supernatant were removed and vacuum dried. The pellet was dissolved in double distilled water and stored at –20° C.

cDNA Synthesis

cDNA was synthesized from the total RNA. The cDNA was synthesized by oligo dT and M-MuLV, the following mixtures were pipetted together in to an eppendorf tube: 4 µl RT buffer (5× conc: 250 mM Tris/Cl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µl 0.1 M DTT, 2 µl 10mM dNTP, 1µg oligo(dT)$^{12–18}$, 2.5 mg RNA, H_2O to a total volume 18.5 µl, 0.5 µl RNAguard (40 U/µl), 1 µl M-MuLV (200 U/µl). The mixture was incubated for 1 hour at 37° C. The synthesis was stopped by heating the sample for 5 minute at 95° C. The mixture was centrifuged for a few second. The cDNA was diluted to a total volume of 100 ml. The cDNA from both groups were diluted 2, 25, and 125 times. *MtRop1*, *MtRop2*, *MtRop3*, *MtRop5* and *MtRop6* were amplified using specific primers (Table 1.) by standard PCR protocol. A 10 ìl PCR product was electrophoresized on agarose gel then blotting, hybridization, and washing were carried out.

Table 1. Primers for RT-PCR (Isogen)

Primer name	Direction	Sequence
MtRop1 3Uf	5 → 3	TCCTCTTCTGCTGAAGAAG
MtRop1 3Ur	3 → 5	ATGTTAGAAAAAGGTGGG
MtRop2 3Uf	5 → 3	TTCCCCTCCCCTTCTTTTC
MtRop2 3Ur	3 → 5	GAGTTGACAATAATAATTCATAG
MtRop3 3Uf	5 → 3	AGGATGACATGACAGTAGC
MtRop3 Ur	3 → 5	TCAGCCAGAGACAAATAGC
MtRop5 5Uf	5 → 3	TTGCCTTATTAAATGCTTCACC
MtRop5 5Ur	3 → 5	GATGGAAACAATAAACGGTAG
MtRop6 5Uf	5 → 3	TCTTCATTTACTTCACACTCTTC
MtRop6 5Ura	3 → 5	GGTGATGGTGCTGTTGGAAAAA

RESULTS

To examine the expression of *MtRop*s in young nodules, total RNA were isolated from inoculated and uninoculated *M. truncatula* ecotype Sickle plants. Then cDNA was synthesized from the total RNA. The RT-PCRs of the resulted cDNA was summarized in Figure 1.

As can be seen from Figure 1., it is apparent that the expression of *MtRop5* and *MtRop6* increase significantly after inoculation, while *MtRop2* is slightly increase. On the contrary, *MtRop3* and *MtRop1* expression were maintained at the same level. A control hybridization with actin probe has been included to show that RNA loadings were approximately equal.

DISCUSSION

So far, the eleven *Arabidopsis thaliana Rops* (*AtRops*) are the best characterized among the plant Rops. Phylogeneticaly, *AtRop*s are divided into four groups which seem to be functionally different (reviewed in Yang, 2002). Group I has only one member that does not have any known function. Group II has been involved in stress responses since over expression of this group causes an alteration of ABA responses or H_2O_2 production (Yang, 2002; Kawasaki *et al.*, 1999). Group III from the cotton Rops is involved in H_2O_2 production (Potikha *et al.*, 1999). Six out of eleven Arabidopsis Rops belong to group IV which is also the

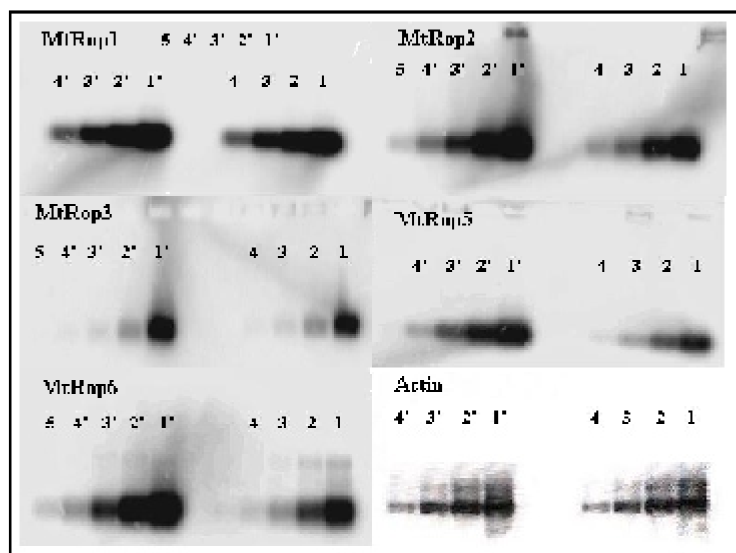

Figure 1. The Expression level of MtRops in the young nodule.

1: uninoculated, undiluted; **2**: uninoculated, 5 × diluted;**3**: uninoculated, 25 × diluted; **4**: uninoculated, 125 × diluted; **1'**: inoculated, undiluted; **2'**: inoculated,5 × diluted; **3'**: inoculated, 25 × diluted; **4'**: inoculated, 125 × diluted; **5**: water control

largest group, i.e., *AtRop1*, *AtRop2*, *AtRop3*, *AtRop4*, *AtRop5* and *AtRop6*. *AtRop1*, *AtRop3* and *AtRop5* are expressed in pollen tubes and are required for pollen tube growth (Kost *et al*., 1999; Li *et al*., 1999); while *AtRop2*, *AtRop4* and *AtRop6* are expressed in vegetative cells and are involved in both root hair tip growth (Molendijk *et al*., 2001; Jones *et al*., 2002) and expansion of cells in various tissues (Li *et al*., 2001; Fu *et al*., 2002).

Previous research has indicated the occurrence of 7 different *Rop* genes in *Medicago truncatula* (Mirabella, unpublished data). Compared to *AtRop*s that are the best characterized Rops in plants, *MtRop7* and *MtRop6* have the highest homology to *AtRop*s of group II, the group which appear to be involved in stress responses. *MtRop4* and *MtRop5* are comparable to group III. The other 3 Rops, *MtRop1*, *MtRop2* and *MtRop3* correspond to the *AtRop*s of group IV. In addition, *MtRop1* and *MtRop2* have the highest resemblance to *AtRop1* (96%) and *AtRop5* (95%) respectively.

From the EST data, it is suggested that *MtRop1*, *MtRop3* and *MtRop5* are expressed in both root and nodules (Table 2.). Meanwhile, *MtRop2* is expressed in root but not in nodules and on the contrary, *MtRop6* is expressed in nodules but not in root. Moreover, both *MtRop4* and *MtRop7* are unlikely to be expressed in root or nodules (Mirabella, unpublished data). Because of that reason, in examining the expression of *MtRop*s in young nodules, this study was focused in *MtRop1*, *MtRop2*, *MtRop3*, *MtRop5,* and *MtRop6*.

Table 2. The results of blast search for *MtRop* genes (Mirabella, unpublished data)

Origin of banks	Nr. of banks	Nr. of ESTs	TC51785/ MtRop1(%)	TC51784/ MTRop2 (%)	TC44847/ MtRop3 (%)	TC54755/ MtRop4 (%)	TC45588/ MtRop5 (%)	TC56250/ MTRop6 (%)	BF647926/ MtRop7 (%)
Roots various	9	29766	0.17	0.1	0.1	0	0.067	0	0
Roots mycorhiza	2	15196	0.31	0	0	0	0	0	0
Roots rhizobium	3	10066	0	0	0	0	0	0	0
Nodules	5	20641	0.096	0	0.096	0	0.096	0.048	0
Stems	1	10371	0	0	0	0.19	0.19	0	0
Leaves various	4	22356	0.044	0.132	0.132	0	0.044	0	0
Leaves fungal	1	5987	0.02	0	0	0.02	0	0	0
Leaves insect	1	10002	0	0.01	0	0	0	0	0
Planlets	2	13588	0.22	0	0	0	0	0	0
Flowers	1	6656	0	0	0.15	0.15	0	0.15	0
Seeds/Pods	4	8577	0.23	0	0.115	0.115	0	0	0
Cell cultures	1	9178	0.1	0	0	0	0	0	0.1

The RT-PCR of *MtRop*s showed that *MtRop5* and *MtRop6* expression were significantly induced in young nodules, it is suggested that these Rops may have specific function during nodule formation. Therefore, in the future, it is worthy to do phenotypic analysis on *MtRop2*, *MtRop5* and *MtRop6* during nodule development.

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