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Review

Gene expression analysis of arbuscule development and functioning

Philipp Franken [a,*], Katrin Donges [b], Ulf Grunwald [c], Gerhard Kost [b], Karl-Heinz Rexer [b], M'Barek Tamasloukht [d], Astrid Waschke [a], Dorit Zeuske [a]

[a] *Institute of Vegetable and Ornamental Crops, Theodor-Echtermeyer-Weg 1, 14979 Großbeeren, Germany*
[b] *Philipps-University Marburg, Department of Biology, Mycology, Karl-von-Frisch-Strasse, 35043 Marburg, Germany*
[c] *MPI for Terrestrial Microbiology and Philipps-University Marburg, Department of Biology, Laboratory for Microbiology, Karl-von-Frisch-Strasse, 35043 Marburg, Germany*
[d] *Federal Institute of Technology (ETH), Zurich, Institute of Plant Science, Experimental Station Eschikon 33, CH-8315 Lindau, Switzerland*

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Abstract

The arbuscular mycorrhiza (AM) is characterized by specific morphological structures of the fungus and the plant and by physiological adaptations which are mostly beneficial for both partners of the symbiosis. This review describes approaches to study the molecular basis of the interaction. RNA accumulation patterns have been monitored in *Pisum sativum* to analyse the plant response to arbuscule development. In a direct approach, the *Mtha*1 gene from *Medicago truncatula* was cloned which is expressed in arbusculated cells of *M. truncatula*. The gene putatively encodes an H^+-ATPase involved in the improved plant nutrition during mycorrhization. Finally, a tripartite system between *M. truncatula*, *Glomus mosseae* and *Aphanomyces euteiches* was established, in order to study bioprotection. Analysis of the transcriptome has been started to analyse the interaction between the plant, the pathogen and the AM fungus.

Keywords: *Medicago truncatula*; *Pisum sativum*; Pea; Arbuscule development; Plant nutrition; Root pathogen; Gene expression

Contents

1. Introduction

The arbuscular mycorrhizal (AM) symbiosis is generally characterized by a typical morphological structure, the arbuscule. This structure is common to all associations of this type of mycorrhiza, although roots of many different plant species are able to interact with the so called AM fungi. Two different forms of arbuscules exist (Smith and Smith, 1997): In the Arum type, intercellular hyphae penetrate cell walls at the inner root cortex and ramify in the apoplast finally forming one highly branched tree-like

* Corresponding author. Tel.: +49 33701 78215; fax: +49 33701 55391.
E-mail address: franken@igzev.de (P. Franken).

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structure. Alternatively, hyphae grow from cell to cell developing intracellular coil-like structures in the whole root cortex with numerous very small intercalated arbuscules per cell. This is referred as the Paris type. The genetic background of the plant species and the fungus determine the type of mycorrhiza that is formed, while the environment seems to have no influence (Smith and Smith, 1997). Arbuscules and the adaptations of the surrounding plant cells do not represent the final developmental stage. They are part of the dynamic interaction between the plant and the fungus and decay after approximately 10 days (Alexander et al., 1988). This is associated not only with plant cell functions, but also by specific fungal activities (Dickson and Smith, 2001).

The transfer of mineral nutrients from the fungus to the plant is probably the major reason for the plant growth promoting effect of the symbiosis which can be observed under certain environmental conditions. It concerns phosphate, nitrogen and a number of trace elements (Marschner and Dell, 1994) and it was suggested to be based on the active uptake of these minerals from a plant fungus interface involving specific transporters and proton pumps (Smith and Smith, 1990). Cytochemical studies located H^+-ATPase activities at the periarbuscular membrane surrounding the fungal arbuscules (Gianinazzi-Pearson et al., 1991). This indicated that the arbuscules are not only the name giving structure of the symbiosis, but also play a central role in the improved nutrient supply of the plant.

The supply of minerals is one important aspect in the application of AM fungi to plant production systems (Gosling et al., 2006). Beyond being biofertilisers, it has been shown that these specialized microorganisms can also act as biocontrol agents conferring resistance against soilborne parasites (Azcon-Aguilar and Barea, 1996). This effect is not confined to regions of mycorrhizal colonisation as the use of split root systems indicated (Cordier et al., 1998). In contrast to the systemic induced resistance by certain rhizobacteria (Vallad and Goodman, 2004), biocontrol by AM fungi is only evident in the roots and, mycorrhizal plants often possess an increased susceptibility to leaf pathogens (Borowicz, 2001). Interestingly, it was shown that roots having a high number of arbuscules are needed to delimitate the spread of a root pathogen (Slezack et al., 2000). This indicates that arbuscule formation and the corresponding plant response is also crucial for the biocontrol function of AM fungi.

In order to get an insight into the molecular mechanisms of mycorrhizal development and functioning, one strategy is the non-targeted recording of RNA accumulation patterns of expressed sequence tags (ESTs) followed by the identification and annotation of regulated genes (Franken and Requena, 2001; Franken and Krajinski, 2006; Küster et al., this issue). This has been first carried out on the plant site using the model legume *Medicago truncatula*(Liu et al., 2003) and was shown to be very successful especially for such a difficult biological system as the obligate biotrophic fungal partner of the AM symbiosis (Requena et al., 1999, 2000, 2002; Lammers et al., 2001; Lanfranco et al., 2002; Tamasloukht et al., 2003; Breuninger and Requena, 2004). An alternative to this is the cloning of a specific gene and its subsequent analysis. Targets for this approach have been numerous in the case of AM fungi (for review: Franken et al., 2002; Ferrol et al., 2004). Here it will be summarised, how we have used targeted and non-targeted approaches in frame of the priority program 'Mycorrhiza' of the German Science Foundation (http://www.genetik.uni-bielefeld.de/MolMyk/) for the identification and analysis of mycorrhiza-regulated plant genes with the focus on arbuscule development and functioning.

2. Arbuscular mycorrhizal development

The first molecular analyses of the AM were targeted to genes which were known from interactions with other microorganisms. This showed that plant defence-related gene expression was only weakly induced or even repressed (Gianinazzi-Pearson et al., 1992; Harrison and Dixon, 1993; Lambais and Mehdy, 1993; Franken and Gnädinger, 1994). However, overlapping RNA accumulation patterns and promoter activities could be detected for genes encoding nodulins (Frühling et al., 1997; Gianinazzi-Pearson and Dénarié, 1997) or mycorrhiza-regulated genes of the same gene families were identified (Roussel et al., 1997; Krajinski et al., 2000; Kaldenhoff et al., this issue). Non-targeted approaches using differential screening of cDNA libraries or differential RNA display resulted in the beginning in only a few cDNA fragments belonging to genes induced or repressed during AM development (Tahiri-Alaoui and Antoniw, 1996; Martin-Laurent et al., 1997; Burleigh and Harrison, 1997; Lapopin et al., 1999). In order to circumvent the necessity of the time consuming cloning step, we established in our laboratory the technique of suppressive subtractive hybridisation (SSH) for AM research. This method finally resulted in collections of a higher number of cDNA fragments from mycorrhiza-regulated genes. Wulf et al. (2003) established an SSH library and sequencing of 1805 inserts lead to the identification of 600 singletons and 290 tentative consensus sequences (TCs) of genes expressed during the symbiosis between *M. truncatula* and *Glomus intraradices*. Reverse Northern blot analyses indicated that two-thirds of the ESTs belonged to AM induced or repressed genes. The transcripts of *MtGst*1 encoding a glutathione *S*-transferase were detected in arbusculated cells, but also in the surrounding region of the cortex colonised by the AM fungus. Brechenmacher et al. (2004) identified in their SSH library six genes from *M. truncatula* induced in late stages of symbiotic development, but it was not clear, if the expression was associated with arbuscule formation.

In order to directly target the cloning of genes with arbuscule-related gene expression, the pea mutant line RisNod24 was used for SSH library construction. This particular late stage mutant shows a relative normal pattern of

root infection and extracellular colonisation, but only a very few truncated arbuscules (Engvild, 1987). Sequence and expression analyses resulted in the identification of numerous genes which were only induced when arbuscules could develop normally (Grunwald et al., 2004). Annotation showed that most of the genes encoded proteins involved in defence and abiotic stress response. This is in contrast to the current opinion that such genes are transiently induced during early stages of AM development before arbuscule development, but repressed when the symbiosis is established (Gianinazzi-Pearson et al., 1996; Garcia-Garrido and Ocampo, 2002). A few defence-related genes, however, had already been identified before to show arbuscule associated RNA accumulation (Harrison and Dixon, 1994; Franken et al., 2000; Bonanomi et al., 2001; Hause et al., 2002). The induction of such genes could be associated to the very early stages of arbuscule formation, when the fungal hyphae penetrate the cell wall and spread into the apoplast. Alternatively the gene products might be involved in the process of arbuscule degradation. This has been postulated for *Prp*1 of potato encoding a glutathione *S*-transferase which could be necessary for the removal of the remains from the fungal arbuscules (Franken et al., 2000). In addition, hydrolytic enzymes like specific chitinases, which are induced during late stages (Salzer et al., 2000) could be also involved in this process.

Further analyses of the *Pisum sativum* genes with an arbuscule-related expression profile by promoter–reporter gene constructs and by the RNAi technology was hampered by the fact that the plant is difficult to transform. Because the synteny to *M. truncatula* is, however, very high (Kalo et al., 2004), it was hypothesised that homologous genes in this legume could be used for such analyses. Moreover, *M. truncatula* seemed to be suitable, because it is used as a model for mycorrhizal research (Harrison, 1997) and also as a cover crop in rotations (Sheaffer et al., 2001). The TIGR database for *M. truncatula* was therefore screened for similar genes with related expression profiles. In some cases, not only one gene, but gene families were found and their members showed different degrees of similarity to the ESTs from pea (examples in Table 1). The *in silico* expression analysis indicated that the most homologous family members can be those with AM related RNA accumulation pattern, but sometimes also genes with a lower similarity were mycorrhiza-regulated (Table 1). This implies that the transfer of results in the other direction from the model legume *M. truncatula* to the agriculturally important crop *P. sativum* has to be handled with caution. For one pea gene putatively encoding a trypsin inhibitor, a gene family was detected in *M. truncatula* where all members seemed to be induced by mycorrhiza (Grunwald et al., 2004). This was verified by quantitative real-time PCR and, arbuscule-related expression was shown in transgenic roots carrying promoter–reporter gene constructs for the gene *MtTi*1 (Grunwald et al., 2004). This analysis showed that at least in the case of the trypsin inhibitor the model legume *M. truncatula* can be used to study the regulation and the function of genes which could play a role for the AM development in the economically important grain legume crop *P. sativum*.

3. Nutrient transfer

The site of mineral nutrient transfer in the AM symbiosis is probably the plant–fungus interface including the fungal arbuscule membrane, the matrix and the plant periarbuscular membrane. This has been substantiated by the finding of phosphate transporter genes which are specifically expressed in plant cells containing fungal arbuscules (Rosewarne et al., 1999; Rausch et al., 2001; Harrison et al., 2002). Such transporters need an electrochemical gradient for their activity, which is usually supplied by a proton pumping ATPase. Mycorrhiza-related RNA accumulation for H^+-ATPase has been detected in several plants (Murphy et al., 1997; Gianinazzi-Pearson et al., 2000; Ferrol et al., 2002). In order to get an insight into the situation in *M. truncatula*, a targeted approach was followed and three different genes for H^+-ATPases

Table 1
Putative expression patterns of *Medicago truncatula* genes showing homology to ESTs from *Pisum sativum* genes with arbuscule-related RNA accumulation

Putative gene products of arbuscule specific expressed genes in pea	Similar *M. trunacatula* genes (TIGR identifiers)	Significance of similarity	Results of *in silico* expression analysis of *M. truncatula* genes
Translational elongation factor 1β	TC106815	$3.5e^{-86}$	No differential expression
	TC106814	$2.9e^{-81}$	No differential expression
	TC94335	$1.7e^{-35}$	AM induced
	TC106816	$1.2e^{-29}$	No differential expression
	BG644352	$1.1e^{-28}$	Only in nodules
	AL388180	$2.1e^{-23}$	Only in AM
Metallothionein	TC93926	$5.7e^{-34}$	AM induced
	TC94086	$2.4e^{-33}$	No differential expression
	TC93919	$1.1e^{-26}$	Stress and AM induced
Non-photosynthetic ferredoxin	TC94189	$1.7e^{-20}$	No differential expression
	TC107246	$2.9e^{-29}$	No differential expression
	TC107245	$9.2e^{-09}$	AM induced

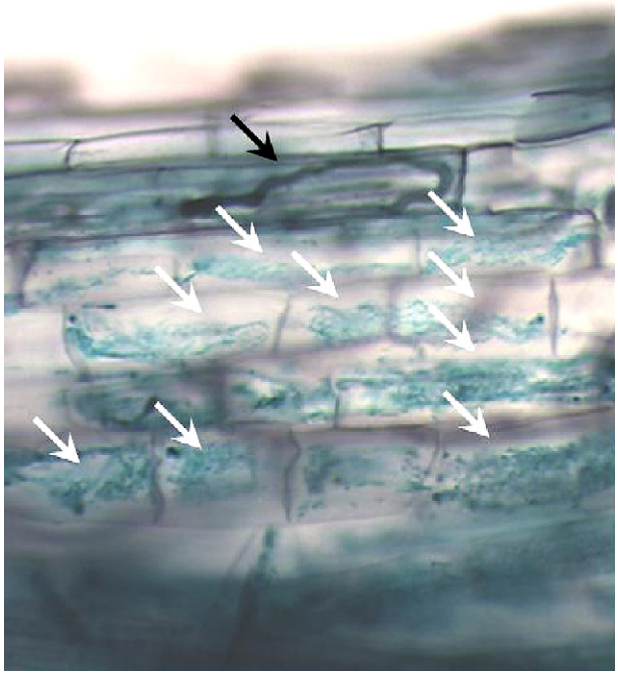

Fig. 1. *Mtha*1 promoter activity in potato mycorrhiza. The promoter of the *Medicago truncatula* H^+-ATPase gene *Mtha*1 was cloned in front of the β-glucuronidase (GUS) reporter gene. Potato roots were transformed with the construct and inoculated with the AM fungus *Glomus mosseae*. After 4 weeks, fungal hyphae were stained with trypan blue (black arrow), and GUS reporter gene activity could be detected in cells filled with fungal coils and small intercalated arbuscules (white arrows).

were cloned (Krajinski et al., 2002). While *Mtha*2 and *Mtha*3 were constitutively expressed, *Mtha*1 transcripts were only detected in mycorrhiza and *in situ* hybridization showed their specific appearance around the fungal arbuscules. This is in contrast to the situation in tobacco, tomato or barley, where the transcripts were also observed in other tissues (Gianinazzi-Pearson et al., 2000; Ferrol et al., 2002) or could be induced by phosphate (Murphy et al., 1997). Screening the TIGR data base also showed that ESTs from this fragment can not be found in non-colonized tissues. However, two ESTs indicated that the gene is also expressed in nodules. This finding was verified by array analysis and real-time PCR using RNA from *M. truncatula* roots inoculated with *G. intraradices* or *Sinorhizobium meliloti* indicating that *Mtha*1 could play a role in both root symbioses (Manthey et al., 2004). In order to study the regulation of the expression, the promoter was cloned in front of a GUS reporter gene (Krajinski, Grunwald and Franken, unpublished) and the construct is currently being studied. Interestingly, GUS activity could not only be observed in *M. truncatula* mycorrhiza, but also in potato cells surrounding fungal coils with intercalated arbuscules (Fig. 1) showing that the same regulatory elements also work in a non-legume. This is similar to the *Vflb29* promoter, which was found to be active not only in nodules and mycorrhiza of legume plants, but also in arbusculated cells of tobacco (Vieweg et al., 2004).

4. Root–pathogen interaction

Aphanomyces euteiches causes the most serious root rot disease of legumes including *P. sativum* (Hagedorn, 1989). Today no effective strategy for the control of this pathogen is available, but it has been shown that mycorrhizal pea plants are at least partially protected against this pathogen (Rosendahl, 1985; Thygesen et al., 2004). In order to study the molecular basis of the interaction between legumes and *A. euteiches*, it was our aim to introduce *M. truncatulas* a model. First studies showed that *A. euteiches* zoospores attached the roots a few hours after inoculation and about 1 week later the root cortex was colonised and haustoria could be observed (Fig. 2). First oospores were also detected at this time point and increased in number until the root was filled with these propagules 4 weeks after inoculation. This indicated that the pathogen is at least a hemibiotroph. In further experiments *M. truncatula* roots were inoculated either with *G. mosseae* or remained free of inoculum. After 4 weeks, when the mycorrhiza was fully established, a zoospore suspension of the pathogen was delivered to the root systems. Comparison of mycorrhizal and non-mycorrhizal roots both infected with the pathogen showed that colonisation with the symbiont obviously reduced the disease symptoms. Moreover, analysis of pathogen development confirmed that the colonisation with the AM fungus significantly reduced the number and spread of *A. euteiches* oospore and that the phenomenon of biocontrol was dependent on arbuscule development. In summary, *M. truncatula*, *G. mosseae* and *A. euteiches* can be used as a model on the one hand to compare pathogenic and mutualistic biotrophic interactions, where haustoria or arbuscules are central structures of nutrient transfer, and on the other hand for analysing the mycorrhiza-induced resistance against root diseases.

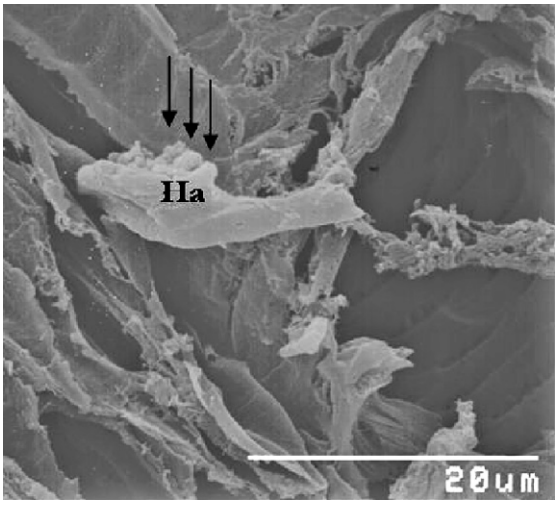

Fig. 2. Haustorium of *Aphanomyces euteiches* in *M. truncatula* root cells. Roots of *M. truncatula* were inoculated with zoospores of *A. euteiches*, harvested after 2 weeks and analysed by raster electron microscopy. Finger-shaped protuberances of the haustorium (Ha) are indicated with arrows.

In order to get a first insight into the molecular mechanisms of the interaction between *M. truncatula and A. euteiches*, an SSH cDNA library with 560 ESTs was established. This library was enriched for cDNA sequences from transcripts present in higher amounts in roots six days after inoculation with the pathogen compared to the corresponding controls (Nyamsuren et al., 2003). Numerous sequences showed similarities to already known *M. truncatula* genes, but also new plant genes with no homologies to any entry in the *M. truncatula* sequence collection and one fungal gene were identified. Some of the results could be substantiated by a proteome analysis (Colditz et al., 2004). In this analysis, proteins like PR10 or gene products from abscisic responsive genes were detected to be induced by the pathogen which corresponds to transcripts present in the SSH library. RNA accumulation was compared in further experiments between roots inoculated or not with the AM fungus *G. mosseae* or with the pathogen *A. euteiches* by establishing SSH cDNA libraries and by screening of the 16 K microarray of *M. truncatula* (Hohnjec et al., 2005). These experiments resulted in a collection of genes which are characterised by an induced expression level, only when both microbes colonises the plant compared to the situation in roots neither inoculated with AM fungi nor pathogens or roots inoculated only with *G. mosseae* or *A. euteiches*. Further experiments are currently being carried out where the role of particular genes for plant defence is analysed in transgenic root organ cultures.

5. Conclusion

In summary, genes have been identified by targeted and non-targeted approaches which show arbuscule-related expression profiles in pea and in *M. truncatula*. On the one hand, genes like *Mtha*1 encoding an H^+-ATPase probably play a role in nutrient transfer, one key characteristics of the mycorrhizal symbiosis. On the other hand a number of genes were identified which are known from plant pathogenic interactions. Since arbuscule development seems to be the prerequisite of the *G. mosseae*-induced resistance in *M. truncatula* against the root pathogen *A. euteiches*, the expression of such genes could be the basis for bioprotection, the second important function of the mycorrhizal symbiosis.

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Philipp Franken studied Biology in Aachen and Cologne. He did is Diploma and PhD thesis at the MPI for Plant Breeding Research on the molecular regulation of anthocyanin biosynthesis in maize. In 1991 he switched to the field of arbuscular mycorrhiza. He analysed the expression of defence-related plant genes during fungal colonisation of parsley and potato roots. In 1993–1995 he developed methods at the INRA in Dijon to study RNA accumulation patterns in AM fungi and used non-targeted approaches in order to identify symbiosis-related genes in *Pisum sativum*. He obtained in 1995 a position at the MPI for Terrestrial Microbiology in Marburg where he established a group working on the molecular biology of the arbuscular mycorrhiza. He used mainly the model organism *Medicago truncatula* for the analysis of the symbiotic transcriptome, but also studied the molecular basis of AM fungal development. He habilitated in 1999 in microbiology and botany at the Philipps-University in. Since 2002 he is head of the plant nutrition department at the Leibniz Institute of Vegetable and Ornamental Crops conducting projects which cover basic research and applied aspects of interactions between plant roots and beneficial microorganisms.