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Review

Towards the elucidation of AM-specific transcription in *Medicago truncatula*

Franziska Krajinski *, André Frenzel

Institute for Plant Genetics, University Hannover, Herrenhäuser Strasse 2, D-30419 Hannover, Germany

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Abstract

Roots of most plants form a mutualistic interaction with arbuscular mycorrhiza (AM) fungi. During the symbiosis, drastic morphological and physiological changes occur in the host plant root system. These changes are likely to be controlled by a specific genetic program of the plant. The legume *Medicago truncatula* is a model system widely used to elucidate this program. A number of loci required for AM-development have been identified by the analysis of *M. truncatula* mutants; however, the genes identified are also required for the Legume-Rhizobium symbiosis, since all *M. truncatula* mycorrhizal mutants so far reported are also nodulation mutants. We have focused on the identification and analysis of AM-specific genes as a further means to gain insight into the molecular background of the AM symbiosis and the molecular regulation of this tight symbiosis. Here, we describe the identification of AM-specific genes and the analysis of their transcriptional regulation. The identification of promoters and regulatory elements mediating the mycorrhiza-specific transcription provides a starting point to identify the corresponding specific transcription factors. This enables the identification of further upstream elements of the regulation cascade, and thus the elucidation of the molecular mechanisms controlling the AM-development within plant roots.

Keywords: *Medicago truncatula*; Arbuscular mycorrhiza; Transcription; Promoter

Contents

1. Introduction

Plants roots are able to form a wide variety of interactions with organisms of the rhizosphere. The arbuscular mycorrhiza (AM) is a mutualistic interaction formed between roots of flowering plants and AM-fungi. This plant–fungus association represents a unique example of a highly compatible interaction between eukaryotes, and it occurs in terrestrial ecosystems throughout the world. Bidirectional nutrient exchange represents the main beneficial

* Corresponding author. Tel.: +49 511 7625548; fax: +49 511 7624088.
E-mail address: krajinski@lgm.uni.hannover.de (F. Krajinski).

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effect for both partners of this mutualistic association. The fungi involved in AM symbiosis are obligate biotrophs that have been traditionally grouped to the Zygomycetes; phylogenetic analyses have shown that they have an ancient origin, and no close relationship to other Zygomycetes exists. They have thus been placed into a new phylum, Glomeromycota. During the symbiotic interaction, the AM-fungi germinate and develop a presymbiotic mycelium with limited growth. After contact with a host root, they form an appressorium and the colonization of the root system then commences. The growth pattern of the fungus within the host root can be divided into two major types, depending on the species involved. In Arum-type interactions, the fungus starts to enter the cells of the inner cortical cell layers and forms the so-called arbuscules, the site of phosphate transfer from the fungus to the host plant. Although the fungus also delivers other mineral nutrients to the plant, the improved phosphate nutrition represents the main benefit for the host plant, and the AM-association has a global impact on plant phosphorous nutrition. The fungus also benefits from this interaction; it obtains a considerable amount of carbohydrates from the plant. Numerous modifications in root cells occur during the development of arbuscules. The fungus does not directly enter the host protoplast; instead, the fungal structures remain surrounded by the periarbuscular membrane originating from the host plasma membrane. The periarbuscular membrane completely surrounds the arbuscule, exhibiting an up to fourfold increase in surface area. Moreover, the periarbuscular membrane exhibits several new properties relative to the plasma membrane of non-colonized cells. A number of proteins involved in the nutrient transfer between the organisms, such as phosphate transporters (Bonfante and Perotto, 1995; Harrison et al., 2002; Rausch et al., 2001) or H^+-ATPases (Gianinazzi-Pearson et al., 1991) are located in this new membrane. Colonization by an AM-fungus also induces considerable changes in the shape and the number of the cortical cells' organelles. Specifically, the central vacuole becomes fragmented the cytoplasm volume as well as number of cell organelles increase (Bonfante and Perotto, 1995) and the nucleus moves into a central position and shows enhanced transcriptional activity (Gianinazzi-Pearson, 1996). Taking into account these striking cytological changes and the fact that more than 90% of a root system can be colonized by AM-fungi, it is clear that a specific genetic program must control the AM-symbiosis. All developmental stages of an AM-symbiosis including the perception of a so-far unknown fungal signal molecule and the expression of mycorrhiza-specific genes, are likely to underlie tight control mechanisms of the host plant (Kistner and Parniske, 2002; Parniske, 2000). Hence, one step towards understanding of the molecular regulation of the AM-symbiosis is the elucidation of the AM-specific genetic program through the analysis of transcriptional changes using a model plant and the subsequent analysis of the mechanism of AM-specific transcription.

2. *M. truncatula* as one model plant in AM research

With the development of high-throughput technologies, untargeted approaches became very efficient for the identification of plant genes induced during AM-development. One prerequisite for a successful application of genome-research based high-throughput technologies is the availability of model systems. In the last decade, two legumes have been established as plant model systems in AM research.

While the well-known model plant *Arabidopsis thaliana* interacts only antagonistically with microorganisms and has no known mutualistic symbionts, *M. truncatula* and *Lotus japonicus,* like almost all legumes, have the potential to establish two different mutualistic interactions in their roots: namely, with AM-fungi or with Rhizobia, leading to nodule formation and nitrogen fixation. Considerable insight into the signaling underlying AM-development in roots was obtained from the analysis of plant mutants unable to form AM-associations. The analysis of legume nodulation mutants showed that a number of these mutants are also impaired in their ability to form the AM-symbiosis. Hence, although the microsymbionts of these symbioses are different types of organisms, they both establish endosymbiosis with plant roots, and a wide range of molecular data confirm the existence of a genetic program that regulates the accommodation of microsymbiont inside plant cells (Parniske, 2000). In *M. truncatula* one mycorrhizal phenotype, represented by three mutants, has been described so far. In the symbiosis mutants *dmi1* (does not make infections), *dmi2*, and *dmi3*, the fungal growth is arrested at the early epidermal cell layer; they only very rarely have infections of root cortex cells (Sagan et al., 1995). These mutants still show the very early response to Nod factors, signal molecules produced by Rhizobia during the initial phase of the root nodule symbiosis. In the last few years, the corresponding genes were cloned in *M. truncatula* and other legume species. DMI2 and its ortholog in *L. japonicus* encode receptor-like kinases (Endre et al., 2002; Stracke et al., 2002). DMI1 encodes a putative channel protein that acts downstream of DMI2 (Ane et al., 2004), and DMI3 encodes a putative calcium/calmodulin-dependent protein kinase (Levy et al., 2004; Mitra et al., 2004). Interestingly, all of the genes encode proteins involved in signal transduction, but how these components are incorporated in the global mycorrhiza signaling pathway is still unknown.

The use of genomic approaches for the model legumes *M. truncatula* and *L. japonicus* has led to significant advances in our knowledge about the molecular changes in the plant partner during the AM interaction. The genomes of both plants have been physically and genetically analyzed and sequenced. In addition to the genomic sequencing projects data, further sequence data are available in freely accessible databases. This is especially true for *M. truncatula*; more than 220.000 (release 8.0, January 2005) expressed sequence tags (ESTs) were contributed to

the *M. truncatula* gene index (MtGI at www.tigr.org) by a number of research groups. Currently, this EST collection includes ESTs from five comprehensive cDNA libraries constructed from mycorrhizal tissues (Journet et al., 2002; Liu et al., 2003; Frenzel et al., 2005) including libraries enriched for AM-induced genes (Wulf et al., 2003). The *M. truncatula* EST collection provides a unique resource for performing electronic Northern analysis (Fedorova et al., 2002; Journet et al., 2002) and for constructing cDNA arrays (Hohnjec et al., 2005; Küster et al., 2004; Liu et al., 2003).

3. Identification of AM-specific *M. truncatula* genes by targeted approaches

Since the development of mycorrhizal symbioses leads to significant changes in plant cell morphology and physiology, one can expect that the transcriptome of mycorrhizal roots will differ dramatically from non-mycorrhizal roots. Genes appearing with different frequencies in the transcriptome of mycorrhizal and non-mycorrhizal tissues are considered to be differentially expressed in response to mycorrhiza development. The identification of these differentially expressed genes is the aim of transcriptome approaches in mycorrhiza research since the collection of genes, up- or downregulated upon mycorrhiza development provides insight into the molecular mechanisms of the AM-symbiosis. Furthermore, plant genes specifically induced in response to mycorrhiza development serve as starting points for investigating the molecular regulation of the symbioses. Targeted approaches undertaken mainly in the pre-genomics area were focused on genes that might be involved in nutrient transport processes or defense responses. Plant genes involved in defense responses have been shown to be transiently induced at early stages of the AM-development in different plants. Different *M. truncatula* chitinases, which constitute part of the inducible plant defense response, were analyzed for their RNA accumulation in AM. These analyses led to the conclusion that class III chitinase genes are strongly induced in mycorrhizal roots, and that they exhibited almost no basal expression in non-mycorrhizal roots (Salzer et al., 2000). Besides the genes involved in defense response, *M. truncatula* genes that might contribute to the nutrient transfer between plant and fungus in AM-symbiosis have been the focus of further studies. H^+-ATPases might be responsible for the generation of a proton gradient, which enables the transfer of different molecules across membranes from the fungal microsymbiont to the host plant. One member of the *M. truncatula* H^+-ATPase family shows a strong transcriptional induction in roots upon AM-colonization and is localized in arbuscule-containing cells (Krajinski et al., 2002). A further gene family that was screened for AM-inducibility is the family of membrane intrinsic proteins, integral membrane proteins that facilitate the movement of small molecules across membranes. Members of this protein group might function specifically in transport processes across the arbuscular interface. The *M. truncatula* membrane intrinsic protein *MtAqp*1 is strongly induced in AM, but shows strongest sequence similarity to members of the subfamily of tonoplast intrinsic proteins (Krajinski et al., 2000). Hence, the MtAQP1 protein is predicted to function during the fragmentation of cell vacuoles during arbuscule development.

4. Identification of AM-specific *M. truncatula* genes by untargeted approaches

In the era of genomics, and with the establishment of model legume systems, untargeted approaches became very efficient in obtaining a comprehensive view of the transcriptomic changes taking place in a root system upon mycorrhizal development. The generation of ESTs represents a first step for various techniques aimed at the identification of induced or repressed genes. On the one hand, these sequences can be used for electronic Northern analysis. By comparing the EST distribution among cDNA libraries generated from mycorrhizal roots, root nodules and root tips, a number of genes were identified that are either up- or downregulated in mycorrhizal tissue (Journet et al., 2002). The suppression subtractive hybridization (SSH) technique (Diatchenko et al., 1996) enables the generation of cDNA libraries that are enriched for sequences overrepresented in a certain RNA sample. Within the MolMyk project, we have constructed a cDNA library enriched for genes upregulated in mycorrhizal tissue (Wulf et al., 2003), an approach leading to the identification of a significant number of AM-induced genes. None of these genes were expressed in non-mycorrhizal roots or leaves. Electronic data obtained by comparing the cDNA sequences to EST sequences from a wide range of cDNA libraries in the *M. truncatula* EST database supported the mycorrhiza specificity of the corresponding genes, since the TC and EST sequences in the MtGI that were found to match the identified SSH-cDNA sequences were originating exclusively from AM cDNA libraries. Hence, this SSH-cDNA population represents a source for selecting candidate genes for subsequent promoter analysis.

Further SSH-libraries have been utilized to clone genes that are specifically induced during early and late stages of the AM development (Brechenmacher et al., 2004; Grunwald et al., 2004).

Apart from representing the starting point and prerequisite for electronic Northern analyses, ESTs have been also used to construct cDNA arrays for subsequent hybridization aimed at identifying genes whose transcription levels are altered in mycorrhizal roots. A macroarray was constructed using 2268 unique sequences of a random cDNA library from mycorrhizal roots of various developmental stages, and hybridization analysis showed that 3% of the genes showed significant changes in transcript levels during AM development (Liu et al., 2003). Surprisingly, the

percentage of the AM-induced genes that were also regulated by high phosphorous nutrition was relatively low, indicating that the observed transcriptional changes are a direct consequence of the symbiosis development, and not due to the improved phosphorous availability during the AM symbiosis. Transcriptional profiling using the Mt6k-RIT (Küster et al., 2004) that contains approximately 6000 genes of the *M. truncatula* root transcriptome led to the identification of 75 the so-called symbiosins (Kistner et al., 2005), genes upregulated in mycorrhizal tissues that are also induced in root nodules (Manthey et al., 2004). In order to identify mycorrhizins, AM-specific genes that are not transcribed in any other tissue represented by cDNA libraries in EST databases, we screened our TC sequences for clusters that are exclusively composed of ESTs of mycorrhizal libraries (Frenzel et al., 2005). This search revealed 115 AM-specific TCs. For the majority of these TCs with sequences similarities to plant genes, the AM-specific expression was verified by quantitative reverse transcription-PCR. Annotation of the novel genes induced in mycorrhizal roots suggested their involvement in various transport and signaling processes and also identified a novel family of AM-specific lectin genes.

Gene regulation upon a mycorrhizal colonization could also be influenced by the AM-fungal species. Although AM-fungi are rather unspecific in their host range, in addition to a large set of genes co-regulated in response to two different *Glomus* species, significant differences were found in the *M. truncatula* root transcriptome in response to both fungi (Hohnjec et al., 2005).

5. Promoter reporter gene studies

The detailed analysis of promoter sequences is one way to elucidate the regulatory mechanism underlying a specific transcriptional regulation. This approach and the subsequent identification of upstream regulatory mechanisms represent a possible means to elucidate regulatory mechanisms underlying the formation of a functional AM-symbiosis. Similar functional promoter studies have been carried out with the so-called late nodulin genes. These genes are strongly and specifically expressed in nitrogen-fixing nodules during the Rhizobium–legume symbiosis. Promoter analysis of some late-nodulins identified several nodule-specific consensus motifs (Andersson et al., 1997; Stougaard et al., 1987) in the promoters of genes expressed in mature, nitrogen-fixing nodulins; the motifs were, however, not present in the promoter sequences of the so-called early nodulins, where other specific promoter-motifs were detected (Hansen et al., 1999; Vijn et al., 1995).

To date, no promoter driving the expression of an early mycorrhiza-specific gene has been described. But it might be anticipated that genes regulating the initial steps of the AM would be specifically or predominantly expressed in appressorium-adjacent cells.

Among the first AM-specific genes identified were those encoding phosphate transporters mediating the host plant uptake of fungus-delivered phosphate (Harrison et al., 2002; Paszkowski et al., 2002; Rausch et al., 2001). The promoters of these AM-specific transporter genes exhibit strong expression in arbuscule-containing cells, demonstrating that these structures are involved in phosphate transfer between both symbiotic partners. The promoter sequences of the AM-phosphate transporter of different plant species were applied to address whether the molecular events leading to the formation of an AM-symbiosis is conserved between different plant species. Plant transformations using heterologous phosphate transporter-reporter gene fusions suggested that signal perception and transduction mediating the mycorrhiza-specific expression of specific phosphate transporters is conserved in the eudicots (Karandashov et al., 2004).

So far hundreds of *M. truncatula* genes that are exclusively expressed in mycorrhizal roots have been identified, but little is known about the promoter elements that mediate this specific transcription. Recently, the promoter activity of several mycorrhiza-specific genes has been demonstrated through the use of reporter gene fusions (Table 1). Interestingly, the expression patterns of the identified mycorrhiza-specific promoters can be divided into two classes as proposed by Harrison (2005). The first group of genes is specifically expressed in arbuscule-containing cells, showing no expression in the vicinity of arbuscule-containing cells or in adjacent cell layers. A second class of mycorrhiza-specific promoters drives, not only, strong expression in arbuscule-containing cells, but also, weaker expression in adjacent cells or cell layers. This suggests that at least two signals activate the expression of mycorrhiza-specific genes. One signal is likely to operate in a cell-autonomous fashion and to activate the expression of the arbuscule-containing cell specific genes; a second signal appears to operate in a local, non-cell-autonomous fashion, and induces the expression of the second class of genes. Table 1 summarizes the *M. truncatula* promoter sequences of AM-specific genes of both subclasses identified to date.

6. Identification of putative upstream signaling mechanisms

Although the full-size promoters of several mycorrhiza-specific genes have been identified, little is known about the promoter elements that mediate AM-specific gene activation. Genes that share identical expression pattern in mycorrhizal roots are likely to be co-regulated by identical regulatory factors. Hence, it might be anticipated that their promoter sequences share common motifs; sites where regulators factors bind to the promoter and orchestrate the initiation of the transcription. In recent years, a few putative regulatory sequences have been described in the promoters of AM-induced plant genes. For example, analysis of the promoter of MtENOD11, an early nodulin gene that also shows an infection-related expression in mature

Table 1
Expression pattern driven by mycorrhiza-specific promoters of *M. truncatula* identified by promoter-reporter gene fusions in transgenic roots

Name	Current annotation	Assumed function	Reference
Arbuscule-containing cells specific expression			
MtPt4	Phosphate transporter	Plant phosphate uptake	Harrison et al. (2002)
MtScp1	Serine-carboxypeptidase	Signaling	Liu et al. (2003)
MtTi1	Protease-inhibitor	Signaling	Grunwald et al. (2004)
MtLec5	Lectin	Storage	Frenzel et al. (2005, 2006)
MtLec7	Lectin	Storage	Frenzel et al. (2005, 2006)
MtChit3-3	Chitinase	Signaling	Elfstrand et al. (2005)
Expression in arbuscule-containing and adjacent cells			
MtGst1	Glutathione-*S*-transferase	Unknown	Wulf et al. (2003)
MtCel1	Endo-1,4-β-D-glucanase	Cell wall modifications	Liu et al. (2003)
MtBcp1	Blue copper binding protein	Electron transfer	Hohnjec et al. (2005)

nodules and during the AM-symbiosis, revealed that 257 bp upstream of the start codon are sufficient to mediate this infection-related expression. Within this promoter area, an AT-rich motif, already described by Hansen et al. (1999) as a conserved motif of early nodulin promoters, was shown to be related to the gene expression in AM-tissue. Mutations within this motif, especially in an AATAA stretch, led to reduction in this endosymbiotic gene expression (Boisson-Dernier et al., 2005). The *VfLb*29 gene of *Vicia faba* is another nodulin that is also induced in arbuscule containing cells. An 85 bp sequence of the *VfLb*29 promoter is necessary for the expression in arbuscules; whereas this element is not involved in the expression in nodules. Within this 85 bp sequence, a palindromic sequence, also present in the promoter of the AM-specific phosphate transporter *StPt*3, was identified (Fehlberg et al., 2005).

The identification of conserved DNA motifs within upstream sequences of co-regulated genes is one of the major challenges in functional genomics and bioinformatics. Computational approaches at first view represent a rather fast way to identify common motifs within promoter sequences, and many different approaches have been developed for this purpose (Rombauts et al., 2003). But the reliable predication of such motifs is still intricate due to the structure of such motifs. Hence, a consistent prediction of conserved motifs within AM-specific promoter sequences is not yet available, also due to the small numbers of available promoter sequences. Database researches revealed a putative mycorrhiza- and resistance related element in the promoter of the mycorrhiza-specific phosphate transporter StPt3 of potato and a similar sequence was reported in the promoter of the AM-specific phosphate transporter of *M. truncatula*, MtPt4. But so far this element was not reported to be conserved in promoter sequences of other mycorrhiza-induced genes. Phylogenetic footprinting has not only led to the identification of candidate regulatory elements within the promoters of different AM-specific phosphate transporters and *MtGst*1, but it also remains to be elucidated whether these motifs, alone or in combination, are definitely related to mycorrhiza-specific transcriptional activation (Karandashov et al., 2004).

The finding that several members of the *M. truncatula* lectin family are AM-specifically transcribed (Frenzel et al., 2005) gave us the opportunity to analyze promoter sequences of paralogous co-regulated genes that are likely to share similar functions during the AM-symbiosis. Deletion studies showed that he promoter of one lectin, *MtLec*5, contained 150 bp located at −300 to −150 upstream of the transcriptional start which are necessary for the arbuscule specific expression (Frenzel et al., 2006). The same is true for the *MtLec*7 promoter. It also contains a 150 bp element, located at −300 to −150, that is required for the gene expression in arbuscules (Frenzel et al., unpublished). Deletion of these areas results in a complete abolition of promoter activity. The similar expression patterns of both genes suggest that these two promoter areas might share at least one common sequence motif, one which might be recognized by a postulated regulator protein. A computational comparison of the two sequences revealed a 10 bp motif common to both sequences. This sequence motif (AACATTTTGT) is located on the + strand on p*MtLec5* and on the − strand in p*MtLec*7 (Frenzel et al., unpublished). This common sequence shows no homology to any other known regulatory promoter element and may represent a new mycorrhiza specific potential TF-binding site. Screening of the promoter sequences of the mycorrhiza-specific genes of Table 1 revealed that none contains this motif. Hence, it is unlikely the common sequence motif detected in the two *MtLec*-promoters is a general motif for transcriptional induction in arbuscules.

In addition, promoter deletions and reporter gene analyses, the identification of specific protein-binding sites represents a further means to gain information about the mechanism of AM-specific transcription. Deletion studies defined a region of about 150 bp of the *MtLec*5 promoter that is necessary for the gene expression in arbuscule-containing cells, suggesting the binding of regulators mediating the transcriptional activation to this area. To strengthen this hypothesis, studies on specific DNA-protein bindings have been carried out. Gel Shift Assays revealed that this promoter sequence contains at least two binding sites that are specifically recognized by proteins present in mycorrhizal roots (Frenzel et al., 2006).

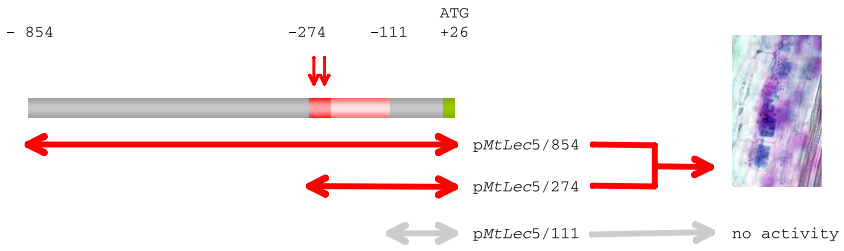

Fig. 1. Schematic representation of the mycorrhiza-specific *MtLec*5 promoter. Deletion studies showed that 274 bp of the *Mtlec*5 upstream region is sufficient to mediate the transcription in arbuscule-containing cells. Further deletion down to −111 upstream from the transcription start revealed a total abolition of the promoter activity, indicating binding of positive regulators between −274 and −111 (shown in red). Gel Shift Assays showed the binding of at least two proteins, which were present in mycorrhizal roots but undetectable to the −274/−231 region of the promoter (indicated by dark red colour and red arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A summary of the results of the *MtLec*5 promoter analysis is shown in Fig. 1. Summarizing the results of the analyses of the *MtLec*5 promoter obtained so far, we hypothesize that a mycorrhiza-specific regulator protein exists, one that is undetectable in non-mycorrhizal roots.

7. Outlook

The application of model systems and transcriptome profiling have considerably improved the understanding of the molecular basis of AM symbiosis. So far hundreds of AM-induced plant genes have been identified. One major challenge is to identify molecular mechanisms leading to this AM-specific gene expression. Preliminary results of the analyses of several mycorrhiza-specific promoters revealed that relatively small regions of the upstream areas are responsible for mycorrhiza-specific transcriptional activation. The identification and characterization of the corresponding transcriptional regulators mediating the mycorrhiza-specific transcription activation is surely a key focus of future research. This and a subsequent analysis of upstream-acting regulatory mechanisms will offer new insights into the molecular regulation of this tight symbiosis.

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Franziska Krajinski heads the research group Molecular Biology at the Institute for Plant Genetics at the University Hannover. She studied Biology at the Universities Greifswald and Marburg and obtained her Ph.D. in Microbiology from Marburg University in 2000. Subject of the thesis was the analysis of mycorrhiza-regulated genes in Medicago truncatula. In June 2000, she established a working group at the University Hannover working on plant gene regulation during mutualistic and parasitic root-microbe associations. She received her habitation at the Faculty of Biology, Hannover University in 2004.