



mRNA extraction of *Xanthomonas fragariae* in strawberry and validation of reference genes for the RT-qPCR for study of bacterial gene expression

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Abstract

This is the first study which describes a unique procedure of isolating of high-quality, intact RNA from strawberry leaves of *Xanthomonas fragariae*, three most suitable reference genes, crucial for the normalization of RT-qPCR data for this pathogen and accurate expression analysis of target genes. In our study, various mathematic algorithms: NormFinder geNorm, Best-Keeper, the delta CT method, RefFinder were adopted for validation of most stable reference genes from nine candidate genes (*flh*, *glyA*, *gyrA*, *gyrB*, *proC*, *pykA*, *recA*, *rpoB*, *rpoD*). The analyses allowing to select three most suitable pioneer reference genes, *gyrB*, *flh*, and *pykA*, that we recommend for the normalization of RT-qPCR data and for the study of the expression of target genes in *Xf*. Moreover, their combination as references allowed for an accurate expression analysis and computation of the fold change of the *flhF* and *iroN2* genes in *Xf*. These two genes are important for the success of the colonization of plant tissue and pathogenicity and sequences of primers designed to study these genes, are presented.

Keywords *Xanthomonas fragariae* · RNA isolation · RT-qPCR · Reference genes · Gene expression

Introduction

Xanthomonas fragariae (*Xf*) is a Gram-negative, slow-growing bacterium and a causal agent of angular leaf spots (ALS) on strawberries (*Fragaria* spp) worldwide [29]. In 1986, *Xf* was classified as a quarantine organism on the A2 list by the European and Mediterranean Plant Protection Organization (EPPO) as well as the European Union (EU) [9]. Currently, its phytosanitary categorization is EPPO A2 list no. 135, EU Annex II/A2. The natural hosts of *Xf* are *Fragaria × ananassa*, its parents *Fragaria chiloensis* and *Fragaria virginiana*,

and various wild strawberry species [9]. Early disease symptoms of ALS are tiny, angular water-soaked areas on leaves, leading to the development of angular spots on the lower leaf surface, that enlarge over time, become reddish necrotic irregular spots on the upper leaf surface and become surrounded by a chlorotic halo. The spots are translucent when using transmitted light but are seen as dark green when using reflected light. Under favourable conditions—moist air and at a temperature of 20 °C, a yellow bacterial exudate is visible on the lower leaf surface and calyces [1, 23].

Occasionally, ALS may cause significant damage to stock of planting material as well as in strawberry production [35], leading to fruit yield losses of about 10% [51], however, usually crop losses are low. The occurrence of ALS was first reported in Minnesota, U.S.A. in 1959 [29]. Since 1970, ALS has been reported in many European countries and has spread globally [9, 30]. Reports on the newest occurrence of ALS are from China and Iran [27, 67]. A comparative genomics approach using genome sequences of different *Xf* strains has been published and indicate high homogeneity of the sequenced *Xf* strains, although some differences have been detected in plasmids [14, 18, 64]. The role of plasmids in the pathogenicity of *Xf* has not been documented.

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Although colonization of and movement in strawberry of *Xf* have been described [66] and critical pathogenesis-related gene clusters have been reported for the *Xanthomonas* genus, e.g., those encoding the structural elements of the Type III Secretion System (T3SS) as well as the Type II Secretion System (T2SS) in *Xf* [14, 18, 64], nothing is known about the expression of bacterial genes during strawberry infection. Understanding the pathogenic process, including changes at the transcription level, is important from the cognitive point of view and it may lead to the development of new disease control strategies.

To study the expression differences of pathogen genes, molecular techniques, such as RNA sequencing (RNA-seq) based on next-generation sequencing (NGS) platforms or quantitative reverse transcription PCR (RT-qPCR), which require a good quantity and quality, of unimpaired RNA, are necessary. To analyze the bacterial transcriptome in plants, crucial steps have to be carried out: (i) correct inoculation of a host plant species and appropriate sampling methods; (ii) selection of adequate RNA isolation and purification methods for the studied organism, allowing for isolation of RNA that is intact and free of contamination. Although laboratory techniques for RNA isolation/extraction using a manual method have already been published and used [33, 37, 42, 52], commercial kits that allow for fast extraction of RNA to be used in the high-throughput analysis are most often used by scientists [22, 25]. From previously published studies, it is known that depending on the type of sample, different RNA extraction kits vary in terms of the yield, quality, and integrity of the obtained RNA [8, 21, 53]. Therefore, to avoid the influence of low-quality RNA on the results of downstream applications, scientists must perform additional work and consider adapting the methodology for the studied organism [19]. Independent of the RNA isolation method selected, it is necessary to consider the need to eliminate contaminating genomic DNA with DNase I, which can adversely affect the results of several applications. Because the majority of bacterial RNA in the bacterial cell is not polyadenylated, as it is in eukaryotes, an additional intermediate step to remove abundant rRNA transcripts from total RNA, which constitutes approximately 90% of the RNA species, is necessary for NGS/transcriptome analysis [6, 15, 55].

Currently, quantitative, reverse transcription PCR (RT-qPCR) is one of the most efficient and robust methods for studying gene expression differences between studied samples e.g. bacteria, plants and for validating data obtained from RNA-seq [5, 32, 42, 45, 49]. Although RT-qPCR is highly specific, sensitive, convenient and rapid in execution [3], it also has certain limitations and requires technical skill to attain precision in the pre-analytical steps, which can influence the Ct values, i.e., obtaining high quality RNA good reverse-transcription and PCR amplification as well as avoiding pipetting errors [21, 61, 63, 65]. Another issue

is the minimization of the variation between samples. To achieve this, an appropriate choice of reference genes that are stably expressed under different environmental conditions for the normalization process is necessary [3, 16, 42]. However, up till now a universal set of genes for normalization doesn't exist. Although a few reference genes, including those for 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin and tubulin, are often used to normalize gene expression for eukaryotic species, it has been reported in numerous studies that the expression levels of these housekeeping genes vary under changing conditions [54, 57, 59, 71]. Moreover, in prokaryotic species, there is no agreement regarding which genes can be used as a universal reference set. Additionally, there are no studies on the selection of the most suitable reference genes in *Xf*. Studying the expression of this pathogen genes would be very interesting in the context of better understanding the pathogenic mechanism. For validation, several mathematic algorithms, which are commonly applied for data normalization [2, 44, 58, 63, 70], have been developed and are supported by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines for more accurate and error-free Analysis of Expression [4, 5].

The purposes of our study were: (i) to select the most efficient method of inoculating strawberry with *Xf*; (ii) to select the optimal RNA extraction method in terms of its ability to obtain high quantity, pure total RNA and intact mRNA from *Xf in planta*; and (iii) to determine the suitability and validate the most appropriate *Xf* reference genes to determine quantitative level of expression of the bacterial genes *flhF* and *iroN2* which are important for the successful colonization of plant tissue and pathogenicity after its inoculation of strawberry leaves employing different mathematic algorithms.

Materials and methods

Inoculation methods

Young potted plants of strawberry cv. Elsanta, after the full development of three true leaves, were inoculated under greenhouse conditions with the *Xanthomonas fragariae* (*Xf*) strain IPO 3485 (IPO = Instituut voor Plantenziektenkundig Onderzoek) isolated in 2011 in the Netherlands, in late spring (May/June). To check the possibilities of successful plant inoculation and symptoms development different methodologies were used. Initially, in the first trial, which was conducted for 4 weeks, the conditions were: 20–25 °C with a relative humidity (RH) of 30–50% under natural light and naturally maintaining humidity conditions. For inoculations of strawberry plants, we used four different methods, each in two versions, the latter being (a) covering the plants

with plastic bags for 48 h to maintain the high-humidity conditions in the first phase of infection (2 days) and (b) not covering the plants and maintaining the above mentioned greenhouse conditions. The four different methods were: (1) high-pressure spraying of the abaxial and adaxial surfaces of leaves with a 10^6 cfu mL⁻¹ bacterial suspension in sterile water of a 48 h culture, grown on Wilbrink's medium [31]; (2) leaf infiltration with a 10^6 cfu mL⁻¹ bacterial suspension using a syringe; (3) treatment of the strawberry leaf surface with carborundum powder and low-pressure spraying of the abaxial and adaxial surfaces of leaves with a 10^6 cfu mL⁻¹ bacterial suspension in sterile water of a 48 h culture, grown on Wilbrink's medium; and (4) treatment of the strawberry leaf surface with carborundum and high-pressure spraying on both sides of leaves with a 10^6 cfu mL⁻¹ bacterial suspension. In the second trial, we tested all inoculation methods described above, but in only one version—plants were not covered with plastic bag—to increase low RH in the greenhouse we 'built' special plastic tent which allows keeping higher humidity level throughout the duration of the experiment. In the second trial, plants were maintained in a quarantine greenhouse at higher temperature 25–30 °C with an RH level of 70–90% under natural light conditions and were systematically watered. Negative control plants were treated similarly, however instead of the bacterial suspension, sterile distilled water was used. RNA isolation was performed to choose the best method for plant inoculation based on the RNA amount obtained. Each technique was tested on 2 sets of 3 plants each. The development of the disease was observed within 4 weeks after inoculation.

Total isolation of total RNA, DNase treatment and depletion of rRNA for NGS

For total RNA extraction from the *Xf* pure culture strain IPO 3485 grown overnight in liquid Wilbrink's medium [31] and from diseased plant material prepared as above, two commercial kits, were used: (1) the TRIzol® Max™ Bacterial RNA Isolation Kit with own modifications, and (2) the Total RNA Purification Kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer's instructions.

For inoculated plant material 10, 15 and 18 days after inoculation (25–30 °C, RH 70–90%), samples were processed and total RNA was isolated. At each time point, RNA was isolated separately from the leaves of each sample inoculated by the four different methods used.

In our first trials, RNA isolation from bacteria *in planta*, independent of the inoculation method (size/amount of symptoms) and RNA kit used, the amount of bacterial RNA obtained was very low (only a few to approx. 15 ng/μL), although a few independent repetitions of isolation were performed. Therefore, the RNA isolation step had to be preceded by additional procedures to better release

bacteria from the plant material. Leaves with disease symptoms were collected, weighed (from 1.5 to 2.5 g were taken), cut into fragments and immediately incubated with RNeasy® Stabilization Solution (ThermoFisher Scientific) for 25 min with shaking in a Excella E24 Incubator Shaker Series (New Brunswick Scientific, Edison, New Jersey, USA) at 26 °C. Subsequently, the mixture was centrifuged, and the pellet was suspended in nuclease-free, sterile H₂O. The amount of water added depended on the weight of the plant material used for RNA isolation and was calculated according to: 2.5 ml of water per 0.5 g of leaf material. Then, the Genomic Mini AX SOIL Spin kit (A&A Biotechnology, Gdynia, Poland), which has high efficiency for isolating genomic DNA from soil, was used. Briefly, the water-leaf-bacteria mixture was vortexed for 30 s at RT and incubated on ice for 5 min. During the incubation time, 1500 μL or more (depending on the weight of the plant material, 500 μL per 0.5 g of material) of a so-called R separation solution (Genomic Mini AX SOIL Spin, A&A Biotechnology, Gdynia, Poland) was poured into a 15 ml Falcon tube and kept on ice for 5 min. The water-leaf-bacteria mixture was slowly and carefully poured over the R solution layer and the tube was carefully placed in a tilting rotor and centrifuged at 4500×g for 10 min. The entire supernatant (two layers) was transferred to a new 15 ml Falcon tube, and nuclease-free H₂O was added up to 30 ml or more (depending on the weight of the plant material and buffer amounts used in the procedure, up to 10 ml per each 0.5 g of material); then, the mixed sample was centrifuged at 4500×g for 10 min. The supernatant was poured out carefully, and the pellet was used for the total RNA isolation procedure with the selected kit.

For the removal of DNA contamination, what is essential for downstream applications, two commercial DNase were used: DNase I (ThermoScientific) and the TURBO DNA-free™ Kit (Life Technologies). The efficiency of removing DNA was tested by PCR with the primers 245A and 245B [46], which are complementary to the genome of *Xf*, preceded by the use of a reverse transcription kit, the iScript cDNA synthesis kit (Biorad, Hercules, CA). The obtained RNA free from DNA was assessed according to the RNA integrity number (RIN) [56] where 10 is recognized to be the most unimpaired pure RNA and 1—the lowest value means the most degraded RNA sample [38]. Determination of the RIN, quality and concentration was performed on an Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA) using the Agilent RNA 6000 Nano LabChip® kit. The procedure according to the manufacturer's instructions was used. Based on our previous experience focused on obtaining of high quantity and quality of bacterial mRNA for next-generation sequencing, depletion of rRNA is an essential step. Therefore, based on our previous experience with removing of rRNA from *Xf* RNA samples, the Ribo-Zero™ Magnetic

Kit (Gram-Negative Bacteria, <http://www.illumina.com>) was used according to the manufacturer's instructions.

Candidate reference gene selection and primer design

Based on a screen of literature data concerning the most often used genes for validation as reference ones in other bacterial species [7, 12, 26, 36, 50], nine reference genes i.e. *gyrB* (DNA gyrase B), *ffh* (signal recognition particle protein), *glyA* (serine hydroxymethyltransferase), *gyrA* (DNA gyrase A), *pykA* (pyruvate kinase), *proC* (pyrroline-5-carboxylate reductase), *recA* (recombinase A), *rpoB* (DNA-directed RNA polymerase subunit beta) and *rpoD* (encoding sigma factor 70), were used for determination of their usefulness as reference for the study of virulence gene expression in *Xf*. To minimize the possibility of co-regulation between the genes, candidates with various biological functions were chosen. Primers were designed with the PrimerSelect program of the LASERGENE package (DNASTAR) based on the gene sequences of strain *Xf* IPO 3485 (=NBC 2815; NBC = Naktuinbouw Bacterial Collection) genome (LT853880, LT853881) (Gétaz et al. 2017). The selected primers were synthesised by Genomed

S.A. (Warszawa, Poland). The specificity of the designed primers and their usefulness were examined twice using conventional and real-time PCR in the manner described by Kałużna et al. [26].

Reverse transcription, qPCR and determination of PCR efficiency

From the total RNA, 150 ng/μL was used for cDNA synthesis by applying an iScript cDNA synthesis kit (Biorad, Hercules, CA). The reaction was conducted in a thermocycler Bio-Rad CFX96 using SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA). The prepared reaction mixture (20 μL in total volume), contained: 15 ng of cDNA, 1 × SYBR Green Supermix and 0.5 mM of each primer pair (Table 1) performed in a separate PCR for each gene. Nuclease free water was used as negative control. The PCR conditions used were as previously described [26] with slight modification, including the use of temperature of 60 °C instead of 59 °C. The amplification efficiency of each reference gene was determined and calculated as described previously [26].

Table 1 Primers sequences for nine candidate reference genes in *Xanthomonas fragariae* for studies of expression with indication of parameters obtained in RT-qPCR

Gene name	Primer sequence (5'–3')	Amplicon length (bp)	T _m (°C)	PCR efficiency (%)	Regression coefficient (r ²)	Reference
<i>ffh</i>	Forward: ATCAACCCGACCGAGACACTT TTC Reverse: GCCGTCGGTCTTGGTCAGCACTAC	123	90	95.7	0.992	This study
<i>glyA</i>	Forward: AGCCGGGCGACACCATTCTG Reverse: AGGCCCTGCGCGTTGACAC	124	89	102.8	0.998	This study
<i>gyrA</i>	Forward: TGGCCGACATCGACAAGGAGAC Reverse: GGCCATACCCACCGCAATACC	134	87	98.5	0.989	This study
<i>gyrB</i>	Forward: TCC ACT ACG AAG GCG GCA TCC Reverse: GGT AGG CGT CGG TCC ATTG	141	87.5–88	104.2	0.998	This study
<i>proC</i>	Forward: GCGCGATGCCCAACACG Reverse: GTCCTCGATCCAAACGGTCAC	137	90.50	109.2	0.993	This study
<i>pykA</i>	Forward: TGACCGAGCGGACAAGGAG Reverse: AGAACGACACTGCGATGAAG	72	86	93.6	0.999	This study
<i>recA</i>	Forward: CGGAATCCTCGGGCAAAAC Reverse: ACAGCAGCAGGTCGTCAAC	150	88.5	93.1	0.999	This study
<i>rpoD</i>	Forward: CTCAAGCGCAAGCAAAAAGTG Reverse: GATCTCGGCCAGGGTCAAGTAG	117	86.5	99.9	0.998	This study
<i>rpoB</i>	Forward: GAGCGTCTGCGTGGTGA AAC Reverse: TGACGTGACGCGCAGTGATG	94	87.0	93.00	0.999	This study
<i>iroN2</i>	Forward: GCTTGACGTCTGCGCTGCTTTTC Reverse: TGAGGCTGCCACGGATACC	153	91.50	99.5	0.995	This study
<i>flhF</i>	Forward: CTCTGGAGCTAATGGACGACTACG Reverse: GACAGCAGGCCCCAGCATCAACC	118	87.50	105.6	0.997	This study

Expression data and stability of the reference genes

The data concerning expression for the reference genes were calculated as the quantification cycle (Cq) values obtained from the RNA originating from a pure culture and the RNA of bacteria *in planta*. Five programs as in our previous paper [26], NormFinder [2], geNorm [63], BestKeeper [44], the delta CT method [58] and the RefFinder [70] were used to determine the level of stability of the candidate reference genes. The methodology of data preparations and Cq conversions for particular programs and formula used for efficacy calculations are described in Kałużna et al., [26].

Expression analysis of the pathogenicity—related genes

For expression profile analysis, the following target genes, the products of which are important for the successful colonization of plant tissue and pathogenicity were selected: *flhF*, which is a flagellar biosynthesis regulator, and *iroN2*, which is the TonB-dependent receptor. Although the latter does not play a direct role in pathogenesis it allows Gram-negative bacteria to take up scarce resources from nutrient-limiting environments as well as siderophores, heme etc., [17, 40]. The primers for the target genes (Table 1) were designed using the PrimerSelect program of the LASER-GENE package (DNASTAR) and synthesized by Genomed S.A. (Warszawa).

In order to compute the relative fold changes in gene expression of the studied genes, data from Cfx96 (Bio-Rad, Hercules, CA) were analysed using the comparative $2^{-\Delta\Delta Ct}$ method [34] was used.

Results

Strawberry plant inoculation

Comparing the inoculation efficiency under two temperature/humidity conditions, earlier and more intensive symptoms were observed at higher temperatures 25–30 °C and higher humidity 70–90%. In the temperature range 20–25 °C and at the RH 30–50%, the first symptoms, necrotic lesions were observed quite late, at 14 days after inoculation, on strawberry plants inoculated by infiltration with a syringe. In the case of higher temperature/humidity, the symptoms appeared earlier, with the first symptoms observed 7–10 days after inoculation of plants sprayed under high pressure and after infiltration. Similar symptoms were observed in the case of applying of low pressure but with less intensive symptom formation.

The lesions size slightly increased throughout the experiment with 1–2 mm and a few additional spots developed

due to the secondary spread of the bacteria. In the infiltration method, experiments apart from a water soaked tissue, necrosis of leaf tissue at the infiltration location was also observed (Fig. S1). Disease symptoms were not observed in non-inoculated control plants.

Total RNA isolations, DNase treatment and depletion of rRNA

The quality and quantity of RNA obtained from individual samples largely depended on the method of inoculation. The analysis of the total RNA concentration and integrity (RIN parameter) showed that, the highest concentration ($> 2 \mu\text{g } \mu\text{L}^{-1}$) and best quality RNA (RIN 9.8) were obtained from plants, 15 days after inoculation by spraying under pressure (kept at 25–30 °C with a humidity level from 70–90%) and using total RNA Purification Kit (Norgen Biotek) proceeded by the Genomic Mini AX SOIL Spin kit (A&A Biotechnology, Gdynia, Poland). For pure culture cells of the *Xf* strain IPO 3485 the RIN was 10. When low-pressure spraying was applied, lower concentrations and slightly lower RIN values (lower of 0.5–0.7 lower) were recorded. When using the TRIzol® Max™ Bacterial RNA Isolation Kit, the RIN was approximately 7–8. Smaller amounts and bad quality RNA were obtained from leaf samples infiltrated by a syringe (RIN < 5). This could possibly be due to the dark brown necrotic spots formation in the infiltration points where a rapid host cell death likely leads to activity of reactive oxygen species (ROS) causing some degree of RNA degradation.

Both DNases used were equally effective for the removal of genomic DNA; however, we chose DNase I for our study (ThermoScientific). The Ribo-Zero™ Magnetic Kit used to obtain mRNA for next-generation sequencing allowed us to obtain a good quantity of 30–70 ng/ μL of *Xf* mRNA from all samples.

Selection of reference genes from candidates and design of the primer

All nine candidate reference genes were used to further examination of gene expression stability and their ranking based on the data from conventional and real-time PCRs. All the data concerning the name of genes used, forward and reverse primer sequences, length of amplicons, melting temperatures (T_m), PCR efficiency, and regression coefficients obtained are listed in Table 1. Single product with the expected size from 72 to 150 bp was obtained with the primer pairs designed for each gene and used in PCRs for all genes (Table 1, Fig. S2). The verification of primers by real-time PCR gave melting curves with a single peak, where the T_m varied from 86 to 90.50 °C and no unexpected or additional peaks were noticed (Fig. S3). This confirmed that the

primers were not able to form dimers. The determined efficiencies of the primers designed for all candidate reference genes ranged from 93.1 to 109.2% for all genes depend on each primer pairs/genes. The linear correlation coefficients (r^2) were from 0.989 to 0.999 (Table 1).

Stability and expression levels of the candidate reference genes

Cq values obtained (Fig. 1) for the candidate reference genes were quite variable, indicating varied expression. Therefore, statistical tools were necessary to estimate the stability of genes studied allowing us to determine the most appropriate references for studies of gene expression (Fig. 1). The Cq values obtained from all candidates varied from 20.17 to 31.25. The median and average Cq values varied for all candidate genes studied. The *rpoD* gene had the lowest mean value (24.24), while *proC* the highest one (29.57).

Stability of expression of the reference genes

Statistical algorithms applied in geNorm, NormFinder, BestKeeper, the delta-Ct method, and the RefFinder web-based tool programs, were used to determine the stability of the expression of the candidate reference genes (Table 2).

Based on the geNorm analysis, *gyrB* and *ffh* genes followed by *gyrA* and *rpoB* genes possess the highest stability as they showed the lowest M-values (0.697, 0.740, 0.749 and 0.834, respectively) (Table 2). *RpoD* turned out to be the least stably expressed gene. Based on the geNorm analysis, the *gyrB* and *ffh* followed by *gyrA* and *rpoB* genes had the lowest M-value (0.697, 0.740, 0.749 and 0.834, respectively), what pointing out that these genes have the highest stability (Table 2). Only *rpoD* showed an M-value above the determined default limit of $M < 1.5$ and therefore turned out to be the least stably expressed gene.

The analysis of V-value—pairwise variation determined for the reference genes studied showed that value $V_{4/5}$ was below the default threshold value of 0.15

The Normfinder analysis indicated that the most stably expressed genes were *gyrB*, *ffh*, *rpoB* and *gyrA* (SV: 0.208, 0.244, 0.268 and 0.299, respectively). *rpoD* and *recA* were the least stably expressed genes findings similar to those obtained by geNorm (Table 2). Additionally, it was shown that *gyrA* and *gyrB* are the best combinations of genes for further studies of the expression of interest genes (calculated stability value 0.132).

The results obtained by BestKeeper analysis was different from both: GeNorm and Normfinder and revealed that all genes can be treated as inconsistent as reference genes for RT-qPCR because they have an SD value

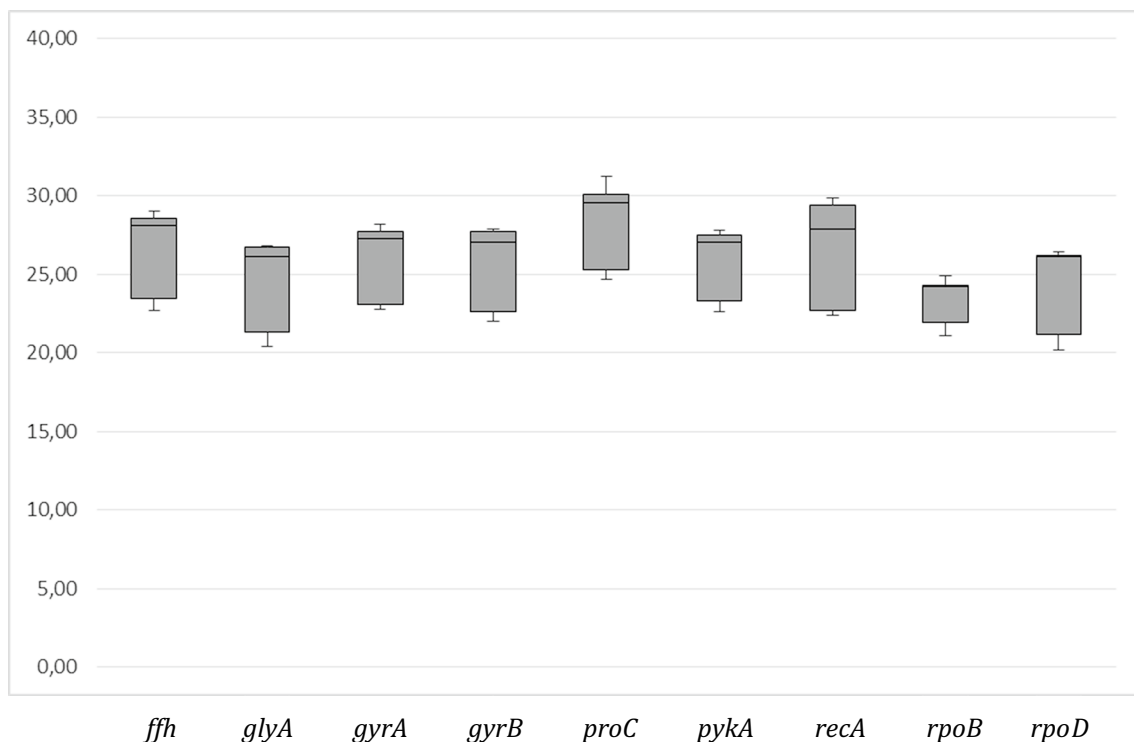


Fig. 1 Cq values (expression levels) for nine candidate reference genes samples. The box indicates the 25th and 75th percentiles while the whiskers caps the maximum and minimum values. A centre line across the boxes indicate the median

Table 2 Stability values and ranking list of the nine candidate reference genes of *X. fragariae* studied obtained of all analysed samples from strawberry leaves cv. Elsanta based on results generated from RefFinder, geNorm, NormFinder, BestKeeper and Delta Ct

Ranking	geNorm		NormFinder		BestKeeper		Delta Ct		Comprehensive ranking	
	Gene	M-value	Gene	Stability value	Gene	SD [\pm CP]	Gene	Average of SD	Gene	Geomean of ranking values
1	<i>gyrB</i>	0.697	<i>gyrB</i>	0.208	<i>rpoD</i>	1.08	<i>gyrB</i>	0.46	<i>gyrB</i>	1.41
2	<i>ffh</i>	0.740	<i>ffh</i>	0.244	<i>gyrA</i>	1.74	<i>pykA</i>	0.52	<i>ffh</i>	2.78
3	<i>gyrA</i>	0.749	<i>rpoB</i>	0.268	<i>pykA</i>	1.85	<i>ffh</i>	0.54	<i>pykA</i>	2.78
4	<i>rpoB</i>	0.834	<i>gyrA</i>	0.299	<i>gyrB</i>	2.01	<i>rpoB</i>	0.56	<i>gyrA</i>	3.81
5	<i>proC</i>	0.853	<i>pykA</i>	0.397	<i>ffh</i>	2.07	<i>gyrA</i>	0.58	<i>rpoB</i>	4.86
6	<i>glyA</i>	0.934	<i>proC</i>	0.427	<i>proC</i>	2.09	<i>glyA</i>	0.59	<i>rpoD</i>	5.20
7	<i>pykA</i>	1.031	<i>glyA</i>	0.497	<i>rpoB</i>	2.10	<i>recA</i>	0.63	<i>glyA</i>	5.58
8	<i>recA</i>	1.278	<i>recA</i>	0.549	<i>recA</i>	2.28	<i>proC</i>	0.73	<i>recA</i>	6.96
9	<i>rpoD</i>	1.500	<i>rpoD</i>	0.821	<i>glyA</i>	2.30	<i>rpoD</i>	1.27	<i>proC</i>	7.44

calculated greater than 1. Of all analyzed candidates the genes *rpoD* and *gyrA* were designated as the most stably expressed with SD: 1.08 and 1.74, respectively followed by *pykA*, *gyrB* and *ffh* (SD: 1.85, 2.01 and 2.07, respectively) (Table 2). *glyA* was found to be the least stably expressed gene.

According to the delta Ct method genes *gyrB*, *pykA* and *ffh*, (SD: 0.46, 0.52, 0.54) were the most stably expressed ones; and *rpoD* the least stably expressed gene, with an SD of 1.27 (Table 2).

Based on the RefFinder web-based tool, the ranking of the candidate reference genes was similar to those obtained by geNorm and NormFinder. The results of BestKeeper were similar when using RefFinder, although the efficiency value was not included as in the original BestKeeper software. The complete ranking from the RefFinder was as follows: the *gyrB*, *ffh*, *pykA* and *gyrA* (GM: 1.41, 2.78, 2.78, 3.81, respectively) were the most stably expressed genes, and *recA* and *proC* were the least stably expressed (Table 2; Table S1).

Expression analysis of the *flhF* and *iroN2*

Based on the analysis of the gene expression differences of the *flhF* and *iroN2* genes normalized with *gyrB*, *ffh*, and *pykA* as the selected reference genes, *flhF* was up-regulated in *planta*; however, *iroN2* was down-regulated. The *flhF* gene was up-regulated by 231.64-fold (SE 42.01) compared with that of a pure bacterial culture; however, for the *iroN2* gene, the opposite result was noted i.e., down-regulation in *planta* (– 16.2-fold; SE 2.072) compared to pure bacterial culture. Compared to the results of transcriptome analysis by RNA seq, the expression values of the *flhF* and *iroN2* genes obtained in this study were similar [48].

Discussion

Xanthomonas fragariae causes damages to plant stock as well as strawberry production worldwide. Therefore, identification of its pathogenicity factors would be of great value from cognitive and practical points of view. Several pathogenesis-related gene clusters have already been described [14, 18, 64], and expression analysis of these clusters in different stages of the infection process would be valuable. The present study is one of a few works focused on the selection and validation of bacterial reference genes for the normalization of RT-qPCR gene expression [20, 26, 50, 62, 69]. To the best of our knowledge, no specific internal references for *Xf* have been described. Normalization of the expression level of the studied genes, against validated, stably expressed reference genes allow to avoid the obtaining of false positive/false-negative results. It also can compensate and equalize variations that can arise due to factors e.g. RNA quality of samples analyzed, cDNA synthesis. Therefore, this study constitutes the first work on the validation of candidate reference genes using a numerous algorithms for use for the relative quantification of *flhF* and *iroN*—target gene expression in *Xf*. RT-qPCR has recently become the method of choice for analyses, allowing the determination of the different expression changes of target genes relative to reference genes [45], because it is simple, reproducible and sensitive [3]. However, RT-qPCR requires technical skills e.g. precise in pipetting) and the development of a few analytical steps before expression study e.g. crucial normalization and validation assays.

Our methodology allowed the selection of the most suitable inoculation method of strawberry leaves with *Xf* based on analyses of four methods of obtaining high-quality RNA, which appeared to be strictly depended on the method of inoculation.

The first disease symptoms appeared in plants inoculated with infiltration 25–30 °C; 70–90% RH. In the case of the pathogenicity tests performed by Kastelein et al. [28], the selected optimal temperature differed from our—it was lower and ranged from 17–20 °C and 80% RH, it gave them under this condition and 10% of the diseased leaves already diseased 7 days post inoculation. Early symptoms of infection in angular water-soaked lesions on the abaxial surface of leaves appeared approximately 8 days after inoculation in the study of Allan-Wojtas et al. [1], they also used lower temperatures 18–22 °C and high relative humidity (> 95%). Wang et al. (1966) inoculated plants with Xf100-GFP mutant strain at 25 °C and 60% relative humidity during the day and 15 °C and 90% RH during the night [66] and first lesions as in our work appeared 7 days post-inoculation. In our case, covering plants for 48 h with plastic bags after inoculation to maintain high humidity but under lower temperature conditions did not positively influence the speed of disease symptom development, and moreover, covering with plastic bags caused significant infestation by fungal pathogens, which interfered with bacterial RNA isolation (data not shown). In Kastelein et al. [28] and Allan-Wojtas et al. [1], air-pressurized spraying onto the abaxial surface of leaves was applied as a successful method of inoculation, similar to the methodology used in our paper. The methodology applied (°C and RH) and results obtained—symptoms appearing—in presented above studies suggest that besides the obvious essential high humidity conditions the use of an optimum temperature is also crucial for the bacteria multiplication and early symptoms development. However, in the work of Kastelein et al. [28] 20 °C was used throughout the entire experiment, and the results anyway appeared quite early. Additionally, similar inoculation methods, which were found in our case to be best for inoculation, such as high-pressure spraying on both sides of leaves with a bacterial suspension in sterile water, were used for pathogenicity tests that were performed to confirm the pathogenicity of the pathotype strain of *Xanthomonas arboricola* pv. *fragariae* (another pathogen of strawberry [10]). In their work only slight differences in symptoms development were observed for inoculations using low-pressure spraying of the bacterial suspension (10^7 cfu mL⁻¹) on the lower surface of leaves [10].

The highest concentration and best quality of total RNA were obtained from plant samples inoculated by spraying under pressure, 15 days after inoculation that were kept at 25–30 °C with a humidity level 70–90%. During the first attempts, the main obstacle was the inability to isolate sufficient quantities of bacterial RNA. In the first few trials we used a procedure that worked well for another bacterium, *Erwinia amylovora* in apple shoots [25], but in the case of *Xf*, the efficacy of RNA isolation was much lower. We supposed that this failure was caused by the inefficient release of bacterial cells from plant tissue. To overcome this problem,

the most crucial step of the total RNA isolation procedure of *Xf* from strawberry leaves consisted of a combination of different systems, namely, the partial adoption of the reagents and methodology of the Genomic Mini AX SOIL Spin (A&A Biotechnology) kit followed by application of the total RNA Purification Kit (Norgen Biotek). Our study demonstrated once more that there is no common isolation method for all types of microorganisms (especially bacteria for different plant material or when used in different environments; thus there is a need to develop the protocol for an individual need and purpose [41, 42]. We assume that the procedure we developed can work for other bacteria, particularly in cases in which the extraction of bacterial cells from a particular tissue is difficult, but that also has to be tested in each individual host–pathogen combination.

From the literature, it is known and recommended that the minimum RNA integrity number (RIN) for analyses should be at least seven; higher than eight is considered to be optimal [11, 24]. Here, we obtained RNA with a RIN near or equal to 10 in all samples. However, so high RINs were not obtained in other bacterial species, such as *Erwinia amylovora*, because apparently the ribosomal RNA ratio is disturbed due to a 99-bp intervening sequence (IVS) insertion within the 23S rRNA gene [47, 60]. It is known from the literature that IVSs are transcribed but next finally removed by RNase III, resulting in fragmented rRNA [47]. The presence of two extra peaks indicates the presence of these fragmented rRNAs, the latter making it impossible to obtain the maximum RIN value.

In our study, nine reference housekeeping genes of *Xf* were chosen and their expression determined. The amplification efficiency of the designed primers for these genes was calculated using the conventional standard curve method. We preferred this method, although it has some disadvantages (it is time-consuming, and requires production of repeatable and reliable standards [43, 72]; its efficiency of reaction differs from the relatively stable in the exponential phase to gradually decreasing later [68]; the efficiency calculated can be over-estimated [50]). This method was also found to be reliable and preferred in our previous expression study and other recently published papers [26, 39, 42, 54].

To evaluate the candidate reference genes stability to use the most stably expressed for normalization of RT-qPCR data and for calculation of the expression of genes involved in pathogenicity and in playing a role in plant tissue colonization (*flhF* and *iroN2*), several different mathematical algorithms that have already been used by several authors were adopted [32, 39, 42, 50, 73]. Based on the results of the algorithms used, the comprehensive range demonstrated that *gyrB*, followed by *ffh*, *pykA* and *gyrA*, were the most stably expressed reference genes. According to the MIQE Guidelines, at least two reference genes are acceptable and required to determine gene expression changes; however,

three reference genes are preferred, and as stated by authors the use of only one is not acceptable [5]. The results of the expression analysis using three reference genes allow to obtaining more precise and trustworthy results. It has been reported that the use of a single reference gene can generate up to a 20-fold error in the expression data [59, 63].

The *gyrB* gene was also identified as the most stably expressed gene in *Aeromonas salmonicida* subsp. *salmonicida* [50] and *Staphylococcus pseudintermedius* [7]; however, it was expressed at varying levels under different experimental conditions in the validation of the reference genes for RT-qPCR analysis in *Burkholderia pyrrocinia* [69]. *GyrA* and *ffh* were the most stable reference genes in normalization studies of reference genes in *Azospirillum brasilense* [36] and *Pectobacterium atrosepticum* [62], respectively. *RpoB* was present in a group of the most stably expressed genes [20] among candidates selected in studies on *Xanthomonas citri* subsp. *citri* genes during the infection of *Citrus sinensis*. In our study, *rpoB* was also one of the most stably expressed genes. The *rpoD* gene was identified as one of three genes whose expressions were stable throughout the growth of *Gluconacetobacter diazotrophicus* strain PAL5 on different carbon sources [13], while in our case, and also for *Aeromonas salmonicida* subsp. *salmonicida* [50], this gene was the least stably expressed gene. The variation in the results obtained in many papers for a particular organism and environment applied, point at the necessity to perform gene normalization for each environment and species.

When analysing *flhF* and *iroN2*, it was found that *flhF*, a flagellar biosynthesis pathway gene, was highly up-regulated, while *iroN2*, which encodes the TonB-dependent receptor, was down-regulated *in planta*. The results of the reference *gyrB*, *ffh* and *pykA* genes in terms of the relative gene expression analysis agree with the results obtained in preliminary analysis of the expression of *flhF* and *iroN2* in transcriptomes obtained after RNA-seq [48]. *GyrA* did not make significant changes in the results obtained (data not shown). Moreover, as was the case in the study of Rivera et al. [50], geNorm analysis showed that at least five reference genes should be used in all studies. In our hands, however, the use of three, for more genes, did not make meaningful changes in the results.

Conclusions

We present in this article the research that led to the development of a novel, procedure for the isolation of high-quality, intact RNA of *Xanthomonas fragariae* *in planta*. The described unique methodology can be adopted for other bacteria, in each individual host–pathogen combination. Also, our results provide the designed and validated primers for the group of reference genes which can be useful for other

studies of gene expression analysis of *Xanthomonas fragariae*. Numerous programs and algorithm analyses enabled the selection and first-time use of three the most suitable reference genes, *gyrB*, *ffh*, and *pykA*, that are crucial for the normalization of RT-qPCR data. The adopted genes used for the normalization of RT-qPCR data allowed an accurate expression analysis and computation of the fold change of the *flhF* and *iroN2* genes which play a role in the successful colonization of plant tissue and pathogenicity by *Xf*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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