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Technical advance

Extraction of high-quality bacterial RNA from infected leaf tissue for bacterial *in planta* gene expression analysis by multiplexed fluorescent Northern hybridization

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SUMMARY

Plant pathogenic bacteria possess a large number of genes that allow them to grow and cause disease on plants. *In planta* gene expression analysis is important to understand the impact of these genes on bacterial virulence. A new mRNA-based approach using multiplexed Northern hybridization was developed. High-quality bacterial and plant total RNA was successfully isolated from leaf tissue infiltrated with *Pseudomonas syringae*. The procedure employs a new extraction buffer formulation containing glycine, sodium dodecylsulphate, cetyltrimethylammonium bromide, high-molecular-weight polyethylene glycol and β-mercaptoethanol. Cell lysis and classical acid–phenol extraction steps followed by LiCl precipitation yielded large amounts of total RNA of high purity and integrity. Multiplexing of DIG and chemically fluorescently labelled RNA probes was developed and expression data were normalized using the 23S rRNA gene as reference. The method was validated by studying *in planta* expression of the *P. syringae* genes *mucD*, *cmaA*, *cfl*, *corR*, *corS* and *corP* comprising a selection of highly expressed biosynthetic and low-expressed regulatory genes. The method was assessed regarding its sensitivity and might by useful for studying a variety of plant–microbe interactions.

INTRODUCTION

Analysis of *in planta* gene expression can significantly contribute to our understanding of the interaction of plant pathogenic bacteria with their plant hosts. For the plant pathogenic bacterium *Pseudomonas syringae*, thus far classical reporter genes such *egfp* or *uidA* have been utilized to measure gene expression inside infected host tissue (Keith *et al.*, 2003; Weingart *et al.*, 2004). Differential gene expression of *P. syringae* on plant surfaces was studied with *gfp*, *lux* or *inaZ* reporter gene fusions (Cirvilleri and Lindow, 1994; Joyner and Lindow, 2000; Marco *et al.*, 2005; Waterhouse *et al.*, 1993). The activity of reporter genes is easy to measure but each investigated gene requires an elaborate study design and introduction of a resistance marker-carrying reporter construct into the bacterial test strain. Expression data can be biased by altered mRNA processing due to reporter gene fusion or the stability of the reporter protein.

Direct measurement of mRNA levels with quantitative RT-PCR or Northern hybridization would be suitable alternatives to gain accurate and reliable *in planta* gene expression data. An important prerequisite is the extraction of high-quality total RNA from infected leaf tissue. Plants produce secondary metabolites, which can strongly interfere with the RNA extraction and enzymatic downstream applications such as RT-PCR. Bacterial mRNA has a shorter half-life and is more unstable than that of eukaryotes because eukaryotic mRNA is capped and polyadenylated. There are several plant RNA extraction protocols available that address the problems of plant tissue rich in polysaccharides or secondary metabolites. The detergent cetyltrimethylammonium bromide (CTAB) was successfully used to separate nucleic acids from polysaccharides (Chang *et al.*, 1993). High-molecular-weight polyethylene glycol (HMW-PEG) was found to eliminate large amounts of phenolic compounds and polysaccharides (Gehrig *et al.*, 2000). The reducing agent β-mercaptoethanol can be used to prevent the oxidation of phenolic compounds (Jaakola *et al.*, 2001) and to inactivate RNases. Extraction protocols for bacterial total RNA use rapid cell lysis under heat with the detergent sodium dodecylsulphate (SDS) followed by repeated organic extractions with acid-phenol (Majumdar *et al.*, 1991; Sambrook *et al.*, 1989).

RNA isolated from soybean leaf tissue infected with *P. syringae* represents a mixture of bacterial and plant total RNA at an unknown ratio. Therefore, normalization of expression data becomes an important issue, especially if generated by Northern hybridization, where equal amounts of total RNA from to-be-compared samples are usually required. For quantitative RT-PCR

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analysis, normalization of data against reference genes is indispensable. Normalization of Northern hybridization data with a reference gene would require two probes, one for the gene of interest and one for a reference gene.

To our knowledge, there are no reports dealing with the simultaneous extraction of bacterial and plant total RNA suitable for Northern hybridization and quantitative RT-PCR experiments. Here we report for the first time the simultaneous extraction of high-quality bacterial and plant total RNA from infected leaf tissue and a refined method of bacterial *in planta* gene expression quantification based on classical and simple non-radioactive, fluorescent Northern hybridization, as demonstrated on selected genes of interest.

In this study, two *P. syringae* pv. glycinea strains were used which differ in the presence of the functional alternative sigma factor AlgT, PG4180 (*algT*$^-$) and PG4180.muc (*algT*$^+$). As a result, PG4180.muc is able to produce large amounts of the exopolysaccharide alginate, whereas alginate production is negligible in PG4180 (Schenk *et al.*, 2006). We investigated the *in vitro* and *in planta* gene expression of *mucD*, coding for a periplasmic serine protease previously implicated in alginate regulation (Schenk *et al.*, 2006), expression of *lsc*, coding for levansucrase required for synthesis of the exopolysaccharide levan (Li *et al.*, 2001), expression of *cmaA* and *cfl*, genes involved in the biosynthesis of the phytotoxin coronatine (Budde *et al.*, 1998), and expression of *corR*, *corS* and *corP*, genes coding for regulators of coronatine biosynthetic genes (Weingart *et al.*, 2004). Several of these genes were previously reported to exhibit a thermoresponsive pattern of expression *in vitro* (Budde *et al.*, 1998). Consequently, two temperatures, 18 and 28 °C, were used. Alginate and levan, and coronatine are important virulence and fitness factors of *P. syringae*, respectively. An appropriate and sensitive method to analyse their potential plant inducibility consequently is the key to a better understanding of their role(s) in the plant–microbe interaction.

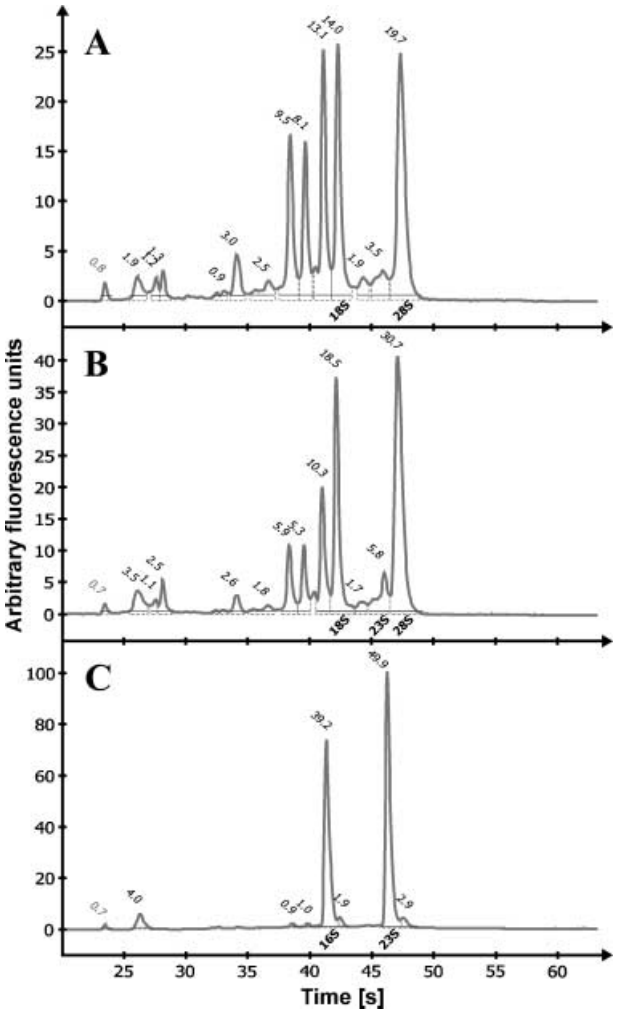

Fig. 1 Electropherograms of total RNA extractions. Total RNA from uninfected soybean leaf tissue (A), soybean leaf tissue infected with *P. syringae* pv. glycinea (B) and *in vitro* grown *P. syringae* pv. glycinea (C) was analysed using a Agilent 2100 Bioanalyzer. Main ribosomal RNA peaks of bacteria (16S, 23S) and plant (18S, 28S) are indicated. Numbers above the peaks show their quantitative percentage within the entire sample.

RESULTS

Extraction of total RNA from infected leaf tissue

The alginate-producing *P. syringae* pv. glycinea strain PG4180.muc and the non-alginate-producing strain PG4180 were inoculated into soybean leaves by infiltration. Infected and uninfected control plants were kept in growth chambers set to 18 or 28 °C. Three days after inoculation, infiltrated leaf regions showed typical symptoms and *in planta* samples were taken. For *in vitro* samples, PG4180.muc and PG4180 were grown in HSC medium at 18 and 28 °C to an OD_{600} of 1.0. Total RNA was extracted using the extraction buffer and method described herein. Yields of total RNA were in the range 75–125 μg per plant tissue sample (150–200 mg plant tissue) and 75–100 μg per bacterial cell pellet (5×10^9 CFU). A_{260}/A_{280} ratios ranged between 2.1 and 2.2. Determined A_{260}/A_{230} ratios above 1.6 indicated high purity and absence of protein and polysaccharide contaminations. Aliquots of the preparations were further analysed using the Agilent 2100 Bioanalyzer (Fig. 1). The isolated RNA was of high quality and integrity, as demonstrated by sharp ribosomal RNA peaks.

23S rRNA probe for normalization of bacterial *in planta* gene expression data

Northern or Spot blot expression data of bacterial total RNA can be directly generated from hybridization signals, as each line or spot contains a defined amount of bacterial RNA. Samples isolated

from infected leaf tissues consist of a mixture of bacterial and plant total RNA and might vary in their bacterial total RNA content. Consequently, normalization of hybridization signals is required to generate bacterial *in planta* expression data. The high abundance and relatively constant expression of the 16S and 23S bacterial ribosomal RNAs make them a suitable target for reference. Potential probe binding sites in the 23S rRNA were identified by alignment of *23S rDNA* gene sequences from *Pseudomonas syringae*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Arabidopsis thaliana* chloroplast, and *Nicotiana tabacum* chloroplast. Two short regions, one located at the beginning (23S#1) and one located at the end (23S#2) of the 2903-bp *23S rDNA* gene from *P. syringae*, showed sufficient sequence uniqueness (data not shown). DIG-labelled RNA probes 23S#1 and 23S#2 were generated and analysed by Northern hybridization with plant total RNA, total RNA isolated from infected tissue and bacterial total RNA (Fig. 2). Both probes showed strong signals at the size of the 23S rRNA in the total RNA isolated from infected tissue and bacterial total RNA samples. No cross-hybridization with total RNA derived from uninfected plants was detectable.

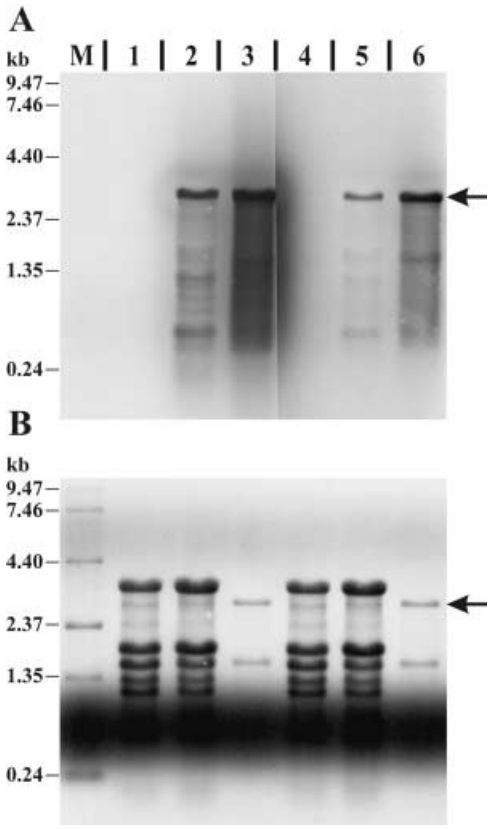

Fig. 2 Evaluation of two 23S rRNA-specific probes. (A) Northern blot membrane hybridized with DIG-labelled probe 23S#1 (lanes M, 1–3) or 23S#2 (lanes 4–6). Lanes contain total RNA extracted from uninfected soybean leaf tissue (1, 4; 5 μg), soybean leaf tissue infected with *P. syringae* pv. glycinea (2, 5; 5 μg), *in vitro* cultured *P. syringae* pv. glycinea (3, 6; 0.5 μg) and an RNA size standard (M; 3.0 μg). Hybridization signals specific to the 23S rRNA are indicated by the arrow. (B) Methylene-blue-stained membrane prior to Northern hybridization for size estimation, control of total RNA quantity and successful Northern transfer. The same region of the membrane as in A is shown.

Multiplexed fluorescent Northern hybridization

Bacterial gene-specific and 23S rRNA signals were analysed using a new multiplexed fluorescent Northern hybridization approach. A DIG-labeled gene-specific and an Oyster-645 fluorescence-labelled 23S#1 RNA probe were multiplexed in a single Northern hybridization reaction. Northern and Spot blotting techniques were evaluated for their usability in our multiplexed hybridization approach. For *mucD* analysis, total RNA samples of PG4180.muc and PG4180 grown *in vitro* and *in planta* at 18 and 28 °C and total RNA from uninfected plants as control were separated by denaturating RNA gel electrophoresis, followed by Northern transfer and hybridization (Fig. 3). The *mucD* Northern blot showed a transcript at a size of ~1.75 kb (Fig. 3A) and sharp bands for the 23S rRNA at ~2.9 kb (Fig. 3B). Neither the *mucD* nor the 23S#1 probe showed cross-hybridization with uninfected plant total RNA controls. Cross-talk of the two fluorescent dyes ECF and Oyster-645 was not observed, as determined by scanning the membrane sequentially on the FLA-3000 phosphoimager with appropriate laser and filter combinations. Signal intensities were always in the linear range of detection. The coronatine genes, *cmaA*, *cfl*, *corR*, *corS* and *corP*, were analysed with PG4180 *in vitro* and *in planta* total RNA samples. Samples were spotted in triplicate and hybridized (Fig. 4). The Spot blots showed signals for *cmaA* (Fig. 4A), *cfl*, *corR*, *corS*, *corP* and the 23S#1 probe (Fig. 4B). Signals obtained for *cmaA* and *cfl* biosynthetic genes were stronger than those for the regulatory genes *corR*, *corS* and *corP*. No cross-hybridizations with the plant total RNA controls were observed.

Quantitative data were acquired for Northern and Spot blot signals using the AIDA V4.00 software package (Raytest). For each hybridization signal, a total volume and local background value was obtained, which led to background-corrected volume values after subtraction. Corrected volume values were normalized to their corresponding 23S rRNA signals by division and, when possible, standard deviations were calculated. For each data set the highest value was set to 100% relative expression and other data were related to this. Figure 5 summarizes the normalized data. Expression of *mucD* was slightly higher *in vitro* than *in planta* and about two-fold higher at 28 than at 18 °C, whereas *in planta* this situation was almost reversed. Both investigated strains exhibited similar expression patterns with slightly lower signals for PG4180.muc than for PG4180. The *in vitro* expression of *cmaA* and *cfl* in PG410 showed a clear temperature-dependent

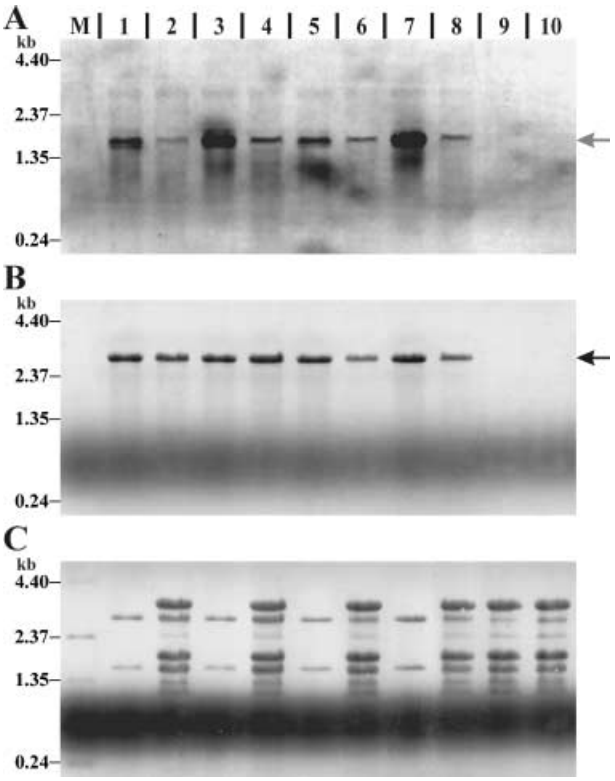

Fig. 3 Multiplexed fluorescent Northern blot hybridization. Northern blot membrane hybridized with a DIG-labelled *mucD* probe (A) multiplexed with an Oyster-645-labelled 23S#1 probe (B). Lanes contain total RNA extracted from *in vitro* cultured PG4180.muc at 18 (1) or 28 °C (3), PG4180.muc-infected soybean tissue at 18 (2) or 28 °C (4), *in vitro* cultured PG4180 at 18 (5) or 28 °C (7), PG4180-infected soybean tissue at 18 (6) or 28 °C (8), uninfected soybean leaf tissue at 18 (9) or 28 °C (10), and an RNA size standard (M). Arrows indicate probe-specific signals. For *in vitro* samples 1.5 μg and for *in planta* samples 6 μg RNA was loaded. (C) Methylene-blue-stained membrane prior Northern hybridization. A, B and C show the same region of the membrane.

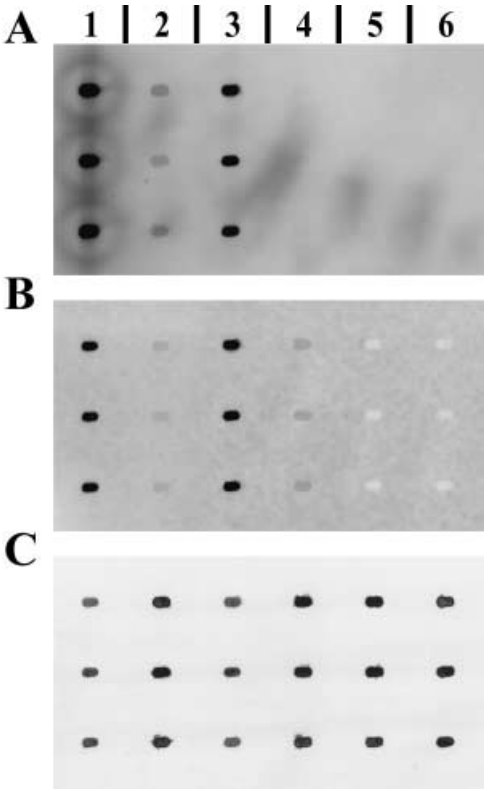

Fig. 4 Multiplexed fluorescent Spot blot hybridization. Example of a Spot blot membrane hybridized with a DIG-labelled *cmaA* probe (A) multiplexed with an Oyster-645-labelled 23S#1 probe (B). Spots contain total RNA extracted from *in vitro* cultured PG4180 at 18 (1) or 28 °C (3), PG4180-infected soybean tissue at 18 (2) or 28 °C (4), and uninfected soybean leaf tissue at 18 (5) or 28 °C (6). For *in vitro* samples 0.2 μg and for *in planta* samples 1 μg RNA was spotted. (C) Methylene-blue-stained membrane prior Northern hybridization. A, B and C show the same region of the membrane.

phenotype with 12-fold higher expression at 18 than at 28 °C. The temperature phenotype was confirmed *in planta* for both strains but at a 50-fold lower overall gene expression level than *in vitro*. PG4180 *corR*, *corS* and *corP* regulatory genes could be quantified *in vitro* and *in planta* with expression values varying independent of temperature and environmental background up to three-fold.

In order to assess the sensitivity of the method described herein, *in planta* detection limits were determined for the moderately expressed bacterial *lsc* gene coding for levansucrase (Li *et al.*, 2001, 2006) (Fig. 6). For this, a very low inoculation density (OD of 0.001) and sampling after 2, 4, 7 and 10 days were used. No signal could be detected with 5.2×10^2 CFU per leaf disc. For the 23S rRNA probe a signal was observed with 5×10^4 CFU per leaf disc (Fig. 6A) and for *lsc* 2.5×10^6 CFU per leaf disc was required to obtain a signal. The data suggested that expression of genes from ~10^6 cells per sample can be monitored by this method.

DISCUSSION

Prerequisite for any gene expression experiment at the RNA level is reliable starting material. For *in planta* experiments, quality and yield are far more important than sample throughput. Therefore, we developed a new extraction buffer formulation and method for the isolation of total RNA from leaf tissue infected with *P. syringae*, yielding consistently large amounts of high-quality total RNA. A combination of SDS and β-mercaptoethanol in the extraction buffer with a short hot lysis step facilitated cell-wall dissolution and recovery of RNA. Addition of HMW-PEG improved integrity and purity of RNA by removing phenolic compounds (Gehrig *et al.*, 2000). Successive organic extraction with acid-phenol and addition of CTAB detergent in the last

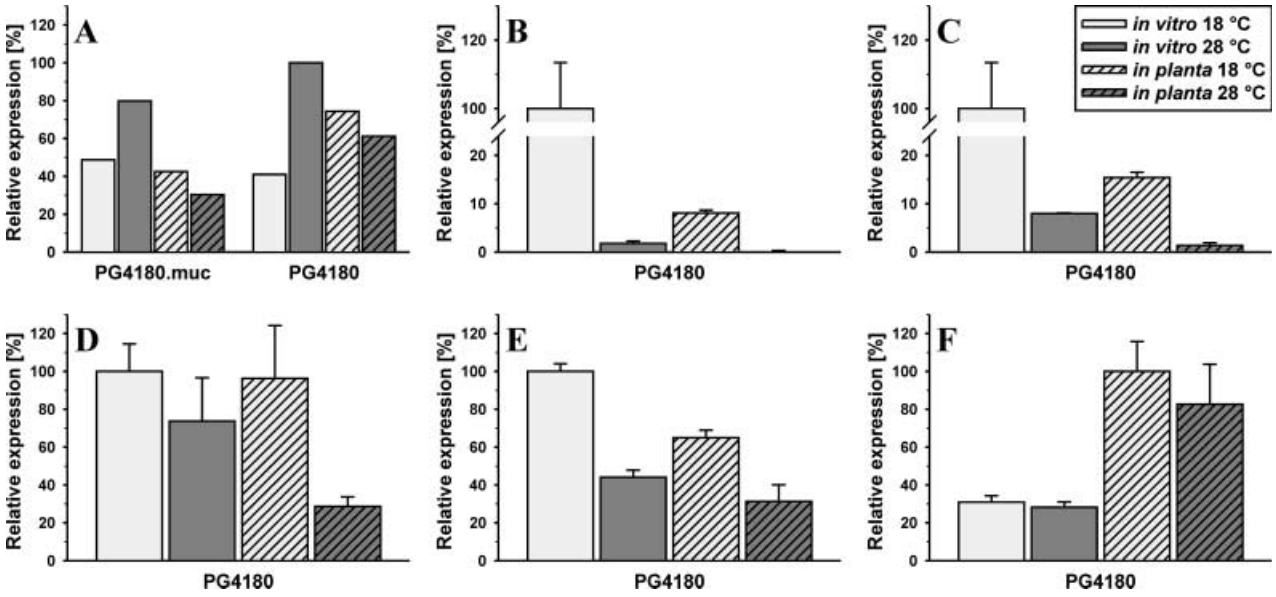

Fig. 5 Quantitative *in vitro* and *in planta* gene expression. Relative abundance of *P. syringae* pv. glycinea *mucD* (A), *cmaA* (B), *cfl* (C), *corR* (D), *corS* (E) and *corP* (F) mRNA *in vitro* and *in planta* at 18 and 28 °C. Data were generated by quantification of gene-specific signals and normalization to the corresponding 23S rRNA signals. Bars in B–F represent the mean of three replica spots and their standard deviation.

extraction step greatly improved separation of RNA from proteins and polysaccharides (Chang *et al.*, 1993; Jaakola *et al.*, 2001; Majumdar *et al.*, 1991). Finally, selective RNA precipitation using LiCl further increased RNA purity. Spectrophotometric and gel electrophoretic analysis demonstrated the high quality of the bacterial and plant total RNA. This RNA was applicable for enzymatic downstream applications such as cDNA synthesis and RT-PCR.

With a yield of high-quality total RNA, we evaluated the usability of well-established, non-expensive, low-tech Northern and Spot blot hybridization methods for bacterial *in planta* gene expression analysis, although the more expensive quantitative RT-PCR method needs to be kept as a sensitive and high-throughput option. For RT-PCR it is essential to have demonstrated *in vitro* that the probe is specific to a transcript of a given size. However, that technique might not detect variable transcript sizes in high-throughput experiments *in planta*. Such variations in transcript sizes might arise from differential regulation in the natural setting. The method described herein circumvents this often neglected potential problem. The quality of the RNA obtained by the extraction procedure described herein is suitable for RT-PCR as determined in a previous study (Schenk *et al.*, 2006).

As total RNA samples isolated from infected leaf tissue represent a mixture in which the bacterial RNA portion is unknown, all expression data had to be normalized to a reference. We took advantage of the 23S rRNA as a target of high abundance and relatively constant expression. Two RNA probes, directed against 23S rRNA, were developed and yielded clear hybridization signals highly specific for *P. syringae* 23S rRNA. To increase data accuracy and to reduce the amount of blots and chemicals, we successfully developed multiplexing of a gene-specific and a 23S rRNA probe. The total RNA samples isolated from infected leaf tissue contained only a low proportion of bacterial mRNA. Sensitivity of gene-specific probes was increased by labelling them with the DIG system. This has the advantage of producing an amplified ECF-fluorescence signal mediated by an antibody binding cascade and alkaline phosphatase action. In contrast to gene-specific mRNAs, the abundance of 23S rRNA in total RNA samples is high. Therefore, the 23S rRNA probe was directly labelled by chemical linkage of an activated fluorescent dye. Taking into account that a higher number of potential probe targets demands more RNA probe, large amounts of *in vitro* synthesized probe were generated by large-scale *in vitro* transcription. Development and quantification of the ECF and Oyster-645 fluorescence signals was successfully carried out on a phosphoimager system, utilizing two different laser and filter settings. Cross-talk or fluorescence resonance energy transfer (FRET) between the two fluorophors used was not detectable as excitation and emission spectra of the dyes did not show a significant overlap.

Validation of the method was addressed by *in planta* gene expression analysis of the *P. syringae* genes *mucD*, *cmaA*, *cfl*, *corR*, *corS* and *corP*, which we had investigated in previous studies (Budde *et al.*, 1998; Schenk *et al.*, 2006; Weingart *et al.*, 2004). Sensitivity was assessed by monitoring the moderately expressed *lsc* gene, indicating that gene expression from ~10^6 CFU per leaf disc can be detected. *In vitro* data for *mucD* obtained

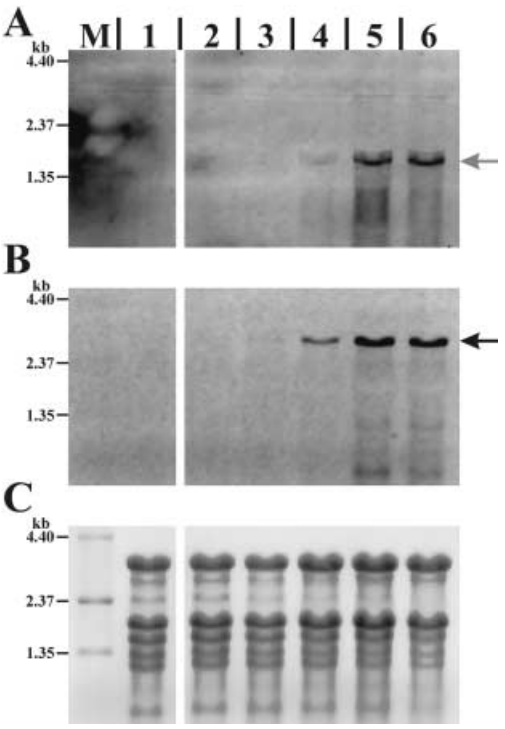

Fig. 6 Evaluation of *in planta* sensitivity of multiplexed fluorescent Northern blot hybridization. Membrane hybridized with a DIG-labelled *lsc* probe (A) multiplexed with an Oyster-645-labeled 23S#1 probe (B). Lanes contain 10 μg total RNA extracted from uninfected soybean leaf tissue (1), soybean leaf tissue infected with *P. syringae* pv. glycinea PG4180 (2–6) and 3 μg of RNA size standard (M). Soybeans were infiltrated with a bacterial suspension of OD 0.001 and leaf discs were sampled for CFU determination and RNA extraction at different time points. Lane 2, 2 days (5.2×10^2 CFU per leaf disc); Lane 3, 4 days (5.0×10^4 CFU per leaf disc); Lane 4, 7 days (2.5×10^6 CFU per leaf disc); Lane 5, 10 days (1.3×10^7 CFU per leaf disc); and Lane 6, 16 days (2.2×10^7 CFU per leaf disc). Arrows indicate probe-specific signals. (C) Methylene-blue-stained membrane prior to Northern hybridization. A, B and C show the same region of the membrane.

from Northern blots confirmed our previously reported data (Schenk *et al.*, 2006). *In planta* data showed that *mucD* transcription in *P. syringae* is not plant-inducible and supported our previous finding that it is not dependent on the sigma factor AlgT, as *mucD* expression was not elevated in PG4180.muc (*algT*$^+$) as compared with PG4180 (*algT*$^-$). The two-fold induction observed *in vitro* at 28 versus 18 °C was not observed *in planta*. *In vitro* and *in planta* data obtained for the biosynthetic genes involved in coronatine biosynthesis, *cmaA* and *cfl*, confirmed our previous findings, which showed a strong temperature dependence but no plant induction in *P. syringae* strain PG4180, in contrast to the situation in strain DC3000 (Weingart *et al.*, 2004). In our previous study, *cmaA* expression was measured using *egfp* as reporter gene. In the present study, we observed about 50-fold lower *in planta* expression than *in vitro*. This can be explained by growth phase differences because *in planta* samples are likely to represent the mid-stationary phase whereas *in vitro* samples correspond to the early exponential growth phase. Highest EGFP accumulation *in vitro* occurred in the stationary phase, whereas highest levels for the *cmaA* transcript were found in the early exponential phase and declined in the stationary phase (Weingart *et al.*, 2004). Reporter gene fusions measure promoter activity over time by accumulation of the reporter protein, whereas mRNA-based methods provide snapshots of mRNA levels at a defined time point.

Sensitivity of the multiplexed fluorescent Northern hybridization is also applicable for *in planta* gene expression analysis of regulatory genes, as demonstrated for *corR*, *corS* and *corP*. The method described herein can provide further insight into bacterial and plant gene expression during their natural pathogenic or symbiotic interaction(s).

EXPERIMENTAL PROCEDURES

Bacterial strains, growth conditions and harvesting of bacteria

P. syringae was routinely maintained at 28 °C on mannitol-glutamate (MG) medium (Keane *et al.*, 1970). For liquid cultures at 18 or 28 °C, bacteria were grown in Hoitink-Sinden minimal medium (HSC) (Palmer and Bender, 1993) and growth was continuously monitored by measuring the optical density at 600 nm (OD_{600}). For three RNA extractions, a culture equivalent of 15 mL OD_{600} of 1.0 (*c.* 15×10^9 CFU) was harvested at a defined OD_{600} by transfer into a falcon tube filled with 15 mL ice-cold killing buffer [20 mM Tris-HCl (pH 7.5); 20 mM NaN_3) and centrifuged for 15 min at 3200 ***g*** and 4 °C. The supernatant was discarded, the pellet resuspended in 3 mL ice-cold killing buffer, distributed to three 1.5-mL centrifuge tubes and centrifuged for 5 min at 6600 ***g*** and 4 °C. The supernatants were carefully removed, the tubes immediately frozen in liquid nitrogen and stored at –80 °C for further RNA extraction.

Plant material, inoculation procedures and harvesting of plant material

Soybean seedlings [*Glycine max* (L.) Merr. cv. Maple Arrow] were grown in shelves equipped with fluorescent lamps at 22–25 °C, 55% humidity, with a 16-h photoperiod (350 μE/m^2/s). Two days prior to inoculation plants were transferred to growth chambers set to 18 or 28 °C, 55% humidity, with a 16-h photoperiod (350 μE/m^2/s). Bacteria were infiltrated into leaves by means of a needleless syringe at an OD_{600} of 0.1 (*c.* 1×10^8 CFU/mL) and plants were kept in growth chambers at 18 or 28 °C. Three days after inoculation 20 discs (7 mm diameter) containing infected

leaf tissue (about 150–200 mg) were excised with a cork borer, transferred into a 2.0-mL centrifuge tube, immediately frozen in liquid nitrogen and stored at –80 °C for further RNA extraction.

Extraction of total RNA from infected leaf tissue

The total RNA was isolated from infected leaf tissue by a combination of several described methods and reagents (Chang *et al.*, 1993; Gehrig *et al.*, 2000; Geraats *et al.*, 2002; Jaakola *et al.*, 2001; Majumdar *et al.*, 1991; Sambrook *et al.*, 1989). All solutions were prepared with 0.1% (v/v) diethylpyrocarbonate (DEPC)-treated and autoclaved water. Prior to use, glassware, mortars, pestles and spatulas were wrapped in aluminium foil and backed at 200 °C for at least 6 h. Additional precautions were taken for isolating total RNA (Sambrook *et al.*, 1989). Chemicals and reagents, if not otherwise stated, were obtained from AppliChem (Darmstadt, Germany) and, if available, at molecular biology grade. The bacteria plant extraction (BPEX) buffer [0.35 M glycine; 0.7 M NaCl; 2% (w/v) polyethylene glycol 20000 (Merck, Darmstadt, Germany); 40 mM EDTA (0.5 M EDTA stock solution, pH 8.0); 50 mM NaOH (10 M NaOH stock solution); 4% (w/v) SDS] and CTAB/NaCl solution [10% (w/v) CTAB; 0.7 M NaCl] was prepared under gentle warming and stirring. Aliquots of the BPEX buffer were supplemented with 100 mM β-mercaptoethanol shortly before use. Total RNA was extracted according to the steps as listed in Table 1.

RNA quantification and quality assessment

The purity and concentration of the purified RNA was determined spectrophotometrically. An aliquot of the total RNA was diluted 1:70 in 10 mM Tris/HCl buffer (pH 8.0), and RNA concentration was measured by its absorbance at 260 nm (A_{260}) in a Microvolume cell and a Ultrospec 2100 pro UV/Visible Spectrophotometer (Amersham Biosciences, Freiburg, Germany). A_{260}/A_{280} and A_{260}/A_{230} ratios were determined to check for protein and polysaccharide contaminations (Rapley and Heptinstall, 1998). Quality and integrity of RNA was assessed by analysing total RNA aliquots in an RNA 6000 Nano LabChip® Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Denaturating RNA gel electrophoresis, Northern and spot blot

Aliquots of total RNA (1.5 μg bacterial or 6 μg plant/bacterial total RNA per lane) and an RNA size standard [2 μg 0.24–9.5 kb RNA Ladder (Invitrogen, Karlsruhe, Germany)] were separated by denaturing glyoxal RNA agarose gel electrophoresis as described by Burnett (1997) and transferred to a positively charged nylon membrane (Pall, Dreieich, Germany) as described by Ingelbrecht *et al.* (1998). For RNA spot blot analysis, aliquots of total RNA (200 ng bacterial or 1 μg plant/bacterial total RNA per spot) were transferred in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, adjusted with HCl to pH 7.0) to a positively charged nylon

Table 1 Bacteria and/or plant total RNA extraction protocol.

1. This step is only necessary for plant tissue, for bacterial cell pellet start at step 2. Grind the plant tissue sample (150–200 mg) to a fine powder in liquid nitrogen with a pre-cooled pestle and mortar. Transfer the powder to a 2.0-mL centrifuge tube with a pre-cooled spatula. Keep it in liquid nitrogen until BPEX buffer is added.
2. Add 750 μL supplemented BPEX buffer to the grinded plant tissue or the bacterial cell pellet, mix completely by vortexing for 10 s (grinded plant tissue) or pipeting up and down (bacterial cell pellet) and incubate in a pre-heated thermomixer at 900 r.p.m. and 95 °C for 90 s.
3. Add 750 μL water-saturated, stabilized phenol/chloroform mix (5:1; pH 4.0). Shake in a mixer at room temperature for 5 min (speed has to be adjusted until an emulsion is formed). Centrifuge at 16 000 ***g*** and room temperature for 7 min.
4. Transfer 675 μL of the upper phase to a fresh 2.0-mL centrifuge tube, preloaded with 675 μL water-saturated, stabilized phenol/chloroform mix (5:1; pH 4.0). Shake in a mixer at room temperature for 5 min. Centrifuge at 16 000 ***g*** and room temperature for 7 min.
5. Transfer 575 μL of the upper phase to a fresh 2.0-mL centrifuge tube, preloaded with 575 μL water-saturated, stabilized phenol/chloroform/isoamyl alcohol mix (25:24:1; pH 4.0). Shake in a mixer at room temperature for 5 min. Centrifuge at 16 000 ***g*** and room temperature for 6 min.
6. Transfer 495 μL of the upper phase to a fresh 2.0-mL centrifuge tube, preloaded with 550 μL water-saturated, chloroform/isoamyl alcohol mix (24:1) and overlay with 55 μL CTAB/NaCl solution (prewarmed to 55 °C). Shake in a mixer at room temperature for 5 min. Centrifuge at 16 000 ***g*** and room temperature for 6 min.
7. Transfer 435 μL of the upper phase to a fresh 1.5-mL centrifuge tube, preloaded with 145 μL 8 M LiCl solution. Mix by inverting the tube and precipitate RNA at –20 °C for 30 min. Centrifuge at 16 000 ***g*** and 4 °C for 20 min.
8. Decant the supernatant and dab the tube on a fresh paper towel. Wash the pellet by adding 1 mL ice-cold 75% ethanol and inverting the tube several times. Centrifuge at 16 000 ***g*** and 4 °C for 10 min.
9. Optional: Repeat step 8 to remove any LiCl traces.
10. Decant the supernatant and collect ethanol remains by short centrifugation. Remove any ethanol remains by pipeting. Air-dry the RNA pellet briefly at room temperature for 5 min.
11. Resuspend the RNA pellet in 50 μL RNase-free water and store at –80 °C.

Table 2 Oligonucleotide primers used in this study.

Oligonucleotide primer	Nucleotide sequence (5′–3′)*
mucD_fwd	CGAATTTCTCGAGCGCAGCATGC
mucD_revT7	*TAATACGACTCACTATAGGGAGG*GGAGCGGGTAAATATCTGCG
cmaA_fwd	TTTGAGTCGGTCTGCACGCA
cmaA_revT7	*TAATACGACTCACTATAGGGAGG*GCTGTACGTTGTCTACTAG
cfl_fwd	ATGAGTCTGATTTCTGAGTTCCGCA
cfl_revT7	*TAATACGACTCACTATAGGGAGG*TAGTTATTCCTGTGGTGC
corR_fwd	ATGCCGAGCTCTTCGATCTTGC
corR_revT7	*TAATACGACTCACTATAGGGAGG*AGTATCGCCTGGACATGG
corS_fwd	AATACGGCGCGCTGTCAGTT
corS_revT7	*TAATACGACTCACTATAGGGAGG*AATGGATGGCCTAATAGGCG
corP_fwd	ATCACGCCTTGTTCCGTT
corP_revT7	*TAATACGACTCACTATAGGGAGG*TCATTTGCAAATCGAGCAAGATGAGATCG
23SrRNA_fwd#1	ACGTGGACCAGCCCTTAAGTTGTATTG
23SrRNA_revT7#1	*TAATACGACTCACTATAGGGAGG*CCCCCATATTCAGACAAG
23SrRNA_fwd#2	CTTGAGTTCCCTGAAGGGCCGTCGAAG
23SrRNA_revT7#2	*TAATACGACTCACTATAGGGAGG*TGGTCAAGCCTCACGGGC
lsc_fwd	GTCAGTGCGGACTTTCCGGTCATG
lsc_revT7	*TAATACGACTCACTATAGGGAGG*GATCGCGAAAGTTCCAGCTC

*T7 RNA polymerase promoter sequences incorporated in primers are shown in italics.

membrane (Pall) using the Minifold I Spot-Blot System (Schleicher & Schuell BioScience, Dassel, Germany) according to the manufacturer's recommendations. Even and successful transfer of the RNA was verified prior to hybridization by reversible staining of the membrane with methylene blue (Herrin and Schmidt, 1988).

Generation of DIG and fluorescently labelled RNA hybridization probes

RNA probes were generated by *in vitro* transcription of T7-modified PCR products. Gene-specific primers (Table 2) were used to amplify PCR products from genomic DNA of *P. syringae*. The reverse PCR primers carry a T7 promoter sequence at their 5′-end.

DIG-labelled RNA probes were synthesized by using the Strip-EZ™ RNA T7 Kit (Applied Bioscience, Cambridgeshire, UK) and digoxigenin-11-UTP (Roche Diagnostics, Mannheim, Germany), yielding hybridization probes of the following sizes internal to the structural genes: for *mucD*, 511 nt (mucD_fwd, mucD_revT7); *cmaA*, 523 nt (cmaA_fwd, cmaA_revT7); *cfl*, 520 nt (clf_fwd, cfl_revT7); *corR*, 524 nt (corR_fwd, corR_revT7); *corS*, 608 nt (corS_fwd, corS_revT7); *corP*, 143 nt (corP_fwd, corP_revT7); *lsc*, 543 nt (lsc_fwd, lsc_revT7); 23S#1, 146 nt (23S_fwd#1, 23S_revT7#1); and 23S#2, 108 nt (23S_fwd#2, 23S_revT7#2).

Fluorescently labelled RNA probes were generated by incorporation of 5-(3-aminoallyl)-UTP during *in vitro* transcription and a subsequent chemical linkage of an N-hydroxysuccinimide-activated fluorescent dye (Denovo Biolabels, Muenster, Germany). For a 40-μL *in vitro* transcription reaction, 2 μg of T7-modified PCR product adjusted to 20 μL with RNase-free water, 8 μL 5× rNTP-mix [20 mM ATP, 20 mM CTP, 20 mM GTP, 10 mM UTP (Fermentas, St. Leon-Rot, Germany); 10 mM 5-(3-aminoallyl)-UTP (Ambion Europe)], 4 μL 10× T7 RNA Polymerase transcription buffer (Ambion Europe), 2 μL 200 mM $MgCl_2$, 2 μL inorganic Pyrophosphatase [0.3 U/μL, lyophilized inorganic Pyrophosphatase, *S. cerevisiae* (Sigma, Taufkirchen, Germany) dissolved in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA (pH 8.0), 50% (v/v) glycerol], 4 μL T7 RNA Polymerase-Plus™ [20 U/μL (Applied Bioscience Europe)] were combined in a 1.5-mL centrifuge tube, mixed and incubated at 37 °C for 12 h. The PCR template was digested by addition of 2 μL DNase I (RNase-free) [2 U/μL (Applied Bioscience)] and incubation at 37 °C for 15 min. After addition of 5 μL sodium acetate (pH 5.2) and 35 μL 2-propanol, RNA was precipitated at –20 °C for 2 h followed by centrifugation at 16 000 ***g*** and 4 °C for 40 min. Supernatant was removed by pipeting and pellet was washed three times by addition of 1 mL 75% ice-cold ethanol followed by centrifugation at 16 000 ***g*** and 4 °C for 5 min. RNA pellet was briefly air-dried and resuspended in 41 μL RNase-free water. RNA concentration in a 1-μL aliquot was determined.

The *in vitro* synthesized RNA was then chemically labelled with a fluorescent dye. One hundred to 200 μg of the *in vitro* transcribed RNA, adjusted to 40 μL with RNase-free water, was mixed with 40 μL 200 mM sodium bicarbonate buffer (pH 8.5). The Oyster®645-NHS dye [0.2 mg N-hydroxysuccinimide-activated bifunctional pentamethine fluorophor (Denovo Biolabels, Münster, Germany)] was completely dissolved in 20 μL dimethyl sulfoxide by vortexing. The bicarbonate-buffered RNA was immediately added to the dissolved dye and incubated under gentle shaking at room temperature for 45 min. Unbound dye was quenched by addition of 50 μL ethanolamine and gentle shaking at room temperature for 15 min. After addition of 300 μL RNase-free water, 50 μL sodium acetate (pH 5.2) and 350 μL 2-propanol, RNA was precipitated at –20 °C for 2 h followed by centrifugation at 16 000 ***g*** and 4 °C for 40 min. Supernatant was removed by pipeting and pellet was washed twice by addition of 1 mL 75% ice-cold ethanol followed by centrifugation at 16 000 ***g*** and 4 °C for 5 min. RNA pellet was briefly air-dried, resuspended in 50 μL RNase-free water and stored at –20 °C until further use.

Multiplexed fluorescent Northern hybridization and imaging

The Northern or Spot blot membranes were incubated in hybridization solution [50% formamide; 7% SDS; 2% blocking reagent (Roche Diagnostics); 0.1% N-laurylsarcosine; 5× SSC] in roller bottles (Hybaid, Middlesex, UK) at 68 °C for 1 h. The hybridization solution was then discarded and pre-warmed (10 min at 95°C) hybridization solution containing Oyster-645 and/or DIG-labelled RNA probe was added. After hybridization at 68 °C for 16 h, the membranes were washed twice for 5 min at room temperature in 2× SSC/0.1% SDS, followed by two washes for 15 min in 0.2× SSC/0.1% SDS at 68 °C. Hybridization signals for DIG-labelled probes were generated by incubation with anti-digoxigenin-AP Fab fragments (Roche Diagnostics) and ECF substrate (Amersham, Freiburg, Germany) according to the manufacturers' instructions. Fluorescence signals were quantified using a FLA-3000 phosphoimager (ECF: 473-nm laser/520-nm filter; Oyster-645: 633-nm laser/675-nm filter) and the manufacturer's image analysis software package (Raytest, Straubenhardt, Germany).

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