Plant Cell Fermentation of *Cephalotaxus*

Background

What is the potential of Plant Cell Fermentation?

Plants are very proficient in producing secondary compounds. The evolutionary background of these compounds (around 1 million of these compounds are produced by plants) is the interaction (and often manipulation) of other organisms. Therefore, many plant secondary compounds are medically active, which is the molecular base of traditional plant therapy. Most traditional medical systems have been using plants for healing, often as part of sophisticated medical systems. Traditional Chinese Medicine (TCM) and Ayurvedic Medicine represent probably the most elaborate of these systems. The potential of phytomedicine is in the great variation that allows for individualised treatments. The reason, why Phytotherapy has been neglected in Modern Medicine is the difficulty to control and standardise quality. This is partially caused by environmental fluctuations (soil, light, temperature), partially by genetic differences (chemotypes). Often, traditional medical plants are not really defined in terms of taxonomy. For instance, the traditional "Holy Basil" (Tulsi) of Ayurvedic Medicine seems to be a complex of several species (Jürges et al. 2018). Important medical plants have become very rare and their exploitation for medical purposes has shifted some species to the verge of extinction (which fuels prize increases and is a strong motivator for fake and deceipt, so called adulteration). The idea, to use plant cell cultures to produce these valuable compounds, has therefore been popular in the 1980ies. However, the failure to standardise such cultures and to get certain compounds in culture, has discouraged this approach and from the 1990ies, Plant Cell Fermentation has been abandoned by most groups. Only in a few cases, it has been economically successful.

What have we done so far?

The production of Paclitaxel (Taxol), an alkaloid, from cell cultures of Taxus at Phyton Biotech in Ahrensburg provides around half of the world market of around 3000 million € per year. Before, the compound had to be extracted from the bark of the Pacific Yew and the demand for Paclitaxel, which is crucial for the majority of anticancer chemotherapies, had almost caused the extermination of this species. This success story could be extended to other compounds, for instance, the *Vinca* alkaloids produced by *Catharanthus roseus* (Madagascar Periwinkle). The problem is the extreme complexity of this metabolic pathway, which is partitioned to more than five different cell types in the leaf. To biotechnologically mimick this situaton, is far from trivial. In a long-lasting cooperation with the group of Dr. Andreas Guber (Institute of Microstructure Technology, KIT-CN-IMT), a microfluidic chip was developed that allows to combine different cell types in a modular way (Maisch et al. 2016). In the frame of a current BMBF project, we explore the possibility to play "metabolic LEGO" and combine different *Catharanthus* cells to generate Vincristine, the most potent and most costly of the *Vinca* alkaloids. In fact, we have succeeded to get vindoline, the crucial precursor for the final step (Finkbeiner et al. 2021).

Cephalotaxines – potential and challenge

Cephalotaxus fortunei has been used by traditional healers in Fujian province to treat neoplastic diseases (Rui, 1995). The genus of these dioecioius trees is distantly related with *Taxus*, the source of the important anti-cancer drug Paclitaxel, and contains only a few species (currently 9 species are acknowledged by The Plant List) that are found in Southern and Eastern Asia (Lang et al., 2013). *C. hainanensis* occurs partially sympatrically with *C. fortune* (**Fig. 1**) but is limited to sporadic incidences in South China and a last viable population of around 2000 adult trees on Hainan island.

Fig. 1: Distribution of *C. hainanensis* in relation with *C. fortunei* and *C. nana* (from Lang et al., 2013)

Similarily to *C. fortunei*, it is victim of illegal exploitation due to its medical efficacy. Together with its slow growth, a high mortality of young seedlings in *ex-situ* conservation, and abuse as timber (this species can reach up to 20 m in height), this endemic species belongs to the most endangered species in China.

The reason for the anti-cancer efficacy of Cephalotaxus are alkaloids belonging to a specific group, called cephalotaxines, first isolated from *C. fortunei* (Paudler et al., 1963), and soon attracting recognition for their potential against tumours in general, and leukemia in particular. While cephalotaxine itself seems to lack bioactivity, its derivatives harbour anti-tumour and antileukemia potentials.

These include esterified forms, such as harringtonine, isoharringtonine, deoxyharringtonine, and, especially, the cephalotaxine-4-methyl-2-hydroxyl-4-methylpentyl butanedioate ester, called homoharringtonine (Powell et al., 1972). Homo-harringtonine has been approved by the US Food and Drug Administration (FDA) for the treatment of chronic myeloid leukemia in adults, and has been shown to excel therapies based on daunorubicin and cytarabine, so far considered as gold standard for the treatment of acute myeloid leukemia (Jin et al., 2013). The rich ramifications of cephalotaxine metabolism, along with the presence of several species exhibiting this pathway, makes it likely that there exist species specific members of the cephalotaxines. The high therapeutic potential of cephalotaxines in combination with the limited supply coming from endangered, and slowly growing trees, calls for alternative ways of production. In fact, semi-synthetic production of homo-harringtonine through esterification of cephalotaxine from dry leaves of *Cephalotaxus* has been achieved, thus reducing the need for the costly barks (Robin et al.,1999) reducing the requirement for biomass by a factor of 70, and at the same time leading to a purer product. This semisynthetic homo-harringtonine is meanwhile the brand omacetaxine mepesuccinate (ceflatonin, CGX-653, Myelostat) by ChemGenex Pharmaceuticals Ltd. (Menlo Park, CA, USA), in cooperation with StragenPharma (Geneva, Switzerland). Still, the growing demand for homo-harringtonine poses progressive constraints on the production of semisynthetic homo-harringtonine from needle biomass. This situation sets the stage for trying plant cell fermentation as alternative and sustainable approach to produce homo-harringtonines. In fact, recently production of these compounds in suspension cells of *C. manii*, has been reported, although this claim was not confirmed by LC-MS, such that the identity of the compounds has to be seen with care (Li et al., 2014). Cephalotaxine biosynthesis. Like for other alkaloids, the biosynthesis of cephalotaxines is rather complex and the final steps leading to the different active derivatives are not known, as well as the enzymes driving this pathway. By feeding radioactively labelled precursors, a part of the pathway could be elucidated (Parry et al. 1981). This led to a model for the early pathway (**Fig. 2**), where one tyrosine and one phenylalanine fusing to a phenethyl-tetrahydro1-phenethyltetrahydro-isoquinoline (**Fig. 2**, structure 8), which is further converted into an adienone (Fig. 2, structure 9), which is then undergoing a ring contraction to yield cephalotaxine.

Since the pathway initiates from two aromatic amino acids, stimulation of the shikimate pathway will promote the formation of a carbon backbone. Stimulation of this pathway by sodium fluoride was shown to enhance the accumulation of cephalotaxines in cultured cells of *C. manii* (Li, 2014). Likewise, methyl jasmonate, a general inducer of stress signalling, was promotive. Although this elicitation of secondary metabolism seemed to mitigate some limitations in the accumulation of cephalotaxines, the final steps of the pathway, especially those leading to the valuable esters, have remained enigmatic, mostly, because the underlying enzymes catalysing those steps and driving different ramifications leading to different types of cephalotaxines are completely unknown.

Specific information on the project

In order to enable molecular access to the biosynthetic pathway of Cephalotaxus ester alkaloids, the lab of Dr. Fei Qiao in Hainan has performed a high throughput RNA-seq analysis and assembled the **transcriptome** *de novo*. Raw reads comprising 4.3 Gbp were assembled *de novo* into almost 40 000 unigenes, with a mean length of around 1 kb, and a total assembly size of 45.8 Mbp (deposited in the GenBank under accession SRR1509462). The reconstructed unigenes were annotated with respect to their potential function to obtain a platform for subsequent analysis of gene functions (Qiao et al., 2014).

The backbone for the cephalotaxines is 3-4-hydroxyphenylpropanal (**Fig. 2**, structure 8). This phenolic structure probably derives from the aromatic amino acids, **phenylalanine** and **tyrosine**, leading to a metabolic model (**Fig. 3**) where **dopamine** and **4-hydroxydihydrocinnamoyl aldehyde (4-HDCA)** are fused in a so-called **Picket-Spengler condensation**. Keep in mind that this is a model – there are several possibilities as indicated by the parallel pathways.

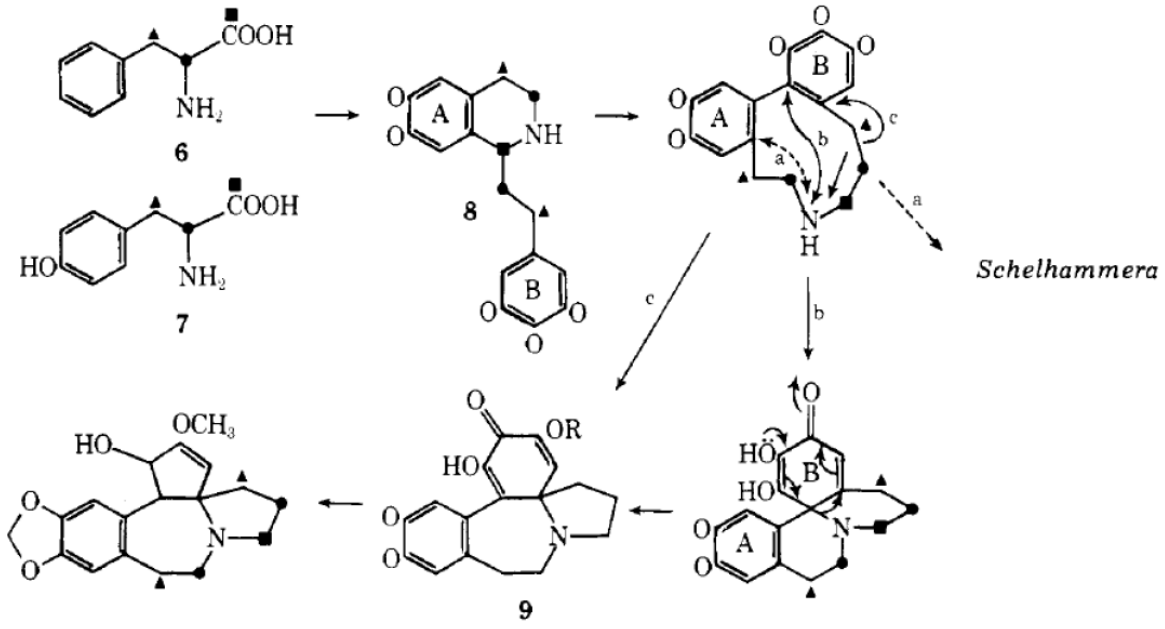

Fig 2. Schematic illustration of biosynthesis of cephalotaxine (lower left), from Parry et al. (1981)

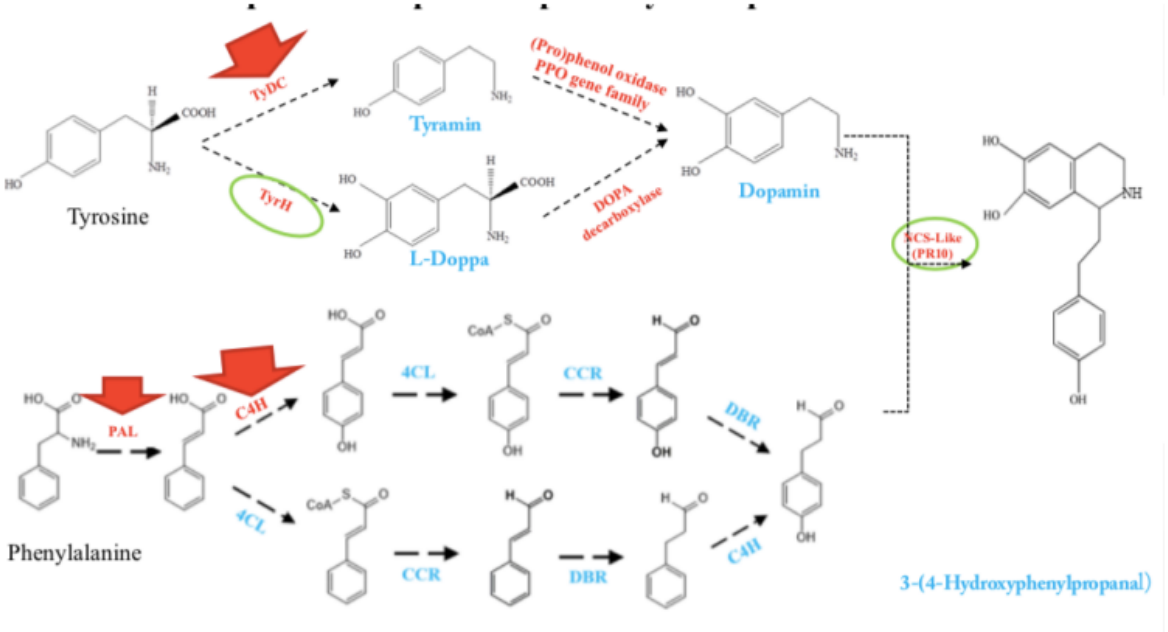

Fig 3. Metabolic model for the generation of 3-4-Hydroxyphenylpropanal, the backbone structure for the cephalotaxine pathway, from the precursors, dopamine (upper pathway) and 4-hydroxydihydrocinnamoyl aldehyde (4-HDCA, lower pathway) by a PR 10 like enzyme driving this so called Picket-Spengler condensation.

During two years of hard work, M. Sci. Nasim Reshadinejad succeeded to clone out most of the enzymes presumably involved in this pathway. Most of them are organised in **small gene families** and harbour small, but potentially relevant differences in the amino-acid sequence, which might mean that some can also drive different, unknown reactions. The full-length transcripts were then introduced into the GATEWAY vector syste that allows for modular shuffling of the insert into different target vectors depending on the desired application. In particular, she generated **GFP-fusions** that were introduced into **tobacco BY-2 cells** as cellular model in a stable manner. The complex state of the art is shown in **Table 1**.

Note: since there are several members of each gene family and we do not know whether they fulfill different functions, it is mandatory that you NEVER mix up the numbers!

The GFP fusions allowed to investigate subcellular localisation (**Table 2**). While most enzymes are found in the cytoplasm, the **cinnamate 4 hydroxylase (C4H)** sticks out, since three of its members are found at the ER, while one is cytoplasmic as well. The ER-binding forms fall into a set of two that are seen at the cortical ER network and one isoform that is seen along transvacuolar strands. Recombinant expression and in-vitro feeding showed that only the two cortical ER forms of this enzyme convert cinnamonic acid. This indicates that, depending on subcellular localisation, these enzymes drive different pathways. In other words: cellular architecture decides over biochemical function.

A similar functional divergence is manifest upon precursor feeding, for instance, two of the PAL expressing lines (PAL2898 and PAL2680) are producing coumaric acid and ferulic acid, indicative of silent metabolic potencies in the host cells (tobacco BY-2).

Gene name	Abbreviation	Full-length cDNA sequence obtained	Names	
Phenylalanine ammonia-lyase	PAL	4	✓ PAL-2344 (2235bp) ✓ PAL-2638 (2271bp) ✓ PAL-2680 (2172bp) ✓ PAL-2898 (2139bp)	
Cinnamate 4-hydroxylase	C4H	4	✓ C4H-656 (1506bp) ✓ C4H-11235 (1551bp) these are active in vitro ✓ C4H-12184 (1566bp) these are active in vitro ✓ C4H-82236 (1500bp)	
4-Coumarate CoA ligase	4CL	2	✓ 4CL-1971 (1629bp) ✓ 4CL-1802 (1671bp)	
Cinnamoyl CoA reductase	CCR	10	✓ CCR-1565 (975bp) ✓ CCR-1323 (966bp) ✓ CCR-1363 (981bp) CCR-1660 (987bp) ✓ CCR-1248 (903bp)	CCR-1615 (969bp) CCR-1703 (999bp) CCR-11512 (1044bp) CCR-111185 (966bp) CCR-11283 (975bp)
NADPH-dependent double bond reductases	DBR	4	✓ DBR-1262 (1047bp) ✓ DBR-1284 or DBR1466 (1032bp-1035bp) ✓ DBR-1450 (1032bp) ✓ DBR-1687 (1047bp)	
Tyrosine / Dopa decarboxylase	TyDC	3	TyDC-1780 (1506bp) TyDC-2534 (1233bp) TyDC-1665 (2073bp)	
Polyphenol Oxidase	PPO	2	✓ PPO-61972 (1674bp) PPO-02009 (1557bp)	
Norcoclaurine synthase-like	NCS-like (PR10-0843)	1	PR10-0843 (483bp)	

Table 1: molecular players potentially involved in the early cephalotaxine pathway and state of cloning and expression in tobacco BY-2 cells as heterologous system (PhD thesis Nasim Reshadinejad)

Sample n°	Signal	Sample n°	Signal
PAL2344	0	CCR1615	1
PAL2638	1	CCR1703	0
PAL2680	1	CCR11283	0
PAL2899	1	CCR111185	1
C4H11	1	DBR1262	1
C4H656	1	DBR1466	1
C4H12	1	DBR1687	1
C4H82	1	4CL1	0
CCR1248	0	4CL2	0
CCR1323	1	PR10_0843	1
CCR1363	1	PPO	0
CCR1565	1	WT	0

1: signal observed; 0: none signal observed

X: cytosolic localisation; X: endoplasmic reticulum localisation

Figure 12: Summary of the localisation of the different enzymes fused with the GFP in tobacco transgenic cell lines observed under the spinning-disk confocal microscope at 4 DO.

Table 2: overview about subcellular localisation patterns observed with the GFP fusions during a EUCOR Master Plant Science internship summer 2021 (Lucas Auroux). In addition, PPO has been seen to be localised in plastids forming stromules.

References

(note: references with a number can be downloaded from www.botanik.kit.edu/botzell/143.php)

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Scope of the study

Objectives of the project and approaches

- If there are silent potencies in the host cells, this might mean that the tobacco homologues of these enzymes are partially active. Can we detect their transcripts?
- Although the GFP fusions are driven by the constitutive CaMV 35S promoter, the fluorescence seems to be variable. Since this cannot be transcriptional regulation, there might be post-transcriptional regulation at work. To interpret the phenotypes observed upon feeding requires knowledge about transcript levels.
- The long-term goal is to combine different cell types to obtain different metabolites ("metabolic LEGO"). This requires that the different elements are in a controlled balance. Again, this is only possible by insight into transcript levels

Note: the different team members will deal with different genes and cell lines. Nevertheless, the workflow is the same. The interpretation of results is, however, specific for the respective gene. Exploit that you are a team, by connecting the data from the individual genes, you can draw a bigger picture.

WP1: Purification of mRNA from the different transgenic lines

High-quality mRNA is the prerequisite for all these objectives. While there are classical (and cheap) recipes for purifying RNA from plant cells, these involve irritating or even toxic solvents such as phenol. Therefore, most labs use commercial kits. Again, not every kit is suitable for every system. Tobacco BY-2 cells are not problematic, however, because they do not accumulate a lot of secondary compounds under normal conditions. Note: these kits are quite costly, so, if you waste, it will become expensive for the lab hosting you.

For the Standard Operation Protocols used in our lab, please refer to:

https://www.botanik.kit.edu/botzell/intranet/1637.php

(access through user: thesus and password: Ariadne)

WP2: Quality control of the extracted RNA and reverse transcription

The mRNA has to be tested for purity (and integrity) and then transcribed reversely. Again, this is done by a kit. A high-quality cDNA can later be used to test many transcripts. So, you have to be sure that your tool is of good quality. In addition to the usual spectrophotometry for purity and yield, you should also run an agarose gel to see, whether you can see the rRNAs. In addition, you should amplify the housekeeping gene by PCR and run the amplicons on a gel to see, whether your cDNA allows this. If not, there is no point in wasting time and money to amplify the other transcripts.

WP3: real-time qPCR and quantification of transcripts

To quantify steady-state transcript levels (which is not the same as transcriptional activity – think about this difference), we use real-time qPCR based on fluorescent labelling. Depending on the initial level of a given transcript, the fluorescence will cross a certain threshold sooner or later. This so-called C_t value is the lower, the more transcript you have in the beginning (because you need then less PCR cycles to reach the threshold) This allows to quantify them. For standardisation a stable so-called reference gene is included as internal standard. Since the number of copies increases exponentially, one needs to take the $\log_2$ to compare transcript levels. A classic paper, you should have read, when you do real time qPCR is

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402-408.

If you are not familiar with real-time qPCR, refer to the course Biological Methods (Bachelor Biology, semester 4)

Note: if you want to measure the induction of transcripts (for instance in response to a signal), one is using the initial value as reference and calculates, by how many times your transcript increases. This is done by the $-\Delta\Delta C_t$ method. In the current case, this does not make sense – you want to compare the different levels of PAL in the different lines, for instance. So, you take here just the level relative to the internal standard (the housekeeping gene), which is done by $-\Delta C_t$. The same approach allows you to compare the transcript levels of different genes (for instance when you want to make sure that the activities of PAL and C4H in a combination experiment are equal.

Organisation of the course

supervision: M. Sci. Nasim Reshadinejad (nasim.reshadinejad@kit.edu), participation in our usual Cell Biology Group meetings, Thu 10-12, seminar room 506-507 is expected (refer to the plan at the blackboard opposite of the escalator).

The course starts with the lecture block. In parallel, experiments can already be launched.

Protocols

1. Protocol RNA extraction (Roboklon Kit)
2. Protocol quality check, reverse transcription and PCR

1.Protocol for RNA Extraction using the ROBOKLON-kit

Suspension cells RNA extraction with Roboklon Universal RNA Kit

1) Sample preparation:

1. collect cells from 1ml suspension with Büchner funnel and vacuum pump, freeze immediately in liquid nitrogen
2. Grind cells to powder in 2ml safe-lock tubes containing steel beads with TissueLyser for 2-3x 30s at 19/s

2) Homogenization:

3. Prepare RL buffer with β-mercaptoethanol: 10µl β-ME + 1ml RL
4. Add 400µl RL + β-ME buffer to cells, vortex and incubate 5min at 24°C while shaking
5. Centrifuge for 1min at 10.000 rpm to pellet cell debris
6. Transfer complete supernatant onto homogenization spin column (purple)
7. Centrifuge for 2min at 12.000 rpm

3) RNA binding:

8. Add 400µl 70% ethanol to the flow-through and mix by pipetting. No vortexing!
9. Transfer complete sample including any precipitates onto RNA binding column (yellow)
10. Centrifuge for 1min at 12.000 rpm, discard flow-through and place back spin-column

4) RNA washing:

11. Add 400µl DN1 wash buffer to spin column
12. Centrifuge for 1min at 12.000 rpm, discard flow-through and place back spin-column
13. Prepare per sample: 50µl DNR buffer + 10µl DNase I (Quiagen)
14. Add 60µl DNR + DNase I buffer onto membrane and incubate for approx. 15min at 24°C
15. Add 400µl RB1 wash buffer onto spin-column
16. Centrifuge for 1min at 12.000 rpm, discard flow-through and place back spin-column
17. Add 650µl RBW wash buffer onto spin-column
18. Centrifuge for 1min at 12.000 rpm, discard flow-through and place back spin-column
19. Add 350µl RBW wash buffer onto spin-column
20. Centrifuge for 1min at 12.000 rpm, discard flow-through and place spin-column into new 2ml collection tube. Do not cab!
21. Centrifuge for 1min at 12.000 rpm to dry spin-column, discard flow-through place spin-column into new nuclease-free 1.5ml collection tube.

5) RNA elution:

22. Add 40-60µl of RNase-free water directly onto membrane.
23. Incubate for 1-2min at 24°C for successful elution!
24. Centrifuge for 1min at 12.000 rpm
25. Analyse quality and quantity by Nanodrop and RNA gel
26. Store RNA at -20°C or 4°C (short term), avoid multiple freezing/thawing

2.Protocol for RNA quality check and RT

- The RNA is DNase treated, to remove remaining genomic DNA (RNAse-free DNAse (Qiagen, Hilden, Germany) for 15 min at 37 °C following the instructions of the producer
- High quality and integrity of RNA was verified via spectrophotometry by NanoDrop (peqlab Biotechnologie GmbH, ND-1000 Spectrophotometer, Erlangen, Germany) and gel electrophoresis.
- Reverse transcription: For cDNA synthesis 2 µg of RNA are added to 1.4 µl of mastermix I (Table below). Total volume of 16 µl is adjusted with nuclease free H_2O (make sure that the water remains pure, you will get your own aliquot, do not use the aliquots of other people!). This approach is kept in a thermocycler for 5 min at 70 °C. Subsequently, 4 µl of mastermix II (Table 2.8) are added and samples returned to the cycler for 60 min at 42 °C followed by 10 min at 90 °C.

mastermix I		
dNTPs (10 mM)	1 µl	New England Biolabs GmbH, Ipswich, USA
oligodT (100 µM)	0.4 µl	Thermo Fischer Scientific, Waltham, USA
Cycler step I		
70 °C	5 min	
mastermix II		
MuIV RT buffer	2 µl	New England Biolabs GmbH, Ipswich, USA
RNase inhibitor	0.5 µl	New England Biolabs GmbH, Ipswich, USA
MuIV RT	0.25 µl	New England Biolabs GmbH, Ipswich, USA
nuclease free water	1.25 µl	Qiagen, Hilden, Germany
cycler step II		
42 °C	60 min	
90 °C	10 min	
12 °C	∞	

- Reverse transcriptase (RT)-PCR is performed in a T-GRADIENT Thermo Cycler, using the cDNA as a template and gene-specific primers (see below) for analysis.

volume [µl]	component
2	Taq Reaction Buffer 10x
1	dNTPs (10 µM)
1	forward primer (10 µM)
1	reverse primer (10 µM)
1.5	template DNA
up to 20 µl	nuclease free H_2O
0.5	Taq DNA polymerase
20	final volume

PCR-conditions: After denaturation at 95 °C for 30 sec, primer annealing was conducted between 50°C-55°C (see below) for 30 sec, and extension at 68 °C for 30

sec. The 30 cycles were preceded by a denaturation step of 95°C for 5 min and followed by a final extension of 68°C for 5 min.

- Amplicons are analysed by gel-electrophoresis using 1,2% agarose gels and staining with SYBR-Safe. Running time is 30 min at 100 V.
- A 100-bp DNA ladder is used to determine the size of the amplicons.

Important: make sure that you get the information on the primers and the PCR conditions before you do the experiment