ОРИГИНАЛЬНЫЕ РАБОТЫ

CONSTRUCTION AND APPLICATIONS OF A MYCORRHIZAL ARBUSCULAR SPECIFIC cDNA LIBRARY

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To exploit the potential benefits of mycorrhizas, we need to investigate the processes that occur in these symbiotic interactions, particularly in the arbuscular compartment where nutrients are exchanged between the plant and the fungus. Progress in this area is restricted due to the intricacy and complexity of this plant-fungus interface and many techniques that have been employed successfully in other plants and animal systems cannot be used. An effective approach to study processes in arbuscules is to examine transcript composition and dynamics. We applied laser capture microdissection (LCM) to isolate approximately 3000 arbuscules from Glomus intraradices colonised Medicago truncatula roots. Total RNA was extracted from microdissected arbuscules and subjected to T7 RNA polymerase-based linear amplification. Amplified RNA was then used for construction of a cDNA library. The presence and level of enrichment of mycorrhiza-specific transcripts was determined by quantitative Real-time and conventional PCR. To improve enrichment a cDNA library subtraction was performed. Complementation of yeast mutants deficient in the uptake of potassium, phosphate, sulphate, amino acids, ammonium and of a Mn^{2+} sensitive strain, demonstrates the functionality of our cDNA library.

Key words: *arbuscule, Glomus intraradices, LCM, Medicago truncatula, mycorrhiza, SSH cDNA library, yeast complementation.*

Introduction. More than 80 % of terrestrial plant species form associations with arbuscular mycorrhizal (AM) fungi [1]. Fossil remains of AM fungi date back to over 400 million years, suggesting that AMs were highly relevant for land colonisation of plants [2]. Today, AM is the most widespread type of mycorrhizal association worldwide. The main feature of this mutual symbiosis is the exchange of nutrients between both partners: the fungus supplies the plant with mineral nutrients from the soil and receives carbohydrates in return. Other features of mycorrhiza-associated plants are increased resistance to root pathogens [3−5] and abiotic stress as imposed by drought and heavy metals [6, 7].

Two classes of AM are described on the basis of structural differences in forming intracellular hyphal branches, the so-called Arum and Paris types [8]. In the Arum type, the AM fungus invades the root cortex and forms intraradical hyphae. Subsequently, these hyphae enter cortex cells and form highly branched tree-like structures, called arbuscules. Arbuscules are believed to be the location where nutrient exchange takes place between plant and fungus.

Arbuscular mycorrhizas can deliver up to 80 % of plant P, 60 % of plant Cu, 25 % of plant Zn and N and 10 % of plant K [9] and have therefore significant ecological and agricultural importance. However, our understanding of the development and maintenance of functional symbioses is still limited: Little is known about the molecular dialog between the two symbiotic partners that leads to the establishment of AMs and also the exact mechanisms of nutrient exchange have yet to be revealed.

A promising approach to gain further insights into these processes is to study the composition and dynamics of the local transcriptome. By examining mRNA populations from arbuscules we should be able to assess which genes are expressed in these compartments while at the same time providing a handle for further downstream applications such as functional analysis of the identified gene products. To gain access to arbuscular transcripts, we employed laser capture microdissection (LCM) which has previously been used to dissect animal tissues [10, 11], plant vascular tissues [12−14], plant embryos [15] and various other plant tissues [14, 16−24]. LCM allows the excision of individual cells or cellular compartments from mixed populations that can be used to study aspects of cell- or tissue-specific processes such as RNA, protein or metabolite composition [25].

We describe here the establishment of protocols for microdissection, RNA extraction and amplification, cDNA preparation and creation of a functional cDNA library using *Medicago truncatula* root sections colonised with *Glomus intraradices*. The mycorrhiza-specific cDNA library was then used to complement 8 different yeast strains deficient in phosphate, K^+, NH_4^+, sulphate, amino acids, and Mn^{2+} homeostasis to demonstrate its functionality.

Materials and Methods. *Plant materials, bacterial strains and yeast strains. Medicago truncatula* L. Gaertn. var. Jemalong A17 was grown in expanded clay (2−5 mm particle size) in 250-mL plastic pots under a 16-h light/8-h dark regime in the green house. Fertilisation was carried out weekly through application of a Long Ashton solution with 10 % of the original phosphate content [26]. Fungal inoculum of *Glomus intraradices* (isolate 49) [27], enriched by previous co-cultivation with leek (*Allium porrum* L.), was used to achieve mycorrhization by co-cultivation of plants with the inoculum mixed with sterile expanded clay (1.5:8.5, v/v). After 5 weeks of inoculation colonised root tissues were used for LCM.

Saccharomyces cerevisiae strains: strain CY162 (trk1/trk2) *MATαhis3Δ200 leu2-3, 112trp1Δ901 ura3-53 suc2Δ9 trk1Δ51 trk2Δ50::lox-kanMX-lox* (Ko & Gaber, 1991) carries deletions in high affinity K^+ uptake transporters; the strain YKR039w (GAP1) *MATα his3Δ 1leu2Δ0 ura3Δ0 YKR039w::kanMX4* carries a deletion in the amino acid permease; the strain YBR296c (PHO89) *MATα his3Δ 1; leu2Δ0 met15Δ0 ura3Δ0 YBR296c::kanMX4* carries deletion in the Na^+/Pi cotransporter; the strain YGR121c (MEP1) MAT α *his3Δ1 leu2Δ1 leu2Δ0 met15Δ0 ura3Δ0 YGR121c::kanMX4* carries a deletion in the ammonium permease 1; the strain YNL081w (MEP2) *MATα his3Δ1 leu2Δ1 leu2Δ0 met15Δ0 ura3Δ0 YNL081w::kanMX4* carries a deletion in the ammonium permease MEP2; the strain 31019b (*mep1Δ mep2Δ::LEU2 mep3 Δ::KanMX2 ura3*) carries deletions in 3 ammonium permeases (1, 3) [28]; the YSD1 strain has a deletion in the sulphate transporter gene *Sul1* [29]; the PMR1 strain (YGL167C) MATα *leu2-3,112 his3Δ1 sod1Δα::URA3 sod2Δ α::TRP1 Gal+ cup1Δ::URA3 pmr1Δ::LEU2* carries a deletion in the P-type ATPase located in the Golgi that is responsible for transporting Ca^{2+} and Mn^{2+} [30]. Transformations of the various yeast strains was carried out with the pYES2 plasmid (Invitrogen, Carlsbad, USA) alone (empty vector, EV), or pYES2 containing the arbuscular cDNA library [31]. Uracil-free, minimal medium (SD) supplemented with galactose was used to select complemented transformants in growth restrictive growth conditions as explained in the text.

Tissue fixation and sectioning. Medicago roots colonized with *Glomus intraradices* were cut into 5 mm pieces. The roots were placed in ethanol : acetic acid (3:1 ratio) and slightly vacuum infiltrated several times for 15−20 min whilst on ice. Fresh ethanol : acetic acid (3:1) was added and then roots were placed in the dark, with gentle shaking at 4 °C for 12−14 h. The roots were processed through a series of ethanol dehydration steps for 1 h (80 %, 90 %, 100 %, 100 %, 100 %) at room temperature. After dehydration, a concentration series of PEG-1000 (Fluka, Buchs, Switzerland) was introduced, using a water bath at 56 °C. The lower PEG percentages were prepared with 100 % (v/v) ethanol. Every hour, fresh PEG solutions with increased concentrations (20 %, 50 %, 70 %, 100 %, 100 %) were replaced. The roots in 100 % PEG were then placed into pre-warmed Dismoulds (7 × 7 × 5 mm, BDH Laboratory Suppliers, Pool, Dorset, UK) with processing cassettes (Agar Scientific, Stansted, Essex, UK) placed on top of the sample. Then fresh, 100 % PEG-1000 was poured into the processing cassette and left in the dark at 4 °C until solidification. Samples were then cut at 12 μm thickness on a rotary microtome (Leica RM 2135) at room temperature. The PEG ribbons were

placed on pre-water sprayed poly-L-lysine and RNAzap treated PEN membrane slides (PALM®) and fixed in 70 % ethanol. To remove the PEG before LCM cutting the sections were treated with 95 % ethanol and left to dry in a laminar hood for 30 min. The samples were used for LCM on the same day.

LCM protocol. Laser capture microdissection was performed using a PALM® MicroBeam − micromanipulation system equipped with a UV-A Laser (P.A.L.M. Microlaser technologies AG, Germany). Briefly, a slide was placed into position on the computer controlled PALM® RoboStage and a RNase-free 0.5 ml collection tube with cap was placed on the PALM® CapMover, which automatically positions the collection cap above the sample to be catapulted. The collection cap contained 20 μl lysis buffer (Ambion). A 40×LD objective was used for magnification and laser energy and focus were adjusted to particular sections and slides but were typically 80 mW and 27 μm and between 10 and 15 sections were processed per 0.5 ml tube cap.

RNA extraction and RNA amplification. Medicago truncatula roots (100 mg fresh weight) were homogenized in liquid nitrogen. Total root RNA was extracted using the Plant RNeasy Extraction Kit (Qiagen, Hilden, Germany). cDNA synthesis was performed using Super SMART™ PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA) according to manufacturer instructions.

RNA from LCM sections was extracted using the RNAqueous-Micro Kit (Ambion, Europe, Ltd, Huntingdon, UK) according to the manufacturer's instructions with slight modifications. Briefly, before RNA extraction the samples embedded in PEG 1000 were heat-treated at 65 °C for 10 min and then the kit protocol was followed. Eluted RNA was stored in 100 % ethanol at −20 °C. cDNA synthesis and RNA amplification were performed using the MessageAmp™ aRNA kit (Ambion) according to the manufacturing instructions.

The aRNA amplification procedure comprises of the following steps: 1st strand cDNA synthesis, 2nd strand cDNA synthesis, and aRNA transcription. After the first aRNA transcription procedure, the newly amplified aRNA could be used as template for the subsequent 2−3 rounds of cDNA synthesis and RNA transcription until the quantity of cDNA was sufficient for further PCR analysis with gene specific primers.

Briefly, a first round of cDNA synthesis was initiated with T7oligo(dT) primer. One μl T7oligo(dT) primer was added to 11 μl RNA, heated to 70 °C for 10 min and to this 2 μl 10× first strand buffer was added (preheated to 42 °C), 1 μl ribonuclease inhibitor, 4 μl dNTP mix and 1 μl reverse transcriptase. After 2-h incubation, second strand synthesis was performed by the addition of 63 μl nuclease-free water, 10 μl 10× second strand buffer, 4 μl dNTP mix, 2 μl DNA polymerase and 1 μl RNase H. The reaction was performed for 2 h at 16 °C. cDNA was purified according to the manufacturer's instruction and concentrated by ammonium acetate − ethanol precipitation in a 10 μl volume. For *in vitro* transcription, 8 μl of the cDNA was mixed with 8 μl NTP mix, 2 μl 10× reaction buffer and 2 μl T7 enzyme mix. The reaction was incubated at 37 °C for 8−12 h and then treated to DNase I digestion. aRNA was purified and resuspended in 10 μl of nuclease free water.

Subsequent rounds of cDNA synthesis were performed as follows: To 10 μl of aRNA 2 μl random primers were added and the reaction was incubated at 70 °C for 10 min. At 42 °C, the following components were added: 2 μl 10× first strand buffer, 1 μl ribonuclease inhibitor, 4 μl dNTP mix and 1 μl reverse transcriptase and the reaction incubated for 2 h. 1 μl of RNase H was added and incubated for 30 min at 37 °C. Second strand synthesis was primed with 5 μl T7oligo(dT) primer. After 10-min incubation at 70 °C the remaining components were added at room temperature: 58 μl nuclease free water, 10 μl 10× second strand buffer, 4 μl dNTP mix, 2 μl DNA polymerase. The reaction was incubated at 16 °C for 2 h. cDNA was purified and *in vitro* transcription performed as described. Typically 2 rounds of RNA amplification were required to produce several micrograms of aRNA.

Construction and subtraction of cDNA library. The adapter (25 μM) from GenomeWalker Universal Kit (Clontech) was ligated to the ends of cDNA fragments (100 ng) using T4 ligase (Clontech) according to the manufacturer's instruction. The GenomeWalker adapter has *MluI*, *SalI* and *XmaI* recognition sites for cloning. The ligation mixture was purified with a QIAquick PCR purification kit (Qiagen) to remove unligated adapter. DNA polymerisation was performed in a 50 μl volume with the following components: 25 pmol of 3′-T7-*SalI* primer (5′-TCTAGTCGACGGC-

CAGTGAATTGT-3'), 1× advantage 2 PCR buffer (Clontech), 200 µM of each dNTP (Clontech) and 1× advantage 2 polymerase mix (Clontech). The reaction was performed in a 50 µl volume with the following programme: preheat at 94 °C for 1 min; 5 cycles at 94 °C for 1 min, 62 °C for 1 min, 72 °C for 2 min; final extension at 72 °C for 5 min. After reaction purification with a QIAquick PCR purification kit (Qiagen), subsequent PCR was performed using 25 pmol of Adapter primer 1 (5'-GTAATACGACT-CACTATAGGGC-3') (GenomeWalker Universal Kit, Clontech) of and 3'-T7-*SalI* primer 5'-TCT-AGTCGACGGCCAGTGAATTGT-3'), 1× advantage 2 PCR buffer (Clontech), 200 µM of each dNTP (Clontech) and 1× advantage 2 polymerase mix (Clontech). The reaction was performed with the following cycling conditions: preheat at 94 °C for 1 min; 30 cycles at 94 °C for 1 min, 62 °C for 1 min, 72 °C for 2 min; final extension at 72 °C for 7 min. The PCR products were purified with a QIAquick PCR purification kit (Qiagen).

1 µl of the PCR product was diluted to 100 µl with nuclease free water, and 1 µl of the diluted DNA was used as template for the second round of PCR. The PCR was performed in a 50 µl volume of the same buffer as described for the 1st round of PCR with 25 pmol of nested adapter primer (5'-ACTATAGGGCACGCGTGGT-3') from the GenomeWalker Universal Kit (Clontech) and 25 pmol of 3'-*SalI* nested primer (5'-GTGAATTG-TAAGTCGACTCAC-3'). The cycling profile was 94 °C for 1 min; 30 cycles at 94 °C for 1 min, 62 °C for 1 min, 72 °C for 2 min; final extension at 72 °C for 7 min. The PCR products were purified with a QIAquick PCR purification kit (Qiagen), digested with *SalI* and *XmaI*. The digests were separated by agarose gel electrophoresis, and fragments of more than 300 bp were collected and purified with a QIAquick Gel Extraction kit (Qiagen).

The DNA was cloned into *SalI* and *XmaI* sites of pBluescript II KS (Stratagene, La Jola, USA) and used to transform the *E.coli* XL10-Gold strain (Stratagene). The pBluescript cDNA library was amplified according to the Stratagene manufacturer's instructions (pBluescript II XR cDNA Library construction Kit, Statagene).

In order to enrich the cDNA for arbuscule-specific clones, the double stranded (ds) cDNA obtained from LCM samples was subjected to subtractive hybridization with cDNA derived from *Medicago* non colonised roots using CLONTECH PCR-Select™ cDNA subtraction Kit. Subtraction was performed to isolate differently expressed ds-cDNA according to the manufacturer's instructions (Clontech). This method selectively amplifies the mycorrhiza related cDNA from *M. truncatula* colonized by *G. intraradices*. The amplification products were cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA).

Using the substitution of the Nested PCR primer 2R from the CLONTECH PCR-Select™ cDNA subtraction Kit (5'-AGCGTGGTCGCGGCCGA-GGT-3') to specially designed *NotI* Adapter primer 5'-ATGCGGCCGCGTGGTCGCGGCCGA-GGT -3') and two rounds of nested PCR, the *Not I* restriction sites were introduced to both sides of subtracted cDNAs. Subsequently, using *Not I* restriction, subtracted cDNA was cloned into the pYES2 vector.

PCR analysis. 20 ng of cDNA obtained from non-colonised roots and 50 ng of library DNA were used as PCR template. PCR was performed in 50 µl volume with 1× advantage 2 SA PCR buffer (Clontech), 200 µM of each dNTP (Clontech) and 1× advantage 2 polymerase mix (Clontech). The cycling profile was: 95 °C for 1 min; 36 cycles at 95 °C for 30 sec, 68 °C for 1 min; final extension at 68 °C for 7 min. The primer pairs used for PCR were as follows: for elongation factor α1 (#TC106485, TIGR): Efα1 forward (5'-AGAGACCCACAGACAAGCC-CCTCAG-3') and Efα1 reverse (5'-CTTGGCAG-CAGCTTTGGTGACTTTG-3'); for the mycorrhiza specific phosphate transporter (*MtPT4*) (#TC85743, TIGR): MtPT4 forward (5'-ACAGCCCGAAGG-GGATTTACTCTGG-3') and MtPT4 reverse (5'-GTTTCCGTCACCAAGAACGTGCAAA-3'); for the mycorrhiza specific H⁺-ATPase (*Mtha1*) (#TC95400, TIGR): Mtha1 forward (5'-TGGAC-TTCGTTCTGGGGTTGCT-3') and Mtha1 reverse (5'-AAGCGGTGCACACCAAAATTGTC-AG-3'); for the *Glomus intraradices β-tubulin 2* (#AY326321, NCBI): βtub2 forward (5'-CCATTA-CACCGAAGGCGCTGAACTT-3') and βtub2 reverse (5'-GTCAATGGAGCAAATCCGACCAT-GA-3'); for *Glomus intraradices* 18S ribosomal gene: 18S forward (5'-CGGTGCGTTGCAATTTTT-GTGATG-3') and 18S reverse (5'-GGAACCAC-ACGATATGGTCGCATCT-3'); for the mycorrhiza specific nitrate transporter (EST clone #TC78158, TIGR), Nittrans1 forward (5'-GCC-

GGAGTCGCTTCGGTTTCAGGTA-3′) and Nit-trans2 reverse (5′-CGATCGGAAGTGTATTCGC-ACGTCCT-3′), for the mycorrhiza specific hexose transporter (*MtST1*), (#TC87421, TIGR) MtST1 forward (5′-GTGGGTCGGATCTTGCTCGGG-TTTG-3′) and MtST1 reverse (5′-CCAACCAA-GAGGACCCCATGACCAA-3′), for the mycorrhiza specific manganese transporter (*MtZIP7*), (# TC88701, TIGR) MtZIP7 forward (5′-CGC-CACTTTCATTGCCGGTGTTTCA-3′) and MtZ-IP7 reverse (5′-TGCAAAAGCGTAGGCTGCG-CATGAT-3′), for the mycorrhiza specific H⁺ pump intrerator (EST clone #TC80954, TIGR) Protp forward (5′-TAATGCTGGTCGGGCGG-TATCTGC-3′) and Protp reverse (5′-CCCTAC-TCAGCTGCCGCATGTCCAA-3′), for the *Glomus intraradices* extensin-like protein, (EST clone # giI1076557IpirIIs54157, SWBIC) ExtGl forward (5′-AGCTTCCCGGGTGACAAAGATGACG-3′) and ExtGl reverse (5′-GCCTGGGGAGGCAGA-TCGTTTGTAGC-3′).

Real-time PCR analysis. Primers for Real-time PCR were designed using the Primer Express™ software (Applied Biosystems, Foster City, USA). A fragment from *MtPT4* transcript was amplified using the primers MtPT4sybr forward (5′-CTG-CATTTCAACCGTTTCGA-3′) and MtPT4sybr reverse (5′-CAGCCGAAGACTAATTGGCC-3′). A fragment from elongation factor α1 was amplified using primers EFsybr forward (5′-CATTT-GTTCCCATCTCCGGA-3′) and EFsybr reverse (5′-TGTCTGTGGGTCTCTTGGGC-3′). The primers Mtha1sybr forward (5′-TGATGCCGGTAA-TATGC-3′) and Mtha1sybr reverse (5′-CGAAGT-CCACGTTCTGCAAA-3′) were used to amplify fragments corresponding to *Mtha1*. Fragments of the *G. intraradices* 18S ribosomal gene were amplified using 18Ssybr forward (5′-GCGAAGTGCGATAA-GTAATGTGA-3′) and 18Ssybr reverse (5′-AATT-GCAACGCACCGATTTT-3′). Real-time PCR was carried out using the ABI Prism™ 7000 sequence detection system, optical caps and optical tubes (Applied Biosystems). PCR amplification mixtures (20 μl) contained 20 ng of obtained cDNA or 50 ng of library cDNA, 2× SybrGreen™ Master Mix buffer (10 μl) (Applied Biosystems) and primers (1 μl). The cycling conditions were chosen according to the manufacturer instructions. They comprised 10 min polymerase activation at 95 °C and 40 cycles at 95 °C for 15 sec and 60 °C for 60 sec.

Each assay was performed in triplicate. Data evaluation was carried out using the ABI Prism software. The threshold cycle number (C_t) was determined according to manufacturer instructions. The differences in C_t values between EF α1 and experimental amplicons (ΔC_t) were normalized to the lowest ΔC_t value ($\Delta \Delta C_t$). Relative numbers of transcripts were calculated using the formula $2^{-\Delta \Delta Ct}$, setting the number of samples with the lowest ΔC_t-value as 100 %.

Results and discussion. To extract viable RNA, tissue sectioning has to be compatible with microscopy demands, laser ablation and the prevention of RNA degradation. For successful capturing of cell material by LCM, the mounted tissue has to be completely dehydrated which frequently obscures structural nuances. Thus, our attempts to visualise arbuscules using cryosections normally failed since it was very difficult or impossible to distinguish arbuscules from surrounding tissue after dehydration of cryosamples. After evaluating various methods we found that tissue fixed with either paraformaldehyde or ethanol : acetic acid (3:1) and embedded in PEG1000 gave the best visualisation of arbuscular material after dehydration. Though no differences were detected between the fixation methods regarding arbuscular identification, ethanol : acetic acid fixation is generally believed to be more compatible with RNA recovery [14]. Using ethanol : acetic acid as a fixation medium and arbuscular autofluorescence as a further aid to select tissue sections, arbuscules were marked and subsequently collected (Fig. 1, see inset).

Total RNA was extracted from approximately 3000 young arbuscules, treated with DNase I, linearly amplified and subjected to synthesis of double-stranded cDNA. Approximately 1 μg of double stranded cDNA was obtained by amplification. Usually, 2 rounds of amplification generated a sufficient amount (approximately 10—20 μg) of aRNA.

Prior to construction of the cDNA library, RT-PCR experiments were performed to determine whether the aRNA was a suitable template and whether mycorrhiza specific genes could be detected. For example, in situ hybridization experiments showed localization of *MtPT4* and *Mtha1* within arbuscule containing cells whereas the *β-tubulin 2* gene is constitutively expressed in *Glomus intraradices* [32—34]. Our RT-PCR experiments on aRNA support these previous findings (data not shown).

To estimate enrichment of mycorrhiza-specific transcripts relative to intact colonised roots of *Medicago truncatula*, the obtained cDNAs were subjected to Real-time PCR analysis. The obtained data demonstrate a significant enrichment of *Medicago Mtha1*, *MtPT4* and *Glomus* 18S ribosomal cDNAs with the level of *MtPT4* transcript increased 3 times, the *Mtha1* transcript 14 times and the level of *Glomus* 18S transcript rose by nearly 78 times in comparison with those values for cDNA from colonised *Medicago truncatula* roots.

A cDNA library was constructed according to the T7 polymerase based RNA amplification protocol with subsequent synthesis of double-stranded cDNAs. The cDNAs were ligated to adaptors to generate *MluI*, *SalI* and *XmaI* restriction recognition sites and then amplified by PCR. The observed PCR products ranged from 100 bp to 4000 bp in size and were fractionated by agarose gel to remove small PCR products, unincorporated primers, mononucleotides and primer artefacts. The cDNAs were cloned into the pBluescript II KS vector and an arbuscule specific primary library was obtained in *E. coli* which after amplification showed a titre of 5·10⁸ cfu per ml.

The cDNA library was tested using Real-time PCR, showing the presence of amplicons for mycorrhiza-specific genes of *Medicago truncatula* (*MtPT4, Mtha1*) and *Glomus intraradices* (*β-tubuline 2*, 18*SrDNA*) [34, 35]. In order to further check the

quality of the primary library, 10 randomly selected clones were subjected to sequencing. Three clones out of 10 showed similarity to *Medicago truncatula* ESTs. None of the clones detected had sequence similarity to *Glomus intraradices*, probably due to the limited amount of sequence information available for arbuscular mycorrhiza fungi in the databases. We therefore proceeded with a cDNA subtraction to enrich the obtained library further.

Five mycorrhiza-specific ESTs were selected to test library enrichment: The *Medicago* high-affinity nitrate transporter, the hexose transporter *MtSt1*, the manganese transporter *MtZIP7*, and the H⁺ pump interactor [36]. For *Glomus* an extensin-like protein and *β-tubulin 2* were tested [34]. The results show that subtraction allowed us to amplify new mycorrhiza specific transcripts that were not amplified before (Fig. 2, *a, b*, see inset). Subtracted cDNAs were cloned into pGEM®-T Easy vector (Promega). Using the two rounds of nested PCR subtracted cDNA with introduced *NotI* restriction sites were cloned into the yeast vector pYES2. pGEM®-T Easy and pYES2 based cDNA libraries were amplified and gave a titre ~ 5 · 10⁸ cfu.

As for the non-subtracted library, 12 randomly selected clones from the pGEM®-T Easy library were sequenced (Table). The sequencing results demonstrate a much higher frequency of sequence similarity to *Medicago* and *Glomacean* ESTs. Eight clones out of 10 showed similarity to *Medicago trun-*

Features of sequences and BLAST analysis from randomly selected cDNA clones from subtracted cDNA library. BLAST analyses against NCBI EST database shows high sequence similarity to specific Medicago or fungal ESTs

Clone number	Similarity	Source	Accession number
1, 2	95 % to gene of uncultured *Glomus 25S rRNA*	Uncultured *Glomus*, genome DNA, root sample, mycelium	AB250019
3	90 % to *Glomus intraradices* telomeric region	*Glomus intraradices*, genome DNA	AJ851840
4, 6, 8	97 % to *Medicago truncatula* mRNA sequence	Phosphate starved leaf *Medicago truncatula* cDNA, clone NFO008A12PL	giI20294998IgbIBQ157941.1
5, 9, 10	89 % to *Medicago truncatula* mRNA sequence	Irradiated *Medicago truncatula* cDNA, clone NFO093HO31H	giI20293590IgbIBQ156531.1
7	93 % to *Medicago truncatula* mRNA sequence	Drought *Medicago truncatula* cDNA, clone NF099D11DT	BG451206.1
10	86 % to *Medicago truncatula* mRNA sequence	*Medicago truncatula*, seedling roots, 3 days post-inoculation with *Sinorhizobium*, cDNA, clone KV3-49N24	CB891346
11, 12	No similarity	Unknown	Unpublished

catula ESTs, 3 clones had a similarity to *Glomacean* ESTs and 2 cDNA clones did not show any similarities.

To test for the presence of full length clones in our library, we attempted to amplify six inserts using primers that would span the entire open reading frame of the *M. truncatula* hexose transporter *MtSt1*, the elongation factor α1 and the phosphate transporter *MtPT4*, and the *G. intraradices* sequences encoding tubulin-2 and an extensin-like protein. We were able to amplify a 1713 bp fragment of the 1730 bp long Hexose transporter *MtSt1* (#TC87421, TIGR) and 1079 bp of 1100 bp long Glomacean *β-tubulin2* (data not shown).

In order to test the functionality of the library, we used it to transform several yeast mutant strains defective in the uptake and compartmentation of various nutrients. Fig. 3 (see inset) shows that library derived clones were capable of complementing yeast deficiencies in the uptake of K^+, sulphate, phosphate, ammonium and the compartmentation of Mn^{2+}. To certify that restored yeast growth was due to genuine transformation events, plasmids were recovered and used to retransform the deficient genotype. In all cases, a restoration of growth was observed that was similar to that obtained from the original transformation with the SSH cDNA library, showing that the arbuscule-specific library derived inserts were responsible for the complementation.

The pYES2-based cDNA library was transformed into yeast manganese sensitive strains and deficient in high affinity K^+, phosphate, sulphate, amino acids, ammonium uptake. The low K^+ sensitive CY162; low sulphate sensitive YSD1; low ammonium sensitive YGR121c (MEP1), YNL081w (MEP2), 31019b (Mep1, Mep2, Mep3); low phosphate sensitive YBR296c (PHO89); amino acid sensitive YKR039w (GAP1) and manganese sensitive PMR1 strains were transformed with the SSH cDNA library. Obtained transformants have shown restored ability to grow on low K^+ condition for CY162 (Fig. 3), on low sulphate condition for YSD1 (Fig. 3), on low NH_4^+ condition for YGR121c (MEP1), YNL081w (MEP2), 31019b (Mep1, Mep2, Mep3), on phosphate depleted condition for YBR296c (PHO89) and low proline NH_4^+ free condition for YKR039w (GAP1). Moreover, we were able to restore ability to grow for manganese sensitive mutant PMR1 with presence of 3mM $MnSO_4$ (Fig. 3). Complemented yeast mutant transformants had growth rates similar to those of wild type cells.

Plasmids isolated from complemented strains were used for retransformation of the same yeast background mutants and for sequencing analysis. After retransformation of 8 different yeast mutants, the restored growth properties were identical to those observed after the initial transformation.

At the present time many processes leading to the establishment, development and functioning of micorrhizas remain unclear. Progress in this area is greatly frustrated by the inaccessibility and intricacy of the plant-fungus interface. Indeed, many approaches and techniques that have been successfully used for other biological systems cannot be applied to the fine structures of arbuscules and intraradical hyphae. However, this study demonstrates the effectiveness of LCM as a novel approach to study AM mycorrhizas. LCM not only allows physical access to arbuscules but, in combination with amplification and subtraction protocols, also allows the construction of specific cDNA libraries that can be used to study the transcriptome of arbuscules and colonised root cells.

To generate an arbuscular cDNA library we excised arbuscules from colonised root cells. LCM has been used previously to isolate subcellular compartments but due to the laser resolution the material surrounding subcellular targets is inevitably sampled as well. Thus, our starting material derived from both fungi and plants but was greatly enriched in mycorrhizal structures. The small size of arbuscules also required a relatively large sample of 3000. Other studies have shown that smaller samples suffice to generate cDNA libraries but these tended to consist of entire cells and derived from cryosections [12, 15]. Our findings show that cryosectioning is not suitable for mycorrhizal root tissue. Indeed, the longer and more complicated PEG tissue preparation may have led to more RNA degradation and hence the requirement for larger samples.

Subtraction allowed us to further enrich our library for mycorrhiza-related clones. Subtraction procedures can provide greater than 1000-fold enrichment and are therefore ideal to optimise the presence of rare transcripts. Moreover, literature data suggest that subtracted libraries can be used as an alternative or as a complementary transcript profiling tool to microarrays, especially for the identi-

fication of novel genes and transcripts of low abundance [37]. The successful amplification of known mycorrhiza-related genes from the constructed cDNA library confirms the specificity of the library and the effectiveness of our approach. The average size of inserts for the primary pBluescript library was around 500 bp. The subtracted cDNA library comprised clones that ranged from 500 bp to 6000 bp. Thus, despite of an aRNA amplification and cDNA synthesis procedures that initiate at the 3' end of mRNA, the size of the obtained inserts suggests the presence of many full length clones. In addition, we were able to amplify full length sequences encoding a mycorrhiza-specific hexose transporter (*MtST1*) and a fungal *β-tubulin 2*. Complementation of 8 different yeast mutants provides additional evidence that our library contains functional inserts that can be used for further analysis. Indeed, the combination of LCM, library subtraction and yeast complementation assays forms a powerful tool for discovering and cloning of new genes.

In summary, the usage of LCM in combination with PEG embedding protocols is a novel approach and provides a promising route to deepen our understanding of mycorrhizal symbioses. This approach could be expanded to develop further new libraries, for example from intraradical hyphae, plant tissues which surround arbuscule containing cells, or from arbuscular fractions of different developmental stages.

СОЗДАНИЕ И ПРИМЕНЕНИЕ СПЕЦИФИЧНОЙ К АРБУСКУЛЯРНОЙ МИКОРИЗЕ БИБЛИОТЕКИ кДНК

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Для того чтобы оценить потенциальную пользу микоризы, нам нужно было исследовать процессы, имеющие место в этих симбиотических взаимодействиях, в особенности это касается арбускулярных компартментов, где происходит обмен питательными веществами между грибом и растением. При исследованиях в этой области сталкиваются с трудностями из-за сложности образования и структуры симбиотических органов (арбускул) и невозможности отделить эти органы от других типов клеток. Поэтому множество методов, применяемых в исследованиях растительных и животных систем, не могут быть использованы в этом случае. Одним из самых эффективных подходов для изучения процессов, происходящих в арбускулах, является определение композиции транскриптов и их динамики. Нами применен метод микроскопии с лазерной микродиссекцией для сбора и выделения около 3000 арбускул из *Glomus intraradices*, который колонизировал корни *Medicago truncatula*. Общая РНК выделена из собранных арбускул, и ее использовали для линейной амплификации мРНК с помощью Т7 РНК полимеразы. Амплифицированная РНК была использована для создания библиотеки кДНК. Присутствие и уровень обогащения специфическими для микоризы транскриптами определяли с помощью ПЦР анализа в реальном времени и обычной ПЦР. Для того чтобы повысить обогащение библиотеки специфичными для микоризы транскриптами, была проведена субтракция библиотеки. Трансформация мутантов дрожжей, имеющих дефекты в поглощении калия, фосфатов, аминокислот аммония и марганца, клонами из библиотеки кДНК свидетельствует о функциональности созданной библиотеки.

СТВОРЕННЯ ТА ЗАСТОСУВАННЯ СПЕЦИФІЧНОЇ ДО АРБУСКУЛЯРНОЇ МІКОРИЗИ БІБЛІОТЕКИ кДНК

С. Ісаєнков, Ф.Й.М. Маатхаус

Щоб оцінити потенційну користь мікоризи, нам потрібно було дослідити процеси, що мають місце у цих симбіотичних взаємодіях, в особливості це стосується арбускулярних компартментів, де відбувається процес обміну поживними речовинами між грибом та рослиною. При дослідженнях у цій області стикаються з великими труднощами через складності утворення симбіотичних органів (арбускул) та неможливості відокремити ці органи від інших типів клітин. Тому багато методів, що успішно застосовувались для досліджень рослинних та тваринних систем, не можуть бути використані у цьому випадку. Одним із ефективних підходів для вивчення процесів, що мають місце у арбускулах, є визначення композиції транскриптів та їх динаміки. Нами застосовано мікроскопію із лазерною мікродисекцією (LCM) для збору та виділення з клітин приблизно 3000 арбускул із *Glomus intraradices,* що колонізував корені *Medicago truncatula.* Загальна РНК виділена із зібраних арбускул, і її застосовували для лінійної ампліфікації мРНК за допомогою Т7 РНК полімерази. Ампліфікована РНК була використана для створення бібліотеки кДНК. Присутність та рівень збагачення на специфічні для мікоризи транскрипти визначали за допомогою ПЛР аналізу у ре-

альному часі та звичайної ПЛР. Щоб підвищити збагачення бібліотеки кДНК специфічними до мікоризи транскриптами, було проведено субтракцію бібліотеки. Трансформація дріжджових мутантів, дефектних за поглинання калію, фосфатів, амінокислот, амонію та марганцю, транскриптами із бібліотеки кДНК свідчить про функціональність створеної бібліотеки.

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