Biotrophic interactions disentangled: In-situ localisation of mRNAs to decipher plant and algal pathogen – host interactions at single cell level.

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Abstract

Plant-pathogen interactions follow spatial and temporal developmental dynamics where gene expression in pathogen and host undergo crucial changes. Therefore, it is of great interest to detect, quantify and localise where and when key genes are active to understand these processes. Many pathosystems are not accessible for genetic amendments or other spatially-resolved gene expression monitoring methods. Here, we adapt single molecule FISH techniques to demonstrate the presence and activity of mRNAs at the single-cell level using phytomyxids in their plant and algal host in lab and field grown material. This allowed us to monitor and quantify the expression of genes from the clubroot pathogen *Plasmodiophora brassicae*, several species of its *Brassica* hosts, and of several brown algae, including the genome model *Ectocarpus siliculosus*, infected with the phytomyxid *Maullinia ectocarpii*. We show that mRNAs are localised along a spatiotemporal gradient, thus providing a proof-of-concept of the usefulness of single-molecule FISH to increase knowledge about the interactions between plants, algae and phytomyxids. The methods used are easily applicable to any interaction between microbes and their algal or plant host, and have therefore the potential to rapidly increase our understanding of key, spatially- and temporally-resolved processes underpinning complex plant-microbe interactions.

Introduction

Thanks to a series of technological advances over the last years, it has become clear that many biological processes within and between organisms are best studied at the single cell level (e.g. Libault *et al.*, 2017; Moor & Itzkovitz, 2017). Single cell approaches provide a revolutionary toolset to study interactions, especially the complex intermingled crosstalk between biotrophic pathogens and their algal or plant hosts. Biotrophs keep their host's cells alive during their development and growth and, therefore, have evolved a multitude of strategies to escape detection but yet to obtain nutrients or water from their host (Kemen & Jones, 2012; Zeilinger *et al.*, 2016). In such interactions, timing is crucial: the pathogen first needs to escape the host defence while establishing itself, but in a subsequent step the pathogen and host communicate about nutrients to be exchanged. Hence, gene expression changes happen quickly at the cellular level, and can differ between neighbouring cells (Buxbaum *et al.*, 2015). Unless they are combined with expensive or time-consuming techniques such as laser-assisted microdissection, widely used methods such as qPCR or RNAseq are not able to account for spatial and temporal heterogeneity between the cells, and therefore are impracticable to study single-cell changes at the scale of organs or macro-organisms. On the other hand, transformation or genetic manipulation remains inaccessible for a wide range of the pathogen or hosts that cannot be grown in the lab and/or for which only very limited genetic information is available (Libault *et al.*, 2017). Furthermore, current work on plant and algal microbiomes makes increasingly clear that functional studies require to take into account complex microbial communities which include a huge diversity of „non-model" organisms, many of which are not accessible to culturing or genome amendment techniques (Egan *et al.*, 2013; Vandenkoornhuyse *et al.*, 2015; Müller *et al.*, 2016). Thus, descriptive approaches such as FISH (Fluorescence In Situ Hybridisation) have a renewed potential to start exploring these communities functionally.

Indeed, few of the single-cell protocols and –omics approaches available to study transcriptomic dynamics of single cells have the potential to be used routinely outside of model organisms. One notable exception is the *in situ* localisation of individual mRNAs via FISH (Wang & Bodovitz, 2010; Misra *et al.*, 2014; Libault *et al.*, 2017). FISH techniques have been utilised to study gene expression patterns and the distribution of mRNA genes in human cell lines (Weibrecht, I *et al.*, 2013), different animal models (e.g.Trcek *et al.*, 2017), fungi and yeasts (e.g.Niessing *et al.*, 2018) and plants (Bruno *et al.*, 2011; Duncan *et al.*, 2016b; Francoz *et al.*, 2016). Recent improvements involving fluorophores, microscopic detection and resolution now allow to localise individual mRNAs of interest (Buxbaum *et al.*, 2015). Patterns of mRNA expression and the subcellular organisation of mRNAs can thus be accessed. Ultimately this leads to a better understanding of the regulatory processes behind the translation of genetic information (Chen *et al.*, 2015), without requiring genetic manipulation of the organism of interest, nor the availability of extensive genetic and molecular data. Additionally, this can be applied to field-collected samples, which opens new research lines for uncultivable organisms once an mRNA sequence of interest is available.

Phytomyxea (Rhizaria, Endomyxa) are a group of economically important plant pathogens, including e.g. *Plasmodiophora brassicae,* the clubroot pathogen, causing a loss of roughly 10 % of the world brassica crop production (Schwelm *et al.*, 2018). Additionally, ten phytomixid species parasitise important marine primary producers, namely brown algae, seagrasses and diatoms, with essentially unknown

ecological consequences (Neuhauser *et al.*, 2011; Murúa *et al.*, 2017). In brown algae, the galls formed by *Maullinia braseltonii* on the Pacific bull kelp (*Durvillea antartica*) affect the commercial value of this locally important food source. Its closely related parasite, *Maullinia ectocarpii*, infects a broad range of filamentous algae spanning at least four orders, including the genome model *Ectocarpus siliculosus* and gametophytes of the giant kelp *Macrocystis pyrifera* (Maier et al., 2000). Its availability in laboratory culture makes it a good model to start deciphering the interaction between phytomixids and their marine hosts.

Despite their importance as plant and algal pathogens, Phytomyxea have been difficult to study, mostly because they cannot be cultured without their host and because they have a complex multi-stage life cycle (Schwelm *et al.*, 2018). Genome and transcriptome data became available only recently for *P. brassicae* (Schwelm *et al.*, 2015; Rolfe *et al.*, 2016). Yet the unavailability of genetic manipulation of the parasite forces all functional studies to be conducted on transformed plant hosts (mainly *A. thaliana*) either focussing on the host side of the response only (e.g. Irani *et al.*, 2018) or by overexpressing *P. brass*icae genes into the host (Bulman *et al.*, 2018) or plant pathogenic fungi (Singh *et al.*, 2018). Likewise, genome resources are available for brown algae (Ye *et al.*, 2015; Cormier *et al.*, 2016), but this group remains inaccessible to transformation, genome editing or RNAi, to the exception of *Fucus* zygotes (Farnham *et al.*, 2013). Apart from taxonomic markers, no molecular information is currently available for *Maullinia ectocarpii* or any other marine phytomixid. In this context, the prospect of monitoring gene expression of phytomyxean parasites in their host, linking the expression of selected genes to specific stages of the life cycle and to specific time points in the development of the pathogen, is to get a better understanding of the interaction.

Here, we developed tools to monitor gene expression of intracellular pathogens and to monitor the response of their hosts (plants and brown algae) upon infection. For this purpose, two different approaches of single molecule mRNA localisation were evaluated: smFISH (single molecule FISH), which is based on a series of fluorescently labelled probes that flank the mRNA of interest (Duncan *et al.*, 2016b) and RCA-FISH (Rolling Circle Amplification-FISH), which is based on in-situ transcription of RNA followed by in-situ-RCA signal amplification (Weibrecht, Irene *et al.*, 2013). The following *P. brassicae* genes were selected (i) the housekeeping gene actin1 (GenBank: AY452179.1) (Archibald & Keeling, 2004) (ii) a SABATH-type methyltransferase from *P. brassicae* upregulated during the later stages of pathogen development (PbBSMT, GenBank: JN106050.1, (Ludwig-Muller et al., 2015), which is able to methylate salicylic acid (SA), benzoic and anthranilic acids. To monitor mRNA expression and localisation in the host, the following genes were tested: (i) the *Brassica rapa* maltose excess protein 1 (MEX1, GenBank: XM_009109278.2) that encodes a maltose transporter, and (ii) the vanadium-dependent bromoperoxidase (vBPO) of *Ectocarpus siliculosus* Ec32m (Genbank: CBN73942.1), a stress-inducible enzyme assumed to halogenate defensive secondary metabolites (Leblanc *et al.*, 2015; Strittmatter *et al.*, 2016).

MATERIAL AND METHODS

Preparation and storage of the biological material

Clubroot-infected Brassica plants

Field sampling

Infected plants of white cabbage (*Brassica oleracea* var. *capitata* f. *alba*), broccoli (*Brassica oleracea* var. *italica*), Chinese cabbage (*Brassica rapa ssp. Pekinensis*) and kohlrabi (*Brassica olearacea* var. *gongylodes*) with well-developed clubroots were collected from a commercial field (Ranggen, Austria, 47°15'24"N, 11°13'01"E). Clubroots were rinsed with tap water and treated as described below.

Inoculation and cultivation of plants

Chinese cabbage (*Brassica rapa* cv. *'Granaat',* European Clubroot Differential Set ECD-05) seeds were germinated on wet tissue paper for three days and then planted in a potting soil mixture (pH ~ 5.7, mixing standard compost, rhododendron soil and sand (4:2:2) to reach this pH). Plants were grown with a photoperiod of 12 h. After 12 days plants were inoculated with 1 mL watery suspension of *P. brassicae* spores (7 x 10^6 spores mL^{-1}), extracted from homogenised galls. Root galls were harvested after six to seven weeks.

Sample Fixation and preparation

Clubroots were cut into smaller pieces (ca. 3 x 4 mm) to allow for a more homogenous fixation. Samples were transferred into Histofix 4% (phosphate-buffered formaldehyde solution, Carl Roth) where they remained for 1 - 12 h depending on sample size. Samples were used directly, or were washed in an ascending ethanol Series for long term storage (50 %, 80 %, 2x 96 %; all dilutions made with DEPC [diethyl pyrocarbonate]-treated water) and stored at -20 °C until use.

Clubroots (or clubroot pieces) were cut with an RNAse free razor blade by hand and washed with 1x DEPC PBS buffer (phosphate-buffered saline, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, DEPC treated water). Making the cuts by hand posed a significantly lower risk of RNase contamination than using a cryotome (Reichert-Jung, Frigocut 2800, Suppl. Note S1).

Growth and maintenance of *M. ectocarpii* infected *Ectocarpus siliculosus Ec32m* and *Macrocystis pyrifera*

Ectocarpus siliculosus (fully sequenced genome strain Ec32m, CCAP 1310/4) was infected with *Maullinia ectocarpii* (CCAP 1538/1), using a clonal culture of *Macrocystis pyrifera* female gametophyte (CCAP 1323/1) as an intermediate host, as described by (Strittmatter *et al.*, 2016, Fig. 1). Cultures were maintained at 15 °C with 12 h photoperiod, 20 micromol photon m^{-2} s^{-1} in artificial seawater (ASW) with half strength modified Provasoli (West & McBride, 1999). Cultures were regularly checked microscopically and samples were harvested and fixed as described above, replacing DEPC-treated water by DEPC-treated ASW. Samples were stored at -20 °C until use.

Single molecule mRNA visualisation

To prevent RNase contamination, experiments were done in RNase-free environment with RNase-free reaction mixtures, for instance with DEPC-treated Milli-Q water. To avoid photobleaching, the samples were protected from light during and after the hybridisation of the fluorescently labelled oligonucleotides. All enzymes and reaction mixtures were kept on ice during use. Reaction tubes were incubated using a PCR cycler with heated lid to avoid evaporation or a thermal block. All incubation and amplification steps were performed in 0.2 mL PCR reaction tubes unless otherwise stated.

RCA-FISH (Rolling circle amplification – Fluorescence in Situ Hybridisation)
RCA-FISH is based on the *in-situ* reverse-transcription of mRNA, a subsequent signal amplification using a loop target and the RCA ability of Phi-Polymerase, followed by FISH detection of the amplified signal (Tab. 1) (Weibrecht, I *et al.*, 2013). Initially, a locked nucleic acid (LNA) primer hybridises to the mRNA target of interest. The LNA-primer – mRNA hybrid serves as template for the reverse transcription using RevertAid H reverse transcriptase, followed by RNAse H digestion of the RNA part of the RNA/DNA hybrid sequence, so that in all subsequent steps, cDNA serves as template. A so-called padlock probe, which binds with a small loop to the cDNA serves as circular DNA template for the rolling circle amplification of a sequence complementary to the FISH probe.

Primer design:

LNA-Primer: LNA primers were approximately 25 bp long and located close to the 3' end of the mRNA. Starting from the 5' end of the LNA primer, every second nucleotide was replaced by its LNA counterpart, in total 7 LNAs (Tab. 1).

Padlock Probe: About 15bp at each of the 3' and 5' ends of the padlock probe are complementary to the cDNA sequence. When these two regions bind to the cDNA, the central region of the probe (ca. 50 bp) forms a loop, similar to the shackle of a padlock. This central region contains a generic detection sequence (~ 23bp) flanked by random filler sequences (Tab. 1). This loop-like structure serves as target for the Phi-Polymerase mediated RCA which is amplifying the detection sequence and consequently the signal. The padlock probe has to be phosphorylated before use. Overall the structure of the padlock probe is: 5´ phosphate – ***15bp complementary to cDNA*** – 5-15bp random filler – detection sequence - 5-15bp random filler - ***15bp complementary to cDNA*** - 3'.

Detection Probe: This is a fluorescent, mono-labeled FISH probe complementary to the detection sequence included in the padlock probe.
To check the secondary structures in all three types of probes, UNAFold (https://eu.idtdna.com/UNAFold) was used. Specificity and potential off-target binding sites were checked by blasting the sequences (https://blast.ncbi.nlm.nih.gov/) against the nr database.

Table 1: RCA-FISH probes. PbBSMT (*GenBank: JN106050.1*) and actin1 *(GenBank: AY452179.1)* are genes of *P. brassicae.* LNA modified nucleotides are shown in orange, padlock probe target-specific parts are shown in italics and bold letters and the detection sequences are shown underlined.

Oligonucleotide	Sequence
PbBSMT (GenBank: JN106050.1)	
LNA primer PbBSMT	5´ C**C**C**G**T**T**C**A**C**C**T**G**G**C**ATGACTATTCG 3‘
Padlock probe PbBSMT	5´ phosphate--***ATAAAACTCGAATAG***TTCGTTTTATTAGGTCAATGTCTGCTGCTGTACTACTCTT***TTAGCGTT CGTTCC*** 3‘
Detection probe PbBSMT	5’ Cy3—GGTCAATGTCTGCTGCTGTACTAC 3‘
actin1 (GenBank: AY452179.1)	
LNA primer actin	5’ C**G**T**A**C**C**A**G**T**C**G**A**T**C**ATGAAGTGCGACG 3‘
Padlock probe actin	5’ phosphate—***AGTCGACGTCGCACT***TTGATTTACTAGGCCAATGTCCTCAGTACTACTACTCTT***ACAGGTCC TTGCGGA*** 3‘
Detection probe actin	5’ Cy3—GGCCAATGTCCTCAGTACTACTAC 3‘

RCA-FISH experimental Method

A tick-off style, step-by-step protocol is provided in Suppl. Note S2. Modifications and notes on filamentous starting material is provided in Suppl. Note S1.

Sections from clubroot samples or algal material were transferred into the reaction tubes were they were rehydrated in PBS-T (0.05% Tween-20 in 1x PBS). Several sections may be treated in one tube, as long as the material is submerged in the reaction mix. For our samples 50 µl of reaction mix were required for consistent results. The PBS-T was removed and the reverse transcription mix (20 U μl^{-1} RevertAid H minus M-MuLV reverse transcriptase, 1 U μl^{-1} RiboLock RNase inhibitor, 1x M-MuLV RT buffer, 0.5 mM dNTP, 0.2 µg μl^{-1} BSA, 1 µM LNA primer) was added. Samples were incubated for 1 h at 37 °C in a PCR cycler. Samples were washed twice with PBS-T and were then incubated in 3.7% PFA (paraformaldehyde in 1 x PBS) for 5 min at room temperature. Samples were washed in PBS-T. This was followed by RNAse H digestion, hybridisation and ligation of the padlock probe in the hybridisation mix (1 U μl^{-1} Ampligase, 0.4 U μl^{-1} RNAse H, 1 U μl^{-1} RiboLock RNase inhibitor, 1x Ampligase buffer, 0.2 µg μl^{-1} BSA, 0.05 M KCl, 20% formamide, 0.1 µM padlock probe). Samples were incubated for 15 min at 45 °C and washed in PBS-T. Rolling circle amplification was done for 45 min at 37 °C (polymerase reaction mix: 1 U μl^{-1} Phi29 DNA polymerase, 1 U μl^{-1} RiboLock RNase inhibitor, 1x Phi29 buffer, 0.25 mM dNTP, 0.2 µg μl^{-1} BSA, 5% Glycerol), followed by a PBS-T washing step. The detection probe was hybridised for 10 min at 37 °C in a detection probe reaction mix (1x hybridisation mix (2x SSC saline-sodium citrate buffer (300 mM sodium chloride, 30 mM sodium citrate), 40% (vol/vol) formamide, 0.1 µM detection oligonucleotide). Samples were washed twice with PBS-T. Samples were carefully transferred and arranged on microscope glass slides, mounted with Vectashield (H-1000, Vector Laboratories) mounting medium and covered with a coverslip. The coverslips were sealed with clear nail polish and the slides were imaged using a laser scanning microscope. To exclude the possibility of false positive signals, control samples were treated without padlock probes.

Single molecule FISH (smFISH)

smFISH relies on a large number of mono-labeled DNA-probes complementary to the mRNA sequences of interest. The protocol described here was adapted from (Duncan *et al.*, 2016a). Probes for smFISH were designed using the Stellaris® Probe Designer (http://singlemoleculefish.com;). A set of 48 different probes was generated, complementary to our mRNA sequences of interest (PbBSMT GenBank: JN106050.1, vBPO GenBank CBN73942.1, MEX1 GenBank: XM_009109278.2, Suppl. Tab. S1-3). To verify the specificity of the oligonucleotides to the target sequence, the sequences were blasted against the NCBI nr database.

smFISH experimental method

A tick-off style step-by-step protocol is provided in the Suppl. Note S3.

Samples were washed in 1x PBS buffer and were transferred to 0.2 mL PCR tubes and incubated for 1 h in 70% EtOH at room temperature. The EtOH was removed and samples were washed twice for three minutes in washing buffer (10% formamide, 2x SSC). Samples were then incubated in 50 µl of hybridisation buffer (100 mg ml $^{-1}$ dextran sulfate, 40% formamide in 2x SSC, 250 nM probe-mix) in a PCR cycler at 37 °C overnight with the lid heated to 100 °C to prevent evaporation. Subsequently, the hybridisation buffer was removed and the samples were rinsed twice with washing buffer, before being incubated in the washing buffer at 37 °C for 30 minutes. Nuclei were counterstained in 50 µl 4,6-diamidino-2-phenylindole (DAPI) solution (100 ng µl^{-1} in washing buffer) for 15 min at 37 °C. Samples were washed in 50 µl 2x SSC for 1 min before being equilibrated in 50 µl GLOX buffer (0.4% glucose in 10 nM Tris-HCl, 2x SSC) for 3 minutes, followed by 50 µl GLOX buffer containing enzymes (1% glucose oxidase and 1% catalase in GLOX buffer) after removal. Samples were carefully mounted using tweezers in the GLOX buffer containing enzymes. The mounted samples were sealed with nail polish and imaged as soon as possible to avoid a reduction of image quality.

Image acquisition and data analyses

Images were acquired with a Leica SP5-II confocal laser scanning microscope and using the LAS AF software (version 2.7, Leica Microsystems, Germany). The microscope was equipped with a hybrid detector and a 20x (0.7 NA) or 63x (1.3 NA) objective lens and with the following lasers: 405 nm, 458 nm, 514 nm, 561 nm, 633 nm. For probes labelled with Cy3 (i.e. actin1, PbBSMT, and vBPO), an excitation of 514 nm was used and the emission was detected at 550 – 585 nm. For probes labeled with FAM (i.e. MEX1**)**, 488 nm excitation was used and emission was detected at 500 – 550 nm. For DAPI, 405 nm excitation was used and emission was detected at 430 – 485 nm. Images were analysed using ImageJ (Schneider *et al.*, 2012) for generating overlay pictures of different channels and for creating maximum projections of the Z-stacks. Resizing and linear brightness and contrast adjustments were performed in GIMP 2.8.22 (www.gimp.org). The original unprocessed images are available upon request.

For the automated counting of FISH signal in image stacks a "find stack maxima" macro in Fiji (Schindelin, 2012), a distribution of the free image analysis software Image J, was applied. Preliminary noise tolerance values were evaluated to best fit the fluorescence signal and regions of interest (ROI) were marked (Suppl. Fig. S1, S2), measured and saved. Finally, automated counts of every slice in a stack within a ROI were summarised and normalised to volume and corrected for stack slice thickness. Results were presented in FISH signal maxima per µm³.

RESULTS

Initially, three *P. brassicae* genes were analysed using RCA-FISH: the SABATH-type methyltransferase PbBSMT and Actin1. In contrast to the latter two genes, a spatiotemporal expression pattern has already been hypothesised for PbBSMT a (Ludwig-Muller *et al.*, 2015); thus we also analysed its expression using smFISH to compare the performance of both methods, qualitatively and quantitatively.

Qualitative and quantitative comparison of smFISH and RCA-FISH

Localisation of the PbBSMT mRNAs resulted in the same spatio-temporal pattern of dotted signals, when smFISH or RCA-FISH were used (Fig 1a-b, see details in Suppl. Figs. S1, S2, S3, S4).

The hypothesised spatiotemporal expression pattern of PbBSMT was identified with RCA-FISH (Fig. 2 d-f) and smFISH (Suppl. Fig. S4). Specifically, mRNAs were visualised once sporogenic plasmodia started to develop into spores (Fig. 2d); the signals appeared dot-like in the individual planes, while in the maximum projection their arrangement outside the forming cell wall of the developing spores was already visible. With progressing spore formation, the intensity and number of signals intensified around the outside the developing spores, both in individual layers, where the signals were still visible as separated dots, and in the maximum projection were mRNA signals appeared to cover the surface of the developing spores (Fig. 2e, see 3D reconstruction in Suppl. Video S1). Subsequently, the signals became fewer and smaller until they disappeared once the spores were fully developed (Fig. 2f). This pattern indicates that the presence of PbBSMT or its mRNA plays a role during the transition from plasmodial growth to sporogenesis in *P. brassicae*. In the control without padlock probe, no signal could be detected (Suppl. Fig. S3).

The number of signal maxima per µm³ was higher in RCA FISH (median 4,47 x 10^{-02} +/- RSD 3,01 x 10^{-02} signal maxima µm^{-3}, n=9) (median 9,38 x 10^{-03} +/- RSD 5,26 x 10^{-02} signal maxima µm³, n=37 Suppl. Tab. 4). However, when smFISH was used, the number of cells in which signals could be detected was considerably higher and more consistent across different samples. Additionally, the detection efficacy of smFISH was higher: more cells did produce useful results (Suppl. Fig. S1, S2). Therefore, we concluded that for our pathosystem smFISH is more suitable, due to compensation of the reduced number of signal maxima µm^{-3} by a 5-10-fold increased number of cells that can be analysed per sample. For this reason, transcripts of *Brassica oleracea* (MEX) and *Ectocarpus siliculosus* (vBPO) were analysed with smFISH only.

Localisation of *P. brassicae* Actin 1 by RCA-FISH

The actin1-gene mRNA was detected in all life cycle stages of *P. brassicae* (Fig. 1c-e, Suppl. Fig S4), as bright, dot-like structures. Signals were spread evenly within cells (Fig. 1c-e) and could be visualised in individual planes (Suppl. Fig. S5c-d) while in the maximum projection of all optical slices these dots often accumulated to signal hotspots (Fig 1c, Suppl. Fig. S5 e-f). In the maximum projection, dots were sometimes well separated (Fig 1c) while in other cells the signal was less defined appearing “blurred” and filling most of the cell without clear structural pattern (Fig 1e). The control, an unlabelled infected sample, did not show any of the characteristic signals (Suppl. Fig. S3 c-e, Fig. S5 a-b).

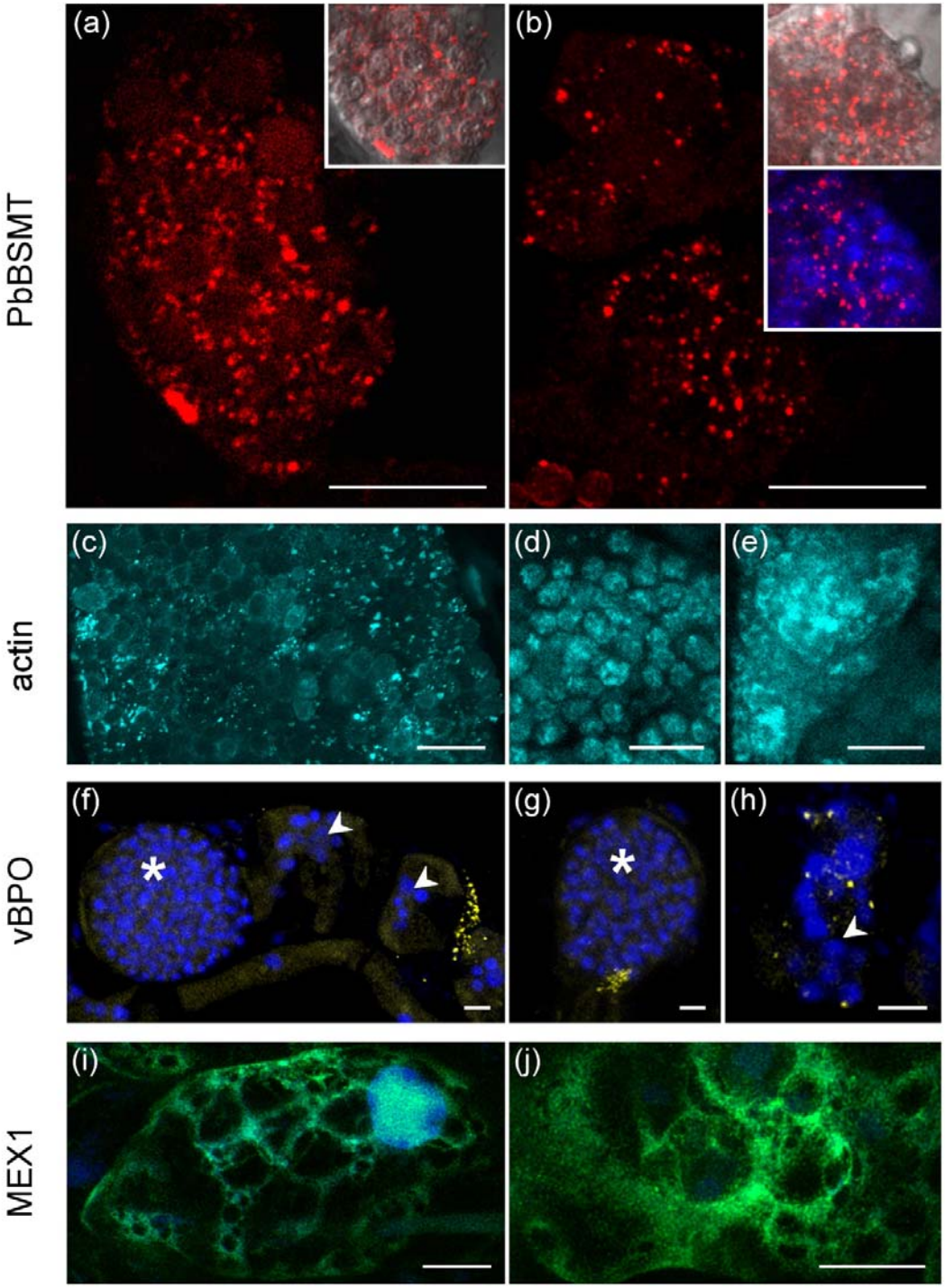

Figure 1: mRNA-transcript localization in P. brassicae (a-d), Ectocarpus Ec32m (f-h) and Brassica rapa (i-j). a, b Red signals are localized mRNAs of P. brassicae PbBSMT using RCA FISH (a) and smFISH (b) during spore formation. The inserts show an overlay of the signal with bright field (a, b) and DAPI (b). c-e: Cyan signals are localized actin mRNAs of P. brassicae using RCA FISH, during the onset of resting spore formation (c), in the developing resting spores (d) and in actively growing sporogenic plasmodia (e). f-h: Localisation of Ec32M vBPO mRNAs (yellow signal) using smFISH close to (f) and inside (g, h) of M. ectocarpii infected cells. arrowheads: early infection, plasmodia containing several nuclei; asterisks: later infection stage, large plasmodia with numerous parasite nuclei. i, j: Green signals localizing Mex1 in the cytosol of unifected B. rapa root cells using smFISH, Mex1 mRNAs are accumulating around the amyloplasts (black areas). Bars = 10 µm. Images of the controls of these experiments are provided in Supplementary Fig. S3.

Localisation of plant and algal transcripts during infection by phytomixids

Brassica rapa maltose excess protein 1 (MEX1, smFISH)

MEX1 encodes a transporter located in the chloroplast envelope; it is essential for the transport of maltose, the main product of starch breakdown, from the amyloplast to the cytosol (Stettler *et al.*, 2009). MEX1 transcripts were be detected everywhere in the cytosol of the Brassica root cells (Fig 1i-j). A signal accumulation was observed around the amyloplasts of cells infected with *P. brassicae*, recognised by the presence of multiple DAPI-stained nuclei. Also, uninfected cells neighbouring the *P. brassicae* infected cells did result in a positive detection of the MEX1 mRNA, while the signal was not present in cells filled with spores and cells that did not contain amyloplasts (Suppl. Fig. S6). In contrast to the previously described genes no localised detection ("dots") could be seen in the individual optical slices and the maximum projection images, although in the latter brighter areas became visible, indicating that mRNAs for MEX1 are present in the whole cytosol of the plant. No signals were detected in the unlabelled infected control sample (Supplement Fig. S3i-j).

Ectocarpus siliculosus vanadium-dependent bromoperoxidase (vBPO, smFISH)

mRNA detection in brown algae required adaptations of the protocol. The filamentous growth of *E. siliculosus* and of *M. pyrifera* gametophytes made it necessary to use small tufts of algal material, which are difficult to handle and do not attach well to L-Polylysine-coated slides. Therefore tubes were used to incubate and wash the samples (see Supplementary Information (Note S1).

The vBPO has previously been shown to play a role in the response of *Ectocarpus siliculosus* 32m against pathogens (Strittmatter *et al.*, 2016) and more generally, in the responses of kelps to elicitors (Cosse *et al.*, 2009). RCA-FISH was tested without success on the algal material (data not shown), but smFISH allowed detection of vBPO mRNAs in Ec32m cells infected with the phytomyxid *Maullinia ectocarpii*, recognised by the presence of DAPI-stained pathogen nuclei (Fig. 1f-h, Suppl. Fig. S7 g-i). Likewise, signals were detected in infected *M. pyrifera* cells (Suppl. Fig. S7 a-f). In both cases, signals were dot-like, yet much more confined and smaller than in the plant material tested previously. Signals were nearly invisible in the individual planes, therefore Z-stacks of at least 10 optical slices with a thickness of ca. 0.5 – 0.8 µm each were sampled and viewed in maximum projection mode (Suppl. Fig. S7d-f). A higher number of thinner optical slices improved both the signal intensity and localisation (Suppl. Fig. S7a-c).

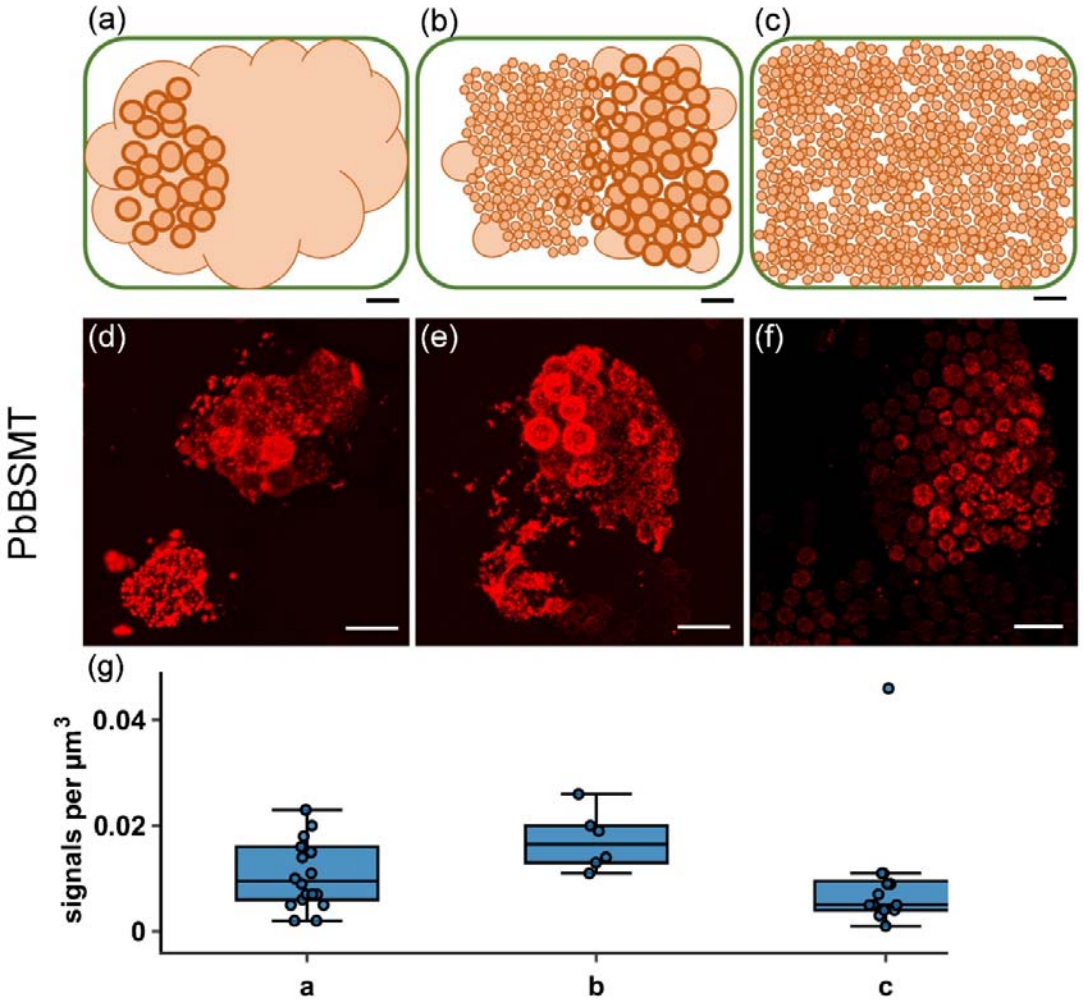

Figure 2: Localization and quantification of mRNA signals in P. brassicae. a-c: diagrammatic overview of P. brassicae spore formation. (a) onset of spore formation in sporogenic plasmodia, first spore like aggregations of the plasmodium become visible. b: young resting spores can be identified, but cells are still enlarged and do not show a fully developed cell wall. c: resting spores are fully developed and fill the entire cell. d-f: Localization of P. brassicae PbBSMT in sporogenic plasmodia. (d) PbBMST mRNAs populate the area surrounding the developing spores once the first spore like aggregations become visible. (e) The signal peaks with spore formation. (f) PbBMST mRNAs get less and start to disappear once resting spores are fully developed (f). Bars = 10 µm. (g) Signal maxima µm-3 of smFISH PbBMST signals (n=37) highlighting a peaking of PbBMST mRNAs during spore formation. Labelling on the x-axes corresponds to the life cycle stages in images (a) – (c). No significant correlation between the three sample pools was identified. One extreme outliner at 0.088 signals µm-3 is not displayed to improve figure layout.

Discussion

mRNA localisation is a powerful tool to study pathogen – host interactions

Here, we demonstrate the feasibility of localising mRNA transcripts of phytomyxid pathogens and their plant and algal hosts. This permits the identification and analyses of spatiotemporal pattern of mature mRNAs in the cytoplasm. Both methods tested here (smFISH, RCA-FISH) are very accessible once a gene of interest in biological interactions is identified. Using mRNA FISH allows to overcome important technical bottlenecks that hamper research on important non-model pathogens, plants or algae (Schwelm *et al.*, 2018). Our protocols therefore have huge potential to move forward our knowledge of these interactions beyond the limitations of RNAseq and organisms for which genetic amendments are possible (Buxbaum *et al.*, 2015). Using clubroots from commercial fields, we directly show the applicability of the methods to investigate host-pathogen interactions in wild type populations. This will greatly increase our understanding of subcellular gene expression pattern, similar to the leap in knowledge that happened in model organisms during the last years (Gaspar & Ephrussi, 2015; Lee *et al.*, 2016; Medioni & Besse, 2018).

RNA stability and accessibility are crucial – or how do you get the protocol to work?

Here we show that both smFISH and RCA-FISH methods are suitable for *in situ* mRNA monitoring in the host and the pathogen. While analysing three totally different biological systems (plants, algae, phytomyxids) it became clear that the overall success and efficiency of the method is determined by the fixation of the mRNAs in the sample material and the permeability of the cell walls, both factors that differ across organisms. Fixation methods and the duration of storage post-fixation influenced the number of cells in which signals could be detected; however, this was different across sample types and genes and no clear duration of storage could be established. Since decreasing signal intensities can be caused by RNA degradation during storage, we recommend to use the samples as soon as possible after harvesting. However, we found that samples kept in an RNAse-free environment give good results after more than one year. Another reason for decreasing signals can be formaldehyde mediated covalent interlinking of mRNAs and the RNA-binding proteins which mediate the transport of the RNA to the site of translation (Foley *et al.*, 2017; Niessing *et al.*, 2018), or mRNA secondary structures that do not permit the binding of the probes to the sites of interest (Ding *et al.*, 2013). Because of this, we recommend not to store the samples in the formaldehyde containing fixative, but to move them to pure ethanol for long term storage. RNA-binding proteins and mRNA secondary structure can impact on the success of the detection method without the fixation bias mentioned above, because both can limit the accessibility of the target site for probes (Foley *et al.*, 2017).

On the other hand, the permeability of cell walls and plant tissue as a whole is a constraint for FISH-based methods. In this study, RCA-FISH was not adaptable to study interactions in filamentous algae, and we hypothesise that this was because the enzymes needed could not permeate the cell walls. Cutting of the algae in an RNAse free environment is very difficult and often destroys the cell arrangement, resulting in a loss of spatial information. All tested permeabilisation efforts were not successful. Also in plants the efficacy of the mRNA detection was lower in RCA-FISH, as signals were only visible in cells which were cut open. The small probes used for smFISH could be used on all samples without additional permeabilisation steps.

Choosing the most suitable protocol for your question

FISH is – as any nucleic acid based method – very adaptable to the needs of the user. A multitude of protocols are available, which are variations of the two protocols tested here, or use a different mode of action (reviewed by van Gijtenbeek & Kok, 2017). The most important things to keep in mind are (i) the nature and type of biological material, (ii) the size of the involved chemicals, as smaller molecules will permeate cell walls more easily than large proteins, (iii) the length of the mRNA analysed, (iv) expression levels of the mRNA, and (v) timing and (vi) costs (Tab. 2).

Table 2: Advantages and disadvantages of RCA-FISH and smFISH. Prices can vary and are estimates based on Austrian prices 2017 including 20 % VAT.

	RCA FISH	**smFISH**
Pros	▪ **Signal amplification** in theory allows to monitor single copy mRNAs also in samples with a high fluorescent background. ▪ **Specificity**: Highly specific probes can be designed to distinguish between paralogues or SNPs . ▪ **No gene length limitation:** short (<500 bp) genes can be analysed. ▪ **Modular**: Possibility of combination with in situ proximity ligation assays to collect information on mRNA-protein interactions or post-translational modification (not tested in the scope of this paper).	▪ **Signal amplification** in theory allows to monitor single copy mRNAs in samples with a high fluorescent background. ▪ **Complexity**: easy probe design, few steps minimising contamination risks. ▪ **Cell permeability**: small probes, no large functional molecules involved. ▪ **Cost of reagents:** most reagents needed are standard lab consumables. ▪ **Time**: Fast protocol, ca. 1 day from sample to image, hands on time ca 3 h.
Cons	▪ **Complexity**: many steps increase the risk of RNAse contamination or errors. ▪ **Cell permeability**: Involves multiple steps requiring large enzymes which need to reach the cytoplasm. This is a major drawback for use in the plant/algal system. ▪ **Costs of probes**: The expensive LNA- and Padlock probes need to be designed for each gene (ca. 400€ per gene for ~200 reactions). ▪ **Costs of reagents:** pricey reagents and enzymes are used (ca 75€ per reaction). ▪ **Time**: ca. 2 days from sample to image, of which there are ca 6 h hands on time.	▪ **Specificity:** using standard FISH probes in a “string of beads” manner opens possibility of false positives for isoforms, orthologues or conserved gene families. ▪ **Gene length limitation:** Short mRNA sequences (< 500 bp) are not suitable for smFISH. ▪ **Costs of probes**: 24-48 labelled probes are needed for each gene. Some companies offer discounts on smFISH probes (ca. 500-800€ depending on the number of probes and the fluorophore, ~1000+ reactions).

mRNA localisation sheds light on phytomyxid biology

In this study, we determined the expression and localisation pattern of three pathogen genes, one plant and one brown algal gene. Although the function and putative expression pattern on a cellular level of all these genes was known, results obtained while adapting this protocol are already improving our understanding of the biological interaction. The *P. brassicae* actin1 (Archibald & Keeling, 2004) was evenly expressed during all stages of the life cycle of *P. brassicae* (Fig. 1c-e) similar to previous microarray studies (Siemens *et al.*, 2009).

More interesting was the expression pattern of PbBSMT, a SABATH-type methyltransferase produced by *P. brassicae*. This methyltransferase has structural similarities to plant methyltransferases and qPCR analysis showed, that its expression during clubroot development is highest when the concentration of salicylic acid in the roots peaks, which is why a role in disease development of this gene is discussed (Ludwig-Muller *et al.*, 2015; Bulman *et al.*, 2018). Here, PbBSMT mRNAs started to appear when spore formation of *P. brassicae* was initiated. The number of FISH signals and their intensity peaked when young, immature resting spores were visible (Fig 2). Looking at the position of the signals, especially in older cells, it became clear that PbBSMT mRNAs accumulate on the outside of the developing resting spores. These resting spores contain chitin in their cell wall (Cavalier-Smith & Chao, 2003), which is one of the best studied elicitors of plant defence and induces (amongst others) salicylic acid (SA) production (Fesel & Zuccaro, 2016). On the other hand, methylsalicylate is a more volatile and membrane-permeable form of SA and is either excreted via the leaves or an inducer of systemic responses in plants (Vlot *et al.*, 2017). Therefore, this localised pattern of mRNAs around the sites of spore formation during which (presumably) the most chitin is produced is intriguing: this allows to speculate that *P. brassicae* might produce PbBSMT to inactivate the SA signal produced by the host in response to chitin, by making it more mobile and thus evading a strong local defence reaction. Combined with findings from previous studies (Ludwig-Muller *et al.*, 2015; Bulman *et al.*, 2018), our observations strongly support the role in host defence suppression of PbBSMT, making it a very interesting target for future studies.

Localisation of mRNAs in the hosts infected with phytomyxids allowed for the first time to confirm a direct interaction and interference of phytomyxids on the transcriptional status of infected cells. Roots of Brassicas infected with *P. brassicae* show a marked accumulation of starch (Ludwig-Müller *et al.*, 2009). MEX1, a gene coding for a maltose transporter essential for transporting maltose from the amyloplast to the cytosol (Stettler *et al.*, 2009) could be localised around the amyloplasts in cells in which plasmodia of *P. brassicae* could be seen (Suppl. Fig. S6). The absence of a dot like pattern of the detected MEX1 mRNAs indicates a high density of mRNAs surrounding the amyloplast where many mRNAs are present permitting their individual detection. A high density of mRNAs is an indicator of ongoing synthesis of a host protein, because of the short half live of mRNAs in vivo (Merchante *et al.*, 2017). Therefore, it can be hypothesised that based on the location MEX1 is activated upon pathogen infection and that it mediates energy supply from the host to the pathogen.

In brown algae the expression of vBPO has previously been linked to stress response (Leblanc *et al.*, 2015; Strittmatter *et al.*, 2016). Here we could confirm that vBPO mRNAs are located in *E. siliculosus* and *M. pyrifera* cells which show early infections with *Maullinia ectocarpii*, while in cells where the infection has advanced to sporangial development no signals were identified. This confirms that brown algae show a localised stress response to plasmodiophorid pathogens. Whether or not this

type of stress response is a general pattern in brown algae or if this is specific for infections with phytomyxids was beyond the scope of this study.

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Author Contribution

JB and SN designed the research, collected, analysed and interpreted data and wrote the manuscript with feedback from all co-authors; JB performed the research; CG provided algal material and algal data; AMS analysed the images. All authors read and contributed to the final MS.

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