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Identification of a set of widely expressed genes in grape (*Vitis vinifera L.*) and its functional characterisation: a multi-evidence based study

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Summary

Quantitative gene expression data analysis requires efficient normalization to be really informative: as a consequence reference genes that are stably expressed in tested *vs.* control samples are used for results standardization. The identification of tissue-wide-expressed genes makes it easier to highlight the best set of candidate internal controls. While tissue-specific genes are often regulated by microRNA, housekeeping genes, being involved in cell maintenance and thus required in all miRNA expressing cells are not expected to be microRNA targets. In this work we have identified a set of tissue-wide expressed genes in grape which has then been functionally characterised and scanned for the presence *vs.* absence of putative miRNA target sites. The gene list obtained by this multi-evidence based procedure can be helpful to identify appropriate references in grape.

K e y w o r d s : widely expressed genes, housekeeping genes, microRNA non-target genes, grape.

Introduction

Quantitative gene expression data analysis requires efficient normalization to be really informative. Reference genes that are stably expressed in treated *vs.* control samples must thus be used for results standardization (Vandesompele *et al*. 2002).

In particular, the identification of widely-expressed genes makes it easier to highlight the best combination of reference genes to be used (Faccioli *et al.* 2007). While tissue-specific genes are often regulated by microRNA, housekeeping genes, being involved in cell maintenance (Warrington *et al.* 2000) and thus constantly required in all miRNA expressing cells are expected to be microRNA non-target genes. MicroRNAs are small noncoding RNAs that play a fundamental role in post-transcriptional regulation of gene expression in higher eukaryotes. The mechanism of their action is based on the recognition, on mRNA, of specific miRNA complementary sites. In plants such matching requires extensive sequence complementarity and usually leads to mRNA cleavage (Barakat *et al.* 2008).

In this work, with the final aim of identifying a list of potential reference genes in grape and on the basis of the method previously developed by Faccioli *et al.* (2007), TCs (Tentative Consensus) related data have been downloaded from the Grape Gene Index and mined by a simple plain frequency count to find out which TCs are present in a remarkable number of different cDNA libraries. At a later stage, these genes have been characterised for their expression stability and for the presence *vs* absence of microRNA target sites on the corresponding mRNA.

Results and Discussion

F i n d i n g b r o a d l y e x p r e s s e d g e n e s : The list of genes obtained according to our method is reported in Tab. 1. Most of the genes collected turn out to code for proteins with a role in cell maintenance: ribosomal genes, ADP-ribosylation factors, thioredoxins, metallothioneins, ubiquitins, aquaporins (Eisenberg and Levanon 2003) and do partially overlap with the ones previously highlighted in other species (Faccioli *et al.* 2007, 2008, Mukhopadhyay *et al*. 2008).

To study the expression stability and mRNA abundance of these genes, quantitative analysis has been carried out by RT-Real-Time PCR on a panel of several grape organs. As reported in the Figure, the tested genes are actually expressed in all samples, with different levels of abundance: the average Ct for a specific TC measured in different samples ranges between 13 and 25. The fact that housekeeping genes comprise both highly and lowly expressed genes has been previously demonstrated both in animals and plants (Zhang and Li 2004, Mukhopadhyay *et al*. 2008). The availability of reference genes expressed at different levels of abundance is very useful for controls with expression levels similar to those of the genes to be quantified, being a well established practice in normalization (Jain *et al.* 2006).

Regarding expression stability of the selected TCs among the tested samples, standard deviations for Ct values range from 0.2 to 2.2 thus including both genes with relatively constant expression levels and genes with quite high variability. Both geNorm and Normfinder analyses were applied to RT-Real Time PCR data. Tab. 2 shows TCs ordered by the two algorithms from the less stable at the top of the list to the most stable at the bottom. The TC rankings obtained with the two analytical approaches are very similar: minimal variations in expression across different tissues have been shown by TC56459, TC70629, TC67872 and TC59398. An "in-silico" co-expression analysis (i.e. co-presence of transcripts) approach was then carried out

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Table 1

Candidate reference genes

Release 4.0	Release 5.0	Number of hosting/total number of libraries	Cluster number	Primer sequences (forward/reverse)
TC38306	TC51721	34/73	631	CCAAGCTTTCATTCCTTCTCAGA/TCAGGCTATGGATCCCTTTGA
TC45122	TC53458	31/73	631	GTCTACGCCGACGGACGTA/TCCCAAAGGTGGGCATGTT
TC45095	TC58751	33/73	239	GACTGTTGCTAAGGAGTGGGAAGT/ACCTTGTCCACTACGTTTCCTTCT
TC38261	TC59319	43/73	239	TTCGGAGTCGACGCTGATG/TGGCTACGCGTGTCATAGCT
TC38333	TC60374	35/73	227	TGATGCCCCTTAGCCATGTC/GCCATTCTTGCGGACTTCAG
TC45112	TC67872	35/73	631	GCCGACGAGAACTTCATCCA/CGGGACCTGCGTTTGC
TC45008	TC68454	41/73	239	GGCTCAGGCCAACATGGT/CGGGTGCCAGGGAAAGT
TC38121	TC69190	34/73	631	GGTTCACTTGGCCACTATTCCT/TAACAGCAGCCCCCAAACTC
TC38313	TC69862	33/73	109	AGCTGAGAACGGATGGAAGTG/GGGAGTGGTTTCTCATTTGCA
TC45076	TC70629	34/73	553	CCAAAGAAGGTGATAGCCCTGTA/CGAACACGCCGCGTTT
TC38304	TC51848	31/73	631	TCCTTACCAGACGCAAAACATG/TCGCATCTCTTTCTTGGCAAA
TC38297	TC53231	35/73	239	CTGTTGTTACAGTGCCTGCCTACT/CACATTGAGGCCTGCAATAACTC
TC38279	TC54117	33/73	631	GAGGACCCTGGCGGACTAC/GCCAACTGACCATTGAAATACTTAAA
TC45173	TC59398	35/73	591	AAGCACATTGGTGGCTGTGA/TCAGTAAGCAAAGGAACAAGTTTCC
TC38309	TC61772	40/73	109	CCTTGTTCGACGAGTTGTAGACA/CCGCCCGGACATGTATGA
TC38120	TC68445	31/73	535	TGCCTGGAAGGCTGTTGAA/TCCAGCAACTGTTTTACCTTGGT
TC44954	TC69388	45/73	239	AGCTATGCTACCGATGGTGTCA/GGGCCTGGGTCATCTGCTA
TC38251	TC70371	34/73	553	TCGTACATGGACGGTAGTTGGA/CGCAAGGGCCAACTTGAT
TC44984	TC56459	38/73	239	ACAGCTCCCGTGTGATCGA/CTACCCTTTGGAAATATCTGCACTTA

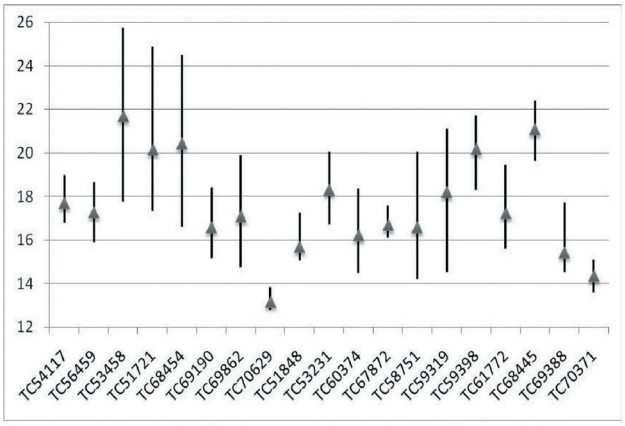

Figure: mRNA quantification expressed as maximum, minimum and mean (triangle) Ct values (Cycle threshold) for the tested TCs in the grape tissues reported in Tab. 2.

on grape TCs (Faccioli *et al.* 2005). After the identification, by the way described in the method section, of co-expressed modules, we examined the distribution of the candidate tissue-wide expressed genes among them which were included in 7 small clusters (see Tab. 1). However most of them were members of just two main clusters (named 239 and 631). To assess the functional significance of such clusters, the enrichment in GO (Gene Ontology) terms within each module has been evaluated and its statistical significance measured using the exact Fisher test (P-value less than 1e-4 from Fisher test). Cluster 239 and 631 were characterized by an over-representation of GO terms mainly related to basic cellular biological process and molecular functions such as cell-cell signalling, protein secretion, amine biosynthesis, nitrogen compound biosynthesis, water and protein transporter activity.

Table 2

TCs ordered from the less stable one at the top of the list to the most stable one at the bottom

All the tissues-NormFinder		All the tissues-GeNorm	
Gene Name	Variability	Gene Name	M-Value
TC53458	2.26	TC53458	1.53
TC51975	2.21	TC51975	1.44
TC51721	1.90	TC51721	1.35
TC58751	1.72	TC68454	1.27
TC59319	1.68	TC59319	1.20
TC68454	1.64	TC58751	1.11
TC69862	1.41	TC69862	1.02
TC60374	1.17	TC58854	0.95
TC58854	1.04	TC61772	0.90
TC68445	0.97	TC60374	0.85
TC53231	0.96	TC68445	0.79
TC61772	0.93	TC53231	0.75
TC51848	0.71	TC51848	0.70
TC69388	0.55	TC69388	0.67
TC54117	0.51	TC54117	0.64
TC70371	0.49	TC70371	0.62
TC67872	0.41	TC69190	0.59
TC69190	0.40	TC56459	0.57
TC59398	0.39	TC59398	0.51
TC70629	0.36	TC70629	0.31
TC56459	0.33	TC67872	0.31

Presence *vs.* absence of miRNA target sites: miRBase (release available at the time of this work 11.0) reports one hundred and forty grape miRNA sequences, grouped in twenty-eight families. Among them, it has been possible to put on evidence 79 different mature sequences (Tab. 3) with some miRNA families presenting a number of possible mature sequences higher than

others. On the basis of sequence complementarity, the target prediction server named miRU (Zhang 2005) has been used for the identification of candidate microRNA target genes. Despite the fact that we used low cut-offs and consequently permissive parameters in miRU target searching (Schwab *et al*. 2005, Lu *et al.* 2008), at least for the tested microRNAs, none of the TC listed in Tab.1 has been identified as potential (conserved or non-conserved) target by miRU. The same result has been obtained by comparing the list of TCs with the data on grape miRNA targets from a recently published work of Lu *et al.* (2008).

Interestingly, recent studies on animals report evidences of evolutionary microRNA target site avoidance for highly expressed genes involved in basic cellular processes: the word "antitargets" has been coined for defining this particular group of genes (Bartel and Chen 2004, Stark *et al.* 2005). In conclusion, by using a multi-evidence based approach for internal controls selection we were able to highlight a set of genes that can be more confidently entered into a shortlist, in order to identify appropriate reference genes across a variety of tissue types in grape.

Dry-based procedures: Identification of candidate housekeeping genes: The DFCI Grape Gene Index (release 4.0) was screened with a Python-based script to find out the TCs that are present in a remarkable number of different cDNA libraries and to order them on the basis of their frequency thus following the procedure reported in Faccioli *et al.* (2007).

Grape microRNA sequences and potential target genes: Sequences for grape miRNA (http://microrna.sangr.ac.uk/sequences) were collected from mirBase release 11.0. Mature sequences from

Table 3

79 different microRNA mature sequences tested with miRU for target finding

Group1	CAGCCAAGGAUGACUUGCCGG	Group41	CUUGGAGUGAAGGGAGCUCUC
Group2	CAGCCAAGAAUGAUUUGCCGG	Group42	UUUGGAUUGAAGGGAGCUCUA
Group3	CAGCCAAGGAUGACUUGCCGA	Group43	CUUGGACUGAAGGGAGCUCCC
Group4	UGAGUCAAGGAUGACUUGCCG	Group44	AUUGGACUGAAGGGAGCUCCC
Group5	CGAGUCAAGGAUGACUUGCCG	Group45	UUUGGACUGAAGGGAGCUCCU
Group6	UGAGCCAAGGAUGGCUUGCCG	Group46	UGACAACGAGAGAGAGCACGCU
Group7	GAGCCAAGGAUGACUGGCCGU	Group47	UGACAGAAGAGAGUGAGCAC
Group8	GAGCCAAGGAUGACUUGCCGU	Group48	UGACAGAGGAGAGUGAGCAC
Group9	GAGCCAAGGAUGACUUGCCGC	Group49	UUGACAGAAGAUAGAGAGCAC
Group10	GAGCCAAGGAUGACUUGCCGG	Group50	UGACAGAAGAUAGAGAGCAC
Group11	AAGCCAAGGAUGAAUUGCCGG	Group51	UGACAGAAGAGAGAGAGCAU
Group12	UAGCGAAGGAUGACUUGCCUA	Group52	UGACAGAAGAGAGGGAGCAC
Group13	UAGCCAAGGAUGACUUGCCUA	Group53	UCCAAAGGGAUCGCAUUGAUCC
Group14	UAGCCAAGGAUGACUUGCCUG	Group54	UUGGCAUUCUGUCCACCUCC
Group15	UGCCAAAGGAGAGUUGCCCUG	Group55	UCAUUGAGUGCAGCGUUGAUG
Group16	CGCCAAAGGAGAGUUGCCCUG	Group56	UGCCUGGCUCCCUGUAUGCCA
Group17	UGCCAAAGGAGAAUUGCCCUG	Group57	UGCCUGGCUCCCUGAAUGCCA
Group18	UGCCGAAGGAGAUUUGUCCUG	Group58	UGGCUCUGAUACCAAUUGAUG
Group19	UGCCAAAGGAGAUUUGCUCGU	Group59	AGGCUCUGAUACCAAUUGAUG
Group20	UGCCAAAGGAGAUUUGCCCCU	Group60	UAGCUCUGAUACCAAUUGAUA
Group21	UGCCAAAGGAGAUUUGCCCGG	Group61	CCUACUCCUCCCAUUCC
Group22	AAGCUCAGGAGGGAUAGCGCC	Group62	UCGAUAAACCUCUGCAUCCAG
Group23	UCGGACCAGGCUUCAUUCCCCU	Group63	UCUUGCUCAAAUGAGUAUUCCA
Group24	UCGGACCAGGCUUCAUUCCUC	Group64	UCUUGCUCAAAUGAGUGUUCCA
Group25	UCGGACCAGGCUUCAUUCCUG	Group65	AUCUCCCUCAAAGGCUUCCAA
Group26	UCGGACCAGGCUUCAUUCCCCC	Group66	UGGAGAAGCAGGGCACGUGCA
Group27	UUCCACAGCUUUCUUGAACUA	Group67	UGGAGAAGCAGGGCACAUGCU
Group28	UUCCACAGCUUUCUUGAACU	Group68	UGAAGCUGCCAGCAUGAUCUA
Group29	UUCCACAGCUUUCUUGAACUG	Group69	UGAAGCUGCCAGCAUGAUCUC
Group30	AUGCACUGCCUCUUCCCUGGC	Group70	UGAAGCUGCCAGCAUGAUCUG
Group31	CUGAAGUGUUUGGGGGAACUC	Group71	UUAGAUUCACGCACAAACUCG
Group32	CUGAAGAGUCUGGAGGAACUC	Group72	UUGAGCCGUGCCAAUAUCAUG
Group33	UGAAUCUUGAUGAUGCUACAU	Group73	UUGAGCCGUGCCAAUAUCAUC
Group34	UGAAUCUUGAUGAUGCUACAC	Group74	UUGAGCCGUGCCAAUAUCACG
Group35	GGAAUCUUGAUGAUGCUGCAG	Group75	UUGAGCCGCGCCAAUAUCACU
Group36	AGAAUCUUGAUGAUGCUGCAU	Group76	UUGAGCCGAACCAAUAUCACC
Group37	UGUGUUCUCAGGUCGCCCCUG	Group77	UGGUUGAGCCGCGCCAAUAUC
Group38	UGUGUUCUCAGGUCACCCCUU	Group78	UGAUUGAGCCGCGCCAAUAUC
Group39	UGUGGUAUUGGUUCGGCUCAUC	Group79	UGAUUGAGCCGCGUCAAUAUC
Group40	UCGCUUGGUGCAGGUCGGGAA		

each miRNA family were aligned and every unique mature miRNA sequence was then tested on possible microRNA targets by miRU (http://bioinfo3.noble.org/miRNA/miRU.htm) which explores TIGR Grape Gene Index 4.0. Default settings for mismatches tolerance were applied.

Identification of groups of co-expressed genes: For this work we used the set of 73 cDNA libraries originating from different tissues and/or developing stages and publicly available through Grape Gene Index, release 4.0 (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=grape), (Quackenbush *et al.* 2000).

For all pairs (TC_1, TC_2) of Tentative Consensus we calculated the binary asymmetric distance as a quantitative measure of dissimilarity between the expression profiles. Therefore we considered the expression profile of each TC as a string of as many bits as the number of libraries, with a bit being 1 if the TC is represented in the corresponding library and 0 otherwise. The binary asymmetric distance between two TCs is defined as the ratio of the number of bits that are 1 in only one of the two TCs over the total number of bits that are 1 in at least one of the two TCs. A hierarchical clustering algorithm was used to determine groups of co-expressed genes. Significance of the relationship was related to a P-value that indicates the probability that the co-expression is due to chance.

The functional characterization of the clusters of co-expressed genes were analysed by looking for overrepresented Gene Ontology terms in each group. P-values were computed with a standard exact Fisher test. Database query and analysis were performed using R packages and a set of Perl programs.

Wet-based procedures: Plant samples: A panel of grape tissues were collected from nine varieties grown in greenhouse-controlled conditions or in vineyards (Tab. 4) and immediately frozen in liquid N_2.

Extraction and quantification of messenger RNA: RNA was isolated from the plant organs (two biological replications) using the modified Li-Cl methods reported by Tattersall *et al.* (2005).

The purified RNA was quantified and equally loaded by standard optical density measurement. The results were also verified using RiboGreen dye (Molecular probes) in a fluorescent-based solution assay (Jones *et al.* 1998), according to the manifacturer protocol.

Gene expression analysis: RNA samples were reverse transcribed and amplified using the SYBR Green PCR Master Mix and TaqMan Reverse Transcriptase Reagents (Applied Biosystems), following the One-Step RT-PCR protocol recommended by the manifacturer. Primers design and their optimization in regard to primer dimer, self priming formation and primer melting temperature was done with Primer Express v.3.0 software (Applied Biosystems). Primer sequences used in RT Real-Time PCR and corresponding to candidate housekeeping genes are listed in Tab. 1.

One hundred ng of mRNA in 50 µl SYBR Green PCR Master Mix 1X with 0.25 U/ml MultiScribe Reverse Transcriptase (Applied Biosystems), 0.4 U/ml RNase Inhibitor (Applied Biosystems), 150 nM forward and reverse primers were subjected to the following thermal profile: one step at 48 °C for 30 min, one step at 95 °C for 10 min, 40 cycles with a denaturation step at 95 °C for 15 s and an annealing/extension step at 60 °C for 1 min. PCRs were performed in the Applied Biosystems 7300 Real Time PCR using MicroAmp optical tubes and caps. The reactions were subjected to a heat dissociation protocol present in the Applied Bio-

Table 4

Grape tissue samples used in the validation step and collected from nine varieties grown in greenhouse-controlled conditions or in vineyards

Sample name	Tissue type	Cultivar	Stage of development	Growing location
Leaf	leaf	Malvasia aromatica from Candia	pre-veraison	Vineyard in Torrechiara (PR, Italy)
Leaf1	leaf	Nebbiolo 142	cuttings	greenhouse
Leaf2	leaf	Moscato CN16	cuttings	greenhouse
Leaf3	leaf	Barbera AT84	cuttings	greenhouse
Leaf4	leaf	Pinot noir 115	cuttings	greenhouse
Tendrils	tendril	Malvasia aromatica from Candia	pre-veraison	Vineyard in Torrechiara (PR, Italy)
Berry1	berry	Malvasia aromatica from Candia	early fruit set	Vineyard in Torrechiara (PR, Italy)
Berry2	berry	Sauvignon	pre-veraison	Vineyard in Torrechiara (PR, Italy)
Berry3	berry	Sauvignon	pre-veraison	Vineyard in Casatico (PR, Italy)
Berry4	berry	Bonarda	pre-veraison	Vineyard in Torrechiara (PR, Italy)
Berry5	berry	Malvasia aromatica from Candia	pre-veraison	Vineyard in Torrechiara (PR, Italy)
Berry6	berry	Cabernet	pre-veraison	Vineyard in Carmiano (PC, Italy)
Rachis0	rachis	Pinot noir 115	cuttings	greenhouse
Rachis1	rachis	Malvasia aromatica from Candia	pre-veraison	Vineyard in Torrechiara (PR, Italy)
Rachis2	rachis	Sauvignon	pre-veraison	Vineyard in Torrechiara (PR, Italy)
Rachis3	rachis	Sauvignon	pre-veraison	Vineyard in Casatico (PR, Italy)
Rachis4	rachis	Bonarda	pre-veraison	Vineyard in Torrechiara (PR, Italy)
Rachis5	rachis	Malvasia aromatica from Candia	pre-veraison	Vineyard in Torrechiara (PR, Italy)
Rachis6	rachis	Cabernet	pre-veraison	Vineyard in Carmiano (PC, Italy)

systems 7300 Real Time PCR software for melting curve analysis and detection of non-specific amplifications: at the end of the final PCR cycle, the amplification products were heat denatured over a 35 °C temperature gradient at 0.03 °C/s from 60 to 95 °C. A negative control without template was run with every assay to assess the overall specificity. The Real-Time PCR data were plotted as the ΔR_n fluorescence signal versus the cycle number. The Applied Biosystems 7300 Real Time PCR software calculates the ΔR_n using the equation $\Delta R_n = (R_n^+)-(R_n^-)$, were R_n^+ is the fluorescence signal of the product at any given time and R_n^- is the fluorescence signal of the baseline emission during cycles 6 to 13. An arbitrary threshold was set at the midpoint of the log ΔR_n versus cycle number at which the ΔR_n crosses the threshold.

GenEx ver.4.3 software package (Multid Analyses AB, Lotsgatan 5A, Gothenburg, Sweden; www.multid.se) were used to manage the RT Real-Time PCR data.

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