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PHYTOCHEMISTRY
Phytochemistry 68 (2007) 8–18
www.elsevier.com/locate/phytochem

Review

Identification and expression regulation of symbiotically activated legume genes

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Received 27 April 2006; received in revised form 20 June 2006
Available online 1 November 2006

Abstract

Legume plants are able to enter two different endosymbioses with soil prokaryotes and soil fungi, leading to nitrogen-fixing root nodules and to arbuscular mycorrhiza (AM), respectively. We applied *in silico* and microarray-based transcriptome profiling approaches to uncover the transcriptome of developing root nodules and AM roots of the model legume *Medicago truncatula*. Several hundred genes were found to be activated in different stages of either symbiosis, with almost 100 genes being co-induced during nodulation and in arbuscular mycorrhiza. These co-induced genes can be associated with different cellular functions required for symbiotic efficiency, such as the facilitation of transport processes across the perisymbiotic membranes that surround the endosymbiotic bacteroids in root nodules and the arbuscules in AM roots. To specify promoter elements required for gene expression in arbuscule-containing cells, reporter gene fusions of the promoter of the *Vicia faba* leghemoglobin gene *VfLb29* were studied by loss-of-function and gain-of-function approaches in transgenic hairy roots. These analyses specified a 85-bp fragment that was necessary for gene expression in arbuscule-containing cells but was dispensible for gene activation in root nodules. In contrast to promoters mediating gene expression in the infected cells of root nodules, the activation of genes in AM appears to be governed by more complex regulatory systems requiring different promoter modules.

Keywords: *Medicago truncatula*; *Vicia faba*; Arbuscular mycorrhiza; *In Silico* transcriptome profiling; Microarray-based expression profiling; Promoter analysis; Root nodule; Symbiosin genes; Transgenic hairy roots

Contents

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doi:10.1016/j.phytochem.2006.09.029

1. Introduction

Legume plants establish two different endosymbioses with soil microorganisms: the nitrogen-fixing root nodule symbiosis and the arbuscular mycorrhiza (AM). Whereas nodulation is almost exclusively restricted to legumes and requires the organogenesis of a root nodule that houses the rhizobial prokaryotes capable of symbiotic nitrogen fixation (Brewin, 1991; Schultze and Kondorosi, 1998; Vessey et al., 2005), more than 80% of terrestrial plants enter an AM with fungi of the phylum *Glomeromycota* (Schüssler et al., 2001).

Arbuscular mycorrhizae support plant development under nutrient-limiting and various stress conditions (Smith and Read, 1997; Graham and Miller, 2005). In AM, fungal hyphae from an extraradical mycelium penetrate the root epidermis via an appressorium and subsequently proliferate in the inner cortex (Harrison, 1997; Strack et al., 2003). In the Arum-type of arbuscular mycorrhiza (Smith and Read, 1997), these intraradical, intercellular hyphae terminate in highly branched, intracellular structures designated arbuscules (Bonfante and Perotto, 1995). Comparable to root nodules, where nutrient exchange takes place across the perisymbiotic membrane surrounding the nitrogen-fixing bacteroids (Day et al., 2001), solute exchange during AM occurs at the perisymbiotic membranes that surround the arbuscules (Gianinazzi-Pearson, 1996; Harrison, 1999; Parniske, 2000). In addition, it is assumed that the intraradical hyphae are a major site for the transfer of nutrients, most importantly for an allocation of carbohydrates to the fungus (Bago et al., 2000). In return for the supply with carbohydrates, fungal microsymbionts transfer minerals, in particular phosphorus, from the soil to the plant (Shachar-Hill et al., 1995; Smith et al., 2001). With respect to phosphorus, arbuscules are the major site of nutrient exchange, and mycorrhiza-specific plant phosphate transporters are exclusively localized at the arbuscular interface (Harrison et al., 2002). It has to be emphasized that although symbiotic microbes colonize root tissues intracellularly during nodule and AM endosymbioses, they stay separated from the plant cytoplasm by the highly specialized perisymbiotic membranes (Provorov et al., 2002).

Considering the apparent analogies in the infection process (Parniske, 2000), including the only recently discovered establishment of an epidermal pre-penetration apparatus during AM that resembles the cortical pre-infection threads formed during nodulation (Genre et al., 2005), an overlap in the activation of gene expression can be expected (Lum and Hirsch, 2002). This overlap is particularly evident for the signal perception and signal transduction cascades that initiate nodulation and mycorrhization (Parniske, 2004). The recent developments in this area are summarized by Limpens and Bisseling (2003), Geurts et al. (2005), and Stacey et al. (2006). Interestingly, studies on legume mutants defective in early stages of both endosymbioses suggest the existence of a Myc-factor that, similar to the action of Nod-factors triggering nodulation, initiates AM formation (Cullimore and Dénarié, 2003). Based on the observation of common signaling cascades, it is tempting to speculate that the root nodule symbiosis where fossil records date back to the late Cretaceaous adopted and subsequently modified more ancient signal transduction pathways leading to AM formation, having been in place already 400 million years ago (Kistner and Parniske, 2002).

In the past few years, different expression profiling strategies were pursued to identify symbiotically induced (symbiosin) genes co-activated during early and late stages of nodulation and mycorrhization. In general, these strategies relied on high-throughput EST sequencing, the construction and analysis of suppressive subtractive (SSH) cDNA libraries, *in silico* profiling of symbiosis-related gene expression by mining comprehensive EST collections, and experimental microarray-based transcriptome profiling (cf. also the reviews by Küster et al. and Krajinski et al. in the present issue). These approaches were facilitated by the selection of two model legumes: *Medicago truncatula* (Barker et al., 1990) and *Lotus japonicus* (Handberg and Stougaard, 1992). In these models and additionally in soybean (Lee et al., 2004), genome and high-throughput EST-sequencing was performed, and different expression profiling tools have been developed to study nodulation and mycorrhization (Colebatch et al., 2002, 2004; Kouchi et al., 2004; Küster et al., 2004; Hohnjec et al., 2005; Lohar et al., 2005).

In this review, we summarize our work on the identification of legume genes transcriptionally activated during nodulation and mycorrhization. Based on *in silico* and experimental transcriptome profiling approaches, novel genes with a symbiosis-enhanced expression were found, and these can be connected to cellular processes relevant for the establishment of either root endosymbiosis. For selected genes, promoter activities have been studied in transgenic roots and minimal promoter regions mediating reporter gene activity in arbuscule-containing cells were specified.

2. *In silico* and experimental expression profiling of different legumes uncover the root nodule transcriptome

To identify genes differentially expressed during the symbiotic interaction of *Sinorhizobium meliloti* and *M. truncatula* leading to nitrogen-fixing root nodules (Fig. 1), both wild-type and mutant plant materials were used to allow not only an identification of genes expressed in developing and mature nodules, but also to detect differential expression of those genes that are regulated by the Nod-factor signaling pathway or require a successful infection for their activation. In case of wild-type tissues, young and mature nitrogen-fixing *S. meliloti*-induced root nodules were taken (El Yahyaoui et al., 2004; Küster et al., 2004; Manthey et al., 2004), whereas whole root systems harvested 3 and 6 days post inoculation (dpi) with *S. meliloti* were chosen from a hypernodulating *M. truncatula* mutant

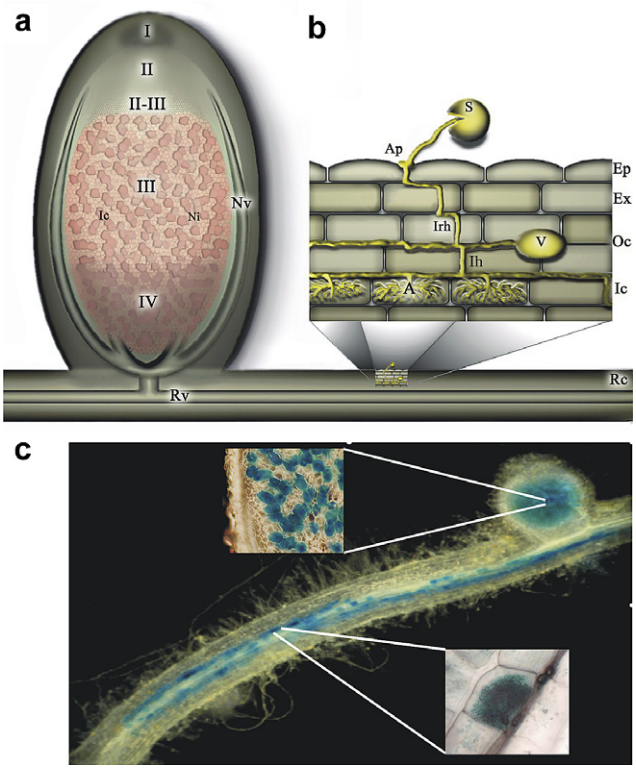

Fig. 1. Schematic representation of mature nitrogen-fixing root nodules as well as arbuscular mycorrhizal roots and cellular expression of the *VfLb29* gene encoding a symbiotically induced (symbiosin) gene. (a) Mature indeterminate root nodule. According to Vasse et al. (1990), the different root nodule zones are referred to as I, meristem; II, prefixing zone; II–III, interzone; III, nitrogen-fixing zone; IV, senescence zone. Ic, infected cells; Ni, non-infected cells; Nv, nodule vasculature. (b) Arum-type arbuscular mycorrhizal root. S, spore; Ap, appressorium; Ih, intercellular hyphae; Irh, intracellular hyphae; V, vesicle; Ep, epidermis; Ex, exodermis; Oc, outer cortex; Ic, inner cortex. Rc, Root cortex; Rv, Root vasculature. (c) The activity of the *VfLb29* promoter was studied by expressing fusions to the *gus*Aint reporter gene in transgenic *M. truncatula* roots. Nodulated and mycorrhized transgenic roots were stained for reporter gene activity to visualize cellular gene expression. It is evident that *VfLb29* gene expression is restricted to the infected cells of root nocules and AM roots, respectively.

(El Yahyaoui et al., 2004). In addition, two infection-defective *M. truncatula* mutants were profiled at 3 dpi: an *nfp* (Nod-factor perception) mutant that due to a mutation in the *nfr5*-type Nod-factor receptor (Stacey et al., 2006) does not respond to Nod-factors, and an *hcl* (hair curling) mutant that still responds to Nod-factors, but is blocked immediately prior to the early root hair curling response (El Yahyaoui et al., 2004). The resulting expression data were evaluated statistically using the EMMA software (Dondrup et al., 2003; cf. also the review by Küster et al. in the present issue) and in total, more than 750 *M. truncatula* genes were found to be differentially expressed during nodule initiation, formation and function. Four major clusters of differentially expressed genes were distinguished: (1) Genes up-regulated strongly in young but also in mature nodules, (2) genes mainly activated in mature nodules, (3) genes transiently induced at 3–4 days post inoculation, and (4) genes down-regulated during nodulation (El Yahyaoui et al., 2004).

In *M. truncatula*, recent studies by Mitra and Long (2004), Mitra et al. (2004), Starker et al. (2006) and Lohar et al. (2005) identified additional genes activated either during the very early and early stages of root nodule development or in response to different *S. meliloti* mutants. Whereas Mitra and Long (2004), Mitra et al. (2004) and Starker et al. (2006) capitalized on an Affymetrix "Symbiosis" Genechip carrying approximately 10,000 *M. truncatula* TC ("Tentative Consensus sequence")-representative probes from the TIGR *M. truncatula* Gene Index 4 as well as probes covering the *S. meliloti* genome, Lohar et al. (2005) relied on a microarray constructed from a 6k unigene cDNA collection of *M. truncatula* TC-representative cDNAs. The authors specified gene activation during the first 72 h of nodule initiation, identifying four stages of the symbiotic interaction on the basis of gene expression profiles, together with potential marker genes for those stages.

Using an *in silico* approach (Fig. 2) in conjunction with targeted macroarrays, Tesfaye et al. (2006) continued the approach of Fedorova et al. (2002) to identify nodule-specific genes, now dissecting the *M. truncatula* nodule transcriptome under different conditions of nitrogen supply, and particularly focusing on genes involved in carbon and nitrogen metabolism. It was found that 81 genes represented by nodule-specific TCs were up-regulated in both effective and ineffective root nodules, thus validating the

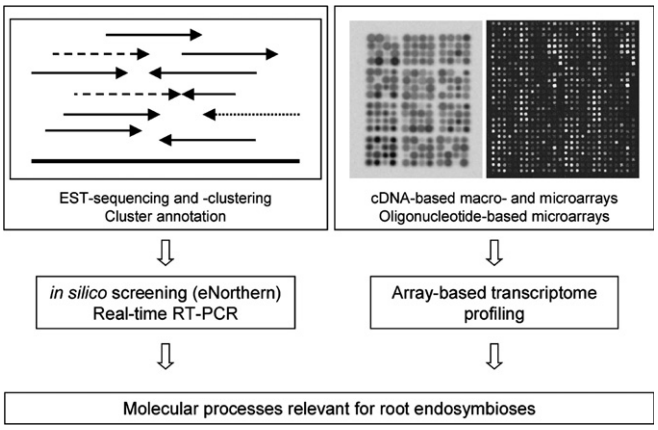

Fig. 2. Transcriptome profiling strategies to identify genes differentially expressed during nodulation and mycorrhization. Based on the generation of EST-clusters (left panel, cluster represented by the black bar) from comprehensive EST collections (left panel, ESTs represented by arrows), *in silico* profiling (eNortherns) can predict genes with a symbiosis-induced expression. These predictions have to be validated, e.g. by real-time RT-PCR. Parallel efforts relying on microarray-based transcriptome profiling (right panel) deliver comprehensive experimental sets of genes activated during root endosymbioses. For the construction of the expression profiling tools and the data mining strategies, cf. also the review of Küster et al. in the present issue. The combination of *in silico* and experimental transcriptome profiles identifies molecular processes activated in root endosymbioses.

results from *in silico* profiles by macroarray hybridizations (Tesfaye et al., 2006).

Comparable efforts to study the transcriptome of developing root nodules were carried out in the closely related model legume *Lotus japonicus*. Here, Colebatch et al. (2004, 2002) used cDNA macroarrays to integrate global changes in the root nodule transcriptome with metabolic differentiations that take place during the developmental switch towards nitrogen fixation.

Together, the application of high-throughput transcriptome profiling in the two model legumes significantly advanced our knowledge on genes differentially expressed during root nodule initiation and organogenesis, allowing not only the high-throughput transcriptional dissection of specific steps in the formation of root nodules but also the identification of metabolic networks activated during symbiotic nitrogen fixation.

3. Global transcriptome profiling specifies legume genes activated in arbuscular mycorrhizal roots

In addition to targeted analyses focusing on selected genes or capitalizing on SSH studies (e.g. Weidmann et al., 2004; Brechenmacher et al., 2004; Wulf et al., 2003; Frenzel et al., 2005; cf. also the review by Krajinski et al. of the present issue), two global microarray-based transcriptome studies were reported that examined gene expression in *M. truncatula* arbuscular mycorrhizal roots (Fig. 2). First, Liu et al. (2003) applied 2.5k macroarrays constructed from the MHAM cDNA library of AM roots. This array was used to identify genes up-regulated in response to *Glomus versiforme* at different stages of AM formation. The authors proposed two distinct temporal gene expression patterns. Genes belonging to the first group showed a transcriptional increase during the early phase of the interaction, with defense- and stress-response genes predominantly present among them. In contrast, genes of the second group displayed elevated transcript levels that correlated with root colonization. Since components of signal transduction pathways were found here, novel signaling pathways were predicted to be activated during the development of the AM symbiosis (Liu et al., 2003).

In a recent study, we applied the more comprehensive Mt16kOLI1 microarrays constructed in the frame of the SPP 1084 "Mykorrhiza" (cf. also the review by Küster et al. in the present issue) to specify the overlapping genetic program activated by two commonly studied microsymbionts, *Glomus mosseae* and *Glomus intraradices* (Hohnjec et al., 2005). In total, 201 plant genes were significantly co-induced at least 2-fold in either interaction. A range of well-known AM marker genes were found to be activated, thus validating the transcriptome data obtained. Amongst these marker genes were those encoding the phosphate transporter MtPt4 (Harrison et al., 2002), the germin-like protein MtGlp1 (Doll et al., 2003), the glutathione *S*-transferase MtGst1 (Wulf et al., 2003), the serine carboxypeptidase MtScp1 (Liu et al., 2003), the hexose transporter MtSt1 (Harrison, 1996), the 1-deoxy-D-xylulose 5-phosphate synthase MtDXS2 (Walter et al., 2002), and the multifunctional Nodulin 26-like aquaporin MtNip1 (Brechenmacher et al., 2004).

Apart from these AM marker genes that were identified by more targeted analyses, more than 150 co-induced genes were reported as AM-induced for the first time. Amongst those were genes specifying novel AM-related nitrate, ion, and sugar transporters, enzymes involved in secondary metabolism, proteases, and Kunitz-type protease inhibitors. In addition, several co-induced genes encoded receptor kinases and other components of signal transduction pathways as well as ten AM-induced transcriptional regulators. Several hundred genes were additionally up-regulated during a sole interaction, indicating that the plant genetic program activated in AM to some extent depends on the colonizing microsymbiont, a finding also evident from other experiments (cf. also the review by Franken et al. in the present issue). Together with the expression profiles from Liu et al. (2003), the transcriptional snapshots obtained by Hohnjec et al. (2005) provided a range of novel candidate genes possibly involved in signaling during AM interactions.

In non-legumes, in particular the study of Güimil et al. (2005) deserves attention. Based on a whole-genome *Oryza sativa* GeneChip, the authors specified genes co-activated during rice AM as well as in the interaction with two root pathogens. This study clearly demonstrated an overlap between the genetic program activated by root-colonizing microbes and thus allowed to track truly symbiosis-related gene expression. The authors were able to relate the expression of several dozen genes induced during rice AM with the transcription profile of the respective *M. truncatula* orthologs (Güimil et al., 2005), thus allowing to correlate data from the monocot rice with the dicot *M. truncatula*.

Together, transcriptome profiling approaches performed in the past few years led to the identification of hundreds of novel AM-activated genes in different plant species (Hohnjec et al., 2006), significantly advancing our molecular knowledge on gene expression in AM interactions.

4. A combination of *in silico* and experimental transcriptome profiling uncovers symbiosis-induced legume genes

Targeted studies demonstrated that genes expressed during root nodule initiation and infection, e.g. the early nodulin genes *Enod2, Enod5, Enod11, Enod12*, and *Enod40*, are transcriptionally activated also during AM (van Rhijn et al., 1997; Albrecht et al., 1998; Journet et al., 2001; Chabaud et al., 2002). Whereas *Enod2, Enod11* and *Enod12* encode proline-rich putative cell wall proteins, *Enod5* specifies an arabinogalactan protein and *Enod40* encodes a small regulatory peptide (Scheres et al., 1990; Schultze and Kondorosi, 1998; Journet et al., 2001).

Prior to an application of comprehensive microarray-based transcriptome profiling in model legumes, a reverse Northern screening for symbiotically induced (symbiosin) genes was performed in *Vicia faba* (broad bean), based on a collection of 44 genes expressed in root nodules (Perlick and Pühler, 1993; Küster et al., 2001). With the leghemoglobin gene *VfLb29*, one of the 20 nodule-specific broad bean genes were specified as AM-induced (Frühling et al., 1997) and in addition, four nodule-enhanced genes were shown to be activated during AM with *Glomus versiforme*. These genes encoded two proline-rich putative cell wall proteins (VfPRP1 and the extensin VfExt1; sequences VfNDS-D and -E in Perlick and Pühler, 1993), a dehydroascorbate reductase (VfDhar1, sequence VfNDS-X26 in Perlick and Pühler, 1993), and the calmodulin-binding protein VfCamBP1 (sequence VfNDS-X8 in Perlick and Pühler, 1993). From the identification of five symbiotically induced genes by the reverse Northern screening of a limited number of genes, it could be expected that a more global non-targeted approach relying on EST-sequencing and the application of microarrays to probe gene expression would reveal an even higher number of symbiosin genes; adding to their identification by targeted analyses (Journet et al., 2001) or by the transcriptome profiling of legume symbiotic mutants (Kistner et al., 2005).

To specify symbiosin genes co-induced during nodulation and AM in the model legume *Medicago truncatula* (Figs. 1 and 2), we applied Mt6kRIT macro- and microarrays (Küster et al., 2004) and Mt16kOLI1 microarrays (Hohnjec et al., 2005), both constructed in the frame of the SPP 1084 ''Mykorrhiza'' (cf. also the review by Küster et al. in the present issue). On the basis of such microarray studies and including the expression data from nodulation and mycorrhization mentioned above, several hundred genes were detected as up-regulated in root nodules and AM, respectively, with a total of almost 100 genes being co-induced in either interaction (El Yahyaoui et al., 2004; Küster et al., 2004; Manthey et al., 2004; Hohnjec et al., 2005). Complementary approaches relying either exclusively (Journet et al., 2002) or in part (Manthey et al., 2004) on *in silico* data mining of the MENS (http://medicago.toulouse.inra.fr/Mt/EST/) database and the TIGR *M. truncatula* Gene Index (http://www.tigr.org/), yielded additional candidate genes with a predicted symbiosis-induced expression (Fig. 1).

Subsets of genes identified by global expression profiling or more targeted analyses (Hohnjec et al., 2003, 2005; El Yahyaoui et al., 2004; Küster et al., 2004; Manthey et al., 2004; Vieweg et al., 2004, 2005) were usually subjected to quantitative real-time RT-PCR for a verification of their symbiosis-induced expression (Bustin, 2000). These experiments allowed the conclusion that microarray-based expression data in general are more reliable than *in silico* predictions, similar to the finding of Fedorova et al. (2002) that a minimal number of ESTs is required for a robust prediction of a nodule-induced expression. Together, co-activation during nodulation and mycorrhization was validated for several dozen *M. truncatula* genes (El Yahyaoui et al., 2004; Küster et al., 2004; Manthey et al., 2004; Hohnjec et al., 2005), most of them having not been reported as being symbiosis-induced before. Selected genes are presented in Table 1, together with their possible function during root endosymbioses.

Although a range of novel genes with a symbiosis-related expression profile were identified, the overlap between expression profiles during nodulation and mycorrhization is somewhat lower than expected (Manthey et al., 2004). The major reasons for this is (1) a dilution effect masking the detection of locally expressed genes in particular during AM and (2) a lack of cellular expression resolution in the global transcriptome profiling approaches that were carried out to date for symbiotic tissues. Moreover, in particular the AM symbiosis is a highly asynchronous process containing a mixture of developmental stages, that way masking the expression of genes under tight temporal or local expression control (Gianinazzi-Pearson and Brechenmacher, 2004).

Nonetheless, the symbiosis-related genes uncovered by transcriptome profiling form a starting point for the functional analysis of the encoded gene products, e.g. by RNAi approaches in transgenic *M. truncatula* roots and plants (Hoffmann et al., 1997; Boisson-Dernier et al., 2001; Isayenkov et al., 2005; Ivashuta et al., 2005) or in the TILLING (Targeting Induced Local Lesions IN Genomes) and *Tnt1* retrotransposon mutant collections (Tadege et al., 2005; Udvardi et al., 2005; Thompson et al., 2005) currently developed for different legumes.

5. Symbiosis-induced genes have similar functions during nodulation and mycorrhization

Based on database searches, the symbiotically induced genes identified were annotated and grouped into functional categories, and some interesting genes summarized in Table 1 are briefly discussed here.

The first AM-activated genes with relevance to arbuscule physiology were two plasma membrane H^+-ATPases from tobacco where the corresponding protein and enzyme activities were localized to the periarbuscular membrane (Gianinazzi-Pearson et al., 2000). In *M. truncatula*, the corresponding H^+-ATPase MtHa1 has been localized to the periarbuscular membrane recently (Valot et al., 2006). In addition to a specific expression in AM roots (compared to non-mycorrhizal roots; Krajinski et al., 2002), MtHa1 was also found to be induced during nodulation, illustrating the common requirement for an acidification of the perisymbiotic space in either root endosymbiosis (Manthey et al., 2004), most probably to facilitate energy-dependent transport across the perisymbiotic membranes.

Amongst the symbiotically induced genes encoding membrane proteins, also the multifunctional aquaporin MtNip1, identified to be mycorrhiza-induced also by Brechenmacher et al. (2004), and a membrane nodulin of

Table 1
Overview of selected genes identified as up-regulated during nodulation and in AM

ID	Gene	Annotation	Proposed function	References
TC94919	MtTubb1	Beta-tubulin	Cytoskeleton rearrangements in infected cells	Manthey et al. (2004)
TC95400	*MtHa1*	Plasma membrane H^+-ATPase	Acidifying the perisymbiotic space	Manthey et al. (2004)
TC95776	*MtAnn2*	Annexin	Regulation of membrane organization	Manthey et al. (2004)
TC96231		Signal peptidase	Cleaving perisymbiotic membrane-specific signal peptides	Hohnjec et al. (2005)
TC100410	*MtSucS1*	Sucrose synthase	Supporting symbiotic sinks	Hohnjec et al. (2003)
TC100436		Cysteine proteinase	Protein processing during symbiosis senescence	Manthey et al. (2004)
TC100851	*MtNip1*	Nodulin-26 type multifunctional aquaporin	Transport of solutes across the perisymbiotic membrane	Manthey et al. (2004)
TC106419	*MtTrHb2*	Truncated hemoglobin	NO-detoxification	Vieweg et al. (2005)
TC106705		Zinc-finger protein	Transcriptional regulation	Manthey et al. (2004)
TC106906		Auxin-regulated GH3 protein	Hormone action in root endosymbioses	Hohnjec et al. (2005)
TC106954	*MtScp1*	Serine carboxypeptidase	Protein processing during symbiosis senescence	Hohnjec et al. (2005)
TC107528		Endo-1,3-1,4-beta-D-glucanase	Modification of extracelluar matrices	Hohnjec et al. (2005)
TC108513	*MtZIP7*	Manganese transporter	Transport across the perisymbiotic membrane	Hohnjec et al. (2005)
TC109472		Polygalacturonase	Modification of extracelluar matrices	Hohnjec et al. (2005)
TC110699		Symbiosome membrane nodulin	Transport across the perisymbiotic membrane	Hohnjec et al. (2005)
	VfLb29	Leghemoglobin	NO-detoxification	Vieweg et al. (2004)

Genes are listed according to their current identifiers in the TIGR *M. truncatula* Gene Index, including gene names where available, updated annotations and proposed functions in root endosymbioses.

unknown function originally identified in soybean symbiosome membranes (Winzer et al., 1999) deserve attention. Together, the up-regulation of different genes encoding symbiosome membrane nodulins indicates that the peribacteroid and periarbuscular membranes share common structural properties.

The second group includes genes related to primary metabolism, amongst those with *MtSucS1* one of the five sucrose synthase genes of *M. truncatula* (Hohnjec et al., 1999). This gene was found to be up-regulated not only in root nodules, but also in AM roots. Based on the expression of reporter gene fusions in transgenic roots, an expression both in the central nodule tissues and in the arbuscule-containing cells of mycorrhizal roots was demonstrated (Hohnjec et al., 2003), in line with *in situ* localizations of sucrose synthase transcripts in arbuscule-containing cells of different plants (Blee and Anderson, 2002). Sucrose synthases catalyze the cleavage of sucrose to UDP-glucose and fructose and thus constitute key determinants of the sink strength in plant tissues (Sturm and Tang, 1999). Whereas the nodule-enhanced sucrose synthase of pea has been shown to be essential for root nodule function (Gordon et al., 1999), data on the functional importance of the symbiotically induced sucrose synthase during AM formation are still lacking. Recent data indicate that a reduction of MtSucS1 expression in transgenic lines expressing *MtSucS1* antisense fusions leads to a significantly reduced root colonization by AM fungi, and this phenotype is accompanied by a reduced phosphate content and by changes in characteristic metabolites (M.C. Baier, H. Küster and N. Hohnjec, Bielefeld University, unpublished data).

With respect to membrane formation, the symbiotically induced annexin MtAnn2 might play a role in calcium-dependent membrane reorganization during the colonization of plant tissues, as proposed for other plant annexins (Manthey et al., 2004). Since the invading microsymbionts are surrounded by membranes of plant origin, such processes are obviously relevant in both the root nodule and the AM symbiosis. The *in situ* localization of *MtAnn2* promoter activity showed an induction of the gene in the nodule primordium and in arbuscule-containing cells (Manthey et al., 2004), an observation consistent with a function during the initiation or establishment of membrane-surrounded endosymbiotic structures.

The co-induction of a polygalacturonase and an endo-1,3-1,4-beta-D-glucanase gene indicates the recruitment of similar cell wall modifying enzymes in root nodules and AM, possibly related to the modification of extracellular matrices surrounding symbiotic structures. Interestingly, also genes encoding enzymes involved in protein processing were activated in both symbioses: the serine carboxypeptidase gene *MtScp1* (Liu et al., 2003), a cysteine protease, and a gene specifying a signal peptidase. The identification of a symbiosis-induced signal peptidase is intriguing, since an involvement of similar proteins in the translocation of gene products across endomembranes, possibly to destine them towards the perisymbiotic spaces, can be hypothesized.

Finally, three genes encoding the leghemoglobin *VfLb29* as well as the truncated hemoglobin genes *MtTrHb1* and *MtTrHb2* were up-regulated during nodulation and mycorrhization. Whereas the *VfLb29* gene was identified in the course of the reverse Northern blot screening for symbiotically induced *Vicia faba* genes mentioned above (Frühling et al., 1997), the two genes encoding truncated hemoglobins were found by *in silico* analyses (Vieweg et al., 2005).

The *VfLb29* gene displays a remarkable expression pattern, since the gene is exclusively expressed in the infected cells of root nodules and the arbuscule-containing cells of mycorrhizal roots. The arbuscule-specific expression was observed not only in *Vicia faba*, but also in other legumes including the model legume *M. truncatula* and in the non-legume tobacco. This indicates a general AM-specific trigger for *VfLb29* gene expression under arbuscular mycorrhizal conditions (Vieweg et al., 2004).

Since an obvious *VfLb29* orthologue was not identified in *M. truncatula*, alternative hemoglobin-type genes were screened by *in silico* approaches for a common induction during nodulation and mycorrhization. That way, two truncated hemoglobin genes were identified that appear to be preferentially expressed in the central tissue of root nodules (*MtTrHb1*) and in arbuscule-containing cells (*MtTrHb2*), respectively (Vieweg et al., 2005). Considering the fact that some truncated hemoglobins from prokaryotes were shown to be involved in the detoxification of nitric oxide (Ouellet et al., 2002), it was proposed that the function of *MtTrHb1* and *MtTrHb2* is related to NO-detoxification rather to the supply of oxygen (Vieweg et al., 2005).

Interestingly, an NO-detoxification function has recently also been shown for non-symbiotic hemoglobins from *Medicago sativa* (Seregélyes et al., 2003) and *Alnus firma* (Sasakura et al., 2006). The existence of NO-detoxifying hemoglobins in other plant species supports the hypothesis that also the VfLb29 protein is primarily related to this process rather than to the binding of oxygen (Vieweg et al., 2004).

6. Functional analysis of legume promoters mediating a specific gene expression in arbuscule-containing cells

The regulation of gene expresion in the infected cells of root nodules requires the presence of short motifs designated OSEs (''organ-specific elements'') approximately 150 to 200 bps upstream of the trancriptional start site (de Bruijn et al., 1994). Originally, OSEs were identified in the promoters of leghemoglobin genes (Stougaard et al., 1987), but it turned out that other genes activated in the infected cells of root nodules also contain such elements (Küster et al., 1999, 2001).

In contrast to the regulation of gene expression in root nodules, defined short promoter motifs mediating expression in arbuscule-containing cells of mycorrhizal roots to date have not been specified. Nevertheless, subsequent to the identification of several hundred AM-induced genes by transcriptomics, full-size promoters of several AM-activated genes were isolated and their cellular expression properties in AM was determined. These experiments led to a collection of promoters exclusively or predominantly expressed in arbuscule-containing cells of mycorrhizal roots (e.g. *MtEnod11*, Journet et al., 2001; *MtPt4*, Harrison et al., 2002; *MtHa1*, Krajinski et al., 2002; *MtCel1* and *MtScp1*, Liu et al., 2003; *MtGst1*, Wulf et al., 2003; *MtTi1*, Grunwald et al., 2004; *MtAnn2* and *MtTubb1*, Manthey et al., 2004; VfLb29, Vieweg et al., 2004; *MtLec5* and *MtLec7*, Frenzel et al., 2005, 2006; *MtBcp1*, Hohnjec et al., 2005; *MtTrHb2*, Vieweg et al., 2005).

In *M. truncatula*, Harrison et al. (2002) and Liu et al. (2003) studied the cellular expression of three AM-induced genes by expressing reporter gene fusions in transgenic roots. Whereas the phosphate transporter *MtPt4* and the endoglucanase *MtCel1* were exclusively expressed in arbuscule-containing cells, the serine carboxypeptidase *MtScp1* was up-regulated in discrete cell files of the roots, even if only some cells contained arbuscules. These differences led Harrison (2005) to propose that gene expression in mycorrhizal roots is signaled both by cell-autonomous (expression in arbuscule-containig cells) and non cell-autonomous signals (expression in arbusculated cell files or additionally in cells surrounding arbuscules).

Even though these signals were not identified up to now, it might turn out that gene expression regulation in AM is more complex than transcriptional regulation in root nodules. A first hint in that direction came from the finding of Harrison et al. (2002) that the *MtPt4* promoter contained a sequence sharing similarity with a mycorrhiza- and resistance-related element (MRR1) from the promoter of the AM-specific*Solanum tuberosum* phosphate transporter gene *StPT3* (Rausch et al., 2001). A cross-species identification of regulatory sequences would obviously make these elements interesting candidates, but so far, MRR1-elements were not reported to be functional in other genes activated in arbuscule-containing cells.

Up to now, the promoters of only a few AM-induced genes were studied in detail by electrophoretic mobility shift assays (EMSA) or loss-of-function analyses. Recently, Frenzel et al. (2006) characterized the promoter of the*M. truncatula* lectin gene *MtLec5* (cf. also the review by Krajinski et al. in the present issue). The authors specified a 150 bp fragment from position −300 to −150 of the transcriptional start site as essential for gene induction during AM. Using EMSA, it was shown that several factors exclusively present in AM roots bind within the minimal promoter region, providing the first experimental evidence for such factors in mycorrhizal roots.

Another promoter mediating expression in AM roots drives the expression of the *MtEnod11* gene encoding a proline-rich protein. This gene deserves specific attention, since it is activated by Nod-factors, *S. meliloti* infection, secreted factors from AM fungi and by AM formation (Journet et al., 2001; Kosuta et al., 2003; Chabaud et al., 2002). Boisson-Dernier et al. (2005) performed a detailed loss-of-function study of the *MtEnod11* promoter, specifying a short promoter region 257 bp upstream of the transcriptional start as sufficient for driving gene expression in AM roots. Interestingly, an AT-rich regulatory sequence found also in other early nodulin gene promoters (e.g. driving *Enod12* gene expression in different legumes) was identified in this region and site-directed mutagenesis of this

element led to a significant reduction of gene expression not only during *S. meliloti* infection, but also during colonization with AM fungi. On the other hand, since the 257 bp promoter region did not respond to Nod-factors, it appears that different modules are responsible for different symbiotic *MtEnod11* promoter activity (Boisson-Dernier et al., 2005).

A similar observation was made for the promoter of the *Vicia faba* leghemoglobin gene *VfLb29* exclusively expressed in the infected cells of root nodules and in the arbuscule-containing cells of mycorrhizal roots of different legume and non-legume plants (Vieweg et al., 2004). A detailed loss-of-function study identified an 85 bp region (position −410 to −326 relative to the start codon) that is necessary for expression in arbuscule-containing cells, although not being required for gene activation in root nodules. To investigate an autonomous function of the 85 bp region and the surrounding sequences in arbuscule-containing cells, different gain-of-function studies were performed using chimeric promoter constructs designed to *trans*-activate the promoter of another leghemoglobin gene that is not expressed in AM roots (Fehlberg et al., 2005). Since *trans*-activation was not achieved, it was concluded that the activation of *VfLb29* gene expression in arbuscule-containing cells of legumes and non-legumes is controlled by a more complex regulatory system requiring at least two promoter modules (Fehlberg et al., 2005).

Clearly, detailed molecular analyses of more AM-induced promoters will be needed to specify core elements responsible for gene expression in arbuscule-containing cells. In this respect, the different global transcriptome profiling experiments delivered a valuable resource of AM-induced genes that, together with information on promoter sequences from the *M. truncatula* genome project, will form the basis for a more genome-wide study of promoter motifs governing gene activation during AM.

7. Concluding remarks

Complementing the analysis of mutants defective in particular during early stages of root endosymbioses, the introduction of high-throughput expression profiling techniques in the past years has uncovered the transcriptome of symbiotic legume roots, both under nodulation and mycorrhization conditions. Although several hundred differentially expressed genes were specified in either interaction, the overlap in gene activation between the root nodule and the AM symbiosis is comparably low. This is on the one hand related to the fact that detailed time course analyses are difficult to perform for the asynchronous AM symbiosis. On the other hand, dilution effects from the use of pooled tissue samples inevitably obscured the expression profiles obtained, leading to a significant loss of information. It will thus be of prime importance to extend transcriptome profiling approaches to a cellular level, combining the high-throughput character of microarray and GeneChip hybridizations with the cellular resolution of low-throughput *in situ* expression or promoter activity studies. It can be expected that a global comparison of gene activation during cellular time courses of root cell colonization with either rhizobial prokaryotes or AM fungi will specify the full transcriptional response of legumes to either microbe, allowing comparative approaches on the basis of cellular, whole-genome expression snapshots.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft in projects Pe-814/1-1, Pe-814/1-2 and KU1478/2-3 (all SPP 1084 ''Mykorrhiza''). Helge Küster and Natalija Hohnjec acknowledge financial support by the International Graduate School in Bioinformatics and Genome Research (Center for Biotechnology, Bielefeld University). We are grateful to our Diploma students Esther M.N. Dohmann and Vera Fehlberg for their contribution to the *VfLb29* promoter analysis and we thank Manuela Meyer and Tanja Thias (Institute for Genome Research, Center for Biotechnology, Universität Bielefeld) for excellent technical support.

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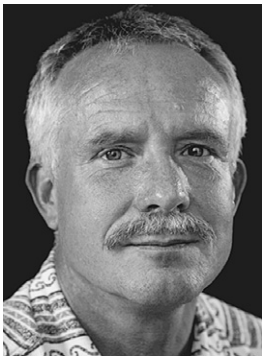

Andreas M. Perlick studied and obtained his doctoral degree at the Chair of Genetics, Faculty of Biology, Bielefeld University. Together with his group, he identified and analysed *Vicia faba* genes involved in the root nodule symbiosis. Subsequently, A.M. Perlick inititated the comparison of gene expression during the two symbiotic interactions of legume plants, i.e. nodulation and arbuscular mycorrhiza. In 2003, he changed his focus and is now engaged in the field of antiviral agents and mechanisms at the Bioengineering Department of Erlangen-Nuremberg University.