Microarray-based uncovering of reference genes for quantitative real-time PCR in potato tuber infected with PVY

Barbara Gerič Stare[1*], Aleš Sedlar[2#], Vladimir Meglič[2]

[1]Plant Protection Department, Agricultural institute of Slovenia, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia
[2]Crop Science Department, Agricultural institute of Slovenia, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia
[#] no longer employed here

*Corresponding author:
Barbara Gerič Stare
Tel: +386 (0)1 280-5276
E-mail: barbara.geric@kis.si
Fax: +386 (0)1 280-52-55

Additional key words: *Solanum tuberosum*, Potato virus Y.

Disclosure of interests: The authors have no relevant financial or non-financial interests to disclose.

Acknowledgements: This study was financially supported by the Slovenian Research Agency (project L4-2400-0401, programme P4-0072).

Author contributions:
Conceptualization: Barbara Gerič Stare; Methodology: Barbara Gerič Stare, Aleš Sedlar; Formal analysis and investigation: Barbara Gerič Stare, Aleš Sedlar; Writing - original draft preparation: Aleš Sedlar; Writing - review and editing: Barbara Gerič Stare, Vladimir Meglič; Funding acquisition: Vladimir Meglič.

Suggested running head: Potato tuber reference genes

Abbreviations:
18S rRNA - 18S ribosomal RNA
adj.p – adjusted p value
APRT - Adenine phosphoribosyl transferase
C_T – threshold cycle
CYP - Cyclophilin
dC_T – delta threshold cycle
EF1-α - Elongation factor 1-α
EIF5A - Eukaryotic translation initiation factor 5A
Hsp20.2 - Heat shock protein 20
KATN – Katanin
L2 - Cytoplasmic ribosomal protein L2
lfc - logarithmic fold change, $\log_2$-fold change
NCLN - Nicalin
PVY - Potato virus Y
PVY^{NTN} – strain of Potato virus Y that induces potato tuber necrotic ringspot disease
RT-qPCR - reverse trancription quantitative polymerase chain reaction
SD - standard deviation
USP - Universal stress protein

ABSTRACT

Stored potato tubers are susceptible to pathogens, such as Potato virus Y, and studies of host/pathogen interactions on a gene transcription level can provide insight into the disease development. A method for studying individual gene expression is reverse trancription quantitative polymerase chain reaction (RT-qPCR) that relies on utilization of reference genes. To select appropriate reference genes with stable expression in our experimental setting, we screened the genome-wide microarray expression data for suitable candidate reference genes rather than to use generally recognised constitutively expressed housekeeping genes as reference genes. Four highly expressed genes with stable expression across several comparisons were selected based on microarray data. Stable expression of these candidate reference genes (Nicalin, Eukaryotic translation initiation

factor 5A, Universal stress protein and Katanin p60 ATPase-containing subunit) was confirmed using RT-qPCR. Our candidate reference genes were more suitable than housekeeping genes often used as reference genes. Additionally, microarray expression data was evaluated for eight previously reported reference genes.

Keywords: Reference genes, RT-qPCR, microarray, *Solanum tuberosum*, tubers.

INTRODUCTION

The most widely used and cost-effective method for gene expression analysis of individual genes is reverse transcription quantitative polymerase chain reaction (RT-qPCR) that is also often used for validation of high-throughput microarray and RNA-seq transcriptomic data. The accuracy of this method relies on reference genes with stable expression in studied conditions that are used for normalisation of gene expression of genes of interest (Vandesompele et al. 2002; Andersen et al. 2004).

A recent review of reference genes for development and stress response studies utilized in expression analysis in 15 model and crop plant species, indicates that housekeeping genes, such as actin, beta tubulin, 18S rRNA, Elongation factor-1α (*EF-1α*) and Eukaryotic initiation factor 1 (*EIF1*), are one of the most widely and traditionally used as internal control (Joseph et al. 2018). However their expression stability can be influenced by abioitic, biotic as well as developmental factors (Joseph et al. 2018).

The established practice for choosing reference genes before the availability of whole transcriptome data was to utilize a set of genes generally regarded as stably expressed, and evaluate them in specific conditions of interest. Such evaluations of reference genes were reported for potato plants of different cultivars as well as tubers exposed to forms of biotic and abiotic stress (Nicot et al. 2005; Lopez-Pardo et al. 2012). *EF-1α* proved as the most stable gene in leaves of potato plants during late blight infection, cold stress and salt stress (Nicot et al. 2005). *EF-1α* and adenine phosphoribosyl transferase (*APRT*) proved to be the most stable in tubers under different cold storage conditions (4°C and 11°C) (Lopez-Pardo et al. 2012). Subsequently, *EF-1 α* has been used as a normalization gene in potato exposed to different forms of biotic and abiotic stress, such as infection with fungus *Fusarium solani* (Charfeddine et al. 2016), and exposure to drought and high light intensity stress (Szalonek et al. 2015).

In recent years the number of microarray and RNA-seq transcriptomic studies on potato plants as well as specifically on tubers is increasing. In addition to tuber development and maturation, studies are also focused on tubers during storage and under different biotic and abiotic stress conditions (Mariot et al. 2015; Sedlar et al. 2018). These studies do not only serve as an excellent starting point for further investigations using RT-qPCR but also represent the opportunity to screen the transcriptome for stably expressed genes in specific conditions of interest. In the screening of whole-genome transcriptome data, genes *C2*, exocyst complex component sec3 (*SEC3*) and *CUL3A* proved the best choice for reference genes for edible tubers of different potato varieties (Mariot et al. 2015).

Strain of Potato virus Y (PVY^{NTN}) induces potato tuber necrotic ringspot disease. In the present study we selected genes with stable expression that can serve as reference genes for potato/PVY^{NTN} pathosystem by evaluating microarray expression data of healthy and PVY^{NTN} infected potato tubers differing in storage conditions and disease stages. The first aim was to evaluate the stability of previously reported reference genes. The second aim was to identify highly expressed genes with stable expression across several comparisons factoring in storage temperature, sampling time, infection status and symptom presence from our experiment. The third aim was to validate chosen candidate reference genes using RT-qPCR and test them for suitability using dCT values, stability scores by three algorithms (GeNorm, NormFinder and BestKeeper), and comprehensive ranking by RefFinder.

METHODS

Tuber production and storage

The study was performed on samples collected during the course of a previously described storage experiment (Dolničar et al. 2011). Tubers of PVY^{NTN} susceptible cultivar Igor were harvested from healthy plants and secondary PVY^{NTN}-infected plants. Tubers without necrotic symptoms from healthy plants and from secondary PVY^{NTN}-infected plants were selected for the experiment and stored in closed cardboard boxes

continuously at 4 ± 1.3°C and 24 ± 2.6°C as well as initially at 4 ± 1.3°C with subsequent transfer to 24 ± 2.6°C as represented in Figure 1.

Sample collection and RNA extraction

Samples were collected from healthy and infected tubers (non-necrotic and necrotic tissue separately), yielding in 14 biological groups differing in infection and symptom status as well as storage conditions (Figure 1, Table 1). At different time points four different tissues were sampled, each in 5 biological replicates: (1) uninfected potato, (2) infected potato without necrosis, (3) infected potato with necrosis, non-necrotic tissue and (4) infected potato with necrosis, necrotic tissue, which resulted in samples of 14 biological groups (Figure 1, Table 1). Sample collection and RNA extraction were performed as previously described (Sedlar et al. 2018).

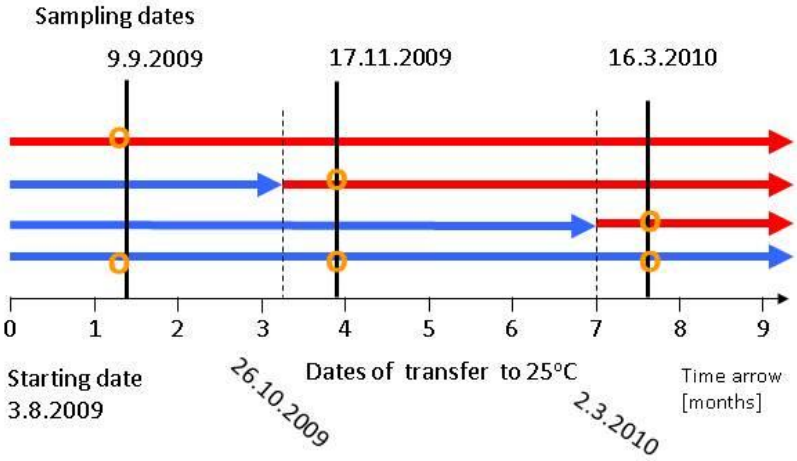


Figure 1: Storage of potato tubers at different temperatures and time points of sampling. Color of horizontal arrows represents different storage temperatures, red: 25 °C, blue – 4 °C. Vertical lines mark times of sampling in the group, while hatched vertical lines mark times of tuber transfer to 25 °C. Yellow circle marks time points of sampling in the group.

Table 1: Description of characters in the 14 biological groups of the sampled potato tubers in the experiment. Five biological replicates were sampled in each of the 14 biological groups.

Biological group	Sampling date	Incubation T (°C)	PVY	Tuber	Tissue type
1	09.09.2009	25	infected	necrotic	non-necrotic
2	09.09.2009	25	infected	necrotic	necrosis
3	10.09.2009	4	uninfected	healthy	healthy
4	10.09.2009	4	infected	non-necrotic	non-necrotic
5	17.11.2009	4-25	uninfected	healthy	healthy
6	17.11.2009	4-25	infected	non-necrotic	non-necrotic
7	17.11.2009	4-25	infected	necrotic	non-necrotic
8	17.11.2009	4-25	infected	necrotic	necrosis
9	17.11.2009	4	uninfected	healthy	healthy
10	17.11.2009	4	infected	non-necrotic	non-necrotic
11	16.03.2010	4-25	infected	non-necrotic	non-necrotic
12	16.03.2010	4-25	uninfected	healthy	healthy
13	16.03.2010	4	infected	non-necrotic	non-necrotic
14	16.03.2010	4	uninfected	healthy	healthy

Microarray processing

Five biological replicates were sampled in each of the 14 biological groups. For all of these 70 samples, RNA quality and quantity were determined using the 2100 Bioanalyzer (Agilent Technologies) and the NanoDrop ND -1000 UV-VIS spectrophotometer (Thermo Fischer Scientific). Four out of five samples for each biological group with the highest RNA quality were selected for further microarray processing. In total, 56 samples representing 14 biological groups, each with four samples as biological replicates were included in the microarray analysis. Microarray processing was performed at IMGM Laboratories GmbH (Martinsread, Germany) using custom 60-mer oligo microarrays (4 × 44; design ID 015425), designed by the Potato Oligo Chip Initiative (POCI) consortium (Kloosterman et al. 2008) and kits and software from Agilent Technologies, as previously described (Sedlar et al. 2018).

Identification of stably expressed genes and stability evaluation of previously reported reference genes

Raw data of the 56 microarray samples were analysed in R statistical environment (RSTUDIO v. 0.98.1102, R v. 3.1.2) using BIOCONDUCTOR v. 3.3 package LIMMA (linear models for microarray analysis) v 3.27.6 (Smyth 2004). The data were analysed using a single emission channel and normalized between arrays using the quantile method. Normalized data were fitted to the linear model and empirical Bayes statistics for differential expression was applied. Ten comparisons were evaluated factoring in sampling time, storage temperature, status of infection and presence of necrosis, and interactions of these conditions (Table 2, Supplementary Figure 1). A table of POCI unigenes (Kloosterman et al. 2008) ranked by $\log_2$-fold change, adjusted P-value and A score, was extracted from the linear model fit. Benjamini-Hochberg correction was used to adjust the P-values for multiple testing. For identification of stably expressed genes, POCI unigenes were filtered for high A scores (mean values in the upper quartile) and low expression level ($\log_2$-fold change between -0.25 and 0.25) across all comparisons. Additionally, genes with high A scores at more stringent condition of $\log_2$-fold change between -0.1 and 0.1 were inspected. POCI unigene sequences were analysed using NCBI BLAST to identify suitable candidate genes for evaluation with qPCR. For stability evaluation of previously reported reference genes, the gene sequences were searched for in NCBI database using GebBank ID numbers. Fasta sequences were searched against the PLAZA database to determine all corresponding potato gene orthologues (Van Bel et al. 2012). GoMapMan database was utilised to identify corresponding POCI unigenes (Ramšak et al. 2013). The raw and processed data as well as determined differentially expressed genes of the microarray experiment are deposited in the ArrayExpress database with accession E-MTAB-1071 (http://www.ebi.ac.uk/arrayexpress/files/E-MTAB-1071).

Table 2: Different studied parameters affecting gene expression in potato tubers.

Parameters' abbreviation	Independent parameters affecting differential expression	# differentially expressed (DE) genes	Cut-off values
T1	The first tuber storage time (Sept 2009 - Nov 2009)	509	*
T2	The second tuber storage time (Nov 2009 – March 2010)	566	*
Temp	The storage temperature (4°C *vs.* 25°C),	4.239	*
T1xtemp	The interaction of the first storage time and temperature	370	*
T2xtemp	The interaction of the second storage time and temperature	982	*
Infect	The infection status of the plant / tuber (PVY infected *vs.* healthy)	191	**
T1xinfect	The interaction of the first storage time and infection status	8	**
T2xinfect	The interaction of the second storage time and infection status	0	*
Tuber	The tuber status (non-necrotic *vs.* necrotic)	78	**
Tissue	The tuber tissue type (healthy *vs.* necrosis)	2.255	*

* - lfc > 2, adj.p < 0.001, B > 4

** - lfc > 1, adj.p < 0.001,B > 4

Quantitative real-time PCR analysis and gene stability evaluation

Sequences representing POCI unigenes of four candidate reference genes were selected by employing NCBI Blast and EBI Ensembl Plants tools. Specific primers and probes were designed by Applied Biosystem service Array on demand on provided sequences (Supplementary Table 1, Supplementary Figure 1). Quantitative real-time PCR analysis was performed as previously reported (Sedlar et al. 2018). TaqMan probes marked with VIC dye were used. Raw C_T data was transformed into relative expression levels based on inter-plate calibrators and efficiency curves. Expression stability was evaluated using delta C_T method (Silver et al. 2006), algorithms GeNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004), as well as comprehensive ranking by RefFinder (Xie et al. 2012).

RESULTS

Evaluation of gene expression stability of previously reported constitutively expressed genes

The gene expression stability of eight previously reported potato tuber reference genes was evaluated utilizing the microarray gene expression dataset (Supplementary Table 2, Figure 2a). Multiple corresponding gene orthologues were determined for each gene, based on the published gene sequences. The number of unigenes on the POCI microarray corresponding to each of these genes ranged from 4 to 31. The most consistent gene expression among all POCI unigenes was observed for beta tubulin, *L2* and cyclophillin however even for these genes, variation was higher for parameters temperature and tissue infection than in other tested parameters. Among these eight tested generally accepted reference genes, genes *EF1-α* and *Hsp20.2* exhibited the highest variation of POCI unigene expression in several conditions, including temperature, tissue infection and sampling time.

Identification of potato genes with high and stable expression in a 56 sample microarray dataset

Among the 44k POCI unigenes 14.381 probes had a raw expression in the upper quartile and a low variation in the differential gene expression analysis (log_2-fold change between -0.25 and 0.25) for all 56 tested samples of 14 biological groups across tested conditions. Four of these genes, coding for highly conserved proteins, namely *NCLN*, *EIF5A*, *KATN* and *USP* were chosen for evaluation with qPCR. For these genes also, multiple corresponding gene orthologues were determined with the number of POCI unigenes ranging from 2 to 5. The expression patterns of POCI unigenes were the most consistent for *NLCN* and *USP* within each condition as well as across all examined conditions (Supplementary Table 2, Figure 2b).

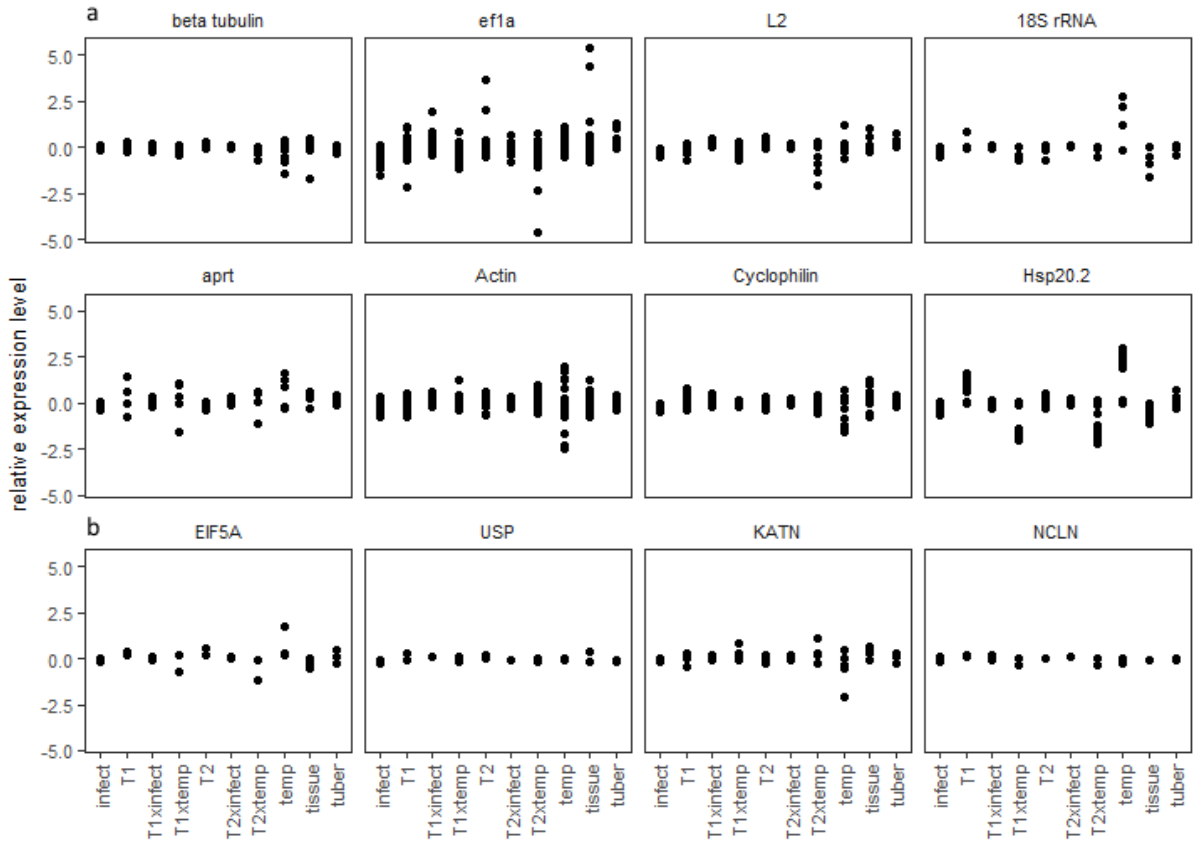


Figure 2: Expression of reference genes (a) from the literature and (b) from novel candidate reference genes across 10 comparisons as determined by microarray analysis. Gene expression of individual POCI unigenes is displayed.

Validation of gene expression stability for candidate reference genes

Expression stability of four selected candidate reference genes was validated by RT-qPCR analysis. The C_T values of four selected genes varied from 21 to 32 and according to the average C_T values, *EIF5A* was the most abundantly expressed gene, followed by *USP*, *NCLN* and *KATN* (Figure 3a).

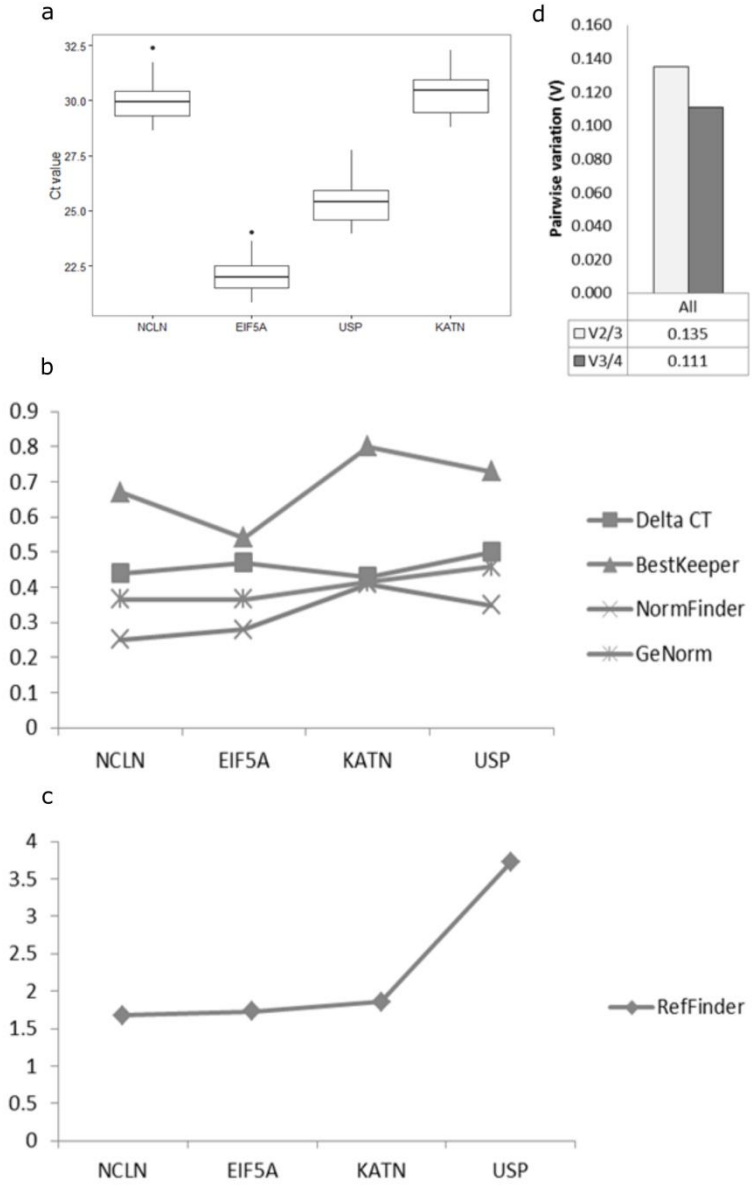


Figure 3: RT-qPCR validation of four candidate reference genes (*NCLN*, *EIF5A*, *KATN* and *USP*); (a) C_T values four candidate reference genes (*NCLN*, *EIF5A*, *KATN* and *USP*), (b) stability values calculated by four algorithms: delta C_T, BestKeeper, NormFinder and GeNorm, (c) as well as the comprehensive ranking by RefFinder are displayed. (d) The pairwise variation between the best two genes (NCLN and EIF5A) with stepwise inclusion of additional genes (V2/3, V3/4) was lower than the proposed cut-off value of 0.15, meaning that the variation between reference genes changes very little by addition of the third gene to the two best genes. Since addition has no significant effect the best two reference genes (NCLN and EIF5A) are sufficient for normalisation of genes of interest. V2/3 represents the pairwise variation of two genes compared to that with three, V3/4 represents three compared to four.

Similarities between the rankings of expression stabilities across all the experimental conditions, determined using four different methods (dC_T, algorithms GeNorm, NormFinder and BestKeeper) and comprehensive ranking by RefFinder were determined (Figure 3b, c). *NCLN* was ranked in the two top stable genes by all individual methods as well as the comprehensive ranking by RefFinder (Table 3). *EIF5A* was ranked in the top two stable genes according to BestKeeper, NormFinder and GeNorm algorithms, as well as comprehensive ranking by RefFinder. *KATN* was ranked in the top two stable genes only by dC_T method (Table 3).

Table 3: Expression stability and ranking of the candidate reference genes evaluated by four algorithms and comprehensive RefFinder ranking.

Gene	RefFinder		Delta C_T		BestKeeper		NormFinder		GeNorm	
	Geomean	Rank	Mean SD	Rank	SD [+/- CP]	Rank	Stability	Rank	Stability	Rank
NCLN	1.68	1	0.44	2	0.67	2	0.251	1	0.365	1
EIF5A	1.73	2	0.47	3	0.54	1	0.28	2	0.365	1
KATN	1.86	3	0.43	1	0.8	4	0.41	4	0.415	2
USP	3.72	4	0.5	4	0.73	3	0.349	3	0.458	3

All evaluated genes passed the validation according to the proposed stability tresholds of GeNorm and BestKeeper algorithms. GeNorm algorithm ranks genes by M-values, with lower values signifying greater stability. According to M-values the stabilities of all four reference genes were below the recommended threshold of 1.5, and even below the more stringent threshold of 0.5. The pairwise variation between the best two genes (NCLN and EIF5A) with stepwise inclusion of additional genes (V2/3, V3/4) was lower than the proposed cut-off value of 0.15 (Figure 3d), meaning that the variation between reference genes changes very little by addition of the third gene to the two best genes. Since addition has no significant effect the best two reference genes are sufficient for normalisation of genes of interest. Bestkeeper employs the pair-wise correlation analysis of raw C_T values for all pairs of candidate genes and calculates the geometric mean of the most suited ones. Most stably expressed genes, exhibiting the lowest variation, are ranked the highest. Any studied gene with the standard deviation (SD) higher than 1 can be considered inconsistent. All examined genes in ours study had SD < 1 and are therefore considered suitable to be used as reference genes.

DISCUSSION

Infection with PVY^{NTN} can result in great loss of harvested potato tubers of susceptible cultivars, however necrotic symptom development can be mitigated by proper post-harvest storage conditions (Dolničar et al 2011). Study of potato-PVY^{NTN} interactions in infected tubers during storage facilitated understanding of the factors contributing to necrosis development and may enable more effective symptom prevention (Sedlar et al 2018). RT-qPCR is an established method for testing gene expression and its accuracy relies on the normalization to expression of constitutively expressed genes. Several reference genes have been used for differential gene expression in potato tubers, and most of them were chosen based on general knowledge of their constitutive expression (Kloosterman et al. 2008; Pompe-Novak et al. 2006; Lopez-Pardo et al. 2012) while data for expression for specific pathogen interactions remains limited.

To find appropriate reference genes for our experimental setup of PVY^{NTN} - potato tuber interaction we screened the potato tuber microarray data set for the potential candidate genes. This enabled us to select genes with lowest variability across all our experimental conditions. The advantage of this method is that it eliminates the need to evaluate a large number of potential reference genes. This is especially important in experimental set-ups, where available RNA quantities are low, and only a limited number of reference genes and genes of interest can be assessed.

The four selected candidate reference genes, *NCLN*, *EIF5A*, *USP* and *KATN*, chosen for validation with RT-qPCR, are genes for highly conserved proteins, respectively involved in functions associated with signalling, plant development, cytoskeleton organisation and defence mechanism. Nicalin is a metazoan nodal signalling antagonist, which is highly conserved also in plants where it may play a similar role (Haffner et al. 2004). EIF5A, originally identified as translation initiation factor, plays a role in plant growth and development (Feng et al. 2007). While USPs are involved in a biological defence mechanism and tend to be over-expressed in plants under stress conditions, different number of copies of the gene exist depending on the species, with little detail on their exact function (Tkaczuk et al. 2013). Katanin is a microtubule severing protein that affects microtubule organisation and dynamics in higher plants (Luptovčiak et al. 2017). Among the four candidate reference genes the lowest variation was observed for *NCLN* and *USP*.

The variation in gene expression of previously reported reference genes was higher than in our four selected candidate reference genes. Among the previously reported reference genes even the overall most stable

genes, such as beta tubulin exhibited variation of POCI unigene expression for our testing parameters temperature and tissue. The high variation of POCI unigenes expression for *EF1-α* and *Hsp20.2* in several of our testing conditions presumably corresponds with their reported role during stress. *EF1-α* was reported as a host factor in viral pathogenesis and is essential for the virulence of soybean mosaic virus (Potyviridae) and the associated unfolded protein response (Luan et al. 2016). Hsp20.2 is a plant heat shock protein induced by various abiotic stressors, such as heat, cold and salinity (Al-Whaibi 2011).

Several algorithms were developed for evaluation and ranking of multiple candidate reference genes across desired conditions (De Spiegelaere et al. 2015). Among the most widely used are GeNorm that utilizes pairwise comparisons and NormFinder based on estimation of intra- and inter-variability of candidate reference genes in samples of interest. Since changes in ranking can be observed between these algorithms their outputs were compared as this is a standard practice (Lopez-Pardo et al. 2012; De Spiegelaere et al. 2015; Mariot et al 2015). In our study, all four selected candidate genes proved suitable reference genes based on dC_T values, stability scores by 3 algorithms (GeNorm, NormFinder and BestKeeper), and comprehensive ranking by RefFinder. Despite different algorithms *NCLN* and *EIF5A* consistently ranked as the top two most stable reference genes according to multiple methods. Therefore they should be considered as the first choice for the study of postharvest tuber gene expression with regard to changing temperature and PVY infection status conditions.

In conclusion, we have demonstrated the utilization of genome-wide expression data for selection and validation of reference genes in potato tubers under different abiotic and biotic conditions. The validated reference genes can be used to analyse gene expression in postharvest potato tubers differing in storage temperature and time, PVY^{NTN} infection status, and symptom presence.

ACKNOWLEDGMENTS

This study was financially supported by the Slovenian Research Agency (project L4-2400-0401, programme P4-0072).

REFERENCES

Al-Whaibi, M. H. 2011. Plant heat-shock proteins: A mini review. Journal of King Saud University - Science 23: 139–150. doi:10.1016/j.jksus.2010.06.022.

Andersen, C. L., J. L. Jensen, and T. F. Ørntoft. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64: 5245–5250. doi:10.1158/0008-5472.CAN-04-0496.

Charfeddine, M., D. Bouaziz, S. Charfeddine, A. Hammami, O.N. Ellouz and R. Bouzid. 2016. Overexpression of dehydration-responsive element binding 1 protein (*DREB1*) in transgenic *Solanum tuberosum* enhances tolerance to biotic stress. Plant Biotechnology Reports 9: 79–88. doi:10.1007/s11816-015-0345-8.

De Spiegelaere, W., J. Dern-Wieloch, R. Weigel, V. Schumacher, H. Schorle, D. Nettersheim, M. Bergmann, et al. 2015. Reference gene validation for RT-qPCR, a note on different available software packages. *PLoS ONE* 10: e0122515. doi:10.1371/journal.pone.0122515.

Dolničar, P., I. Mavrič Pleško, and V. Meglič. 2011. Long-term cold storage suppress the development of tuber necrosis caused by PVY^{NTN}. American Journal of Potato Research 88: 318–323. doi:10.1007/s12230-011-9196-1.

Feng, H., Q. Chen, J. Feng, J. Zhang, X. Yang, and J. Zuo. 2007. Functional characterization of the Arabidopsis Eukaryotic translation initiation factor 5A-2 that plays a crucial role in plant growth and development by regulating cell division, cell growth, and cell death. Plant Physiology 144: 1531–1545. doi:10.1104/pp.107.098079.

Haffner, C., M. Frauli, S. Topp, M. Irmler, K. Hofmann, J. T. Regula, L. Bally-Cuif, and C. Haass. 2004. Nicalin and its binding partner Nomo are novel Nodal signaling antagonists. *The EMBO Journal* 23: 3041–3050. doi:10.1038/sj.emboj.7600307.

Joseph, J. T., N. Jabeen Poolakkalody, J. M. Shah. 2018. Plant reference genes for development and stress response studies. Journal of Biosciences 43: 173-187. doi:10.1007/s12038-017-9728-z.

Kloosterman, B., D. De Koeyer, R. Griffiths, B. Flinn, B. Steuernagel, U. Scholz, S. Sonnewald, U. Sonnewald, G.J. Bryan, S. Prat *et al*. 2008. Genes driving potato tuber initiation and growth: identification based on transcriptional changes using the POCI array. Functional & Integrative Genomics 8: 329–340. doi:10.1007/s10142-008-0083-x.

Lopez-Pardo, R., J. I. Ruiz de Galarreta, and E. Ritter. 2012. Selection of housekeeping genes for qRT-PCR analysis in potato tubers under cold stress. *Molecular Breeding* 31: 39–45. doi:10.1007/s11032-012-9766-z.

Luan, H., M.B. Shine, X. Cui, X. Chen, N. Ma, P. Kachroo, H. Zhi and A. Kachroo. The Potyviral P3 protein targets Eukaryotic elongation factor 1A to promote the unfolded protein response and viral pathogenesis. Plant Physiology 172: 221-234. doi:10.1104/pp.16.00505

Luptovčiak, I., G. Komis, T. Takáč, M. Ovečka, and J. Šamaj. 2017. Katanin: A sword cutting microtubules for cellular, developmental, and physiological purposes. Frontiers in Plant Science 8: 1982. doi:10.3389/fpls.2017.01982.

Mariot, R. F., L. Abruzzi de Oliveira, M. M. Voorhuijzen, M. Staats, R. C. B. Hutten, J. P. Van Dijk, E. Kok, and J. Frazzon. 2015. Selection of reference genes for transcriptional analysis of edible tubers of potato (*Solanum tuberosum* L.). PloS One 10: e0120854. doi:10.1371/journal.pone.0120854.

Nicot, N., J.-F. Hausman, L. Hoffmann, and D. Evers. 2005. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. Journal of Experimental Botany 56: 2907–2914. doi:10.1093/jxb/eri285.

Pfaffl, M. W., A. Tichopad, C. Prgomet, and T. P. Neuvians. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnology Letters 26: 509–515. doi:10.1023/B:BILE.0000019559.84305.47.

Pompe-Novaka M., K. Gruden, Š. Baeblera, H. Krečič-Stresa, M. Kovač, M. Jongsma and M. Ravnikar.2006. Potato virus Y induced changes in the gene expressionof potato (*Solanum tuberosum* L.). Physiological and Molecular Plant Pathology 67: 237-247.

Ramšak, Ž., Š. Baebler, A Rotter, M Korbar, I Mozetič, B Usadel, and K Gruden. 2013. GoMapMan: integration, consolidation and visualization of plant gene annotations within the MapMan ontology. Nucleic acids research 42: D1167-1175. doi:10.1093/nar/gkt1056.

Sedlar, A., B. Gerič Stare, I. Mavrič Pleško, P. Dolničar, M. Maras, J. Šuštar-Vozlič, Š. Baebler, K. Gruden, and V. Meglič. 2018. Expression and regulation of programmed cell death associated genes in systemic necrosis of PVY^{NTN} susceptible potato tubers. Plant Pathology 67: 1238-1252. doi:10.1111/ppa.12818.

Silver, N., S. Best, J. Jiang, and S. L. Thein. 2006. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Molecular Biology 7: 33. doi:10.1186/1471-2199-7-33.

Smyth, G. K. 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Statistical Applications in Genetics and Molecular Biology 3: 1–25. doi:10.2202/1544-6115.1027.

Szalonek, M., B. Sierpien, W. Rymaszewski, K. Gieczewska, M. Garstka, M. Lichocka, L. Sass, K. Paul, I. Vass, R. Vankova *et al*. 2015. Potato Annexin STANN1 promotes drought tolerance and mitigates light stress in transgenic *Solanum tuberosum* L. plants. *PLoS ONE* 10: e0132683. doi:10.1371/journal.pone.0132683.

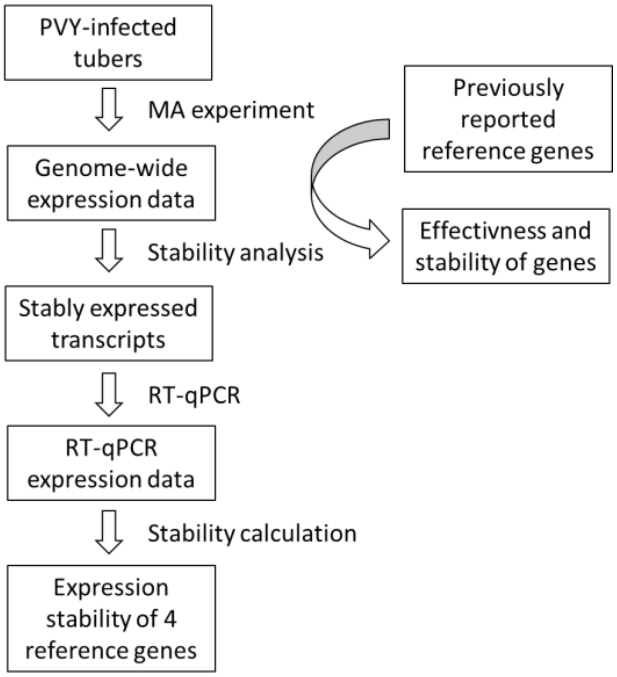
Tkaczuk, K. L., I. A. Shumilin, M. Chruszcz, E. Evdokimova, A. Savchenko, and W. Minor. 2013. Structural and functional insight into the universal stress protein family. Evolutionary Applications 6: 434–449. doi:10.1111/eva.12057.

Van Bel, M., S. Proost, E. Wischnitzki, S. Movahedi, C. Scheerlinck, Y. Van de Peer, and K. Vandepoele. 2012. Dissecting plant genomes with the PLAZA comparative genomics platform. Plant Physiology 158: 590–600. doi:10.1104/pp.111.189514.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3: RESEARCH0034.

Xie, F., P. Xiao, D. Chen, L. Xu, and B. Zhang. 2012. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Molecular Biology

Supplementary material



Supplementary Figure 1: Analysis workflow for identification of stably expressed genes and stability evaluation of previously reported reference genes.

Supplementary table 1: Candidate potato tuber reference genes validated by RT-qPCR.

Gene	Description	ID	Cut-off Log FC	A
NCLN	Nicalin [*Solanum tuberosum*]	Sotub01g010370 micro.524.C1	0.1	8.16
EIF5A	Eukaryotic translation initiation factor 5A	Sotub07g00650 micro.165.C19	0.25	15.43
USP	Universal stress protein	Sotub01g020920 micro.11979.C1	0.25	14.20
KATN	Katanin p60 ATPase-containing subunit	Sotub02g037280 micro.2792.C1	0.25	13.08

ID - gene ID and POCI unigen, respectively
A - Amean / Expression
E - amplification efficiency (%)

Supplementary Table 2: Previously reported (above) and novel (below) reference genes used for gene expression analysis of potato tubers.

Gene	Gene description	Acc. number	No. of POCI unigenes
Beta tubulin	Beta tubulin	609267	7
EF1-α	Elongation factor 1-α	AB061263	31
L2	Cytoplasmic ribosomal protein L2	39816659	8
18S rRNA	18S ribosomal RNA	X67238	4
APRT	Adenine phosphoribosyl transferase	CK270447	5
Actin	Actin	X55749	20
CYP	Cyclophilin	AF126551	11
Hsp20.2	Heat shock protein 20	BQ511516	19
NCLN	Nicalin	MICRO.524.C1	2
EIF5A	Eukaryotic translation initiation factor 5A	MICRO.165.C19	3
USP	Universal stress protein	MICRO.11979.C1	2
KATN	Katanin	MICRO.2792.C1	5