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The reduced mycorrhizal colonization (*rmc*) mutation of tomato disrupts five gene sequences including the *CYCLOPS/IPD3* homologue

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Abstract
Arbuscular mycorrhizal (AM) symbiosis in vascular plant roots is an ancient mutualistic interaction that evolved with land plants. More recently evolved root mutualisms have recruited components of the AM signaling pathway as identified with molecular approaches in model legume research. Earlier we reported that the reduced mycorrhizal colonization (*rmc*) mutation of tomato mapped to chromosome 8. Here we report additional functional characterization of the *rmc* mutation using genotype grafts and proteomic and transcriptomic analyses. Our results led to identification of the precise genome location of the *Rmc* locus from which we identified the mutation by sequencing. The *rmc* phenotype results from a deletion that disrupts five predicted gene sequences, one of which has close sequence match to the *CYCLOPS/IPD3* gene identified in legumes as an essential intracellular regulator of both AM and rhizobial symbioses. Identification of two other genes not located at the *rmc* locus but with altered expression in the *rmc* genotype is also described. Possible roles of the other four disrupted genes in the deleted region are discussed. Our results support the identification of CYCLOPS/IPD3 in legumes and rice as a key gene required for AM symbiosis. The extensive characterization of *rmc* in comparison with its 'parent' 76R, which has a normal mycorrhizal phenotype, has validated these lines as an important comparative model for glasshouse and field studies of AM and non-mycorrhizal plants with respect to plant competition and microbial interactions with vascular plant roots.

Keywords: Arbuscular mycorrhizal symbiosis, *Solanum lycopersicum*, Fast neutron mutagenesis, Common Sym pathway, The Tomato Genome Consortium

Introduction

Sophisticated molecular mechanisms have evolved to facilitate the interactions between plants and their beneficial symbionts (Staskawicz and Parniske 2001). The most thoroughly characterized of the root symbioses, the nod-

ulation symbiosis between legumes and eubacteria, is now accepted to have evolved from the arbuscular mycorrhizal (AM) symbiosis (Wang et al. 2010). The evolution of land plants significantly predated the evolution of nodulation, and available evidence supports the key role of AM fungi in the success of the modern vascular plant lineage (Smith and Read 2008). The hypothesis that an underlying similarity between nodulation genetics and the AM colonization pathway might exist has been well substantiated, first at the genetic level (Duc et al. 1989; Sagan et al. 1995; Wegel et al. 1998) and subsequently at the molecular level (van Rhijn et al. 1997; Sträcke et al. 2002; Manthey et al. 2004; Wang et al. 2010; and others). Characterization of these processes has benefited from the study of model host–microbe interactions and availability of host mutants that lack the capacity to form a functional symbiosis (Peterson and Guinel 2000).

Genetic studies have identified elements of the early molecular pathway that are shared between AM symbiosis and rhizobial nodulation (the common Sym pathway). The identities and proposed roles of seven members of this signaling cascade have been reviewed by Parniske (2008). The most comprehensive analyses have been performed in *Lotus japonicus* and *Medicago truncatula,* and genes identified in those two models have different nomenclatures; consequently, the gene names from both models have been used in this paper. Following fungal signal reception (Maillet et al. 2011), the first identified plant response is Ca^{2+} spiking in and around the nucleus of epidermal root cells (Kosuta et al. 2008). This response requires function of a number of genes: *SYMRK/DMI2* which encodes a NORK receptor kinase and is the first known common Sym pathway member (Endre et al 2002; Sträcke et al 2002), *CASTOR&POLLUX/DMI1* (Harris et al. 2003; Kosuta et al. 2008) that encode a nuclear membrane-associated cation channel (Ané et al. 2004; Riely et al. 2007) and two putative nuclear pore component genes *Nup85* and *Nup133* (Kanamori et al. 2006; Miwa et al. 2006; Saito et al. 2007). Following the initial fungal hyphopodium development and plant nucleus migration towards the forthcoming entry site that has no identified genetic regulation, both epidermal and cortical cells prepare for fungal entry by development of a prepenetration apparatus that requires both *SYMRK/DMI2* and *CCaMK/DMI3* (Genre et al. 2005; 2008). CCaMK encodes a calcium–calmodulin-dependent protein kinase (Levy et al. 2004; Mitra et al. 2004). It is proposed to be the primary sensor and interpreter of Ca^{2+} spiking and modulation (Oldroyd and Downie 2006), and it interacts with and phosphorylates the nuclear localized protein encoded by *CYCLOPS/IPD3* (Messinese et al. 2007; Yano et al. 2008). Maillet et al. (2011) have presented evidence that NSP2, which is a transcription factor that acts downstream of CCaMK/DMI3 in nodulation, is also required for normal abundance of colonization by AM fungi. Other genetic components are undoubtedly involved in early symbiosis events but will be hard to identify by mutagenesis if they have multiple roles in plant functioning as found, for example, with *Nup85* (Saito et al. 2007) and *Nup133* (Kanamori et al. 2006).

Whereas targeted studies focusing on common processes in the AM and rhizobial symbioses provide insights into symbiotic relationships, nitrogen-fixing symbioses in root nodules occur in only ten of the approximately 380 angiosperm families (Soltis et al. 1995). Gutjahr et al. (2008) investigated the consequences of mutations of several common Sym pathway genes in rice. Their detailed transcript analysis showed that several genes previously known to be induced early in AM colonization are not influenced by loss of "common Sym pathway" members. These results are indicative of an additional AM response pathway that is of unknown mutation phenotype. Isolation and characterization of the reduced mycorrhizal colonization mutant (rmc) of tomato (*Solanum lycopersicum* L.) (Barker et al. 1998) has also contributed to the identification of a broader range of genetic resources for investigation of AM symbiosis. The *rmc* mutation is recessive, which is suggestive of a loss of function that is unique in the genome of the affected host, and the colonization phenotype is reflective of a "common Sym pathway" mutation. *Rmc* has been mapped to the short arm of chromosome 8 (Larkan et al. 2007). Therefore, the mutation does not directly affect the *SYMRK/DMI2* gene (Nair and Bhargava 2012) because the tomato homologue of *SYMRK/DMI2* is located on chromosome 2 (http://solgenomics.net/feature/17744127/details).

The *rmc* mutant line has been extensively characterized. It has a variable AM phenotype depending on the identity of the AM fungus with which it has been inoculated (Barker et al. 1998; Gao et al. 2001). Some AM fungal species are unable to colonize the roots (Pen^-), whereas others penetrate the epidermal and hypodermal cell layers but do not colonize the root cortex (Coi^-). One AM fungus WFVAM23, formerly called *Glomus versiforme,* currently identified as *G. intraradices* (J. Jansa, personal communication), colonizes the roots of *rmc* more slowly than roots of wild-type tomato but is able to complete colonization of the cortex and produce a functional mycorrhiza (Myc+) (Gao et al. 2001; Poulsen et al. 2005). The mutant *rmc* does not significantly differ from its near-isogenic parent line 76R in its growth parameters or phosphorus (P) uptake when not inoculated with AM fungi (Cavagnaro et al. 2004; Facelli et al. 2010). The phenotypic similarity between *rmc* and 76R makes them a useful pair for studies of mycorrhizal biology in controlled and field environments (for example, Marschner and Timonen 2005; Hallett et al. 2009; Facelli et al. 2010; Schwarz et al. 2011; Ruzicka et al. 2012). The *rmc* mutation, however, does result in increased susceptibility to *Fusarium* wilt in the absence of AM symbiosis (Barker et al. 2006); a locus for *Fusarium* wilt tolerance has been lo-

calized to this region of chromosome 8 (Bournival et al. 1989). Characterization of the molecular basis of the *rmc* mutation would be highly informative not only for understanding AM symbiosis biology but also for further research on the mechanism of tolerance to *Fusarium* wilt, which is a root disease of world-wide consequence in a range of plant production systems (Alabouvette et al. 2009; Michielse and Rep 2009).

Here we report the results of additional molecular and genetic characterization of the mycorrhizal function that is identified via the *rmc* mutation. It was hypothesized from the mutant phenotype that *Rmc* functions in early recognition stages of the symbiosis (Barker et al. 1998). Another aim was to determine whether the mutation disrupted one or more genes in order to better understand the connection between the AM and the *Fusarium* wilt phenotypes of rmc. Reciprocal graft analyses of 76R and *rmc* seedlings were used to determine whether the *Rmc* function could result from a shoot signal or was autonomous within the root. Direct identification of candidate gene functions was attempted by proteome and transcriptome analyses. With those data in hand, we queried the emerging tomato genome datasets (The Tomato Genome Consortium 2012) which, in combination with our original mapping data (Larkan et al. 2007), enabled the location and sequencing of the *rmc* mutation. This information provides a significant clue to the identity of the *Rmc* function in AM symbiosis.

Materials and methods

Tomato genotypes

The *rmc* line used was generated by a single seed descent from one original mutant (plant number 40), which was identified at the M2 generation from a fast neutron mutagenized population (Barker et al. 1998). Parental wild-type seed "76R" (Rio Grande 76R, courtesy of Peto Seed Company, CA, USA), used in all experiments, was collected from plants grown at the same time to minimize seed source differences in genotype performance.

AM fungal cultures

Two fungi were pot-cultured for glasshouse experiments. One was DAOM 181602, formerly known as *Glomus intraradices* Schenck and Smith. This accession is now identified as a synonym of DAOM 197198 (Sokolski et al. 2010) and currently is formally described as *Rhizophagus irregularis* (Krüger et al. 2012). The other AM fungus used was *Scutellospora calospora* (Nicol. & Gerd.) Walker & Sanders (WUM 12). Pot cultures of these fungi were prepared as described by Larkan et al. (2007). Pot culture material was mixed with pasteurized sand or soil mix in the proportion 1:10 to produce inoculated plant material.

Plant growth conditions and inoculation

Plants grown for grafting experiments

All plant growth media were pasteurized prior to use by steaming twice for 1 h at 80–90 °C with a 1-day resting period between each pasteurization. Uninoculated 76R and *rmc* seedlings were grown prior to grafting in punnets of washed river sand containing 0.025 g/kg $CaHPO_4$ (Gao et al. 2001). Pre-inoculated seedlings were produced by planting as seeds directly into *R. irregularis/Allium porrum* nurse trays. Seedlings of the two genotypes and grafted plants were planted in a randomized order to improve confidence that differences in colonization of grafted plants were not due to patchy distribution of infective mycelium. All plants were grown in temperature-controlled glasshouse conditions and were watered daily with deionized water. Nurse culture leeks and grafted tomato plants were watered three times weekly with deionized water. All plants were fertilized weekly with a 1/2X concentration of modified Long Ashton nutrient solution (MLANS) (Cavagnaro et al. 2001).

Two forms of nurse culture (known as nurse pots and nurse trays) were used to promote rapid colonization in test seedlings. An active AM fungal mycelium in the growth medium was established on *A. porrum* (leek) as nurse plant in all cases. Once the mycelium was established, test plants were transplanted into the soil, and rapid colonization of susceptible plants followed. Nurse pots were prepared with *R. irregularis* or *S. calospora* as described by Rosewarne et al. (1997). Nurse trays are an adaptation of the nurse pot method facilitating the separation of roots of nurse and test plants and allowing larger numbers of plants to be screened (S. E. Smith and T. Edmonds-Tibbett, unpublished). In this investigation, new pots or trays were established for each experiment.

Trays were prepared using seedling trays lined with muslin, in which there were three strips of washed river sand containing 0.025 g/kg $CaHPO_4$ and totaling 2 kg, separated by two strips of inoculated medium consisting of a 1:1 mixture of washed river sand and low P sandy soil from Lancelin, Western Australia, totaling 2 kg. The inoculated 1:1 mix contained 1/10 by weight of dried pot culture inoculum. Lancelin soil was amended before use with 10 ml each of three stock nutrient solutions per 3 kg of sand added separately as follows: solution 1 10.6 g/l KH_2PO_4 , 21.3 g/l K_2SO_4; solution 2 21.3 g/l $CaCl_2$; solution 3 0.64 g/l $CuSO_4 \cdot 5H_2O$, 1.49 g/l $ZnSO_4 \cdot 7H_2O$, 2.98 g/l $MnSO_4 \cdot 7H_2O$, 0.11 g/l $CoSO_4$, 0.06 g/l $Na_2MoO_4 \cdot 2H_2O$, 5.96 g/l $MgSO_4 \cdot 7H_2O$. The two strips of inoculated 1:1 mix were planted with surface-sterilized and pre-germinated leek seeds. Leek plants in nurse pots or trays were grown for a minimum of 8 weeks prior to testing for the presence of an

active hyphal network in the sand strips (Barker et al. 1998). Tomato seeds were planted into the sand strips containing an active hyphal network for the production of pre-colonized plants for grafting. Alternatively, non-mycorrhizal tomato seedlings (grafted or not; see Table 1) were transplanted into the sand strips to produce plants in which grafting and initiation of colonization occurred almost simultaneously (not pre-colonized).

Plants for protein analysis

Root protein extraction and analyses were performed with material harvested from plants grown in University of California mix minus phosphate as described by Larkan et al. (2007) for mapping population studies, except that AM fungal inoculum was omitted and plants were watered to weight weekly with 1/2X MLANS. Plants were harvested at 6 weeks after germination. Roots were washed well to remove soil organic matter then snap-frozen and stored at −80 °C until extraction.

Roots for RNA analyses

The plant materials used for transcriptome analyses came from experiments described by Ruzicka et al. (2012). In summary, glasshouse-grown plant materials were grown in sterile potting mix and were not colonized. Field materials were grown in an organically managed farm and were therefore exposed to a mixed assemblage of root colonizers including several different AM fungi. Root colonization of 76R was 23 % of root length, while colonization was <1 % in *rmc* (Ruzicka et al. 2012). RNA was sampled from roots of 8-week-old tomato plants, rinsed and snap-frozen. RNA extraction and analysis is described in Ruzicka et al. (2010).

Plant material for genomic DNA and leaf cDNA analyses

Seeds were germinated and grown in soil-free plant growth medium (Stringam 1971) containing approximately 11 g/L Osmocote PLUS controlled-release fertilizer (Scotts, Marysville, OH, USA) under controlled temperature glasshouse conditions. Leaves of young plants were harvested and freeze-dried for analysis.

Grafting procedure

The grafting procedure followed the methods of Szymkowiak and Sussex (1992). Grafts were prepared by making a diagonal cut through the cotyledon node of the stem of 6-week-old seedlings that had been well watered 1 h previously. The root portion (stock) of pre-in-

Table 1. Reciprocal graft analysis of AM colonization in *rmc* and 76R. Data presented are the percentage root length colonized for pre-colonized (yes) or non-pre-colonized (no) grafted combinations and ungrafted controls. Data are the combined results for one pre-colonized experiment and three non-pre-colonized experiments from both nurse pot and nurse tray colonization.

Pre-colonized	Stock	Scion[a]	*R. irregularis*				*S. calospora*			
			No. assd.[b]	No. col.[c]	Avg. %[d]	Range (%)[e]	No. assd.[b]	No. col.[c]	Avg. %[d]	Range (%)[e]
No	76R	*rmc*	9	9	20.7	4–68	4	4	8.3	1–16
Yes	76R	*rmc*	9	9	73.2	55–91				
No	*rmc*	76R	13	1	0.1	0–1	3	1[f]	0.7	0–2
Yes	*rmc*	76R	13	0	0.0	0				
No	76R	76R	14	14	25.3	4–63	1	1	11.0	11
Yes	76R	76R	6	6	75.3	60–89				
No	*rmc*	*rmc*	13	0	0.0	0	2	0	0.0	0
Yes	*rmc*	*rmc*	6	2	1.2	0–4				
No	76R	ng	33	33	33.8	4–60	1	1	38.0	38
Yes	76R	ng	1	1	70.0	70				
No	*rmc*	ng	26	1	0.0	0–1	1	0	0.0	0
Yes	*rmc*	ng	3	0	0.0	0				

a. Not grafted control
b. Number of plants assessed for AM colonization 6 weeks after grafting
c. Number of plants observed to have at least one internal AM structure from examination of trypan blue-stained roots
d. Average observed % colonization across all plants
e. Range of observed % colonization among all plants
f. Intraradical hyphae but no arbuscules

oculated seedlings, grown from seed in nurse cultures as described earlier, was not disturbed. For seedlings grown without pre-inoculation, the root system was excised as completely as possible from the sandy growth medium using a wide spatula and shaken free of excess sand prior to grafting then transplanted into nurse cultures. The cut zones were kept moist in deionized water, while the second seedling was cut for the reciprocal graft. Incisions were matched, and the stem was wrapped firmly with Parafilm™ to hold the grafted stock and shoot portion (scion) together. The procedure took 2 to 3 min to complete. All but the top leaf was removed from the grafted scion. Nurse trays were covered with plastic bags to maintain humidity and were moved below the glasshouse bench for 2 to 3 days to reduce light intensity. The trays were then returned to the bench, and after 2 weeks the plastic bag and Parafilm™ were removed from surviving grafted seedlings. The plants were grown for a further 4 weeks then harvested for root colonization assessment. Roots were cleared with KOH and stained with trypan blue; a minimum of 100 grid intersects per sample were counted to determine the percentage of root length colonized (% colonization) and occurrence of arbuscules (McGonigle et al. 1990; Barker et al. 1998). Grafted plants transplanted into nurse pots were covered for 2 weeks with Sunbags (Sigma-Aldrich Pty Ltd, Sydney, Australia) rather than plastic bags; otherwise, they were treated in the same way as the nurse trays.

Design of the grafting experiments

Within each experiment, all four possible genotype combinations were prepared, i.e. 76R stock: *rmc* scion, *rmc* stock:76R scion, 76R stock:76R scion and *rmc* stock:*rmc* scion. Ungrafted controls of both genotypes were also grown. Planting was randomized in the nurse pots and nurse trays to reduce the impact of variation due to external environment on analysis of the different treatments. The first experiment with seedlings that were not pre-inoculated utilized four nurse pots containing *S. calospora* inoculum and four nurse pots containing *R. irregularis* inoculum. All other experiments utilized three nurse trays containing *R. irregularis* inoculum. A total of 30 tomato seedlings were planted in each tray and six in each nurse pot, respectively. Unhealthy plants or those where the graft did not form properly were not assessed for colonization, which reduced the number assessed to between 30 and 50 % of the grafted plants in each nurse culture pot or tray. In total, one pre-inoculation experiment and three experiments without pre-inoculation were performed to obtain sufficient numbers of surviving grafts for a meaningful analysis.

Protein extraction

The method for phenol/chloroform protein extractions was adapted from Wessel and Flugge (1984). Precipitated protein from 500 mg powdered tissue was pelleted with a final centrifugation (10 min, 4,500 rpm) and air-dried before being resuspended in 300 µL immobilized pH gradient (IPG) buffer (8 M urea, 2 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 % IPG buffer (3-10NL or 4-7 L; Amersham Pharmacia, Uppsala Sweden), trace amount of Bromophenol Blue). Any insoluble material was removed by brief centrifugation. Protein concentration was determined by the scaled-down method of Bradford (1976) using acetylated bovine serum albumin as the protein standard. Aliquots of the protein/IPG solution containing 500 µg protein were then mixed with a few grains of bromophenol blue and used for isoelectric focusing (IEF).

Separation of proteins by 2D-PAGE

First-dimension separations were performed by IEF adapted from Berkelman and Stenstedt (1998) using immobilized pH gradient strips (either pH 3-10NL or pH 4-7L 13 cm Immobiline DryStrip; Amersham Pharmacia, Uppsala Sweden). Second-dimension separations were performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 10 % acrylamide with a 37.5:1 ratio of acrylamide to *bis*-acrylamide. The resultant gels were stained with colloidal Coomassie blue stain (17 % $(NH_4)_2SO_4$, 34 % methanol, 3 % phosphoric acid, 1 % Coomassie brilliant blue G-250 in water). The contrast and brightness of the images was improved for printing purposes using Adobe PhotoShop 5.0 software (Adobe Systems Inc, USA).

Protein sequence analysis

Protein sequence, determined by mass spectrometry of trypsin digest products and database Basic Local Alignment Search Tool (BLAST) searches, was performed by Proteomics International Pty Ltd, PO Box 6064, East Perth 6892, Western Australia.

Candidate gene identification

The sequences of the flanking markers for the *rmc* locus, CT88 and EST248494 (Larkan et al. 2007), were used to search the available tomato genome sequence (The Tomato Genome Consortium, accessed from solgenomics.net). Positioning of the markers on chromosome 8 of *S. lycopersicum* allowed the identification of three bacterial artificial chromosomes (BACs) corresponding to the marker interval and provided approximately 240 kb of genomic DNA sequence. Genes within this sequence

were predicted using FGENESH software (softberry.com). The predicted protein sequences were then used to search the National Center for Biotechnology Information non-redundant protein sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for homologous genes via blastp. This work was carried out prior to current annotation of the tomato genome.

Transcript analysis

Transcriptome profiling of *rmc* and 76R root RNA extracts was performed using the Tomato Genome Array Chip (Affymetrix, Santa Clara, CA, USA) as described in Ruzicka et al. (2010). Expression of the *CYCLOPS/IPD3* gene homologue was assessed by RT-PCR as described in Ruzicka et al. (2012), and primer sequences are included in Online Resource 1.

Investigation of candidate locus

After identification of a candidate gene, several pairs of primers were designed in and around the candidate gene locus, based on the candidate BAC sequence (Online Resource 1). Polymerase chain reaction (PCR) was used to survey the locus in 76R and *rmc* and to delineate the *rmc* mutation using genomic leaf DNA extracted by the CTAB method of Murray and Thompson (1980). A PCR fragment produced using primers flanking the mutation site was amplified from *rmc* DNA using high-fidelity Phusion DNA Polymerase (New England Biolabs Inc., Whitby, ON, Canada), cloned into the vector pCR4-TOPO (Life Technologies Inc., Burlington, ON, Canada) and sequenced. Leaf total RNA from 76R was produced using a PureLink RNA Mini Kit (Invitrogen = Life Technologies Inc., Burlington, ON, Canada), and a full-length transcript of the coding sequence of the candidate gene was amplified using "RTfull F & R" primers (Supplementary Resource 1), cloned and sequenced.

Phylogenetic analysis

The *S. lycopersicum CYCLOPS/IPD3* protein prediction was used to identify other homologues deposited in GenBank via BLASTP (http://blast.ncbi.nlm.nih.gov). These sequences were aligned using ClustalW2 to produce a multi-sequence alignment which was then bootstrapped with SEQBOOT. The resulting data set was passed to PROTPARS, which inferred a phylogeny, and then a single bootstrapped tree was produced by CONSENSE and viewed using Tree View. Clustal programme was obtained from the European Bioinformatics Institute (ebi.ac.uk), while SEQBOOT, PROTPARS and CONSENSE are from the PHYLIP suite of programs (Felsenstein 1989).

Results

Reciprocal graft analysis

Reciprocal grafting experiments were performed to investigate whether *Rmc* function involved a signal from the shoot or was autonomous to the root. Table 1 summarizes the colonization results obtained for the reciprocal grafting experiments. The results of all the grafting experiments were clear in showing that the stock (root + hypocotyl) genotype controlled the AM colonization phenotype of *rmc* by both *R. irregularis* and *S. calospora,* regardless of whether the seedling was uninoculated or mycorrhizal at the time of grafting. An occasional *rmc* root sample had a trace of hyphal colonization as has previously been noted (Barker et al. 1998; Gao et al. 2001). This result demonstrated that Rmc does not function via a signal from shoot to root but operates directly within the root.

Proteomic analysis

Since grafting experiments showed that *Rmc* functioned in the root, it was hypothesized that *Rmc* function was constitutive and might be identifiable by comparative analysis of the protein profile of roots from 76R and *rmc* plants grown in the absence of AM colonization. Differentially accumulated proteins were not detected when total protein extracts were compared by 2D-PAGE (data not shown). However, separation of proteins extracted by the phenol/chloroform method identified reduced accumulation of a 24-kDa p*I* 5 protein in *rmc* roots compared to 76R roots (Online Resource 2). This reduced accumulation was reproducibly observed in protein extracts from different batches of plants. The matching protein spots for both 76R and *rmc* were isolated from the gels, and both samples were subjected to mass spectroscopy analysis. The protein was determined to closely match the N terminal region of the ATPase domain of members of the 70-kDa heat shock protein (HSP70) family by comparing fragment mass data generated from trypsin digestion and mass spectroscopy analysis. The protein, which has an estimated length of 220 amino acids, was named HSP24. In particular, it most closely matched the cytoplasmic HSP70s and had a 39 % exact amino acid match to the N-terminal sequence of HSP70-3 from *Nicotiana tabacum* (Q67BD0_TOBAC). HSP24 lacked the protein binding and variable domains of the HSP70 (and HSP54) protein consensus sequences. From its truncated size, it is unlikely to contain a nuclear targeting signal and therefore is most likely a cytoplasmic protein. As the protein was reproducibly found to be reduced in abundance in *rmc* but not absent or detectably mutated, it does not appear to be a prime candidate for the *Rmc* locus.

Tomato genome sequence analysis

Once genome sequence data for tomato chromosome 8 had become publicly available, the availability of BAC sequences containing the closest marker sequences was re-assessed. A contig across the *Rmc* region that contained three BAC clones and consisted of approximately 240 kb of genomic sequence was created. FGENESH gene prediction software (softberry.com) was used to annotate the region and to determine whether any match to known mycorrhiza-regulating genes could be identified. A DNA sequence match to HSP24 (Online Resource 2) was not located in the contig. Several predicted protein kinase and NBS-LRR-type R-gene homologues were identified within the interval between the rmc-flanking markers. Most importantly, one of the three BACs located in this region, "C08Hba0001K22" (Genbank #AP009260), was found to contain a homologue of the *CYCLOPS/IPD3* gene located approximately 80 and 160 kb from the rmc-flanking markers CT88 and EST248494, respectively. This predicted sequence (later annotated as Solyc08g075760) was the most likely *Rmc* candidate gene identified by this sequence scanning.

Transcriptome analysis of field-grown *rmc* and *Rmc* tomato plants

Concomitant with the tomato chromosome 8 genome sequence analysis described earlier, transcriptome analysis of *rmc* and 76R grown in organic cropping conditions was performed using the Affymetrix tomato array. The analysis of *rmc* and 76R roots grown in both the absence (glasshouse) and presence (organic field) of AM fungi identified the absence of two transcripts in *rmc* roots that were expressed in 76R roots. One of these transcripts encodes a putative Xaa-proline dipeptidase (XPD). A BLAST search of the XPD gene sequence (AK322013) was performed, and the gene encoding this transcript was found to be located on tomato chromosome 7 (data not shown). The second transcript mapped to the same region of tomato chromosome 8 as the Rmc function did. This transcript is from the predicted gene sequence AK329636, which shares sequence similarity with a predicted *Arabidopsis* protein of unknown function. Whilst examining the available tomato genome sequence around AK329636, it was realized that the tomato homologue of *CYCLOPS/IPD3* was adjacent. Since the *CYCLOPS/IPD3* gene transcript is not represented on the Affymetrix tomato array, primers were designed for RT-PCR detection of the tomato *CYCLOPS/IPD3* gene transcript, and its expression was detected in both colonized and non-colonized 76R root tissue, but no expression was detected in *rmc* roots (Figure 1).

Sequence of the *rmc* mutation

Two independent approaches had identified a *CYCLOPS/IPD3* homologue in tomato that was located in the previously mapped Rmc region of chromosome 8 and was not detectably expressed in *rmc* roots. To finalize the characterization of the *Rmc* region, the corresponding region of *rmc* was sequenced. Several primer pairs, designed within both the predicted *S. lycopersicum CYCLOPS/IPD3* coding region (CDS) and the neighboring AK329630 CDS, successfully amplified fragments of the expected size from the wild-type 76R but failed to produce fragments from the mutant *rmc* (data not shown). Additional primer pairs were designed from sequence 3, 6, 10 and 22 kb upstream and 15 and 22 kb downstream of the *CYCLOPS/IPD3 CDS* (prefixed "Up" and "Down", Supplementary Resource 1) and used to survey the genomic region surrounding *CYCLOPS/IPD3* in both 76R and *rmc*. All primers were successful in amplifying 76R DNA. The "Up3" primer pair (positioned 3 kb upstream of the *CYCLOPS/IPD3* CDS) failed to amplify a fragment from the mutant *rmc*, though all of the other primer pairs were successful. The primers "Up6 F" and "Down15 R" were used to produce a 2,416-bp PCR fragment which spanned the *rmc* mutation locus. Sequencing of this fragment revealed a deletion of 24,474 bp spanning the entire *CYCLOPS/IPD3* and AK329630 loci and also affecting several neighboring genes. The remaining sequence was a perfect match to the BAC "C08Hba0001K22", with the first 1,329 bp of the PCR fragment matching the BAC at 60,924 to 62,252 bp and the remain-

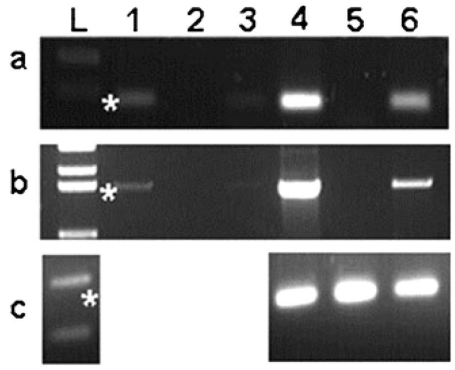

Figure 1. PCR amplification of the *CYCLOPS/IPD3* sequence that lies within the Rmc locus of tomato. Root RNA samples were assessed with two different primer pairs for the presence of *CYCLOPS/IPD3*-like RNA sequence. Input cDNA was assessed at 1:50 dilution (*lanes 1–3*) and a zero dilution (*lanes 4–6*). *Lanes 1* and *4* are from 76R grown in field conditions, *lanes 3* and *6* are from 76R grown in glasshouse conditions and *lanes 2* and *5* are from *rmc* plants grown in field conditions. a. Primer set "RT-PCR sense/antisense" (Supplementary Resource 1) product expected (* indicates right side of DNA marker *lane L*) was 150 bp. b. Primer set "RTfull sense/antisense" (Supplementary Resource 1) product expected (* indicates right side of DNA marker *lane L*) was 1,600 bp. Products were amplified from 76R grown in both sterile soil and field conditions; however, *rmc* samples failed to amplify. c. Detection of actin gene expression (* indicates right side of DNA marker *lane L*) was used as the positive control (Ruzicka et al. 2012). Only the zero dilution is shown (*lanes 4–6*)

ing 1,087 bp matching at 86,728 to 87,814 bp. Figure 2 illustrates the organization of the *Rmc* locus. No additional chromosomal damage or variation from the tomato genome sequence was detected as a result of the mutation. The interval spanned by the deletion in *rmc* corresponds to SL2.40ch08:57039278 to 57063752 in the current iteration of the tomato genome, which partially or fully excises a total of five genes: "Solyc08g075750" to "Solyc08g075790" (Figure 2; Table 2).

The *CYCLOPS/IPD3* transcript coding sequence was amplified from the wild-type 76R leaf cDNA. Sequencing of this transcript confirmed the gene model currently predicted for the tomato genome (Solyc08g075760). The leaf cDNA coding sequence has the Genbank reference JX972196. Additional BLAST analysis of the current iteration of the tomato genome failed to identify a second copy of this sequence elsewhere in the tomato genome (data not shown). The neighbor joining tree (100 bootstraps) of all the full-length *CYCLOPS/IPD3* proteins available in Genbank is presented in Figure 3. The tomato sequence clusters with that of potato in a group that is separate from the Rosid sequences, consistent with broader phylogenetic analyses.

Discussion

Results from the present study indicate that *CYCLOPS/IPD3* is the prime candidate sequence for the *Rmc* function. Research was previously initiated on mutations of tomato that influence development of arbuscular mycorrhizas in order to expand knowledge of AM root biology and to develop materials for research into arbuscular mycorrhizas that are not complicated by nodulation biology (Barker et al. 1998). Identification by chromosome sequence analysis that the *rmc* mutation is a 24-kbp deletion that includes a *CYCLOPS/IPD3* gene (Messinese et al. 2007; Yano et al. 2008) is fortuitous, however, because legume model research has demonstrated the importance of this particular function to AM symbiosis, enabling us to hypothesise that the *rmc* phenotype is at least in part a consequence of the loss of this function. In their phylogenetic analysis of three key common Sym pathway genes including *CYCLOPS/IPD3*, Wang et al. (2010) have confirmed the single gene status of *CYCLOPS/IPD3* across all available genome sequences, supporting the previous Mendelian analysis of the rmc mutation (Barker et al. 1998), and BLAST search of the available tomato genome sequence (data not shown). Horváth et al. (2011) have investigated mutations of the *CYCLOPS* ortholog *IPD3* in *M. truncatula* and conclude, as do Ovchinnikova et al. (2011), that there is an effect of genetic background of the host on the severity of the mutant phenotype. A genetic background effect indicates that there is involvement of other host genes in the AM signalling pathway that may vary in functionality or presence between genetic backgrounds. These conclusions are congruent with present and previous (Barker et al. 1998) observations of the occasional 'leaky' *rmc* phenotype, suggesting that a genetic background effect may also be influencing the *rmc* phenotype. Alternatively, it is worth noting that colonization in *rmc* is significantly increased if the mutant plants are grown in the presence of the wild type (76R), indicating that colonization may also be assisted by a nearby host (Facelli et al. 2010). The grafting results presented here also support the proposal that the *CYCLOPS/IPD3* homologue is the most likely candidate gene for the *Rmc* function in AM symbiosis. Reciprocal genotype grafting or grafting onto *rmc* roots of pre-inoculated 76R shoots might have shown a shoot signal effect if Rmc functioned via a shoot signal such as a microRNA (Gu et al. 2010). However, *CYCLOPS/IPD3* regulates AM symbiosis directly through an intranuclear role in combination with CCaMK (Messinese et al. 2007; Yano et al. 2008), congruent with the grafting results.

Although the grafting results indicate that *Rmc* does not function via a signal that is translocated from the shoot, it was possible to clone and sequence full-length *CYCLOPS/IPD3* from leaf cDNA, indicating that its expression is not root specific. Although Messinese et al. (2007) report the relative expression of *IPD3* in roots of *M. truncatula* to be 40 times higher than in leaves or flowers and Yano et al. (2008) could not detect *CYCLOPS* gene expression in shoots of *L. japonicus,* some other common Sym pathway genes are expressed in other plant organs (Ané et al. 2004; Levy et al. 2004; Kanamori et al. 2006). It will be of interest to examine if genes from the common Sym pathway have been recruited to function in other symbioses, such as those with obligate shoot endophytes (Kuldau and Bacon 2008), given their single or low copy number status and constrained evolutionary freedom

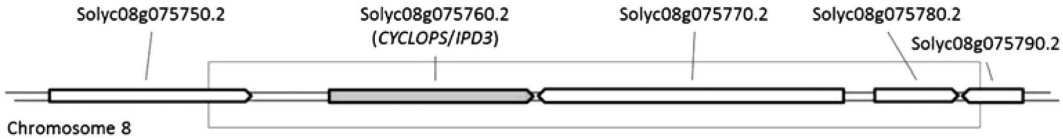

Figure 2. Cartoon image of the *Rmc* locus of tomato. The figure shows the region SL2.40ch08:57033000 to 57065999 of tomato chromosome 8, which harbours the *rmc* mutation. The 24-kbp deletion spanning three intact predicted genes and cleaving two additional genes is indicated by the surrounding box. Arrow boxes designate the location of gene sequences as annotated in the ITAG 2.3 Release, with the *CYCLOPS/IPD3* homologue indicated by the filled arrow box

Table 2. Identity of the five predicted gene sequences that are disrupted by the *rmc* mutation

Gene	Description
Solyc08g075750	ATP-dependent Clp protease proteolytic subunit
Solyc08g075760	*CYCLOPS/IPD3*
Solyc08g075770	Unknown
Solyc08g075780	Ubiquitin-fold modifier 1
Solyc08g075790	Vacuolar sorting protein

(Wang et al. 2010). The bootstrap analysis performed for the full-length *CYCLOPS/IPD3* homologues fits with the available phylogenetic information about *CYCLOPS/IPD3* and is consistent with the evolution of this symbiosis-related gene pre-dating the separation of the land plant clades (Wang et al. 2010).

The *CYCLOPS/IPD3* gene is most certainly a contributor to the non-mycorrhizal phenotype of the *rmc* mutation, matching well with the description of phenotypes for mutations in this function in other host species. It is feasible that the CYCLOPS/IPD3 function is also a contributor to *Fusarium* wilt tolerance. However, Wang et al.

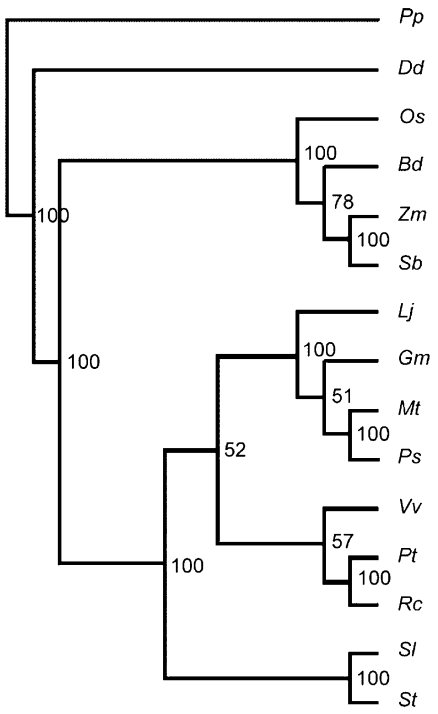

Figure 3. Neighbor joining tree of the tomato *CYCLOPS/IPD3* gene. The tomato sequence was compared with available *CYCLOPS/IPD3* gene sequences from Genbank. The tree obtained is congruent with that of Wang et al. (2010). Species names are as follows: *Pp Physcomitrella patens, Dd Diphasiastrum digitatum, Os Oryza sativa, Bd Brachypodium distachyon, Zm Zea mays, Sb Sorghum bicolour, Lj Lotus japonicus, Gm Glycine max, Mt Medicago truncatula, Ps Pisum sativum, Vv Vitis vinifera, Pt Populus trichocarpa, Rc Ricinus communis, Sl Solanum lycopersicum, St Solanum tuberosum*

(2012) have shown the opposite consequence to oomycete colonization in mutation of a function that operates downstream of *CYCLOPS/IPD3*. They report that mutation of the *M. truncatula* gene *RAM2*, which encodes a glycerol-3-phosphate acyl transferase and is involved in cutin biosynthesis, reduces colonization of roots by *Phytophthora palmivora*. Therefore, it cannot be excluded that one or more of the other genes in the deletion also contribute to mycorrhizal symbiosis and/or tolerance to *Fusarium* wilt, particularly as the *rmc* deletion includes a second root-expressed gene with unknown function. Complementation analysis where each transcript is individually expressed in the *rmc* background will enable precise confirmation of the contribution of each gene to mycorrhizal symbiosis and tolerance to *Fusarium* wilt.

Secondary consequences of the *rmc* mutation can be inferred from the proteomic (Supplementary Resource 2) and transcriptomic (data not shown) results. Both have uncovered gene expression consequences of the *rmc* mutation that do not co-locate with the *Rmc* locus on chromosome 8. The mutant line material used in this research has been derived by single seed descent over six generations from an original single plant chosen from self-progeny of a mutant population parent. In early analysis of the *rmc* line, it was crossed to 76R to demonstrate that a single locus was responsible for the non-mycorrhizal mutation phenotype (Barker et al. 1998). Therefore, while there is a possibility that additional mutations from the original mutagenesis remain fixed in the *rmc* background, only the chromosome 8 mutation locus has been linked to disruption of the mycorrhizal phenotype. Attempts to amplify a genome sequence for the XPD transcript from *rmc* DNA have been unsuccessful (data not shown), which is suggestive of a second deletion. However, the location of this gene on chromosome 7 of tomato (data not shown) along with the clear 3:1 (F2) and 1:1 (BC) Mendelian segregation of the AM function that is lost in *rmc* (Barker et al. 1998; Larkan et al. 2007) indicates that this gene probably does not directly function in the regulation of AM symbiosis. Likewise, the fact that neither the HSP24 protein (Supplementary Resource 2) nor a potential progenitor protein is encoded within the *Rmc* region shows that this protein also is not likely to play a primary role in symbiosis. Since the gene sequence of this protein has not been investigated, no comment can be made on whether it is another secondary genetic mutation in the *rmc* line or, alternatively, is affected in its expression in roots as a consequence in trans of the *rmc* deletion. However, the observed changes to XPD and HSP24 might indicate flow-on effects from one of the other disrupted genes. For example, one disrupted gene is predicted to encode a protease and another is predicted to have a role in the ubiquitin pathway. These potential mutation "side effects"' may well be resolved by the complementation studies mentioned earlier.

The *rmc*/76R genotype pair is a useful genetic resource for AM research in both controlled environment and field studies. The *rmc* genotype and its near-isogenic parental breeding line Rio Grande 76R have been distributed over the last decade to more than 30 different research groups around the world. The near-isogenic pair has enabled successful analysis of a broad range of topics involving AM symbiosis (for example, Cavagnaro et al. 2004; Marschner and Timonen 2005; Cavagnaro et al. 2007; Hallett et al. 2009; Facelli et al. 2010; Schwarz et al. 2011; Ruzicka et al. 2012; Watts-Williams and Cavagnaro 2012). The availability of a near-isogenic pair differing in AM symbiosis but with so very few other identifiable side effects enables the study of plant functions in soil that has not been sterilized of all natural biota and allows research on the role of AM symbiosis in sustainable agriculture and in plant competition. However, the presence of several genes disrupted by the *rmc* mutation may confound some analyses.

It is also important to consider that plants carrying *rmc* allow some AM intracellular colonization by particular AM fungal species. For example, when *rmc* was grown in field soils from California and Arizona, colonization was only in the epidermis and was typically 1 to 3 % (Cavagnaro et al. 2006; 2008; 2012; Ruzicka et al. 2012). Colonization of 76R was throughout the root cortex and ranged from 20 to 29 %. Fungi were not identified, but the long-term management of these soils without fungicides would suggest that a diverse mycorrhizal community was present. In glasshouse experiments, *Glomus* sp. WFVAM23 slowly forms typical cortical colonization with arbuscules, and *S. calospora* forms abnormal intracellular structures in exodermal cells (Barker et al. 1998; Gao et al. 2001). In both cases, colonization is accompanied by functionality (limited in the case of *S. calospora*) in terms of P transfer to the plant and AM fungal growth, presumably supported by sugars from the plant (Poulsen et al. 2005; Manjarrez et al. 2008; Manjarrez et al. 2010). The comprehensive functional and sequence information presented here should however allow an informed analysis of past and future results obtained by using the *rmc* genotype as a research tool.

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Supplementary materials follow the **References.**

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article title The reduced mycorrhizal colonisation (*rmc*) mutation of tomato disrupts five gene sequences including the *CYCLOPS/IPD3* homologue.

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Online Resource 1 Sequence of PCR primers used to demarcate, clone and sequence across the *rmc* mutation, and to perform RT-PCR analysis of *CYCLOPS//IPD3* gene expression.

Primer identity	Primer sequence
RTfull-F	ATGGAAATGGAGGGAAGAGGTTATTCA
RTfull-R	AAACACGTCTGAAGCTGCTAAAGAGTAACA
Up3-F	TTCATTGAGAGTGGATGCTTGGTGATG
Up3-R	CGAGCCAATAGATTTCAATAGCCAAGAA
Up6-F	TTGATCTATCCATTCTGAAGGCTTGAGTG
Up6-R	GTTCATATGATCGTCCAGTGGAATTGG
Up10-F	TCGAAGCTCTACTGTAGACTTCACGACAGA
Up10-R	CATTGCGTCTCCTTCAGCACCAC
Up22-F	GCCTCCTTGCATAAGTAACACCAACTGA
Up22-R	TTAAGTCCATCGCCATGTTATACCTATCAA
Down15-F	TTCTCGAGGTCTTACGCTATGAACATTGA
Down15-R	GAGGGCTAAAAGTTTACCAAATTGTGACAT
Down22-F	TAGATTACTCACTGACTGGCTACCAACTGC
Down22-R	TGTTGATGATCAAGTGAAGGAATATGGTTC
RT-PCR sense	AAGTCGGATATGGAAGGAGCTCAG
RT-PCR antisense	CGCTCCACCCTTCTCTTCTTCTTA

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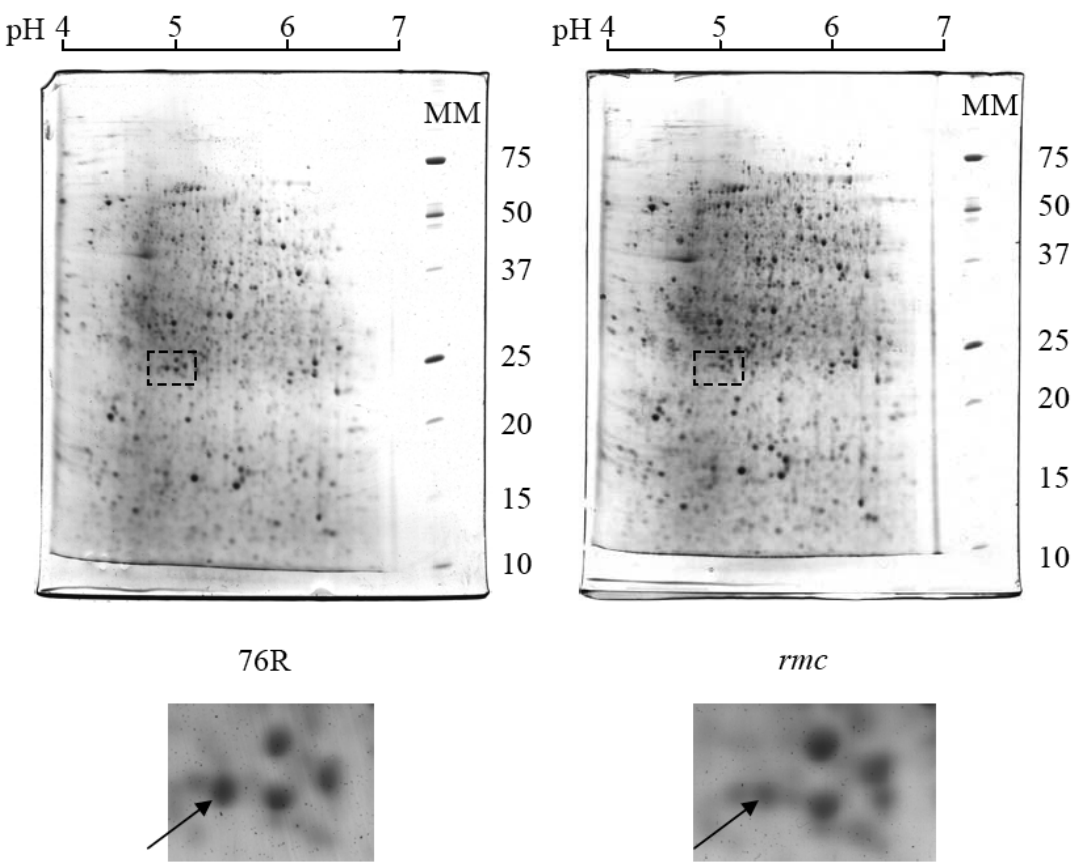
Online Resource 2 2D-PAGE analysis of 76R and *rmc* root proteins. Proteins were separated using 4-7L IPG strips and 10% polyacrylamide gels. Boxes indicate regions enlarged below gels. Arrows indicate differentially accumulated 24 kDa protein taken for sequence analysis. MM mass marker, sizes in kDa. Additional protein analysis is presented in Larkan (2006).



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