VALIDATING INTERNAL CONTROLS FOR *Cucurbita pepo* REAL-TIME PCR STUDIES

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INTRODUCTION

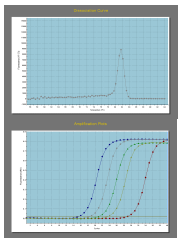
Members of Cucurbitaceae family (pumpkin, squash, cucumber and watermelon) make a significant contribution to our intake of vitamins and minerals. Among them, squash (*Cucurbita pepo*) is an economically important species because of its nutritional quality, relative low price and year-round supply. Several studies have been developed in squash in order to study both quality and stress response aspects. Understanding patterns of expressed genes during squash development may provide insight into complex regulatory networks and could contribute to the breeding process of the species.

A need for reference genes in C. pepo has emerged. For this reason, we have carried out an extensive evaluation using the BestKeeper program (Pfaffl et al. , 2004) with the aim of studying the transcripts stability of eight commonly used housekeeping genes in thirteen C. pepo samples.

This work presents the details and findings from our validation of *18S, elongation factor (elf), actin, tubulin, ubiquitin, protein phosphatase 2A(PDF), helicase (HELI) and glyceraldehyse-3-phosphate dehydrogenase* genes in different *C. pepo* tissues and time points (root, leaf, shoot, flower and fruit) as well as under salinity, cold and drought stresses. The normalization strategy presented here is a prerequisite to accurate real-time PCR expression profiling that opens up the possibility of developing reliable experiments in squash.

PREVIOUS STEPS

1. Selection of commonly housekeeping genes
2. Cloning of specific PCR fragemnt of *C. pepo* gene sequences not available from the public database
3. Specific Real-time PCR primers desing
4. Correct for primer-dimers
5. Correct for PCR efficiency
6. Confirmation of amplification of a specific transcript
7. Avoidance of possible genomic DNA contaminations



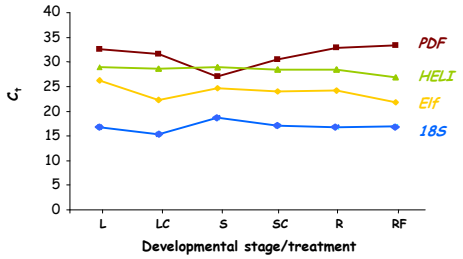
Primer sequences for Real-time PCR of four housekeeping genes studied at the moment. The amplification length obtained as well as PCR efficiencies (E) calculated with LinRegPCR program (Ramakers et al., 2003) are shown

Gene	Primer	Forward & reverse primers 5'→ 3'	Amplicon size (bp)	E
18S		18S primer pair (QuantumRNA 18S, Ambion)	315	1.98
Elf	oli-elf 1	AAG CTA GGA GGT ATT GAC AAG	170	1.99
	oli-elf 2	ACT GTG CAG TAG TAC TTG GTG		
PDF	oli-pdf1	CCA CAT TAC CTG TAT CGG ATG ACA	108	1.98
	oli-pdf2	GAG CCC AGA ACA GGA GCT AAC A		
HELI	oli-heli1	ACA CTG GTC CCT CCC ACA CA	71	2.00
	oli-heli2	GCG GGC ACT TGG AGA TTA TC		

FINDING OPTIMUN REFERENCE GENES

✓Ct values for *PDF* were usually higher than those of other genes, indicating a relatively low level of *PDF* transcription in most life stages of *C. pepo*.

✓ Gene encoding *18S rRNA*, in contrast, was found to display lower Ct values



RNA transcription levels of housekeeping genes tested, presented as Ct mean value in the different samples: (L) leaf, (LC) leaf/cold stress, (S) stem, (SC) Stem/cold stress, (R) root, (RC) root/cold stress.

BestKeeper software statistic for housekeeping genes based on cycle threshold (C_t) values. CP: equivalent terminology for C_t; N: number of samples; n: number of genes; GM (CP): the geometric mean of CP; AM (CP): the arithmetic mean of CP; min (CP) and max (CP): the extreme values of CP; S.D. (± CP): the standard deviation of the CP, CV (%CP): the coefficient of variance expressed as a percentage of the CP level; Min (x-fold) and Max (x-fold): the extreme values of expression levels expressed as an absolute x-fold over or under regulation coefficient; S.D. (± x-fold): standard deviation of the absolute regulation coefficients (Pfaffl et al. 2004).

Data of candidate housekeeping genes (n=4)

Factor	*18S rRNA*	*elf*	*PDF*	*HELI*	BestKeeper (n=4) all genes	BestKeeper(n=3) excl elf	BestKeeper(n=3) excl PDF	BestKeeper(n=2) 18S + HELI
N	5	5	5	5	5	5	5	5
GM (CP)	16.90	23.86	31.25	28.39	24.46	24.66	22.54	21.90
AM (CP)	16.93	23.91	31.32	28.40	24.46	24.66	22.56	21.92
Min (CP)	15.37	21.86	27.09	26.95	23.60	24.04	21.42	20.97
Max (CP)	18.71	26.24	33.37	29.01	25.33	25.05	23.76	23.30
S.D. (± CP)	0.64	1.20	1.69	0.48	0.45	0.29	0.73	0.52
CV (%CP)	3.78	5.04	5.40	1.70	1.84	1.19	3.22	2.38
Min (x-fold)	-2.89	-4.00	-17.83	-2.71	1.81	1.54	2.18	1.91
Max (x-fold)	3.50	5.20	4.36	1.54	1.84	1.31	2.34	2.63
S.D. (± x-fold)	1.56	2.30	3.23	1.40	1.37	1.23	1.65	1.44

✓ When BestKeeper (Pfaffl et al., 2004) was used to compare C_t values in various combinations of the candidate genes (groups of n= 4 or 3), most variation was assigned to *PDF and elf* as well as most stability to *HELI* .

CONCLUSIONS

Taking into account the best housekeeping genes described in plants, they show different stability patterns depending on the species and even the tissue, and the results obtained for one plant species can not be extrapolated to another one. In this regard, what was known about housekeeping genes suitable for Real-studies in *C. pepo* has been very scanty. For this reason, the normalization strategy presented here is a prerequisite for accurate Real-time PCR expression profiling.

In this study, the *C. pepo* genes *18S rRNA, elongation factor, actin, tubulin, ubiquitin, protein phosphatase 2A, helicase and glyceraldehyse-3-phosphate dehydrogenase* will be compared in terms of expression stability using the *BestKeeper* program. Among these genes only *18S rRNA, Elf, PDF* and *HELI have* been studied at the moment and we recommend *HELI* as the most stable gene to obtain the most accurate normalization in *C. pepo* gene expression studies under cold stress.

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