utmost importance in RT-qPCR as they allow the corrections of PCR reactions due to inaccurate quantification of RNA or problems related to RNA quality and purity [1,11].

RT-qPCR data normalisation is usually performed using the expression of an internal control gene [4,6,12,13]. Such a gene is also referred as a "reference gene" or sometimes a "housekeeping gene" i.e. a gene whose expression does not change under the different conditions or tissues under investigation [1,14,15]. Unfortunately no such gene exists, and the universality of such an ideal gene is not valid, since the transcript levels of all genes show some degree of variability under different experimental conditions [5-7,16]. Initially, gene stability was assessed with the ΔCq method. In this method, the Cq value of the gene of interest (either target or reference) is related to a control/calibrator [17]. In order to overcome these flaws, statistical algorithms such as GeNorm [6] and NormFinder [18] have been developed to evaluate the best suited reference gene or a combination of genes for normalisation of RT-qPCR data in a specific set of biological data [6].

The identification of suitable reference genes can be difficult. Several attempts have been made, all with different outcomes. Studies usually allocate different "model" genes to be used in data normalisation. Usually the allocated genes vary with the plant species, as well with the experimental conditions. Also the method to select the reference candidate varies with the availability of data for the plant species under study. Studies regarding reference genes often employ a variety of methods to chose possible reference genes; such as searching the bibliography for published references [19], using orthologs of Arabidopsis reference genes [20], cDNA libraries [21] or analysis of EST libraries [22]. Genes that are frequently identified to be good references include elongation factors 1-α (*eEF-1α*) [23-26], actin (*act*) [8,15,21,23], ubiquitin (*ubiq*) [8,25,27-29], glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) [7,25,27,28], ribosomal proteins [7,21,25,30-32], SAND family protein (*SAND*) [20,27,33] and, other less common genes have been identified to be particularly good references in very specific contexts.

Czechowski et al. [5], using data from Affymetrix ATH1 whole-genome GeneChip, proposed not only the typical reference genes for RT-qPCR but also new ones. After that study, microarrays, when available, have been used for identifying reference genes [34,35], with new genes being found for RT-qPCR data normalisation.

In grapevine, several attempts have been made in identifying reference genes. Gamm et al. [36] indicated two genes (V-type proton ATPase 16 kDa proteolipid subunit and 60S ribosomal protein L18) as being optimal reference genes for the study of the expression of genes involved in pterostilbene synthesis in grapevine leaves infected by *P. viticola* and berries infected by *B. cinerea*. Reid et al. [23] also tested possible reference genes specifically suitable for use in grapevine berry development studies and suggested *GAPDH*, *act*, *eEF-1α* and *SAND* as the most stable.

Whatever the method used for choosing possible reference genes for data normalisation, stability analysis should always be performed in the optimal conditions.

Grapevine (*Vitis vinifera* L.) is a sessile organism and therefore cannot avoid abiotic stress. Plants have been developing mechanisms to cope with environmental changes and help to overcome them. The most relevant abiotic stresses that can affect the production of a Mediterranean crop such as grapevine are: drought, excessive light and excessive heat. In fact, climate models predict an intensification of extreme conditions, which can reduce production to below the threshold for optimal grapevine growth [37]. This represents a serious challenge for Mediterranean agriculture.

The pattern of gene expression in response to abiotic stress has been monitored in *ex vitro* grapevine plants by comparing the use of the grapevine Affymetrix GeneChip with extensive RT-qPCR analysis [38]; and in greenhouse and field plants through genechip microarray (results under analysis).

In this paper we report several putative reference genes chosen from a grapevine microarray analysis and identify the genes to be used as references for RT-qPCR normalisation, after obtaining the "optimal combination" of reference genes using three different methods. We also apply the three "optimal combinations" obtained to quantify the expression of a set of stress-marker genes.

Results and discussion

Choice of reference genes

Candidate reference genes were chosen from a microarray analysis using an array composed of 23 096 unigene sequences [39]. All the genes that did not meet the selection criteria (100% presence in control and in stress samples and both probesets present in all the stress samples of the array) were discarded. Fold-change was then analysed and all probesets outside the fold-change interval of −1.25 to 1.25 were also discarded. This range was the minimum interval in which an acceptable number (eighteen) of possible reference genes could be found (Table 1).

From those eighteen genes, six were selected for the study (Table 2). The selection was performed by choosing genes previously described or belonging to gene families commonly used for RT-qPCR data normalisation, such as Translation initiation factors; but also included grapevine genes that had not been completely described or with unknown functional categories. Well known and

Table 1 Possible reference genes retrieved from the microarray analysis

Probeset ID	NCBI Reference	WS fc	HS fc	Annotation
VVTU3078_at	XM_002274960	1.04	1.02	growth-on protein GRO10
VVTU5951_at	XM_002276120	1.13	1.12	F-box protein 7
VVTU21677_at	XM_002278540	1	1.23	ATSLY1
VVTU38193_s_at	XM_002284329	1.16	1.19	RAB GTPase ARA3
VVTU775_at	XM_002283960	1.21	1.16	Aladin
VVTU38174_at	XM_002274483	−1.11	−1.04	DnaJ homolog, subfamily B, member 4
VVTU39962_s_at	XM_002265755	−1.02	−1.12	ubiquitin-like domain containing CTD phosphatase 1
VVTU291_at	XM_002273137	−1.06	−1.11	peptidylprolyl isomerase PAS1 (PASTICCINO 1)
VVTU15254_at	XM_002275607	−1.05	−1.07	DNA polymerase eta subunit
VVTU15763_at	XM_002282403	−1.02	−1.03	translation initiation factor eIF-3 subunit 4
VVTU16514_at	XM_002278163	−1.11	−1.04	Protein kinase PKN/PRK1
VVTU3178_at	XM_002271296	−1.05	−1.05	translation initiation factor eIF-2B alpha subunit
VVTU12062_at	XM_002274698	−1.01	−1	RNA-binding protein Musashi
VVTU6197_at	XM_002269673	−1.13	−1.06	plectin (myosin-like)
VVTU2620_at	XM_002282316	−1.08	−1.03	DNA repair protein RAD23
VVTU3027_at	XM_002266331	−1.09	−1.21	ankyrin repeat family protein
VVTU5961_s_at	XM_002284235	−1.22	−1.09	ribosomal protein L27
VVTU1226_at	XM_002277764	−1.03	−1	Plastid-specific 50S ribosomal protein 6

Probeset ID, NCBI Reference, Water Stress fold change (WS fc), Heat Stress fold change (HS fc) and annotation of the 18 genes retrieved from the microarray analysis [39]. The presence call is 100% in all genes in both treatments.

described reference genes were also added to the study for comparison, *act* and *L2* (Table 2).

Selection of the best reference genes

The first approach used to verify the stability of the eight reference genes was the ΔCq method [17] (Table 3). In this method, the control/calibrator can be any sample, *e.g.* a real untreated control, or the sample with the highest level of expression (lowest Cq value). The method generates raw (non-normalised) expression values, which need to be normalised by dividing with a proper normalisation factor. The ΔCq method has several advantages, namely, it allows an easy inclusion of multiple reference genes for normalisation. In this research, the best reference genes for RT-qPCR data normalisation obtained with this method were *PADCP*, with *act* ranked second, whilst *aladin-related* was the worst choice.

The GeNorm application for Microsoft Excel determines the most stable reference genes from a group of genes. The application also calculates a normalisation factor of gene expression for each sample, based on the geometric mean of a user-defined number of reference genes. It is based on the assumption that the expression of two ideal reference genes will always have the same ratio among samples regardless of the experimental conditions [6]. This average expression stability value (M) is calculated using the expression data for each gene. M is the average pairwise variation (V) of one gene compared

Table 2 Primers used for the reference genes chosen after analysis of Table 1

Probeset ID	Name	Sense primer	Anti-Sense primer	pb
VVTU3078_at	PADCP	5' ATTCATTAAAGTACCTTTCTTT 3'	5' AACACCCAAAAGATGTCGTA 3'	240
VVTU39962_s_at	*ubiq*	5' CAATTTCCTGAGTTCTACAGTT 3'	5' CCTCATTGTATGACTCCCAGT 3'	229
VVTU15763_at	*TIF*	5' AAAGCAGAAGAAACCAAGATT 3'	5' TTGCCAGTGCCTGTAGTAGCC 3'	206
VVTU3178_at	*TIF-GTP*	5' AGCAGCACAGAATAAGAAACT 3'	5' CCATCAGCCCCAACAAATACC 3'	177
VVTU3027_at	*VH1-IK*	5' CAGGGATTATGATAGTAGGA 3'	5' TTGTTTGGTAGAGGAGGTGG 3'	252
VVTU775_at	*aladin-related*	5' CCTACACTTATTCATCTTCG 3'	5' ACTTGTGGCGGTTGCTCTGC 3'	224
-	*act*	5' TGGATTCTGATGGTGTGAGTC 3'	5' CAATTTCCCGTTCAGCAGTAGTGG 3'	167
-	*L2*	5' TCTACTTCAACCGATATGC 3'	5' CAACCTGTCCGACTG 3'	196

Probeset ID (of all but *act* and *L2* that were not chosen from the microarray), gene name, sense primer, anti-sense primer and transcript length of the product obtained.