Reduced mycorrhizal colonization (*rmc*) tomato mutant lacks expression of SymRK signaling pathway genes

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Comparison of the expression of 13 genes involved in arbuscular mycorrhizal (AM) symbiosis was performed in a wild type tomato (*Solanum lycopersicum* cv. 76R) and its reduced mycorrhizal colonization mutant *rmc* in response to colonization with *Glomus fasiculatum*. Four defense-related genes were induced to a similar extent in the mutant and wild type AM colonized plants, indicating a systemic response to AM colonization. Genes related to nutrient exchange between the symbiont partners showed higher expression in the AM roots of wild type plants than the mutant plants, which correlated with their arbuscular frequency. A symbiosis receptor kinase that is involved in both nodulation and AM symbiosis was not expressed in the *rmc* mutant. The fact that some colonization was observed in *rmc* was suggestive of the existence of an alternate colonization signaling pathway for AM symbiosis in this mutant.

Arbscular mycorrhizal (AM) symbiosis is widely distributed in the plant kingdom, unlike rhizobial symbiosis that is observed only in four orders of the eurosid dicots.[1] AM symbiosis involves provision of mineral nutrients to the plant by the fungus, which in turn derives carbon compounds from the plant.[2] Other benefits of the symbiosis to plants include improved water relations and tolerance to some plant diseases.[3] The fungal hyphae penetrate the root epidermis, spread inter-cellularly in the cortex and form arbuscules in the root cells of the inner cortex. Arbuscules are highly branched hyphal structures that are separated from the plant cytoplasm by a perifungal membrane.[4] They are the main sites where nutrient exchange between the symbiotic partners occurs and their formation signifies the establishment of functional symbiosis.

Molecular events occurring during AM symbiosis have been studied in legumes, which also show an elaborate signaling pathway for establishing nodulation symbiosis.[5] Two genes, *NFR1* and *NFR5* encoding receptor-like serine/threonine kinases with LysM domains, are involved in nodulation (Nod) factor perception in *Lotus japonicus*.[6] Several downstream components of the Nod factor signaling cascade include the leucine-rich-repeat receptor kinase SYMRK, which is known to be involved in AM symbiosis besides nodulation symbiosis and is thought to act near the junction of fungal and rhizobial signaling cascades.[7] Activation of *SYMRK* causes a transient increase in intracellular calcium levels. Downstream components of this signaling pathway include a calcium/calmodulin dependent protein kinase (*CCAMK*) and a protein *CYCLOPS*, whose function is not known. It is suggested that the evolutionarily more recent nodulation symbiosis has recruited this signaling pathway from the more ancient AM-symbiosis, since non-legumes like rice, tomato and Casuarina show orthologs of the legume genes involved in symbiosis signaling.[8-10]

A number of AM induced genes have been identified, which show expression only in AM colonized roots. Many of these are genes associated with defense responses of plants and it is reported that the initial stages of colonization by the fungal symbiont and biotrophic pathogens are similar.[11] Some genes are associated with nutrient exchange that occurs between the two symbiont partners and include a low affinity phosphate transporter and genes involved in sugar and nitrogen metabolism.[12] A few transcription factor genes are also induced by AM colonization, which are either related to regulation of expression of defense genes or genes involved in alteration of growth patterns in mycorrhizal roots.[13]

Symbiosis pathway mutants have been identified in leguminous (*Lotus japonicus*),[14] as well as non-leguminous plants like *Oryza sativa*[8] and *Casuarina glauca*.[10] In these mutants, infection by AM fungi is either aborted before or after hyphal penetration of root cells. However, several AM induced genes were expressed in the *SYMRK* pathway mutants, suggesting that another pathway may be involved in AM signaling.[15] A reduced mycorrhizal colonization mutant *rmc* has been identified in tomato,[16] in which reduced symbiotic association was attributed to lack of penetration, inability to colonize the root cortex or a slower but successful colonization, depending on the species of the fungal interacting partner.[17] The mycorrhizal phenotype of *rmc* mutants

resembles that of *dmi* mutants of *Medicago trunculata* suggesting the possibility that *RMC* may be an ortholog of *DMI* genes, one of which codes for a symbiosis receptor kinase (DMI2).[18] Though the *RMC* locus has been identified and is known to lie on chromosome 8, the function(s) encoded by this locus is not known.[19] In this paper we have compared the expression of a few AM induced genes in the tomato *rmc* mutant and its wild type parent 76R. Our results indicate that reduced colonization in *rmc* could be attributed to the lack of SYMRK signaling pathway in this mutant.

Seeds of *Solanum lycopersicum* cv. 76R and its reduced mycorrhizal colonisation mutant (*rmc*) were procured from Dr. Susan Barker (University of Western Australia, Australia). A soil based AM fungal inoculum of *Glomus fasciculatum* consisting of spores, colonized root pieces and the surrounding soil was supplied by Dr. Joseph Bagyaraj, (Centre for Natural Biological Resources and Community Development, Bangalore, India). The seeds were treated with 1% HCl for 15 min, rinsed with water and sowed in autoclaved (121°C, 1.034 bars, for 1 h) soil in seedling trays. Seedlings were carefully transplanted after 20 d to polythene bags containing about 2 kg autoclaved soil each, ensuring minimum root damage during transplantation. Inoculation with AM fungus was done at the time of transplantation by adding about 1 g inoculum to the soil cavity in which the seedling was transplanted. Controls consisted of plants to which no AM inoculum was applied. Three replicates were used per treatment.

Roots were carefully harvested from 52 dpi plants by completely immersing the polythene bags in water. Roots were carefully washed to remove all particulates, cleared using 10% hot KOH solution and stained with 0.5% trypan blue (invam.wvu.edu/methods/mycorrhizae). Extent of colonization was measured from 30 root segments of 2 cm length per plant using the computer program "Mycocalc".[20] Student's t test was applied to compare the colonization parameters in 76R and *rmc*.

Genes induced during mycorrhizal colonization were identified by searching for tomato orthologs of the rice AM-specific genes[8] and from the microarray data available on AM colonized tomato plants[9] (**Table 1**). RNA isolation was performed using root tissue (pooled from three plants) with Trizol reagent (Sigma Aldrich, cat. no. 93289) from 52 dpi plants and their respective controls. Extracted RNA (1 μg) was used as a template for reverse transcription with ImProm-II reverse transcriptase (Promega, cat. no. A3802). Gene specific primers were designed using primer Blast (blast.ncbi.nlm.nih.gov) and transcript abundance in the cDNA was studied at three dilutions by PCR amplification. Gene expression was normalized using a constitutively expressed *Elongation factor1α* (*EF1α*) gene. Cycling conditions used were: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, varying annealing temperatures (**Table 1**) for 0.45 min and 72°C for 1 min. Final extension was performed at 72°C for 10 min. Two genes *AQP* and *GRAS4*, showed saturation of PCR at 35 cycles and so 30 cycles of amplification were used. Similarly for *EF1α*, 25 cycles of PCR amplification were used so as to get transcript abundance-dependent differential expression. All amplified products were sequenced to confirm the transcripts amplified. Gel images were captured using a gel documentation

system (Bio Rad, Gel Doc XR⁺, USA) and intensities of the bands were recorded.

Real-time RT PCR using the QuantiFast SYBR Green PCR kit (Qiagen, cat. no. 204054) was performed to confirm the differential expression of *SYMRK* using thermocycler (Eppendorf, RealPlex 2, Germany). Primers used were as shown in **Table 1**. Total amount of *SYMRK* cDNA in each reaction was normalized by co-amplification of the constitutively expressed *EF1α* gene.

Colonization of tomato roots by *Glomus faciculatum* was seen in 52 dpi plants in the form of intercellular and extra-radical mycelia as well as arbuscule and vesicle formation. The wild type cultivar 76R and its reduced mycorrhizal colonization mutant (*rmc*) showed the presence of normal arbuscules and extra radical hyphae (**Fig. 1**), but the frequency of mycorrhiza in root system, intensity of mycorrhizal colonization and arbuscule abundance varied. All the colonization parameters were significantly lower in *rmc* as compared with 76R and arbuscule abundance was most severely reduced (**Table 2**). The colonization pattern in tomato roots (76R and its *rmc* mutant) has been reported to be similar for *Glomus fasciculatum* and *Glomus intraradices* interactions.[17]

Expression pattern of 13 AM induced genes in 76R and *rmc* were analyzed from the band intensities in (A) AM roots/control roots for 76R and *rmc* respectively and (B) 76R AM roots/*rmc* AM roots (**Fig. 2 and Table 3**). Comparative analysis of AM induced gene expression in 76R and *rmc* mutant revealed two patterns of gene expression. Some genes were AM-specific and were not expressed in non-colonized roots, while others though induced in response to AM colonization, also showed some basal level expression in non-colonized roots. Functionally these genes could be broadly categorized into (A) defense related genes, which included a fungal endoglucanase inhibitor (*FGI*), a cysteine protease (*CP*), a phenolic glycosyltransferase (*PGT*) and an IAA amidosynthetase (*IAAS*) (B) signal transduction and transcription regulation genes, which included a calcium dependent protein kinase (*CDPK*), an ethylene response element binding protein (*EREBP*) and a GRAS domain transcription factor (*GRAS4*) (C) transporters and primary metabolism genes, which included a low affinity phosphate transporter (*LePT4*), an aquaporin protein (*AQP*), sucrose synthase (*SUSY*), vacuolar invertase (*INVA*) and a cell wall invertase (*INVCW*) (D) nodulation symbiosis pathway gene, symbiosis receptor kinase (*SymRK*).

Of the defense genes studied *FGI*, *CP* and *PGT* showed expression only in the mycorrhizal roots while *IAAS* was expressed in both the mycorrhizal as well as control roots. The defense related genes *IAAS*, *CP* and *FGI* showed similar levels of expression in the AM roots of 76R and *rmc* (**Table 3**). The products of these genes could be classified as pathogenesis-related (PR) proteins, since they did not show any expression in non-AM roots of 76R or *rmc*. Plants are known to develop similar defense responses to biotrophic pathogenic and symbiotic fungi.[11] For example, rice roots colonized by AM fungi were known to produce PR proteins like chitinases, which can hydrolyse fungal cell walls.[21] *PGT* has been reported to be induced in response to systemic pathogen infection in tomato.[22] An IAA amido synthetase that has been reported to play a role in expression of basal immunity in rice[23] was seen to have about 2-fold expression in response to

Table 1. Genes used for studying AM-induced expression in tomato cv. 76R and its reduced mycorrhizal colonization mutant *rmc*

Gene name	Accession number	Primer sequence (5'-3')	Annealing temperature (°C)	Product size (bp)
Xyloglucan-specific fungal endoglucanase inhibitor	AY155579.1	GTG TTC ACT GGG CGG AGC GT TGA CCT GAT GAA AAG GCT GAG GC	59	492
Cysteine protease	AF172856.1	GCT GGT GGC AGA GAC TTC CAG C AGC AAG TGG TGC CGA CAG CG	59	343
UDP-xylose phenolic glycosyltransferase	AJ889012.1	TGG TGG TCG CGA TCA AGC AGG ACC CCA TGC CAA TTC TTC CAT TTG C	59	682
Indole acetic acid amido synthetase	AC215447.2	AGG ACC CGG CTA ACC CAC CC TCA ACG TCG TCG TTC TGG AGT CC	63	314
Ethylene response element binding protein	BT013241.1	GTT CGG AAG AGG CCA TGG GGG TTG CTG ACG TGG CGG TCT CG	61	336
GRAS family transcription factor	DQ399826.1	TCC ATG AGG GCT GGG GGA CC GTC CTC GTC CTC GCG GGG AA	59	450
Low affinity phosphate LePT4	AY804012.2	ATG GTT TGA CTT TCT TCT TTG TAG AAA GCA CAA GGC GTA G	57	418
Aquaporin	AB211518.1	AAG CGG CCT TGG CGG AGT TC ATC CCG TGC TCA TGC CAC CG	59	330
Sucrose synthase Sus 3	AJ011319.1	GCC ATG AGC TGC GGT TTG CC GCT AGT GTC AAC AGC CGG TCG G	61	248
Invertase (Vacuolar)	Z12027.1	CCC CGA AAA CTC CGC CTC TCG AAA CAC CTC TTG ACG GCG GC	63	200
Invertase (Cell wall) Lin6	AB004558.1	AGG ATG GGC CGG GGT TCA CC GGC CCA AGA CCA CCT TGA ACC G	63	309
Symbiosis receptor-like kinase	AY940041.1	For **semi-quantitative RT-PCR** GCC GGC CAG ACT TTC CAT TGC ACC ACT CTC CAC AGC GCC TCA **For Real-Time RT-PCR** AGC TTG GTT GAA TGG GCG AAA CC ACC ACT CTC CAC AGC GCC TCA	59 59	453 107
Calcium dependent protein kinase	AB530160.1	GCT CGG GTG CCG GAT GAC TC ACC GCA TCC TCA TAA GCC CCT	59	491

Primer sequences, primer annealing temperatures and the expected product sizes are given.

AM colonization indicating its probable role in attaining IAA homeostasis after the establishment of symbiosis.

The AM fungi have to evade host defense mechanisms in order to establish successful mycorrhizal colonization. Defense genes like extracellular acidic β-1,3-glucanase, PR-1 and chitinase, though induced during the early stages of colonization showed lower expression than mock-inoculated controls or *rmc* at 42 d post-colonization.[24] However an induction of the defense genes studied by us was observed in both, the wild type cultivar 76R and its *rmc* mutant. Expression of the defense genes appeared to depend not on the extent of colonization, but on the colonization event itself, which was indicative of a systemic response. Split-root experiments with tomato colonized by *Glomus mosseae* revealed a systemic bio-protective effect in roots where the non-mycorrhizal portion of the mycorrhizal root system also exhibited resistance.[25]

The transcription factors EREBP and GRAS4 showed higher expression in AM roots of both 76R and *rmc*, while CDPK, a signaling intermediate was expressed only in AM roots of 76R. Ethylene responsive transcription factors are widely known to play a role in regulating gene expression in response to biotrophic and necrotrophic pathogens.[26] CDPKs have also been reported to play a role in AM signaling in *Medicago trunculata*.[27]

The *LePT4* and sugar metabolism genes, *SUSY*, *INVA* and *INVCW* were induced in response to AM colonization, in both 76R and *rmc*, but the level of expression was at least 2-fold higher in 76R. The fungal symbiont is known to provide phosphate to the AM roots and acquire sugars from the roots in exchange. Expression of symbiosis-specific Pi transporters in AM roots has been reported in reference 28. An increase in the activities and expression of sucrose metabolizing enzymes, invertase and sucrose synthase has also been reported in the AM roots of *Trifolium repens* and tomato, which was independent of the improved phosphate nutrition of colonized roots.[29,30] That arbuscules are the sites of nutrient exchange is known from various studies. For
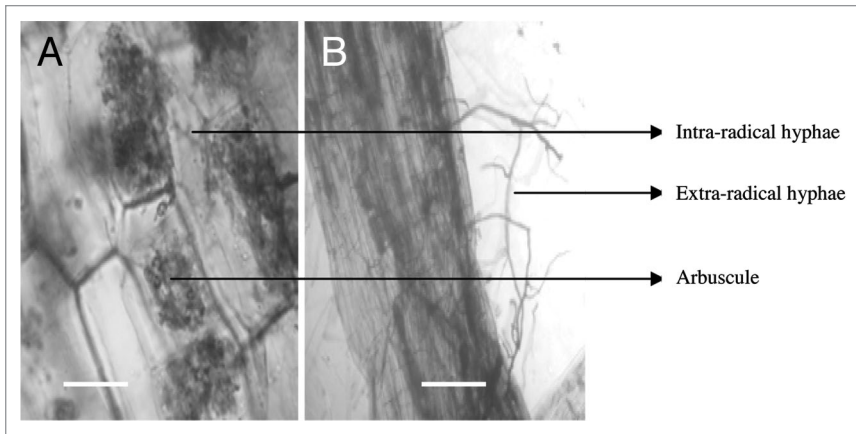
Figure 1. *Solanum lycopersicum* (76R) roots colonized with *Glomus fasciculatum* showing (**A**) arbuscules and (**B**) extraradical hyphae. Both these features were also observed in the colonization deficient mutant (*rmc*) but at a much lower frequency (see **Table 2**). The roots were cleared and stained with Trypan blue, 52 d after inoculum application. Bar = 25 μm (**A**), 150 μm (**B**).

Table 2. Measurement of the extent of *Glomus fasciculatum* colonization in roots of *Solanum lycopersicum* cv. 76R (wt) and its colonization deficient mutant (*rmc*)

Colonization parameter	Mean values %	
	76R	***rmc***
Frequency of mycorrhiza in root system	91 ± 3.2	62 ± 6.2*
Intensity of mycorrhizal colonization in root system	44 ± 3.1	19 ± 4.2*
Intensity of mycorrhizal colonization in root fragment	52 ± 0.7	25 ± 2.9*
Arbuscule abundance in mycorrhizal part of root fragment	37 ± 2.6	23 ± 2.4*
Arbuscule abundance in root system	16 ± 2.9	2 ± 0.6*

The colonization parameters were calculated using Mycocalc software and represent mean % values of 30 root fragments per plant for 3 plants per treatment, along with standard deviations. *Means differ significantly between 76R and *rmc* (p < 0.05).

example, a symbiosis-specific phosphate transporter (*MtPT4*) was shown to localize to the periarbuscular membrane.[31] Likewise a sucrose synthase (*MtSucS1*) knockout mutant showed defects in arbuscule development and maintenance.[32] Hence unlike the defense genes that were induced in response to systemic signals provided by the fungal symbiont, expression of the sugar metabolism and phosphate transport genes correlated with the extent of arbuscular development in AM roots. The water channel protein AQP, which is also known to transport other small molecules like NH_4^+ ions, showed higher expression in AM roots, but in this case, AM roots of *rmc* showed higher levels of expression than those of 76R.

The nodulation symbiosis pathway gene homolog in tomato (*SYMRK*) was expressed in AM roots of 76R but not in the poorly colonized *rmc* roots (**Fig. 2**). Transcript abundance of *SYMRK* was about 200-fold higher in AM colonized roots of 76R as compared with AM colonized roots of *rmc* (**Fig. 3**). SYMRK is a key component of the symbiosis signaling pathway, common to nodulation, actinorrhizal and mycorrhizal symbiosis.[10] During nodulation, Nod factors are known to be perceived by specific receptor-like serine/threonine kinases with LysM domains, which bring about the activation of SYMRK. However receptors for Myc factors, which have been identified as small, lipophilic molecules with chitin backbone, are not known.[33] Activation of SYMRK brings about calcium spiking, which is important for the expression of downstream genes involved in the establishment of symbiosis.[34] In tomato, the *CDPK* gene was expressed only in AM colonized 76R but not in *rmc* roots suggesting that its expression may be dependent on calcium spiking caused by *SYMRK*.

The *rmc* mutant of tomato, which lacked *SYMRK* expression, did however show some extent of colonization, which indicated the possibility of an alternate, SYMRK-independent pathway being employed for establishing symbiosis
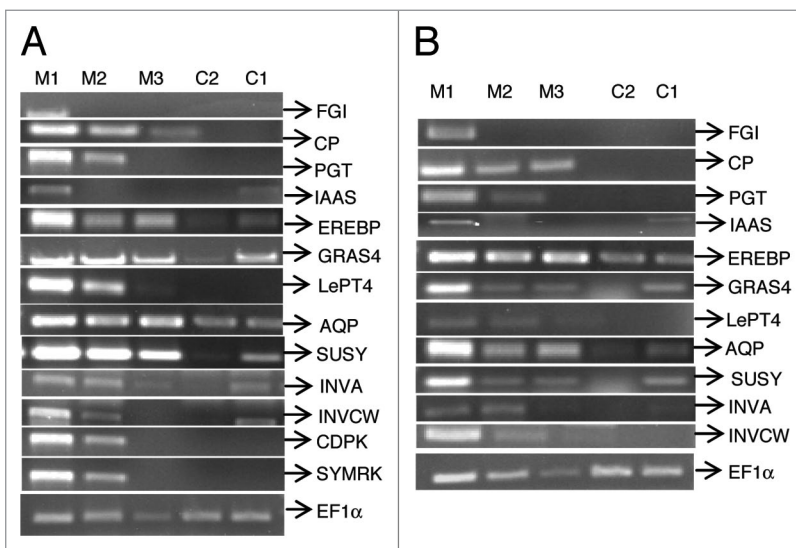
Figure 2. Expression of AM induced genes in *Solanum lycopersicum* cv. 76R (**A**) and its colonization deficient mutant (*rmc*) (**B**), 52 dpi with *Glomus fasciculatum*. Transcript abundance was analyzed using semiquantitative RT-PCR. M1, M2 and M3 represent 0, 5 and 10-fold dilutions of the cDNA prepared from AM roots and C1 and C2 represent 0 and 5-fold dilution of the cDNA prepared from non-colonized roots. Constitutively expressed EF1α was used as a loading control for comparing expression levels. The genes studied were: FGI (*Fungal endoglucanase inhibitor*); CP (*Cysteine Protease*); PGT (*Phenolic glycosyltransferase*); IAAS (*IAA amido synthetase*); EREBP (*Ethylene response element binding protein*); GRAS4 (*GRAS family transcription factor*); LePT4 (*Low affinity phosphate Transporter*); AQP (*Aquaporin*); SUSY (*Sucrose synthase*); INVA (*Vacuolar Invertase*); INVCW (*Cell wall Invertase Lin6*); CDPK (*Calcium dependent protein kinase*); SYMRK (*Symbiosis receptor-like kinase*).

Table 3. Fold expression of 13 a.m.-induced genes in *Solanum lycopersicum* cv. 76R and its reduced mycorrhizal colonization mutant (*rmc*) analyzed using semiquantitative RT-PCR

Product	Fold-expression in AM roots/non-AM roots		Fold-expression in 76R AM roots/rmc AM roots
	76R	**rmc**	
Xyloglucan-specific fungal endoglucanase inhibitor	Only expressed in AM colonized roots	Only expressed in AM colonized roots	1.1
Cysteine protease	Only expressed in AM colonized roots	Only expressed in AM colonized roots	1.2
UDP-xylose phenolic glycosyltransferase	Only expressed in AM colonized roots	Only expressed in AM colonized roots	3.0
IAA amido synthetase	2.6	2.5	1.3
EREBP	5.5	3.4	1.2
GRAS4 transcription factor	3.6	3.5	1.1
Phosphate Transporter	Only expressed in AM colonized roots	Only expressed in AM colonized roots	4.0
Aquaporin	3.7	6.6	0.7
Sucrose synthase	5.2	3.0	2.0
Vacuolar invertase	1.5	1.5	2.6
Cell wall invertase	1.7	1.3	1.5
Calcium dependent protein kinase	Only expressed in AM colonized roots	Not expressed in AM colonized roots	—
Symbiosis receptor-like kinase	Only expressed in AM colonized roots	Not expressed in AM colonized roots	—

The band intensities (see **Fig. 2**) were normalized using constitutively expressed EF1α. Fold-expression was determined as the ratio of normalized intensities in (1) AM colonized roots (M1 in **Fig. 2**) and non-colonized roots (C1 in **Fig. 2**) for 76R and *rmc* respectively and (2) AM colonized roots of 76R (M1 in **Fig. 2A**) and AM colonized roots of rmc (M1 in **Fig. 2B**).

in the mutant. The *sym15 and castor* mutants of *Lotus japonicus*[14] and the *dmi* mutants of *Medicago trunculata*,[18] which showed mycorrhizal colonization in spite of a non-functional SYMRK pathway also suggested the presence of an alternate pathway for symbiosis signaling. Lack of *SYMRK* expression could be a probable cause for the fewer arbuscules detected in *rmc*, since the kinase is known to play an important role during penetration of the inner cortical cells by the fungal symbiont partner as seen in Casuarina.[10] This is a first report offering a functional explanation for the mycorrhizal colonization deficiency observed in *rmc* mutants. It is possible however that up stream components of the *SYMRK* pathway, like NFP in *Medicago trunculata*[35] or NFR1 and NFR5 in *Lotus japonicus*[6] could be responsible for the lack of expression of *SYMRK* in the tomato reduced mycorrhizal colonization (*rmc*) mutant.

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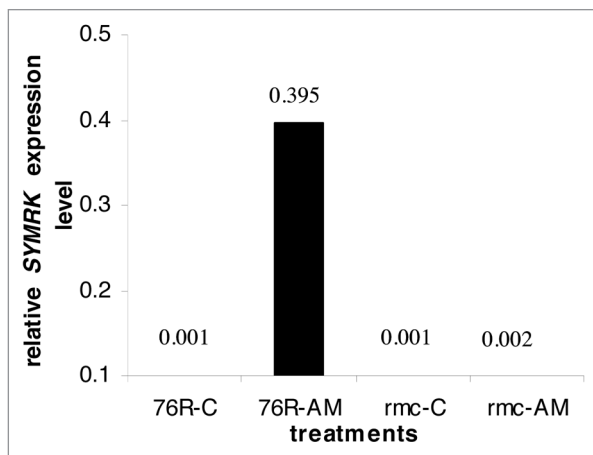
Figure 3. Real-time PCR analysis of transcript abundance of *SYMRK* in 76R and *rmc*. Values represent mRNA levels of SYMRK normalized to transcript abundance of the constitutively expressed gene *EF1α*.

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