Scientific Report of Gianluca Lucchetta on the STSM
(ref. code: ECOST-STSM-FA0807-151111-012373)
at NIB, National Institute of Biology,
Department of Biotechnology and Systems Biology, Ljubljana, Slovenia
15/11/2011 – 30/11/2011

Innovative techniques for diagnostic and gene expression studies of grapevine phytoplasmas

The aims of this training were the following:

a) comparing the real time PCR protocols used in the two groups, Italian and Slovenian, for the diagnosis of grapevine phytoplasmas;
b) comparing the real time PCR protocols used in the two groups for the gene expression studies
c) learning the practical and theoretical basis of the microarray analyses used at NIB to study gene expression in the interaction grapevine-phytoplasma.

a) Real time PCR diagnostic protocols

The first objective of this training was the comparison of the real time PCR protocols used in the two groups, Italian and Slovenian, for the identification of grapevine phytoplasmas and for the gene expression studies. The protocol comparison was done with some grapevine leaf samples collected from symptomatic and healthy plants. In the first phase, real time PCR assays used by the two groups for routine diagnostic of grapevine phytoplasmas were performed on the two batches of samples (Angelini et al., 2007; Hren et al., 2007); in the second phase, performance of the endogenous grapevine genes used as reference genes in transcriptomic studies by the two groups (Hren et al., 2009a; Hren et al., 2009b, Bertazzon et al., 2011) was compared too.

For the diagnostic purpose, leaf aliquots were freeze-dried powdered and nucleic acid extraction was performed according to the protocol in use in the two groups (Angelini et al., 2007, Pirc et al., 2009, with minor modifications). In the first one, CTAB extraction buffer-based method was used, whilst in the second one an automated DNA-extraction method based on magnetic beads (QuickPick™) was employed. DNA amplification was performed using real time quantitative PCR with TaqMan probes for both the methods.

In the Italian protocol (Angelini et al., 2007) group-specific probes and primers for the diagnosis of phytoplasmas target the 16S rRNA genes of 16SrI (AY), 16SrV (FD) and 16SrXII (BN) group phytoplasmas, respectively. Primers and probe for real-time PCR endogenous control target the grapevine chloroplast chaperonin 21 gene. TaqMan® probes were labelled at the 5′-end with 6-carboxyfluorescein (FAM) and at the 3′-end with 6-tetramethylrhodamine (TAMRA) as fluorescent quencher (Tab. 1).

In the Slovenian protocol (Hren et al., 2007) primers and probes for the universal detection of phytoplasmas and for specific detection of BN and FD were used (Tab. 1). Endogenous control targets 18S rRNA gene (eukaryotic 18S rRNA TaqMan endogenous control, Applied Biosystems) and amplified plant DNA co-extracted with phytoplasmic DNA. TaqMan® MGB probes were labelled with 6–carboxyfluorescein (FAM) at the 5′ end and a non-fluorescent quencher (NFQ) with minor groove binder (MGB) at the 3′ end.

Table 1. Characteristics of primers and probes used in the two protocols.

Origin	Specificity	Location	Target	amplicon size (bp)	Reporter dye	Quencer dye	Efficiency (E)	Reference
Phyto.[a]	Aster Yellow	16S rRNA	16s rRNA I	102	FAM	TAMRA	0,99	Angelini et al., 2007
Phyto.	Flavescence dorée	16S rRNA	16s rRNA V	103	FAM	TAMRA	0,95	Angelini et al., 2007
Phyto.	Bois noir	16S rRNA	16s rRNA XII-A	98	FAM	TAMRA	0,97	Angelini et al., 2007
Grape. (ref.)[b]	Chaperonine 21	chloroplastic genome	Chaperonine 21	90	FAM	TAMRA	0,99	Angelini et al., 2007
Phyto.	UniRNA	16S rRNA	16s rRNA V	73	FAM	MGB	1,00[d]	Hren et al.,

								2007
Phyto.	UniRNA	16S rRNA	16s rRNA XII-A	73	FAM	MGB	0,93[d]	Hren et al., 2007
Phyto.	FDgen	sec Y gene	16s rRNA V	85	FAM	MGB	0,89	Hren et al., 2007
Phyto.	BNgen	Stol11 genomic fragment	16s rRNA XII-A	71	FAM	MGB	1,02	Hren et al., 2007
Eukar. (ref.) [c]	18S	18S rRNA	18s rRNA	187	VIC	MGB	0,89	Hren et al., 2009

Legend:
a: Phytoplasma; b: Grapevine (reference gene); c: Eukaryiotic (reference gene); d: target diluted in water

All real-time PCR reactions were performed on an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems) in optical 384-well plates with optical adhesive covers (both Applied Biosystems) using universal cycling conditions (2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C, with 9600 Emulation mode), which allowed running of all reactions on the same plate. Real-time PCR was performed in a final reaction volume of 10 µL containing 2 µL of sample DNA, 900 nm primers, 250 nm probe and 1× TaqMan® Universal PCR Master Mix (Applied Biosystems), which includes ROX as a passive reference dye. Each sample DNA was tested with all three amplicons for phytoplasmas and an amplicon for an endogenous control (*18s*). All reactions were performed in three replicate wells in 10-fold dilution on the same 384-well plate. The software **SDS 2.3** (Applied Biosystems) was used for fluorescence acquisition and calculation of threshold cycle (Ct). For this calculation, the baseline was automatically set and the fluorescence threshold was manually set to intersect with the linear part of amplification curves of all amplicons, resulting in the final Ct value for each well. Amplification efficiency for each primer for the both method was listed in Tab. 1.
The two protocols were compared calculating the main performance criteria, which were based on the qualitative results. The following calculations were performed for both diagnostic protocols:

- N: number of samples analyzed;
- PA, Positive Accord in the detection of infected (positive) samples;
- NA, Negative Accord in the detection of healthy (negative) samples;
- PD, Positive Deviation in the detection of infected (positive) samples (false positive);
- ND, Negative Deviation in the detection of healthy (negative) samples (false negative);
- AC, relative accuracy: proportion of accords between the results obtained with a tested method and reference results on identical samples: AC=100x(PA+NA)/(NA+PA+PD+ND);
- SE, relative sensitivity: capability of the tested method to detect the infected samples (based on the positive samples): SE=100xPA/(ND+PA);
- SP, relative specificity: capability of the tested method to not detect the healthy samples (based on the negative samples): SP=100xNA/(NA+PD);
- Repeatability: level of agreement between replicates of a sample tested under the same condition.

The results for each protocol are displayed in the following table (Table 2):

Table 2. Performance criteria for detection methods of Flavecescence dorée and Bois noir phytoplasmas

Phytoplasma	FD		BN	
Performance	Angelini et al., 2007	Hren et al., 2007	Angelini et al., 2007	Hren et al., 2007
NA	15	16	6	6
PA	16	16	17	21
ND	0	0	9	5
PD	1	0	0	0

N	32	32	32	32
Relative Accuracy (%)	96,88	100,00	71,88	84,38
Relative Sensitivity (%)	100,00	100,00	65,38	80,77
Relative Specificity (%)	93,75	100,00	100,00	100,00

Both the protocols for FD phytoplasma detection showed high accuracy, sensitivity and specificity, particularly the Slovenian protocol. The situation was slightly different for BN phytoplasma detection, for which, in both the protocols, accuracy and sensitivity values were lower than in FD detection. In conclusion, the Italian protocol showed a slightly lower performance than the Slovenian one, both for FD and BN detection; moreover, both real time PCR assays on BN detection could be improved.

b) Real time PCR protocols for gene expression studies

Afterwards, on the same samples, performance of the endogenous grapevine genes used as reference genes in transcriptomic studies by the two groups (Hren et al, 2009a; Hren et al, 2009b; Bertazzon et al., 2011) was compared.

The aliquot for the total RNA extraction and gene expression analysis, stored in liquid nitrogen and maintained at -80°C, was extracted with RNeasy Plant Mini Kit (Qiagen), spectrophotometrically quantified and quality checked.

In the Slovenian protocol (Hren et al., 2007) two endogenous controls (reference genes) were evaluated for the normalization process, i.e. cytochrome oxidase (*Cox*) and 18S ribosomal RNA, and an exogenous control gene represented by the luciferase (*lux*), to check the efficiency of the RT reaction.

In the Italian protocol, otherwise, a set of five commonly used *V. vinifera* reference genes, namely actin (*Act*), cytochrome oxidase (*Cox*), pyruvate decarboxylase (*Pdc*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and 26S ribosomal RNA (*26S*) were used to normalize the expression data (Bertazzon et al., 2011). Primers for *Cox* in the two protocols were the same (Weller et al., 2000).

RT-qPCR assays were then run according to the protocols used in the two labs and specific for each gene set (Hren et al., 2009a, Bertazzon et al., 2011). Efficiency and average Ct values of the seven reference genes were compared, as showed in the following table (Tab. 3).

Table 3. Sequences of primers used for RT-qPCR.

Target gene	Primer sequences	Efficiency	Average Ct	Reference (Efficiency, Ct)
Cox	**F: CGTCGCATTCCAGATTATCCA[a]** **R: CAACTACGGATATATAAGAGCCAAAACTG**	0,914	15,8-24,5	Hren et al., 2009a
18S rRNA	**F: see ref. Applied Biosistem Inc.** **R: see ref. Applied Biosistem Inc.**	0,887	26,1-34,0	Hren et al., 2009a
Act	**F: ATGTGCCTGCCATGTATGTTGCC[b]** **R: AGCTGCTCTTTGCAGTTTCCAGC**	0,995	26,0-38,0	Bertazzon et al., 2011
Cox	**F: CGTCGCATTCCAGATTATCCA[a]** **R: CAACTACGGATATATAAGAGCCAAAACTG**	0 941	22,6-32,9	Bertazzon et al., 2011
Pdc	**F: GCTTGCGTCGTCACCTTCAC[c]** **R: TGCCGTAGTCGTTGGAGTTGG**	0,823	23,6-35,2	Bertazzon et al., 2011
Gapdh	**F: AATGAAGGACTGGAGAGGTGGAAG[c]** **R: CCGACACATCAACAGTAGGAACAC**	0,851	22,2-33,6	Bertazzon et al., 2011
26S rRNA	**F: TCCCACTGTCCCTGTCTACTATCC[c]** **R: TGGTATTTCACTTTCGCCGTTTCC**	1,009	9,0-20,0	Bertazzon et al., 2011

Reference for the primer sequences:
a: Weller et al., 2000; b: Bezier et al., 2000; c: Bertazzon et al., 2011.

Efficiencies were very good for all the reference genes. On the opposite, the mean Ct values were quite different, according to the endogenous expression of each gene, as expected. Indeed, qPCR on 18S and 26S rRNA genes showed very low mean Ct values, while for the other genes the Ct values were much higher. These differences are useful when designing analyses on expression of specific target genes, as the reference genes with constitutive expression level more similar to the target genes can be used.

c) Microarray analyses for gene expression

Due to the presence of an already performed microarray experiment, related to the research activity of the Slovenian group, it wasn't prepared an *ad hoc* new experiment and the applicant was trained only in the theoretical principles of the experiment and afterwards in the statistical analyses of the data, according to the methodologies used at NIB (Hren et al., 2009b).

Data analysis procedures were performed in R environment for statistical computing (R Development Core Team, 2007). The final list of differentially expressed genes was imported into the MapMan visualization tool, where genes are organized in graphically represented metabolic pathways and the corresponding log2-fold change represents the difference in level of gene expression between infected and healthy samples.

Whole genome transcriptomic analysis is a very powerful approach because it gives an overview of the activity of genes in certain cells or tissue types. However, biological interpretation of such results can be rather tedious. MapMan is a software tool that displays large datasets (e.g. gene expression data) onto diagrams of metabolic pathways or other processes and thus enables easier interpretation of results.

MapMan (Thimm et al., 2004) introduced an ontology which removes redundancies, and displays metabolic maps including many processes at once, thus immediately highlighting important pathways. Moreover, statistical tools are included in this software package to get an unbiased overview of changed pathways or processes. The ontology was originally built for the model species *Arabidopsis thaliana*, and furthermore extended to other species included grapevine (Rotter et al., 2009). MapMan ontology consists of a set of 34 hierarchical functional categories, or BINs, constructed around central metabolism, as well as other categories (e.g. stress, cell etc.). Original BIN assignments were based on publicly available gene annotation within TIGR (The Institute for Genomic Research). The training on MapMan will be useful for the applicant in the future gene expression studies based on microarray technology which will be carried out at CRA-VIT.

References

Angelini E., Bianchi GL., Filippin L., Morassutti C., Borgo M., 2007. A new TaqMan method for the identification of phytoplasmas associated with grapevine yellows by real-time PCR assay. J. Microbiology Methods 68, 613-622.

Bertazzon N., Raiola A., Castiglioni C., Gardiman M., Angelini E., Borgo M., Ferrari S., 2011. Transient silencing of the grapevine gene *VvPGIP1* by agroinfiltration with a construct for RNA interference. Plant Cell Report DOI: 10.1007/s00299-011-1147-2.

Bezier A., Lambert B., Baillieul F., 2002. Study of defense-related gene expression in grapevine leaves and berries infected with Botrytis cinerea. European J. Plant Pathology 108, 111–120.

Hren M., Boben J., Rotter A., Kralj P., Gruden K., Ravnikar M., 2007. Real-time PCR detection systems for *Flavescence dorée* and *Bois noir* phytoplasmas in grapevine: comparison with conventional PCR detection and application in diagnostics. Plant Pathology 56, 785–796.

Hren M., Nikolić P., Rotter A., Blejec A., Terrier N., Ravnikar M., Dermastia M., Gruden K., 2009b. « *Bois noir* » phytoplasma induces significant reprogramming of the leaf transcriptome in the field grown grapevine. BMC Genomics 10, 460.

Hren M., Ravnikar M., Brzin J., Ermacora P., Carraro L., Bianco P.A., Casati P., Borgo M., Angelini E., Rotter A., Gruden K., 2009a. Induced expression of sucrose synthase and alcohol dehydrogenase I genes in phytoplasma-infected grapevine plants grown in the field. Plant Pathology 58, 170–180.

Pirc M., Ravnikar, M., Tomlinson J., Dreo, T., 2009. Improved fireblight diagnostics using quantitative real-time PCR detection of *Erwinia amylovora* chromosomal DNA. Plant Pathology 58, 872–881.

R Development Core Team, 2007. A language and environment for statistical computing Vienna: R Foundation for Statistical Computing.

Rotter A., Camps C., Lohse M., Kappel C., Pilati S., Hren M., Stitt M., Coutos-Thévenot P., Moser C., Usadel B., Delrot S., Gruden K., 2009. Gene expression profiling in susceptible interaction of grapevine with its fungal pathogen *Eutypa lata*: Extending MapMan ontology for grapevine. BMC Plant Biology 9, 104.

Thimm O., Bläsing O., Gibon Y., Nagel A., Meyer S., Krüger P., Selbig J., Müller L. A., Rhee S. Y., Stitt M., 2004. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J., 37, 914-939.

Weller S. A., Elphinstone J. G., Smith N. C., Boonham N., Stead D. E., 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied Environmental Microbiology 66, 2853–2858.