Short Term Scientific Mission within the framework of COST Action 863 *Euroberry research: from genomics to sustainable production, quality and health*.

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STSM title: Analysis of expression profiles of a range of disease resistance genes in strawberry (*F.×ananassa*), using Real-Time PCR.

Objectives of the visit

The purpose of Short Term Scientific Mission at East Malling Research was to improve the knowledge about conducing gene expression analysis experiments aimed to obtain the expression profiles of a set of disease resistance genes in strawberry plants infected with *Verticillium dahliae*. Real Time reverse transcriptase PCR is a powerful tool for RNA quantification, and has recently become a routine to monitor gene expression in plants. Besides its unquestionable advantages, like high specificity, sensibility and efficiency in signal detection, the technique suffers from certain pitfalls, of which the most important are: dependence of the results on template quality, quantity and optimal assay design, as well as the fact, that data analysis – if carried out improperly - can easily become very subjective.
The main objectives of the visit were therefore to learn the principles of designing and performing Real-Time reverse transcription polymerase chain reaction (RT-qPCR) analyses as well as interpretation of results obtained.
During this STSM the special emphasis was put on the experimental design features crucial for the RT-qPCR assays performance, i.e.: method of RNA extraction from plant tissues and reverse transcription for cDNA strand synthesis, proper designing of primer pairs for RT-qPCR, choosing the appropriate candidates for reference genes and finally – validation of reference genes' stability under the experimental conditions, which is of great importance for the accuracy in relative gene expression analyses.

Description of work carried out during the visit

During the STSM the complete procedure of RT-qPCR analysis was performed in order to familiarize the participant properly with experimental design and all procedures. Wild strawberry plants (*Fragaria vesca*) were used as a training material for practising the RNA extraction and all following steps leading to gene expression profiling. Plants of two octoploid strawberry cultivars (*Fragaria x ananassa*) differing in susceptibility to Verticillium wilt: 'Dukat' and 'Elsanta', were artificially infected with *Verticillium dahliae* prior to the visit and used for the proper experiments on genes expression during the STSM.
The work carried out consisted of the following steps:

a) total RNA extraction from plant tissues;
Total RNA from root tissue of 'Dukat' and 'Elsanta' plants was extracted using protocol described by Zeng and Yang (*RNA Isolation From Highly Viscous Samples Rich in Polyphenols and Polysaccharides,* Plant Molecular Biology Reporter 20: 417a–417e, December 2002)

b) the reverse transcriptase-dependent synthesis of cDNA on the template of RNA;
cDNA synthesis on the obtained mRNA template was performed using Qiagen Omniscript kit, according to protocol provided by the manufacturer.

c) designing the primers for RT-qPCR
The full length sequences of potential resistance genes, as well as reference genes for the relative quantification, were estimated based on published genome sequence and gene function database for *Arabidopsis thaliana* (ref?). As the entire sequence of octoploid strawberry (*Fragaria x ananassa*) genome remains unknown, chosen *Arabidopsis* sequences were compared (BLAST) against the diploid *Fragaria vesca* genome database, in order to extract the gene analogues for strawberry *sensu largo*. To confirm the presence of corresponding gene analogues in octoploid strawberry genomic DNA, the full length product primers were designed based on obtained *F. vesca* sequences and then used in PCRs on the template of *Fragaria x ananassa* gDNA. PCR products were subsequently cloned and sequenced. Sequences obtained were processed using GenScan software tool in order to estimate exons' number and positions. Potential introns were then removed, and resultant cDNA sequences were used as templates to design appropriate Real-Time PCR primers. All oligonucleotides for both: full-length product and Real-Time polymerase chain reactions (PCR) were designed using Primer3 software tool.

d) calculation of RT-qPCR reactions' efficiency
The efficiency calculation run was performed for all reference and target genes in order to determine the values of variables in the standard curve equations obtained. These values are essential for choosing the appropriate method of relative expression analysis, as well as for the evaluation of reference genes stability using geNorm software. Separate efficiency runs were performed for cDNA template from *Fragaria vesca* and from *Fragaria x ananassa.* In order to create the standard curves, for each primer pair RT-qPCR assay undiluted as well as 2x, 4x, 8x and 16x diluted cDNA templates were used.

e) selection of the most appropriate reference genes
To evaluate the stability of reference genes expression under the experimental conditions, RT-qPCR run on the cDNA templates obtained from the plants put under different treatment and untreated control plants was performed. Basing on the mean cycle threshold values (Ct) together with standard curves' slope and intercept values received from efficiency calculation run, the input data for geNorm software tool were prepared. The geNorm calculations allowed us to determine the genes for which expression remained unchanged within the course of experimental treatment.

f) relative quantification of target genes against chosen reference genes
Several RT-qPCR assays were performed on the cDNA templates obtained from infected and control strawberry plants of two cultivars differing in susceptibility to Verticillium wilt. In all assays two genes with stable expression profiles were included, against which the target genes expression values were normalized. The ddCt method was used for the relative quantification of target genes.

Description of the main results obtained

a) RNA extraction and RT

Total RNA was successfully extracted from both wild and cultivated strawberry root and leaf tissues. RNA presence and integrity were confirmed using agarose gel electrophoresis and ethidium bromide staining. RNA quantity was measured using a NanoDrop spectrophotometer.
Synthesis of cDNA strand by reverse transcription was confirmed by conventional PCR with different primer pairs designed for specific product amplification in F. vesca. PCR products were visualised after agarose gel electrophoresis using ethidium bromide staining.

b) designing primers for *F.x ananassa*

According to the procedure described above, primer pairs for 8 reference genes and for 2 target genes were designed. Based on sequencing results for octoploid strawberry, five out of eight reference and one out of two target genes were chosen for further investigation.
Chosen set of genes is listed in table below.

Gene name	**RT-qPCR function**
Fragaria vesca anthocyanidin reductase (FvANR) putative gene	Target gene
Fragaria vesca glyceraldehyde 3-phosphate dehydrogenase (FvGAPDH) putative gene	Reference gene
Fragaria vesca 60S ribosomal protein (Fv60S) putative gene	Reference gene
Fragaria vesca actin (FvAct) putative gene	Reference gene
Fragaria vesca polypirimidine tract-binding proteine (FvPTB) putative gene	Reference gene
Fragaria vesca UDP-galactose/UDP-glucose transporter-related (FvUUg) putative gene	Reference gene

c) RT-qPCRs' efficiency calculation

As a result of the efficiency calculation assay on a series of diluted cDNA templates, the standard curve equations were obtained for each amplification product. The components of standard curve equation are:

$$y=kx+n$$

where: “y” is mean threshold cycle value
“k” is curve's slope
“x” is a logarithm of cDNA quantity
“n” is Y-intercept

The standard curve equations obtained for chosen reference and target genes are listed in table below.

Gene	**Standard curve equation**
FvANR	y=-3,706016x+35,434654
FvGAPDH	y=-3,337868x+27909000
Fv60S	y=-3,078293x+30,300295
FvActin	y=-3,270438x+35,896954
FvPTB	y=-3,405552x+36,145771
FvUUg	y=-3,509534x+37,140278

d) reference genes stability calculation

As a result of the RT-qPCR assay performed on cDNA samples obtained from plants put under different experimental conditions, mean threshold cycle values (Ct) for each potential reference gene were calculated. Based on Ct values combined with slope and intercept values received from the efficiency run, cDNA quantities were calculated for each gene and each template type. Quantity values were then exported to geNorm software tool, where the most suitable reference genes were found for given experimental design.

Below an exemplary table with the geNorm output data is shown. The "M" value (in bold) indicates the stability of expression. In this example, two genes (Fv60S and FvActin) had been calculated as the most stable expressed within given set of cDNA samples.

Sample ID	Fv60S	FvActin	Normalisation Factor
Fv cDNA untreated 1	2.10E-01	1.70E-01	**0.6201**
Fv cDNA treated 1	2.50E-01	2.80E-01	**0.8684**
Fv cDNA untreated 2	2.40E-01	1.50E-01	**0.6227**
Fv cDNA treated 2	2.40E-01	2.10E-01	**0.7368**
Fv cDNA untreated 3	9.70E-01	1.00E+00	**3.2325**
Fv cDNA treated 3	4.30E-01	3.60E-01	**1.2913**
Fv cDNA untreated 4	1.00E-01	9.00E-02	**0.3114**
Fv cDNA treated 4	1.00E+00	9.00E-01	**3.1137**
M < 1.5	**0.250**	**0.250**	

e) relative gene expression analyses

Several RT-qPCR analyses were run for the relative expression calculation of FvANR gene in uninfected and *Verticillium dahliae* infected strawberry plants of 'Dukat' and 'Elsanta' cultivars. However, due to the poor performance of target gene amplification on given cDNA templates, relative expression analysis of FvANR gene was not fully successful. In such case the primers for this product need to be redesign and the whole procedure needs to be repeated in order to obtain more reliable results.

Both - participant and host agreed, that due to the STSM time limitations, this part of work will be accomplished by the participant after finishing the mission.