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Application of current *in situ* hybridization techniques for grape phylloxera (*Daktulosphaira vitifoliae,* Fitch) and grapevine (*Vitis* spp. L.)

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Summary

***In situ* hybridization and *in situ* PCR directly localize specific DNA and RNA sequences in tissues. To exactly focus on the processes occurring on cell- or tissue level, *in situ* techniques can be efficiently employed. Recent advances in viticultural research in the fields of genomics, proteomics and metabolomics are likely to employ these techniques to link DNA- or mRNA sequence information to physiological traits and processes occurring in the grapevine. In this paper, we present a range of possibilities for *in situ* techniques that can be applied in grapevine research. Two examples covering *in situ* PCR of grapevine roots and *in situ* hybridization of grape phylloxera will be given for illustration. Moreover, key steps of the techniques are discussed, which may be helpful to researchers aiming to employ *in situ* hybridization or *in situ* PCR.**

K e y w o r d s : *in situ* hybridization, *in situ* PCR, grapevine, grape phylloxera.

Introduction

With the help of *in situ* hybridization techniques and nucleic acid probes target DNA sequences can be localized in their native tissue or cell environment. The combination of histological methods with *in situ* PCR techniques allows for detecting and quantification of specific DNA or RNA sequences in a sample. The development of non-radioactive methods for nucleic acid labelling simplifies the application of this technique which is employed for a range of biological and ecological scientific questions: e.g. description of bacterial species in biofilms (Okabe *et al.* 1999), the composition of ecosystems (Zheng *et al.* 1996), the localization of viral material in plant tissues (Singh and Nie 2002) or the localization of secondary symbionts in insects (Harada *et al.* 1996). In viticultural sciences, first approaches were made by Haas *et al.* (1994) and Haas and Alleweldt (2000) employing *in situ* hybridization techniques for karyotype studies of *Vitis vinifera* (L.) and by Sohier *et al.* (1998) detecting bacteria in fermentation processes of wine.

This article presents applications of *in situ* techniques for current grapevine research. *In situ* hybridization was successfully applied to localize associated bacteria within tissue sections of grape phylloxera (Vorwerk *et al.* 2007). Furthermore, activity tests of specific genes involved in host-parasite interactions in root tips of grapevine were modelled using new *in situ* RT-PCR techniques. These two examples together with a critical discussion of key steps of the techniques may be helpful to researchers aiming to employ these techniques.

Material and Methods

E x a m p l e 1 : L o c a l i z a t i o n o f s t a r c h s y n t h e s i s i n v o l v e d g e n e s i n p h y l l o x e r a - i n f e c t e d r o o t s : 'Teleki 5C' (*V. berlandieri* x *V. riparia*) rootstocks were propagated from two-node dormant cuttings and infected with eggs of a parthenogenetic grape phylloxera population collected at Bingen, Germany (Forneck *et al.* 2000). Nodosities of three different stages were collected (Figure) and stored at -20 °C. Root-tips of non-infected rootstocks 'Teleki 5C' were employed for control reactions. Fixation, embedding and sectioning was performed according to Lillie *et al.* 1965 applying the following modifications: Serial sections were adjusted to 8 µm. Pepsin (2 $mg \cdot ml^{-1}$ in 0,01 M HCl for 60 min at 37 °C) was employed instead of proteinase K in order to make cell walls permeable for the penetration of labelled probes. Since this experiment was based on RNA, no RNAse was employed for digestion, but DNA was digested using 20 U DNAse in 40 µl of PCR buffer per sample. Special care was taken to work under RNAse free conditions, reagents were prepared using DEPC-treated water (Applichem, Heidelberg, Germany), slides and experimental material were autoclaved and benches were treated with RNAse-OFF (Applichem, Heidelberg, Germany). cDNA was synthesized using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (MBI Fermentas, St. LeonRot, Germany) with an oligo dT_{13} primer (0,5 µg/µl) (Tab. 1). The RT *in situ* PCR was conducted using 25 mM $MgCl_2$, 2 mM dNTPs, 20 $pM \cdot \mu l^{-1}$ of each primer, 10x PCR buffer, 5 $U \cdot \mu l^{-1}$ Taq-Polymerase (Invitrogen, Germany) and 20 % Roti-stab (Roth, Karlsruhe, Germany) in a total volume of 50 µl. Special frames for *in situ* PCR (Eppendorf, Hamburg, Germany) were used to keep the PCR mix sealed on the sample during the PCR reaction. Ten cycles of "touch-down PCR" (annealing temperature 50-45 °C) were applied, followed by 20 cycles of standard PCR (annealing temperature 45 °C). Cycles for *in situ* PCR were extended to 60 s each. Samples were very gently dipped

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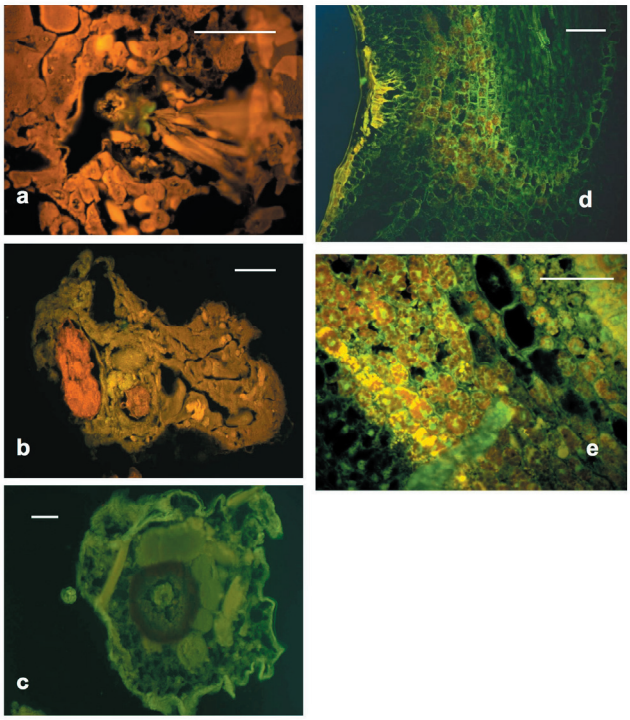

Figure: 16S rDNA of grape phylloxera associated bacteria detected with a fluorescein-labelled oligonucleotide probe in the salivary pump of an adult parthenogenetic grape phylloxera (transversal cross-section) (a). 16S rDNA of grape phylloxera associated bacteria detected with a rhodamin-labelled oligonucleotide probe in the eggs inside an adult parthenogenetic grape phylloxera (longitudinal section) (b). 16S rDNA of *Buchnera aphidicola* detected with a rhodamin-labelled oligonucleotide probe in specific cells near the gut lumen (mycetocytes) of *Aphids fabae* (cross-section) (c). *In situ* RT PCR in nodosities of phylloxera infected root material of Teleki 5C, employing primers for invertase and pyrophosphorylase. Gene activity was monitored around the feeding site (d, e). Bars represent 100 µm.

into 2x SSC buffer in order to eliminate unbound primers prior to microscopic examination.

Example 2: Localizing insect-associated bacteria inside grape phylloxera: Four leaf galling grape phylloxera populations were collected from different locations in Europe (Bingen, Gundelsheim (GER), Rouffach (F), Turino (I), including samples of *Aphis fabae* (Scopoli) and *Myzus persicae* (Sulzer) bearing endosymbiotic *Buchnera* as positive controls. For each sample, 50 adult aphids were prepared from leaf galls and washed shortly in 70 % ethanol. Insect samples were fixed in Johansen solution and formalin-propionic acetate and further cleared through an ethanol-xylene series before being embedded in paraffin at 65 °C. Serial sections were adjusted to 6-7 µm for pre-cooled insect tissue samples. Sections were deparaffinized through a xylene-ethanol series (Lillie *et al.* 1965) prior to each hybridization.

For bacteria a specific 54 bp oligonucleotide probe, developed from *P. agglomerans* sequenced fragments (Vorwerk *et al.* 2007), 5'end labeled with digoxygenin, was applied. Another 54 bp 16S rDNA fragment hybridizing to *Buchnera* but not to *Pantoea* was chosen for application to the control samples (Tab. 1). After deparaffinization, samples were treated with RNAse A (100 µg·ml^{-1} in 2x SSC (saline-sodium citrate buffer)) for 30 min at 37 °C and then gently washed in 2x SSC to eliminate RNA templates and prevent non-specific bindings. Samples were further treated with proteinase K (2.5 µg·ml^{-1} in 2x SSC) for ten minutes in order to make cell walls permeable for the labelled probe and then fixed in proteinase K stop buffer and 4 % formaldehyde. The hybridization mix consisted of 50 % formamide, 10 % dextransulphate, 1 ng·ml^{-1} labelled DNA probe, 250 ng·ml^{-1} herring sperm DNA, 1,25 % SDS (sodium dodecyl sulphate buffer) in 2x SSC. 40 µl were applied and samples were covered with coverslips and placed in humid chambers. The hybridization reaction comprised a denaturation step of ten minutes at 95 °C and the hybridization step itself overnight (ten to twelve hours) at 37 °C. After hybridization, tissue sections were washed twice in washing buffer (50 % formamide in 2x SSC) at 42 °C for three minutes before being incubated in detection buffer containing 2 µg·ml^{-1} anti-digoxygenin-antibodies conjugated to either fluorescein or rhodamin (both dyes were tested). Finally, slides were treated with 40 µl DABCO antifading solution and covered with new coverslips for microscopic examination. Samples were examined directly after hybridization using epifluorescence microscopy with an Axioplan microscope equipped with an UV-light source and UV-filters (exitation 450-490 nm, FT 510, LP 520).

Table 1

Probes and sequences employed for *in situ* techniques in examples 1 and 2

Study	Sequence name	Sequence 5´-> 3´
Endosymbiotic bacteria of grape phylloxera	54 bp oligonucleotide probe for Pantoea agglomerans (PAG)	CGC ATA CAA AGA GAA GCG ACC TCG CGA GAG CAA GCG GAC CTC ACA AAG TGC GTC
	54 bp oligonucleotide probe for Buchnera aphidicola (BAP)	TTT ATA CAA AGA GAA GCA AAT CTG CAA AGA CAA GCA AAC CTC ATA AAG TAA ATC
Starch synthesis in phylloxera infected roots	Invertase 1, forward	GCC CAG TGT ATC ACA AGA TT
	Invertase 1, reverse	GGA GAT GAA GCC ACT CTA TG
	Pyrophosphorylase, forward	TGA AAG CTA TGA AGG TCG AT
	Pyrophosphorylase 3, reverse	CGG TCA TAG AAG CTG AAA TC

Results were documented using digital image processing (Axiocam, Carl Zeiss, Germany) and AxioVision 3.1.

Results and Discussion

In situ hybridization techniques were successfully applied in two different fields of grapevine research. The two examples presented in this paper clearly show the benefits of the applied methods. For localization of starch synthesis involved genes in phylloxera-infected roots, *in situ* PCR has been applied. Due to permanent stimulation, the feeding sites accumulate starch globules (Forneck *et al.* 2002). In this experiment, the activity of genes involved into starch synthesis in phylloxera-infected roots was examined using RT *in situ* PCR with three specific primer pairs amplifying 350-400 bp of the invertase and ADP-glycose-pyrophosphorylase gene (Tab. 1). The use of cDNA in this case as template for hybridization is advantageous since the activity of a specific gene can be monitored by evaluating the strength of the hybridization signal. Results of the first strand synthesis revealed high cell activity around the penetration site. Second strand synthesis, employing two specific primer pairs in RT *in situ* PCR on the samples, revealed clear and strong signals of gene activity radially spread around the feeding site, but not at the opposite side of the pericycle. Both primer sets revealed stronger hybridization signals in root tips of early infection stages than in the older ones. This may be due to the fact, that older infected root tips might already have declined cell activity and that the PCR reaction might have been more difficult to perform in partly lignified cells. Non-infected root tips, employed as a control reaction did not show any hybridization signal with none of the primer sets, but autofluorescence was clearly visible on the exodermis.

In the second example insect-associated bacteria of the genus *Pantoea* inside grape phylloxera were described and identified (Vorwerk *et al.* 2007). In order to elucidate their function and transmission specific 16S rDNA probes were hybridized on ultra-thin sections of adult leaf gall grape phylloxera. Signals were detected inside the salivary pump of the insect and also within eggs inside the insect body (Figure). Positive control samples revealed signals within the gut of *A. fabae* and *M. persicae*, pointing to the presence of *Buchnera* bacteria. Using the same probe concentration, control samples presented notably stronger signals than grape phylloxera samples. Negative controls, employing a hybridization mix without 16S rDNA probes did not reveal any signal.

The connection of molecular techniques with direct localization and visualization in the tissue sample make *in situ* techniques advantageous compared to other methods of investigation. Direct *in situ* hybridization is straightforward, but requires a sufficient copy number of target sequences, whereas its combination with PCR techniques allows detection of low copy numbers of the target sequence which can be quantified if RT PCR techniques are employed.

Every *in situ* experiment consists of the histological preparation, the choice of adequate probes and the hybridization technique itself. Histological preparation (accurate embedding, high-quality sectioning) significantly affect the results. For sample fixation, FAA (formaline and acetic acid), FPA (formalin-propionic acetate) or Karnovski solution (Lillie *et al.* 1965) are commonly used. In the experiments presented, FAA and FPA fixation worked equally, only Karnovski solution was found to show insufficient results, possibly due to the very strong binding structures produced. When working with lignified plant tissues (Lillie *et al.* 1965) or also insect tissues (containing trachea and chitinized structures) (Fukatsu *et al.* 1998), careful elimination of air vesicles from the samples must be achieved to rescue intact sample structures for exact signal interpretation. Paraffin embedded samples are recommended *in situ* hybridization, since embedding procedures are simple, fast and non-toxic. Thin sections on silane-coated slides (2 % aminopropylethoxysilane in acetone) allow multiple buffer treatments during experimental steps. Plastic embedding produces ultra thin slides, however tissue structures can be affected (Osamura *et al.* 2000) and become useless for nucleic acid *in situ* experiments according to our expertise.

The length of specific probes for sequence detection may range from 20 to 1000 base pairs. The longer, the more specific, however very long sequences are difficult to penetrate through cell walls (Nuovo 1996). Therefore, efficient probes are as short as possible without loosing specifity, which can be analysed by using online alignment services in common nucleotide databases. 5' labeling is recommended since it rarely interferes with the binding of probe and target sequence. Probes may be labeled with biotin or digoxygenin, which serve as reporter molecules and can be detected via antibodies. The antibodies are conjugated to fluorescent molecules like fluorescein or rhodamin. Antibodies can also be conjugated to alkaline phosphatase that catalyses a non-fluorescent reaction (NBT-BCIP). In previous experiments, no differences were found between directly labeled and digoxygenin-labeled probes (data not shown). High levels of autofluorescence may be present in tissue samples, especially in tissues of lignified root tips, due to the presence of lignin and cell walls high in phenolic content, but also in grape phylloxera adult bodies, which contain chitin and high amounts of fat. The labelling or detection mode should therefore be chosen to contrast well with the background of the sample. Rhodamin was observed to contrast well in both root and grape phylloxera tissue samples.

For optimum detection of hybridization signals adequate pre-treatments are essential. A nuclease treatment to eliminate either RNA or DNA is useful to reduce background signals resulting from unspecific bindings. To facilitate permeabilization proteases (e.g. pepsin or proteinaseK) should be employed. Here, concentrations and application times need to be adapted. Stringent experimental conditions (e.g. hybridization temperature, addition of formamide, salt concentrations and time factors (Baldino *et al.* 1989)) are necessary to prevent false hybridization signals. Additionally, Denhard´s solution and sonicated herring sperm DNA were employed for the detection of 16S rDNA (see Example 1) as competitive ingredients and probes were let to hybridize for at least 12 h over night. Finally, appropriate control experiments are crucial for the

interpretation of the results (e.g. Tab. 2). *In situ* techniques should be applied, when direct localization of specific sequences is essential. As already applied for the detection of lactic acid and other bacterial species in vine (Sohier *et al.* 1998, Stender *et al.* 2001), the use of differently labeled probes allows identification of multiple DNA products simultaneously in a tissue and even monitor their development over time (Amann *et al.* 1995, Amann *et al.* 1996). This could be a useful application when thinking of monitoring growth stages or also different stages of infection in pest and disease diagnostics. Also for karyotyping, *in situ* techniques represent a solid basis, especially for grapevine, possessing very small chromosomes, as shown by Haas and Alleweldt (2000).

Table 2

Choice of control experiments for *in situ* hybridization and *in situ* PCR

Control experiment	Result
No-probe control	Use of ddH_2O instead of oligonucleotide probe, no signal should be detected
RNAse/DNAse digestion	After digestion of the target sequences (RNA/DNA) no hybridization should be possible
Housekeeping control probe	Using a universally active gene sequence as a target
Housekeeping control „aphids and endosymbionts“	Using aphid tissue that is proved to contain endosymbiotic DNA or alternatively using a universal bacterial probe which hybridises with most bacterial 16S ribosomal sequences (e.g. EUB 338, Amann 1990)

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