Cloning and Expression Analysis of 1 L-*myo*-Inositol-1-phosphate Synthase Gene from *Ricinus communis* L.

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A novel 1 L-*myo*-inositol-1-phosphate synthase (MIPS, EC 5.5.1.4) gene, designate *rcMIPS*, was cloned from *Ricinus communis*. It contained an open reading frame (ORF) of 1669 bp coding for a peptide of 510 amino acids with a molecular mass of 56 kDa. Sequence anaylsis showed high homology compared to other plant MIPS genes, because it contained typical domains owned by MIPS enzymes. The transcript levels of the *rcMIPS* gene in leaves, stems, and roots were examined after drought stress for 24, 48, and 72 h. The transcript levels in the leaves, stems, and roots increased significantly compared to the control. Results of the enzyme assay showed a significant correlation between the changes of enzyme activity and the transcript levels of the *rcMIPS* gene in different organs. Decreased relative water contents (RWC) and increased malondialdehyde (MDA) contents in the leaves represented a stress response against drought stress. Our findings suggest that MIPS plays an important role in the defensive mechanisms of *R. communis* against drought stress.

Key words: Ricinus communis L., 1 L-*myo*-Inositol-1-phosphate Synthase (MIPS), Gene Expression, Drought Stress

Introduction

1 L-*myo*-Inositol-1-phosphate synthase (EC 5.5.1.4, MIPS), an evolutionarily conserved enzyme protein, catalyzes the conversion of D-glucose-6-phosphate into 1 L-*myo*-inositol-1-phosphate (MIP), which is the first and rate-limiting step in the biosynthesis of all inositol-containing compounds (Ray Chaudhuri *et al*., 1997; Abid *et al*., 2009). MIPS is found in diverse organisms, both eukaryotic and prokaryotic, suggesting that the pathway for 1 L-*myo*-inositol-1-phosphate from D-glucose-6-phosphate arose early in the evolution of life. The properties and generally accepted catalytic mechanisms of the enzyme are similar in all organisms where such assessment has been undertaken (Bachhawat and Mande, 2000).

MIPS-encoding sequences represent multi-gene families in some plant species. The multiple MIPS genes in plant species may be applied to attune its differential expression to specific physiological functions (Abid *et al*., 2009). MIPS-coding sequences have been isolated and characterized from a number of plant species, such as chickpea (Kaur *et al*., 2008), *Arabidopsis* (Johnson and Sussex, 1995), *Phaseolus vulgaris* (Johnson and Wang, 1996), and *Sesamum indicum* (Chun *et al*., 2003). Recently, some experimental data have demonstrated that the expression levels of the MIPS gene might be up-regulated and the MIPS activity increased when plants were exposed to strong light treatment and salinity stress as well (Ray Chaudhuri and Majumder, 1996; Ishitani *et al*., 1996; Chun *et al*., 2003). In addition, studies of MIPS in *Arabidopsis* and *Glycine max* using an RNAi approach revealed the critical role of the MIPS genes in early seed development through the regulation of endosperm formation (Nunes *et al*., 2006; Chiera and Grabau, 2007; Mitsuhashi *et al*., 2008). Although MIPS has been characterized in some plant species, identification of its gene and function is still to come.

Ricinus communis L., belonging to the Euphorbiaceae family, is widely distributed in tropical and partially in subtropical areas. Various parts of the plant hold potential for the use as a source of lubricating oil and chemical materials (Sujatha *et al*., 2008). As shown by a previous review of the literature, little information concerning the MIPS gene and its transcription level in *R. communis* L. under drought stress is available. In the present study, a MIPS cDNA from *R. communis* L. was

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Table I. The primer sets used for PCR and detection of the mRNA levels.

Primer	Sequence (5'→3')	Base pair
Oligo(dT)$_{18}$	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)$_{18}$	20-mer
MipsL1	TGGAC(A,G)GC(A,C)AACAC(A,T)GA(G,A)(A,C)G	20-mer
MipsR2	TT(C,T)TG(C,A)AGCAT(A,T)GC(C,A)C(G,T)CTGC	21-mer
MIPS3'-1	GATAGCAAGAGAGCTATGGATG	22-mer
MIPS3'-2	GTTGATGACATGGTCTCTAG	20-mer
MIPS5'RT	GTTCTGTGGGCTTCCATTAATG	22-mer
MIPS5'-1	GATACAAGCCAAAGCATACAAG	22-mer
MIPS5'-2	CACAACAACATTACTGTAC	19-mer
ML	GTGAGCTTGCAATCTCAATC	20-mer
MR	GTATGTTTCACTTGTATTC	19-mer
MRL	ATGTTTATTGAGAATTTCAG	20-mer
MRR	TCACTTGTATTCCAAAATCATG	22-mer
18S1	AGAAACGGCTACCACATC	18-mer
18S2	CCAAGGTCCAACTACGAG	18-mer
RMRL	CTACTCTCACCGGTGGTGTTATAG	24-mer
RMRR	CCAAAGTAATTTGCTTGCTGC	21-mer

cloned and characterized, and its transcription levels and activity as well as the changes of relative water contents (RWC) and malondialdehyde (MDA) contents in leaves exposed to drought stress were examined.

Material and Methods

Plant materials and treatments

R. communis seeds were germinated in seedbeds containing perlite, peat moss, and sandy loam soil mixture (1:1:1, v/v/v), and irrigated daily with half-strength Hoagland solution. After a 20-d culture, healthy and uniform seedlings were selected, washed with distilled water, and then transplanted to vessels filled with half-strength Hoagland nutrient solution. The nutrient solution in the growth container was continuously renewed every day. After 7 d, the seedlings were separated into two lots. One lot was allowed to grow with half-strength nutrient solution to serve as control. Other lots were exposed to 30% PEG for 24, 48, and 72 h, respectively. Rotten and contaminated embryos were removed promptly. After culturing, the young leaves of *R. communis* were washed with double distilled water, blotted, and immediately frozen in liquid nitrogen or stored at –70 °C for analysis.

RNA isolation, PCR cloning and sequencing

Total RNA was extracted from leaves, stems, and roots using plant RNA isolation reagent (Tiangen Biotech Co., Ltd., Beijing, China) following the manufacturer's instructions. Genomic DNA was extracted from 5 g young *R. communis* leaves following the cetyltrimethyl ammonium bromide (CTAB) method (Sambrook and Russell, 2001). After DNase I treatment, total RNA (2 μg) was synthesized into the first-strand cDNA with oligo(dT)$_{18}$ (First-strand cDNA synthesis kit, TaKaRa Biotechnology Co., Ltd., Dalian, China). The degenerate primers, MIPSL1 and MIPSR2, were designed according to the highly conserved sequences of MIPS. Using three universal primers [oligo(dT)$_{18}$, AP1 and AP2] and five gene-specific primers (MIPS3'-1, MIPS3'-2, MIPS5'RT, MIPS5'-1, MIPS5'-2), the nucleotide sequences of the 3' and 5' ends of *rcMIPS* were amplified by the rapid amplification of cDNA ends (RACE) technique. The full-length cDNA was then amplified with two primers (ML and MR). MRL and MRR were used to amplify the open reading frame (ORF). All products were cloned into pMD18-T vectors (TaKaRa Biotechnology Co., Ltd.) and sequenced (Invitrogen, Shanghai, China). PCR amplifications in the present study were carried out with the specific primers described in Table I.

Fluorescence quantitative RT-PCR

A semi-quantitative RT-PCR method was used to evaluate *rcMIPS* mRNA levels. 18S ribosomal RNA (18S rRNA) (GenBank accession no. AY823528) was used as an internal standard in

the RT-PCR reaction. *rcMIPS* gene-specific primers (RMRL and RMRR) and 18S rRNA gene-specific primers (18S1 and 18S2) are presented in Table I. PCR reactions were carried out under the following conditions: one cycle at 94 °C for 4 min, 25 cycles at 94 °C for 30 s, at 55 °C for 30 s, and at 72 °C for 45 s, and one cycle at 72 °C for extra 7 min. The ratios of the quantity of mRNA for the *rcMIPS* gene to that for 18S rRNA were calculated, and the results were considered to reflect the relative quantities of the *rcMIPS* mRNA level.

Assay of 1 L-myo-inositol-1-phosphate synthase

Leaves, stems, and roots (5 g) of *R. communis* were homogenized in 50 ml ice-cold buffer A [20 mM Tris-HCl (pH 7.5), 10 mM NH_4Cl, 10 mM β-mercaptoethanol, 2 mM PMSF]. The homogenate was centrifuged at 28,000 × *g* at 4 °C for 45 min. The supernatant was stored at 4 °C for the protein concentration assay and enzyme activity assay. The enzyme was assayed colorimetrically by the periodate oxidation method of Barnett *et al.* (1970). One unit of enzyme activity is definite as the amount (in μmol) protein converting 1 mg D-glucose-6-phosphate to 1 L-*myo*-inositol-1-phosphate during 1 h (μmol · h^{-1} mg^{-1}). Protein was quantified by the Bradford method using bovine serum albumin as standard (Bradford, 1976).

Assay of relative water content (RWC)

The RWC was measured in the uppermost, fully expanded leaves of each group as described by Premachandra *et al*. (1994).

Assay of malondialdehyde (MDA) content

The MDA content was determined by the thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). Plant tissues were homogenized with 0.1% trichloroacetic acid (TCA, m/v), and the homogenates were centrifuged at 15,294 × *g* for 15 min. To an 1.0-ml aliquot of the supernatant, 3.0 ml of 0.5% TBA in 5% TCA were added. The mixture was heated at 95 °C for 30 min and then cooled immediately in an ice bath. The reaction mixture was centrifugated at 15,294 × *g* for 10 min, and the absorbance of the supernatant was recorded at 532 nm and 600 nm. Lipid peroxidation was expressed as MDA concentration, in μM, using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical analysis

All values shown in this paper is the mean of at least three replicated treatments for each condition. Data tested at significant levels of *P* value were less than 0.05 using one-way ANOVA. One-way analysis of variance (one-way ANOVA) was used to test the significance levels of data (the significance level was chosen to be 0.05).

Results and Discussion

In plants, MIPS plays important roles in various essential metabolic reactions, which include the metabolism of inositols and inositol lipids, the biosynthesis of phytates in seeds, and salinity-related metabolism (Abid *et al*., 2009). These findings suggested that MIPS has a direct influence on growth and development in plant species. In the present study, an 1669-bp ful length MIPS cDNA, which contains an 1533-bp ORF, was cloned from *R. communis*. Sequence data from this article have been deposited at GeneBank under accession number FJ823976. The ORF of *rcMIPS* encodes 510 amino acid residues, which correspond to a polypeptide with a molecular mass of approximately 56 kDa (Fig. 1). In the 3'-nontranslational region, two putative polyadenylation signal sequences, namely AACATA, are present one base pair upstream of the poly (A) tail. *rcMIPS* shared high homology with other plant species, such as *Zea mays* (88.6%, GenBank accession no. AF056326), *Arabidopsis thaliana* (89%, GenBank accession no. NM120143), *Vigna radiata* (91.6%, GenBank accession no. EU239689), and *Xerophyta viscose* (91.4%, GenBank accession no. EF449773). Sequence analysis showed that no predicted signal or chloroplast transit peptide in the *N*-terminal region of *rcMIPS* is found. Results of an alignment of the amino acid sequences of selected MIPS genes suggested that four amino acid stretches, including GWGGNNG (Domain 1), LWTANTERY (Domain 2), NGSPQNTFVPGL (Domain 3) and SYNHLGNNDG (Domain 4), are detected which are involved in MIPS protein binding and are essential for MIPS functions (Majumder *et al*., 2003). Similarly, four highly conserved motifs in the sequence of the *rcMIPS* gene were also detected, but the sequences of Domain 3 (NGSPQNTFVPGV) and Domain 4 (AYNHLGNNDG) showed some differences compared to those of other MIPS genes.

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1     ATG TTT ATT GAG AAA T TT C AG GTT GAG AGT CCT AAT GTT AAG TAC TCA GAG GAT GAG ATT
1      M   F   I   E   K   F   Q   V   E   S   P   N   V   K   Y   S   E   D   E   I
61    CAC TCT GTG TAT AAC TAT GAA ACA ACT GAG CTT GTT CAT GAG AAC AGA AAT GGT ACC TAT
21     H   S   V   Y   N   Y   E   T   T   E   L   V   H   E   N   R   N   G   T   Y
121   CAA TGG ATT GTA AAA CCC AAA ACA GTC CAA TAT GAA TTC AAG ACT GAT GTC CAT GTT CCT
41     Q   W   I   V   K   P   K   T   V   Q   Y   E   F   K   T   D   V   H   V   P
181   AAA TTA GGG GTT ATG CTT GTG GGT TGG GGT GGA AAC AAT GGT TCT ACT CTC ACC GGT GGT
61     K   L   G   V   M   L   V   G   W   G   G   N   N   G   S   T   L   T   G   G
241   GTT ATA GCG AAC AGA GAA GGA ATC TCC TGG GCT ACC AAA GAC AAA GTG CAG CAA GCA AAT
81     V   I   A   N   R   E   G   I   S   W   A   T   K   D   K   V   Q   Q   A   N
301   TAC TTT GGC TCG CTC ACT CAA GCT TCA TCA ATT CGA GTT GGG TCT TTT AAT GGA GAA GAG
101    Y   F   G   S   L   T   Q   A   S   S   I   R   V   G   S   F   N   G   E   E
361   ATT TAT GCT CCA TTC AAG AGC CTT CTC CCC ATG GTG AAT CCA GAT GAC ATT GTG TTT GGT
121    I   Y   A   P   F   K   S   L   L   P   M   V   N   P   D   D   I   V   F   G
421   GGG TGG GAC ATA AGT GAC ATG AAC TTG GCT GAT GCT ATG GCC AGG GCT AAG GTT CTG GAT
141    G   W   D   I   S   D   M   N   L   A   D   A   M   A   R   A   K   V   L   D
481   ATT GAT CTT CAG AAA CAA TTG CGG CCC TAC ATG GAA TCT ATG GTT CCA CTC CCT GGA ATC
161    I   D   L   Q   K   Q   L   R   P   Y   M   E   S   M   V   P   L   P   G   I
541   TAT GAC CCT GAT TTT ATT GCT GCT AAT CAA GGA TCG CGT GCC AAC AAC GTG ATT AAA GGC
181    Y   D   P   D   F   I   A   A   N   Q   G   S   R   A   N   N   V   I   K   G
601   ACT AAA AAA GAA CAG ATG GAA CAA ATT GTC AAG GAT ATT AGG GAG TTT AAG GAG AAA ACC
201    T   K   K   E   Q   M   E   Q   I   V   K   D   I   R   E   F   K   E   K   T
661   AAA GTG GAT AAA GTC GTT GTG CTT TGG ACT GCC AAC ACA GAA AGG TAC AGT AAT GTT GTT
221    K   V   D   K   V   V   V   L   W   T   A   N   T   E   R   Y   S   N   V   V
721   GTG GGG CTA AAT GAC ACT ATG GAG AGC CTC TTG GCT TCT TTG GAG AGA AAT GAA GCT GAG
241    V   G   L   N   D   T   M   E   S   L   L   A   S   L   E   R   N   E   A   E
781   ATA TCT CCC TCA ACC TTG TAT GCT TTG GCT TGT ATC TTT GAA AAT GTT CCT TTC ATT AAT
261    I   S   P   S   T   L   Y   A   L   A   C   I   F   E   N   V   P   F   I   N
841   GGA AGC CCA CAG AAC ACT TTT GTT CCT GGG GTT ATT GAT TTG GCT ATT AAG GGG AAC AGT
281    G   S   P   Q   N   T   F   V   P   G   V   I   D   L   A   I   K   G   N   S
901   TTG ATC GGC GGG GAT GAC TTC AAG AGT GGT CAG ACC AAG ATG AAA TCC GTA CTG GTT GAT
301    L   I   G   G   D   D   F   K   S   G   Q   T   K   M   K   S   V   L   V   D
961   TTC CTT GTT GGG GCT GGT ATT AAG CCA ACA TCA ATA GTG AGT TAC AAC CAT TTA GGA AAC
321    F   L   V   G   A   G   I   K   P   T   S   I   V   S   Y   N   H   L   G   N
1021  AAT GAT GGC ATG AAC CTT TCA GCA CCG CAA ACT TTC CGT TCC AAA GAA ATA TCC AAA AGC
341    N   D   G   M   N   L   S   A   P   Q   T   F   R   S   K   E   I   S   K   S
1081  AAT GTT GTT GAT GAC ATG GTC TCT AGC AAT GGT ATC CTC TAT GAA CCT GGT GAA CAT CCT
361    N   V   V   D   D   M   V   S   S   N   G   I   L   Y   E   P   G   E   H   P
1141  GAC CAT GTT GTG GTC ATC AAG TAC GTG CCA TAT GTG GGG GAT AGC AAG AGA GCT ATG GAT
381    D   H   V   V   V   I   K   Y   V   P   Y   V   G   D   S   K   R   A   M   D
1201  GAG TAC ACT TCA GAG ATT TTC ATG GGT GGC AAA AGC ACC ATA GTC CTG CAC AAC ACT TGC
401    E   Y   T   S   E   I   F   M   G   G   K   S   T   I   V   L   H   N   T   C
1261  GAG GAT TCC CTC TTG GCT GCA CCC ATT ATT TTG GAT TTG GTC CTA CTT GCT GAA CTC AGC
421    E   D   S   L   L   A   A   P   I   I   L   D   L   V   L   L   A   E   L   S
1321  ACC AGG ATC CAG CTT AAA GCT GAA GGA GAG GGA AAG TTC CAT TCC TTC CAT CCT GTG GCT
441    T   R   I   Q   L   K   A   E   G   E   G   K   F   H   S   F   H   P   V   A
1381  ACC ATT CTC AGC TAC CTC ACC AAG GCT CCT CTT GTT CCA CCA GGC ACC CCA GTG GTG AAT
461    T   I   L   S   Y   L   T   K   A   P   L   V   P   P   G   T   P   V   V   N
1441  GCA CTG TCA AAG CAG CGT GCA ATG CTG GAG AAC ATA TTG AGA GCT TGT GTT GGT TTG GCT
481    A   L   S   K   Q   R   A   M   L   E   N   I   L   R   A   C   V   G   L   A
1501  CCT GAG AAC AAC ATG ATT TTG GAA TAC AAG TGA
501    P   E   N   N   M   I   L   E   Y   K   *
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Fig. 1. The nucleotide sequence and deduced amino acid cDNA sequences of the *rcMIPS* gene. The sequences are numbered at the left. The asterisk (*) represents the stop codon.

The mRNA expression levels and enzyme activities of *rcMIPS* in the leaves, stems, and roots of *R. communis* are shown in Table II. The expression levels of the *rcMIPS* gene in the leaves increased significantly by 42.0%, 86.8%, and 155.3%, under stress conditions for 24, 48, and 72 h compared to the control, respectively. Similarly, the expression levels in the stems and roots were also compared to the control, and the highest levels increased by 222.8% and 291.2%, respectively. In addition, the expression levels of the *rcMIPS* gene in the leaves were higher than those in the stems and roots (Table II). Results of enzyme activity assays suggested that MIPS activities in the leaves,

Table II. The expression levels and relative enzyme activities of MIPS in leaves, stems, and roots of *R. communis* under drought stress for 0, 24, 48, and 72 h.

Tissue	Parameter	0 h	24 h	48 h	72 h
Leaves	mRNA level	80.3 ± 3.21	114 ± 4.8	150 ± 6.7	205 ± 9.15
	Activity	3.13 ± 0.117	3.75 ± 0.138	4.3 ± 0.186	5.9 ± 0.203
Stems	mRNA level	12.7 ± 0.54	41 ± 2.05	35 ± 1.45	20 ± 0.86
	Activity	0.125 ± 0.005	0.177 ± 0.007	0.152 ± 0.006	0.117 ± 0.004
Roots	mRNA level	40.9 ± 1.75	126 ± 5.31	160 ± 7.26	113 ± 4.52
	Activity	1.55 ± 0.058	2.6 ± 0.098	2.7 ± 0.106	1.78 ± 0.075

Data are presented as the mean ± SD (n = 3).

Table III. The relative water contents (RWC) and MDA contents in leaves of *R. communis* under drought stress for 0, 24, 48, and 72 h.

Parameter	0 h	24 h	48 h	72 h
MDA	0.155 ± 0.007	0.183 ± 0.008	0.221 ± 0.01	0.272 ± 0.009
RWC	77.5 ± 3.18	61.5 ± 2.65	50.8 ± 2.04	43.2 ± 1.76

Data are presented as the mean ± SD (n = 3).

stems, and roots enhanced significantly compared to the control, and showed a similar change tread compared to the changes of the expression levels. In the leaves, the activities increased by 19.8%, 37.4%, and 88.5% under 30% PEG stress conditions for 24, 48, and 72 h compared to the control, respectively. The activities in the stems and roots reached the peak at 24 and 48 h of stress, and the highest activity increases were by about 41.6% and 74.2%, respectively. The transcript levels of the MIPS gene and its activity have been studied in different organs, such as leaves, roots, embryos, and cotyledons. Moreover, different forms of MIPS at different stages of development reflected regulatory controls at the transcriptional and translational levels (Abid *et al*., 2009). These results suggested the important role of MIPS not only in the basic metabolism, but also in the establishment of developmental programs. The present findings suggested that the expression levels of the *rcMIPS* gene and rcMIPS activity in the leaves, stems, and roots increase significantly after drought stress (Fig. 2 and Table II). An earlier study suggested that the expression levels of the MIPS gene might be correlated with the accumulation of D-pinitol which is a cyclic sugar alcohol involved in the tolerance of environmental stress (Abreu and Aragão, 2007; Suzuki *et al*., 2007). Thus, our results indicated that the defensive system of plants regulates the expression levels and activity of rcMIPS in *R. communis* plants in order to enhance the defensive function against drought stress.

The RWC and MDA contents in the leaves of *R. communis* are shown in Table III. The RWC in the leaves decreased significantly under stress for 24, 48, and 72 h, compared to the control by 20.6%, 34.5%, and 44.3%, respectively. The RWC

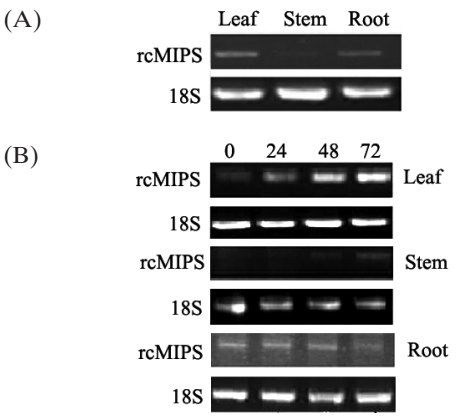

Fig. 2. The differential expression levels of the *rcMIPS* gene from *R. communis* in different tissues. (A) The expression levels in untreated leaves, stems, and roots of *R. communis* (control). (B) The expression levels in leaves, stems, and roots of *R. communis* under drought stress for 24, 48, and 72 h.

is the appropriate measure of plant water status in terms of the physiological consequence of cellular water deficit. The reduction of the RWC under stress conditions might be associated with a decrease in plant vigour and was observed in some plant species (Duan *et al*., 2005; Farooq *et al*., 2008). Our results suggested that a decreased RWC in *R. communis* leaves reflects a stress response of *R. communis* exposed to drought conditions. Moreover, the MDA content in the leaves increased gradually with increasing exposure time by 18.1%, 42.6%, and 75.5%, respectively. The change in the MDA contents, especially in oil-rich seeds, is often used as an indicator of general lipid peroxidation. Under drought stress conditions, higher lipid peroxidation in many plant species has been reported (Duan *et al*., 2005; Farooq *et al*., 2008). Results of increased MDA contents suggested that lipid peroxidation occurs in *R. communis* leaves, and this plant experiences oxidative damage when exposed to drought stress.

In conclusion, our results suggest that the transcript levels of the *rcMIPS* gene in the leaves, stems, and roots of *R. communis* are preferentially expressed under drought stress for 24, 48, and 72 h. This is further supported by the fact that the rcMIPS activities in different tissues of *R. communis* increased significantly. Our results also suggest that increased MDA contents and decreased RWC under drought stress present a drought-resistance response. Thus, the present findings suggest that up-regulation levels of the *rcMIPS* gene as well as increased rcMIPS activities might be involved in the defensive mechanism of drought stress resistance in *R. communis* plants.

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