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Original Paper

Evaluation of internal control genes for quantitative realtime PCR analyses for studying fruit development of dwarf tomato cultivar 'Micro-Tom'

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Abstract Quantitative real-time PCR (qRT-PCR) is widely used to analyze the expression profiles of the genes of interest. In order to obtain accurate quantification data, normalization by using reliable internal control genes is essential. In this study, we evaluated the stability and applicability of eight internal control gene candidates for analyzing gene expression during fruit development in dwarf tomato cultivar Micro-Tom. We collected seventeen different samples from flowers and fruits at different developmental stages, and estimated the expression stability of the candidate genes by two statistical algorithms, geNorm and NormFinder. The combined ranking order and qRT-PCR analyses for expression profiles of *SlYABBY2a*, *SlYABBY1a*, *FRUITFULL1* and *APETALA2c* suggested that *EXPRESSED* was the most stable and reliable internal control gene among the candidates. Our analysis also suggested that *RPL8* was also suitable if the sample group is limited to fruits at different maturation stages. In addition to *EXPRESSED*, *GAPDH* was also applicable for relative quantitation to monitor gene expression profiles through fruit development from pistil to pericarp.

Key words: fruit development, internal control gene, Micro-Tom, qRT-PCR, reference gene.

Introduction

Quantitative real-time polymerase chain reaction (qRT-PCR) is one of the valuable and powerful tools for rapid detection and quantification of gene expressions (Bustin 2000, 2002). qRT-PCR promises high specificity, sensitivity, and reproducibility, and precisely detects the changes in gene expressions in a broad range of species, and various tissue samples collected under different experimental conditions (Gachon et al. 2004). Meanwhile, in order to obtain appropriate and reliable results by qRT-PCR, it is necessary to select the optimal methods to normalize the data you gained (Brunner et al. 2004; Bustin 2002; Freeman et al. 1999). This is because the qualities and quantities of RNA among the samples can easily be altered by the differences in amount of samples collected, transcriptional activities and RNA integrities in individual tissues or cells, recovery rate of RNA, or efficiency of cDNA synthesis for each experiment, and such fluctuations can influence the polymerase chain reactions. There are several ways to conquer such problems, yet the most common and simple method is to apply relative quantitation that normalizes the expression levels of the genes of interest by that of internal control genes (Libus and Štorchová 2006; Thellin et al. 1999). To apply relative quantitation, it is necessary to select rational internal control genes. The usage of inadequate and unstable control genes may cause misunderstandings in expression patterns and relative changes in gene expression, and may mask small differences of gene expressions. Thus, the suitable internal control genes should be expressed constantly and stably in all tissues or cells at every developmental stages irrespective of environmental factors, such as biotic and abiotic stresses (Huggett et al. 2005; Løvdal and Lillo 2009), otherwise multiple internal control genes need to be employed to meet the criteria (Thellin et al. 1999; Vandesompele et al. 2002). Housekeeping

Abbreviations: AP2c, *APETALA2c*; Cq, quantitation cycle; *FUL1*, *FRUITFULL1*; NTC, no template controls; qRT-PCR, Quantitative real-time PCR.
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genes, which are essential to maintain the homeostasis of cellular activities, are often used as internal control genes in many qRT-PCR experiments, however, the expressions of several housekeeping genes are known to fluctuate under different experimental conditions (Czechowski et al. 2005; Gutierrez et al. 2008; Thellin et al. 1999). Therefore, it is prerequisite for one to confirm the expression stabilities of the internal controls in prior to the experiments, even if housekeeping genes that seemed to be indispensable for biological activities are chosen for normalization.

Tomato (*Solanum lycopersicum*) is an important crop in terms of agronomic and economic values in the world. After disclosure of tomato genome information in 2012, tomato researches have rapidly developed, and various reports are being published in recent years (The tomato genome consortium 2012). Numerous tomato cultivars are used for academic researches, while tomato dwarf cultivar Micro-Tom has many experimental advantages. Micro-Tom is a suitable model tomato cultivar, because it is small in size, short in life cycle, and easy to cross with other tomato species. In terms of qRT-PCR analysis in tomato, several internal controls have been examined and reported for some cultivars, such as *S. lycopersicom* cv. Moneymaker, Ciliegia, Suzanne and commercial variety ACE (Argyropoulos et al. 2006; Dekkers et al. 2012; Expósito-Rodríguez et al. 2008; Løvdal and Lillo 2009). Meanwhile, to our knowledge, there is only one report examining for the suitable internal control genes during the fruit development in Micro-Tom (González-Aguilera et al. 2016).

In this study, to validate optimal internal control genes to analyze tissue-specific gene expression patterns during the development of reproductive organs in Micro-Tom by qRT-PCR, we selected eight housekeeping genes as internal control gene candidates, and tested for stability and appropriateness for normalization. We collected samples not only from the developing fruits, as in previous reports, but also samples from the developing flowers (pistil and stamen) and a vegetative tissue (leaf). We also collected the pericarp and the gels including the seeds, separately, and examined the stability of the candidate genes in these tissues. By using geNorm and NormFinder algorithms (Andersen et al. 2004; Vandesompele et al. 2002), we evaluated the gene stability, and then analyzed the expression profile of Sl*YABBY2a*, Sl*YABBY1a*, *FRUITFULL1* (*FUL1*) and *APETALA2c* (*AP2c*) by using our candidates. Our data suggests that *EXPRESSED* and *TIP41* are suitable internal controls to analyze gene expressions at least in the seventeen different samples we collected. Yet *TIP41* was relatively unstable in mature red fruits, thus when performing qRT-PCR analysis in developing fruits, multiple internal controls, including *GAPDH* or *RPL8*, are needed to be employed to accurately evaluate the expression profile of gene of interest.

Materials and methods

Plant materials and growth conditions

Tomato *S. lycopersicum* cv. "Micro-Tom" (TOMJPF0001) plant, which is the dwarf cultivar, was used in all experiments (Saito et al. 2011; Shikata et al. 2016). Seeds of these plants were kept on filter papers for germination in a contained plant cultivation room maintained at 25°C, 16 h/8 h (light/dark), and 300 μmol m^{-2} s^{-1}. After 1 week, germinated seedlings were transferred to hydroculture media Rockwool cubes (Grodan) applying with a nutrient solution Otsuka 1 Gou and 2 Gou (OAT Agrio Co., Ltd. Tokyo, Japan).

Sampling on whole tomato developmental stages

Total of 17 samples from different tissues of different developmental stages were collected (Figure 1). 1 mm bud, 4.5 mm bud, and open flowers were prepared as flower samples (Figure 1A). From 4.5 mm and open flowers, pistil and stamen were collected separately (Figure 1B). Different fruit samples were collected from six different developmental stages of Micro-Tom, namely, "1 cm immature green", "2 cm immature green", "mature green", "breaker", "orange", and "mature red". Collected fruits were divided into pericarps (hereafter referred to as "fruits"), and seed/gel, except for 1 cm fruit (Figure 1C). Leaves were harvested at 30 days after sowing. All samples were immediately frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA extraction was performed by using the QIAGEN RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). RNA was quantified using its absorbance at 260 nm. The integrity of RNA was evaluated by 260 nm/280 nm. cDNA was synthesized from 660 ng of total RNA by using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Synthesized cDNA was diluted to 10-folds to be applied for qRT-PCR experiments.

Quantitative Real-Time PCR

qRT-PCR was performed on 96-wells plates with AriaMx Real-Time PCR system (Agilent, Santa Clara, CA, US). Reactions were prepared in a total volume of 20 μl containing: 2 μl of template, 0.8 μl of each amplification primer sets (final concentration of 200 nM each), 10 μl of KAPA SYBR Fast qPCR Master Mix (2x) ROX Low (Kapa Biosystems, Boston, MA, US). The PCR reaction was performed according to the manufacturer's instructions, in brief, 95°C for 3 min, followed by 40 cycles of 3 s at 95°C (denature), and 20 s at 60°C (anneal and extension). Melting curve analysis was performed under the following condition: 30 s at 95°C, 30 s at 65°C, and 30 s at 95°C. At least three independent experiments were performed by using three biological replicates. Baseline and quantitation cycle (Cq) were automatically calculated by using AriaMX1.2

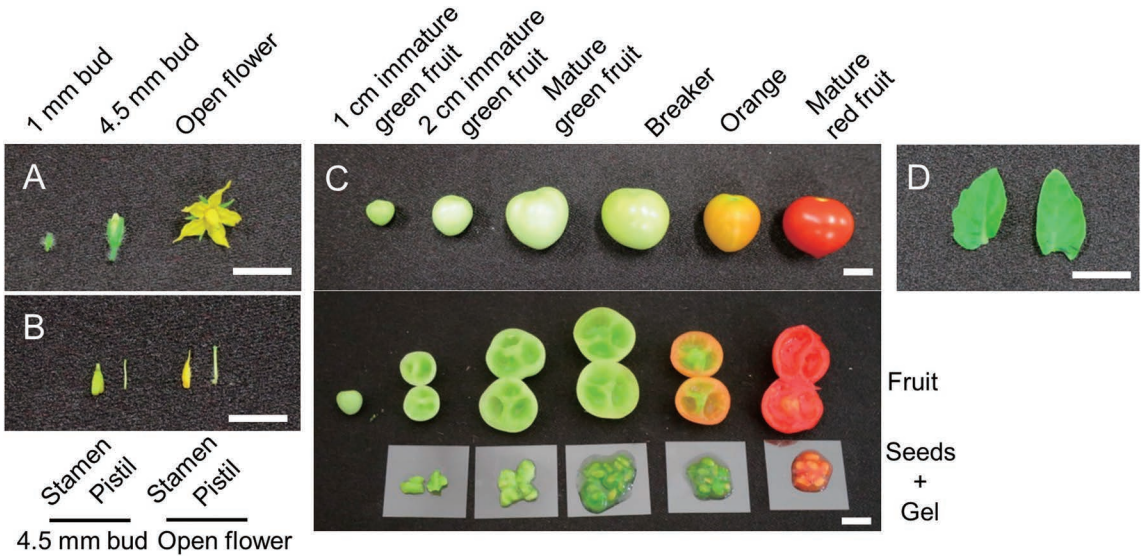

Figure 1. Samples collected for evaluation of internal control gene candidates, and to analyze expression profiles of tissue-specific genes during fruit development in Micro-Tom. Panels show 1 mm bud, 4.5 mm bud and open flower (A), pistil and stamen collected from 4.5 mm bud and open flower (B), individual fruits at different developmental stages (upper panel), and pericarps and seeds/gel portions collected from each stage (lower panel) (C), leaf at 30 days after sowing (D). Bar = 1 cm.

software (Agilent, Santa Clara, CA, US). The primers are constructed as in previous reports (*CAC*, *EXPRESSED*, *RPL8*, *SAND* and *TIP41*; Expósito-Rodríguez et al. 2008, *ACT* and *GAPDH*; Mascia et al. 2010, *PP2a*; Czechowski 2005; Hong et al. 2010, *FUL1* and *AP2c*; González-Aguilera et al. 2016), and listed in Table 2. The primer sets to test for *SlYABBY1a* and *SlYABBY2a* expressions were designed in this study, and listed in Table 2. For all primer pairs, amplification specificities were confirmed by melting curve analysis and gel electrophoresis. In brief, the production of single peak was observed by melting curve analysis to confirm the amplification of single DNA fragment under this PCR conditions. Also, the amplicons were amplified using the mixed cDNA samples as template, and loaded onto 2% agarose gel to confirm for the single bands. The efficiency of PCR reactions was calculated for each primer set by using a dilution series plotted into standard curves.

Evaluation of reference gene stability

The stability of the candidate internal control genes was evaluated by using geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) statistical algorithms. Cq values were converted into relative quantities by using standard curves, then applied for evaluation. The qBase+ software (Biogazelle, Belgium) was installed for geNorm analysis. NormFinder analysis was performed as a free add-in program for Microsoft Excel.

Results and discussion

Sampling of reproductive tissues from Micro-Tom and qRT-PCR analysis of internal control gene candidates

To find internal control genes that are constantly and stably expressed in reproductive tissues (flowers and fruits) at different developmental stages of Micro-Tom, we collected 16 different samples from flowers and fruits (Figure 1). For flowers, pistils and stamens were collected separately, because they are the important organs that fertilize and develop into fruits. For fruits, 11 different samples from six different developmental stages were collected. Except for 1 cm immature green fruits, pericarp (hereafter, referred to as "fruits") and the gel including the seeds were collected separately. As for representative vegetative tissue, 30-day old leaves were collected to check for the genes that are typically expressed in reproductive organs (Figure 1D).

Then, qRT-PCR analysis was performed for 51 cDNA samples (17 samples with 3 biological replicates) by using the primer pairs for eight internal control gene candidates that have been used as reference genes for other studies [*CAC*, *EXPRESSED*, *RPL8*, *SAND* and *TIP41*; (Expósito-Rodríguez et al. 2008), *ACT* and *GAPDH*; (Mascia et al. 2010), *PP2a*; (Czechowski 2005; Hong et al. 2010] (Table 1). Melting-curve analysis, and gel electrophoresis indicated that the primer sets listed in Table 2 successfully amplify the single PCR product of the expected size (Supplemental Figures 1, 2). For the primer sets used to amplify *ACT*, *RPL8*, *SAND*, *TIP41* and *SlYABBY2a*, lower peaks appeared at lower temperatures in controls without templates [no template controls (NTCs); Supplemental Figure 1A, F, G, H, L], and bands corresponding to the nonspecific amplification in NTC appeared in gel electrophoresis analysis (Supplemental Figure 2B). However, such peaks or nonspecific bands were not detectable when template cDNA exists in the reactions. Collectively, the primer sets

Table 1. Description of candidate control genes and validation genes.

Gene symbol	Gene name	Gene ID	Description
ACT	Actin-7	TMCS03g1005280 Solyc03g078400.2.1	Actin protein
CAC	Clathrin adaptor complexes medium subunit	TMCS08g1011650 Solyc08g006960.2.1	Adaptor protein-2 μ-adaptin
EXPRESSED	Expressed sequence	TMCS07g1013650 Solyc07g025390.2.1	Uncharacterized protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase-β-Tubulin	TMCS05g1003510 Solyc05g014470.2.1	Glyceraldehyde-3-phosphate dehydrogenase
PP2a	Phosphatase 2A regulatory A subunit	TMCS05g1002050 Solyc05g009600.2.1	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A β isoform
RPL8	60S ribosomal protein L8	TMCS10g1010670 Solyc10g006580.2.1	60S ribosomal protein L8-like
SAND	SAND family	TMCS03g1010850 Solyc03g115810.2.1	Vacuolar fusion protein MON1
TIP41	TIP41-like protein	TMCS10g1004590 Solyc10g049850.1.1	TIP41-like protein
FUL1	FRUITFULL1	TMCS06g1019990 Solyc06g069430.2.1	agamous-like MADS-box protein AGL8 homolog
AP2c	APETALA2c	TMCS02g1013240 Solyc02g093150.2.1	AP2-like ethylene responsive transcription factor
SlYABBY1a	YABBY 1 of *Solanum lycopersicum*	TMC01g1027040 Solyc01g091010.2.1	YABBY-like transcription factor
SlYABBY2a	YABBY 2 isoform X2 of *Solanum lycopersicum*	TMCS06g1009610 Solyc06g073920.2.1	YABBY-like transcription factor

Table 2. Details of primers of candidate control genes and validation genes and parameters derived from qPCR analysis.

Gene symbol	Prime Sequence Forward/reverse	Amplicon length (bp)*		E (%)**	R^2**
		cDNA	gDNA		
ACT	AGGCAGGATTTGCTGGTGATGATGCT/ATACGCATCCTTCTGTCCCATTCCGA	107	107	102.27	0.982
CAC	CCTCCGTTGTGATGTAACTGG/ATTGGTGGAAAGTAACATCATCG	173	592	95.88	0.992
EXPRESSED	GCTAAGAACGCTGGACCTAATG/TGGGTGTGCCTTTCTGAATG	183	291	91.7	0.996
GAPDH	GGCTGCAATCAAGGAGGAA/AAATCAATCACACGGGAACTG	204	N/A	94.22	0.998
PP2a	TGGCAAAGGTGTTGCAATCC/CTTTCCCCTTTTGCTTCTTCGTG	266	450	99.57	0.996
RPL8	CCGAAGGAGCTGTTGTTTGTA/ACCTGACCAATCATAGCACGA	184	1091	101.89	0.994
SAND	TTGCTTGGAGGAACAGACG/GCAAACAGAACCCCTGAATC	164	3559	107.09	0.989
TIP41	ATGGAGTTTTTGAGTCTTCTGC/GCTGCGTTTCTGGCTTAGG	235	N/A	99	0.995
FUL1	GTTTTGCCACAACAACTGGACTC/CTTGCTGCTGTGAAGAACTACC	106	1124	98.94	0.997
AP2c	CCGTTTCGAATTCAAGTTCA/ACCCAGACCCACCATAGAGA	122	122	107.25	0.981
SlYABBY1a	ACCCAAATGAATCACTCATGCCA/CTTTGATACGTTGGATCTCGTCCT	144	N/A	104.04	0.985
SlYABBY2a	TCTGCAGCACAATTCTTGCG/AATTTGCGCAGTGTCCACAC	84	2346	102.8	0.988

*Estimated form Micro-Tom cDNA/gDNA sequences in TOMATOMICS (http://bioinf.mind.meiji.ac.jp/tomatomics/index.php). **Mean of 3 biological replicates, N/A=Non-amplified.

used in this study are applicable for qRT-PCR analysis.

Schemes to select stable internal control gene candidates

The data obtained by qRT-PCR were subjected to evaluate the expression stability of the candidate genes among the samples we collected. In prior to the analysis, the entire Cq datasets were converted to relative quantity values by using the calibration curves. Then, two representative algorithms, geNorm and NormFinder, were applied (Andersen et al. 2004; Vandesompele et al.

Table 3. Pairwise variation analysis of control genes by geNorm analysis. The $V_{2/3}$ values from all our datasets were below the cut off value of 0.15, indicating that two stable reference genes determined by geNorm are necessary for a reliable normalization.

	All	Fruit	Flower	Seed/gel
$V_{2/3}$	**0.103**	**0.074**	**0.063**	**0.147**
$V_{3/4}$	0.114	0.049	0.08	0.1
$V_{4/5}$	0.116	0.058	0.078	0.116
$V_{5/6}$	0.16	0.072	0.07	0.119
$V_{6/7}$	0.147	0.059	0.118	0.114
$V_{7/8}$	0.119	0.066	0.175	0.128

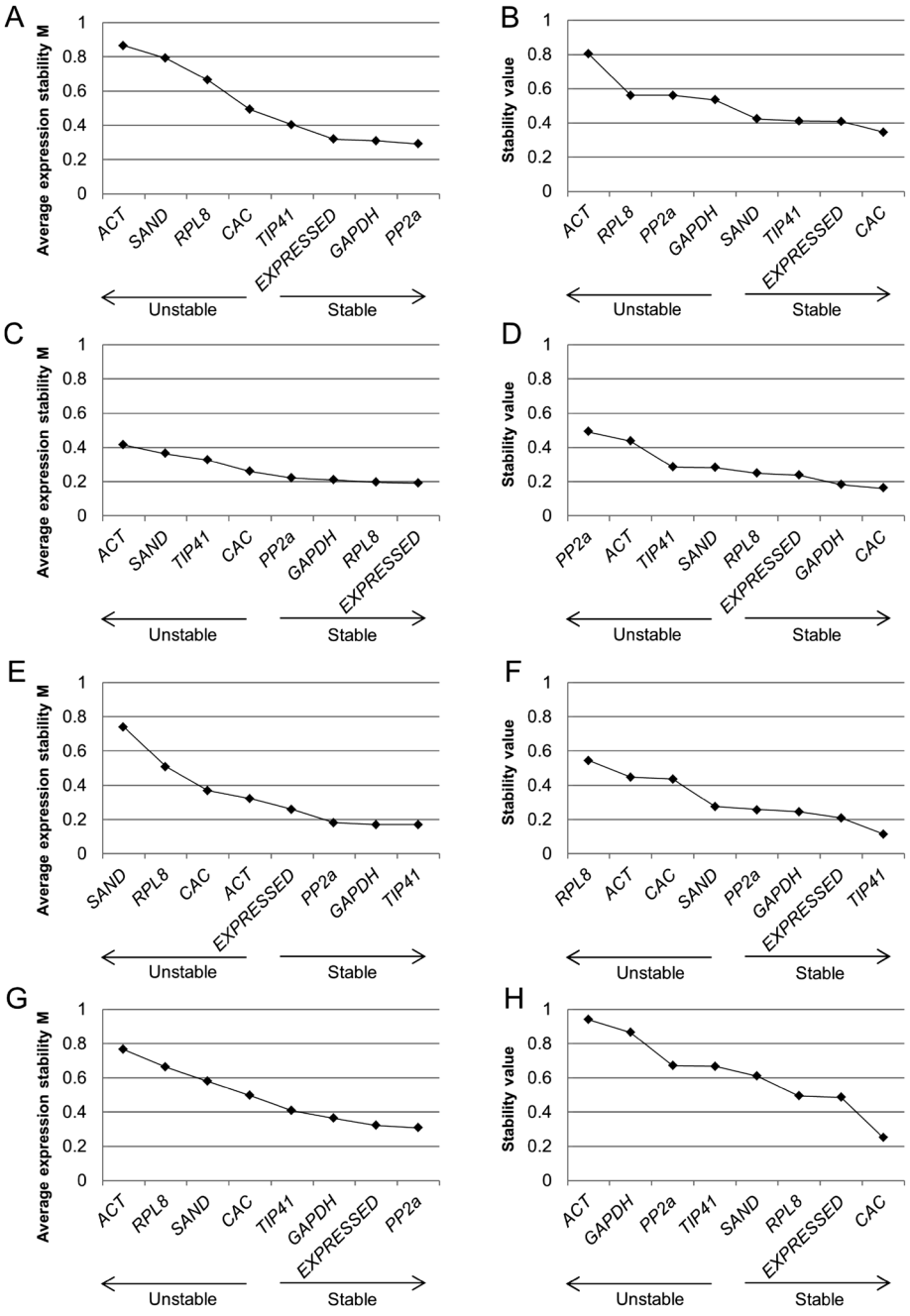

Figure 2. Expression stability of control genes evaluated by geNorm (A, C, E and G) and NormFinder (B, D, F and H) for all datasets (A and B), fruit dataset (C and D), flower dataset (E and F) and seed/gel dataset (G and H). In both analyses, lower values indicate higher stability of the genes. Candidate genes were arranged in the order of the stability on the X-axis.

2002). These algorithms are used to rate the constancy of expressions among samples (Lacerda et al. 2015; Mascia et al. 2010; Müller et al. 2015). The geNorm analysis run by qBase+ software computes an expression stability values (M) for tested genes, as the average pairwise variation among candidates (Vandesompele et al. 2002). The pairwise variation value ($V_{n/n+1}$) suggests for the minimum number of internal control genes employed for normalizations. The pairwise analysis using our datasets indicated that at least two internal control genes should be employed for reliable normalizations (Table 3). NormFinder also calculates stability values for each candidates (Andersen et al. 2004). NormFinder enables grouping of the samples, and calculates the intra- and intergroup variation of candidate genes, and suggests suitable internal control gene. Our data indicated that all candidate genes displayed stability values below 1.5, suggesting for the housekeeping functions of the selected genes (Mascia et al., 2010; Lacerda et al., 2015; Müller et al., 2015) (Figure 2A, B). Meanwhile, the stability values output by geNorm and NormFinder analyses were distinctive for each candidate. For example, *PP2a*, which

was characterized as the most stable gene by geNorm analysis, was ranked to 6th (less stable) by NormFinder analysis (Figure 2A, B). Likewise *CAC*, which was suggested as a good reference gene in other studies (Expósito-Rodríguez et al. 2008; González-Aguilera et al. 2016), was classified as the most stable gene by NormFinder analysis, but less stable gene (5th from the most stable) by geNorm analysis (Figure 2A, B). On the other hand, *EXPRESSED* was ranked as highly stable gene by both analysis. Therefore, we arbitrary grouped the first four genes that were ranked stable by geNorm and NormFinder analyses, and extracted the ones that were commonly classified as highly stable genes by both algorithms (Figure 3). Similarly, we grouped the last four genes that were indicated as less stable, and characterized them as "unstable genes". Table 4 summarizes the results deduced from this classification schemes. Our results indicated that *EXPRESSED* and *TIP41* were grouped into "stable" genes, whereas *ACT* and *RPL8* were classified as "unstable" genes, when qRT-PCR data obtained from all samples (17 different samples with three biological replicates) were subjected for this classification scheme (Table 4).

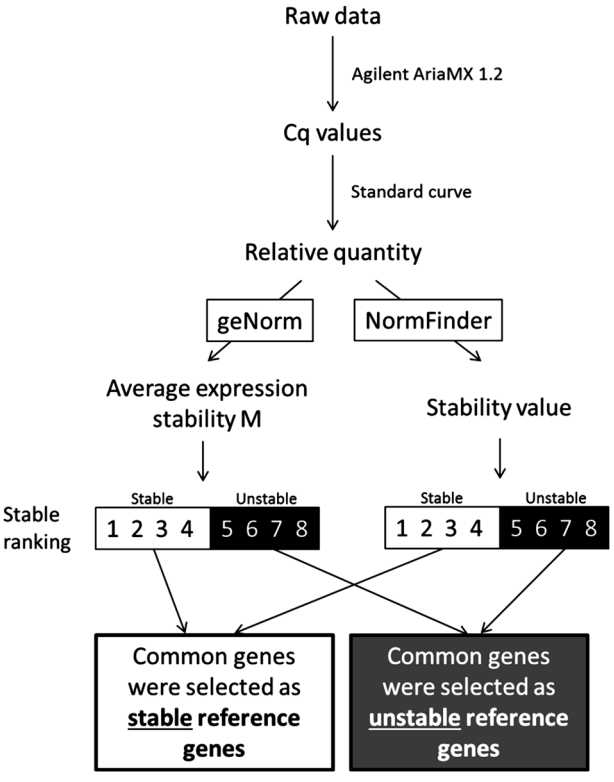

Figure 3. Schemes for combined ranking order. Flow chart represents how the stable and unstable genes were selected in this study.

To see if the optimal candidate genes differ among the tissues, we also classified the candidate genes separately by using datasets only including fruit samples (Figure 2C, D), flower samples (Figure 2E, F), and seed/gel samples (Figure 2G, H). As a result, *EXPRESSED* was classified as the stable gene for every sample groups we analyzed, however, *TIP41* was classified as unstable gene for analyzing fruit samples (discussed later), whereas *RPL8* was classified as "unstable" among flower samples, but "stable" for the group including only the fruits samples (Table 4). *GAPDH* could also be a stable internal control gene candidate, when analyzing and comparing gene expression in developing flowers and fruits. Also, *PP2a* can be employed for normalization when analyzing samples from developing flowers. *EXPRESSED* was the only "stable" internal control candidate for the group including seed/gel samples.

Application of "stable" and "unstable" internal control gene candidates to relative quantitation of gene expressions

To validate the applicability of the selected internal control genes listed in Table 4, we performed further qRT-PCR analysis to see the expression profiles of genes that are specifically expressed during fruit and flower developments (Tables 1 and 2). For this purpose, the expressions of *SlYABBY2a, SlYABBY1a*, *FUL1* and *AP2c* were analyzed by qRT-PCR, and normalized by using the "stable" and "unstable" internal control gene candidates. *YABBYs* encode for the transcription factors which possess Cys_2Cys_2 zinc finger domain and helix-loop-helix motif termed YABBY domain (Golz and Hudson 1999). In *Arabidopsis thaliana*, *INO* and *CRC* are reported as the reproductive *YABBYs* expressed specifically in pistils (Alvarez and Smyth 1999; Villanueva et al. 1999). Tomato has nine *YABBY* genes and eight of which (*SlCRCa*, *SlCRCb*, *SlINO, SlYABBY1a, SlYABBY1b, SlYABBY2a, FAS/SlYABBY2b and SlYABBY5a*) are expressed in floral organs (Ezura et al. 2017; Huang et al. 2013). *SlYABBY2a* and *SlYABBY1a* show notable expression profiles in developing pistils and stamens. The RNA-seq analysis indicated that *SlYABBY2a* was expressed in wide range of floral organs including pistils, stamens, petals, and sepals, and its expression was also monitored in mature green and red fruits (Ezura et al. 2017). In pistils, the expression level of *SlYABBY2a* was shown to elevate, and reached the highest at 7-day-after-flowering stage. Its expression was maintained in mature green fruit stage,

Table 4. List of stable and unstable internal control genes among different datasets.

	All	Fruit	Flower	Seed/gel
Stable	*EXPRESSED TIP41*	*EXPRESSED GAPDH RPL8*	*TIP41 GAPDH EXPRESSED PP2a*	*EXPRESSED*
Unstable	*ACT RPL8*	*ACT SAND TIP41*	*SAND RPL8 CAC ACT*	*ACT*

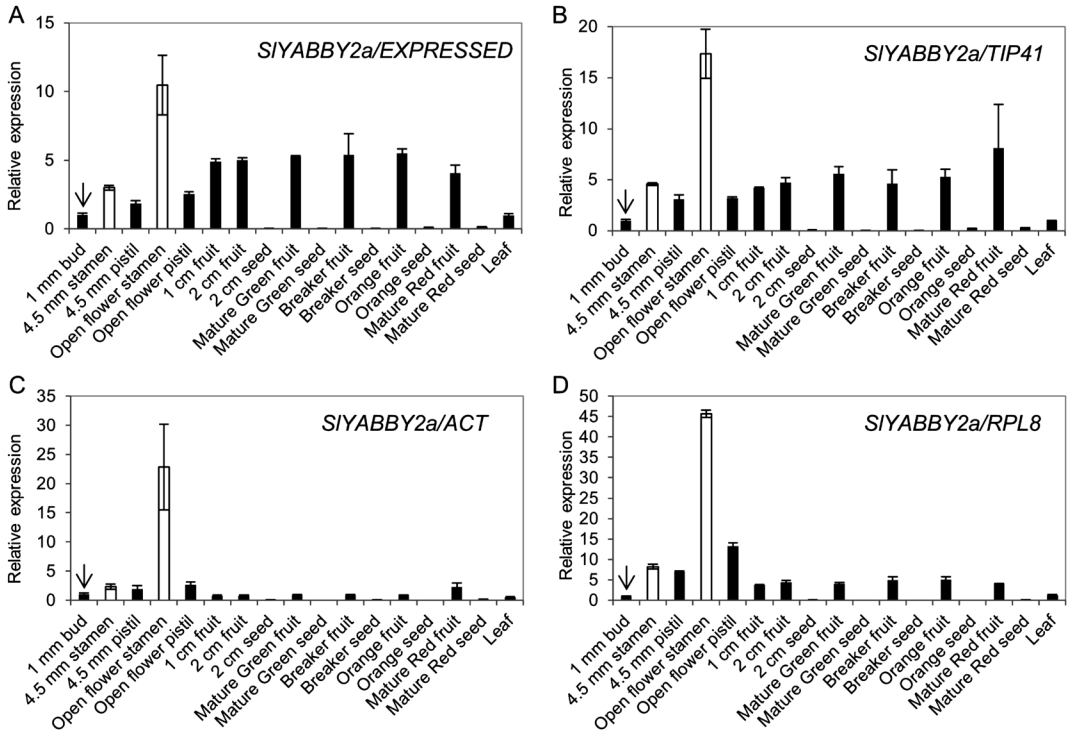

Figure 4. Expression profiles of *SlYABBY2a* normalized with stable genes (*EXPRESSED*; A, *TIP41*; B) and unstable genes (*ACT*; C and *RPL8*; D). For the relative representation, the expression levels at "1 mm bud" stage (arrows) were adjusted to 1. White bars indicate the expression of *SlYABBY2a* in stamens. Error bars represent the standard error of the mean.

and decreased in red fruit stage (Ezura et al. 2017). In comparison to *SlYABBY2a*, *SlYABBY1a* is predominantly expressed in floral organs. The expression of *SlYABBY1a* in pistils was kept high before flowering, and then decreased after anthesis, whereas its expression level in the stamen was elevated after anthesis (Ezura et al. 2017).

To compare the expression profiles normalized by different internal control gene candidates, we firstly performed relative quantitation of *SlYABBY2a* expressions. The RNA-seq analysis indicated that there were approximately 2-folds differences in *SlYABBY2a* expression between young pistils in 1–2 mm bud and mature green fruit (Ezura et al. 2017). When we normalized the expression level of *SlYABBY2a* against *EXPRESSED* and *TIP41*, the stable internal control gene candidates, the fold-changes in *SlYABBY2a* expression from "4.5 mm pistil" to "mature green fruit" stages were 2.8-folds for *SlYABBY2a*/*EXPRESSED* (Figure 4A) and 1.8-folds for *SlYABBY2a*/*TIP41* (Figure 4B). On the other hand, when *SlYABBY2a* expression was normalized against *ACT* (Figure 4C) and *RPL8* (Figure 4D), the unstable internal control gene candidates, its expression looked as though it was decreased during development. The fold-changes in *SlYABBY2a* expression from "4.5 mm pistil" to "mature green fruit" stages were 0.45-folds for *SlYABBY2a*/*ACT* (Figure 4C), and 0.59-folds for *SlYABBY2a*/*RPL8* (Figure 4D). The expression level of *SlYABBY2a* in stamens is reported to elevate by approximately 3-folds upon flowering (Ezura et al. 2017). Our data indicated that the fold changes in relative expression levels of *SlYABBY2a* in stamens collected from 4.5 mm bud ("4.5 mm stamen") and open flower ("open flower stamen") were 3.5-folds for *SlYABBY2a*/*EXPRESSED* (Figure 4A), and 3.8-folds for *SlYABBY2a*/*TIP41* (Figure 4B). However, when the expression of *SlYABBY2a* was normalized against *ACT* (Figure 4C) and *RPL8* (Figure 4D), the fold differences in their expressions were 9.9-times and 5.6-times, respectively.

Similarly, plausible expression profiles of *SlYABBY1a* were obtained by using stable internal control gene candidates. When we normalized the expression level of *SlYABBY1a* against our candidates, the increase in expression levels from "4.5 mm stamen" to "open flower stamen" were 3.6-folds for *SlYABBY1a*/*EXPRESSED* (Supplemental Figure 3A), and 3.9-folds for *SlYABBY1a*/*TIP41* (Supplemental Figure 3B), whereas 10.2-folds for *SlYABBY1a*/*ACT* (Supplemental Figure 3C), and 5.6-folds for *SlYABBY1a*/*RPL8* (Supplemental Figure 3D). It has been demonstrated that *SlYABBY1a*, expressions were increased by approximately 2-folds in stamens (Ezura et al. 2017), thus our data imply that the expression levels of genes in open flower stamen are overestimated when normalized against unstable reference genes.

We further evaluated our internal control gene candidates by analyzing the expression profiles of fruit specific genes, *FUL1* and *AP2c*. *FUL1* is a homolog of

the Arabidopsis MADS domain transcription factor *FUL* (Bemer et al. 2012). In tomato cultivar Micro-Tom and MT-*Rg1*, the *FUL1* expression in fruit is relatively low at immature stages, but gradually increases as the fruit develops and ripens (Bemer et al. 2012; González-Aguilera et al. 2016). *FUL1* was also reported to be expressed in the stamens at lower level (Hileman et al. 2006). *AP2c* was identified as a homolog of Arabidopsis *AP2*, a member of the *AP2/ETHYLEN REPONSE FACTOR (ERF)* transcription factor superfamily that is involved in ethylene biosynthesis and signaling (Karlova et al. 2011). *AP2c* is highly expressed in flowers at anthesis and in immature green fruits, and then its expression level decreases in mature fruits (González-Aguilera et al. 2016; Karlova et al. 2011). When *FUL1* was normalized against *EXPRESSED* (Supplemental Figure 4A) and *TIP41* (Supplemental Figure 4B), we could monitor maturation stage-dependent increase in expression during the course of fruit development and ripening. However, the relative expression level of *FUL1* appeared to be 2.3-folds higher for *FUL1*/*TIP41* compared to *FUL1*/*EXPRESSED* at mature red fruits stage (discussed later). We could also see the expression of *FUL1* in stamen collected from the open flowers. However, unlike the previous report (Bemer et al. 2012), the relative expression of *FUL1* in open flower stamen appeared to be the highest in open flower stamen, when the datasets were normalized against the unstable internal gene candidates (Supplemental Figure 4C, D). In comparison to expression level in "open flower stamen", the *FUL1* expressions appeared to be 2- and 2.2-folds higher than that in "mature red fruit" stage when normalized against *ACT* (Supplemental Figure 4C) and *RPL8* (Supplemental Figure 4D), respectively. Likewise, the expressions of *AP2c* in open flower stamens were over-extrapolated, when their expressions were normalized against *ACT* and *RPL8* (Supplemental Figure 5).

EXPRESSED and RPL8 are suitable internal control genes for qRT-PCR analysis in developing fruits

Interestingly, *TIP41* was classified as "stable" internal control to analyze 17 different samples we employed in this study, but classified to "unstable" gene when we limit the sample groups to fruits (Table 4). Contrarily, *RPL8* was evaluated as "stable" gene among the fruit sample sets (Table 4). Thus, we finely examined the validity of *TIP41* and *RPL8*, as well as *EXPRESSED* and *ACT*, for qRT-PCR analysis using datasets collected from developing fruits (Figure 5). When the expression of *FUL1* was normalized against *EXPRESSED* (Figure 5A) and *RPL8* (Figure 5B), we could monitor the developmental stage-dependent increase in *FUL1* as reported previously (Bemer et al. 2012; González-Aguilera et al. 2016). Notably, the expression level of *FUL1* was reported to double from "mature green fruit" to "breaker" stages, then reached the maxima at mature red fruits stage (Bemer et al. 2012; González-Aguilera et al. 2016). Normalization either by stable and unstable internal control gene candidates indicated such an increase in *FUL1* expression from "mature green fruit" to "breaker" stages (Figure 5A to D; *EXPRESSED*; 2.0-folds, *RPL8*; 2.4-folds, *TIP41*; 1.7-folds and *ACT*; 2.1-folds). However, when *FUL1* expression was normalized against *TIP41* (Figure 5C) and *ACT* (Figure 5D), the expression levels of *FUL1* at mature red fruit stage appeared to be elevated by 6.6- and 13.9-times, respectively, compared to 3.9-folds for *FUL1*/*EXPRESSED* and 4.7-folds for *FUL1*/*RPL8* (Figure 5A, B).

Previous reports indicated that the expression of *AP2c* was gradually lost as the fruit matures and ripens (González-Aguilera et al. 2016; Karlova et al. 2011). Such expression profiles were prominent when *AP2c* expression was normalized against *EXPRESSED* (Figure 5E) and *RPL8* (Figure 5F). On the other hand, when *AP2c* expression was normalized against *TIP41* (Figure 5G) and *ACT* (Figure 5H), *AP2c* expression appeared to decrease from "1cm fruit" to "mature red fruit" stages only by 4.9- and 2.8-folds, respectively, compared to 10.1-folds for *AP2c*/*EXPRESSED* (Figure 5E), and 7.6-folds for *AP2c*/*RPL8* (Figure 5F). Collectively, our results suggest that the expression levels of the target genes may be overestimated especially in "mature red fruit" stages if unstable internal control genes are utilized for normalization. Our data also suggests that *EXPRESSED* and *RPL8* are good internal controls when performing qRT-PCR analysis of the target genes only in developing fruits.

EXPRESSED and GAPDH are ideal internal control genes for qRT-PCR analysis when comparing gene expressions in developing fruits and flowers

Pistils and stamens are important reproductive tissues that develop into fruits, thus the gene expressions in floral organs often need to be monitored in comparison to that in developing fruits. By our classification, *EXPRESSED* and *GAPDH* were found to be the "stable" internal control gene candidates among fruits and flower datasets (Table 4). To test whether these candidates could be the ideal internal controls for qRT-PCR analysis monitoring expression profiles in developing flowers and fruits simultaneously, we performed relative quantitation of *SlYABBY2a* expressions to *EXPRESSED* and *GAPDH*. According to the recent report, the expression of *SlYABBY2a* in female reproductive tissues is kept high in pistils, especially in 7 days after flowering, until mature green fruit stage, then decreased to red fruit stage (Ezura et al. 2017). Such an expression profile was reproduced when *SlYABBY2a* was normalized against *EXPRESSED* (Figure 6A) and *GAPDH* (Figure 6B). When the expression of *SlYABBY2a* was normalized against *TIP41*

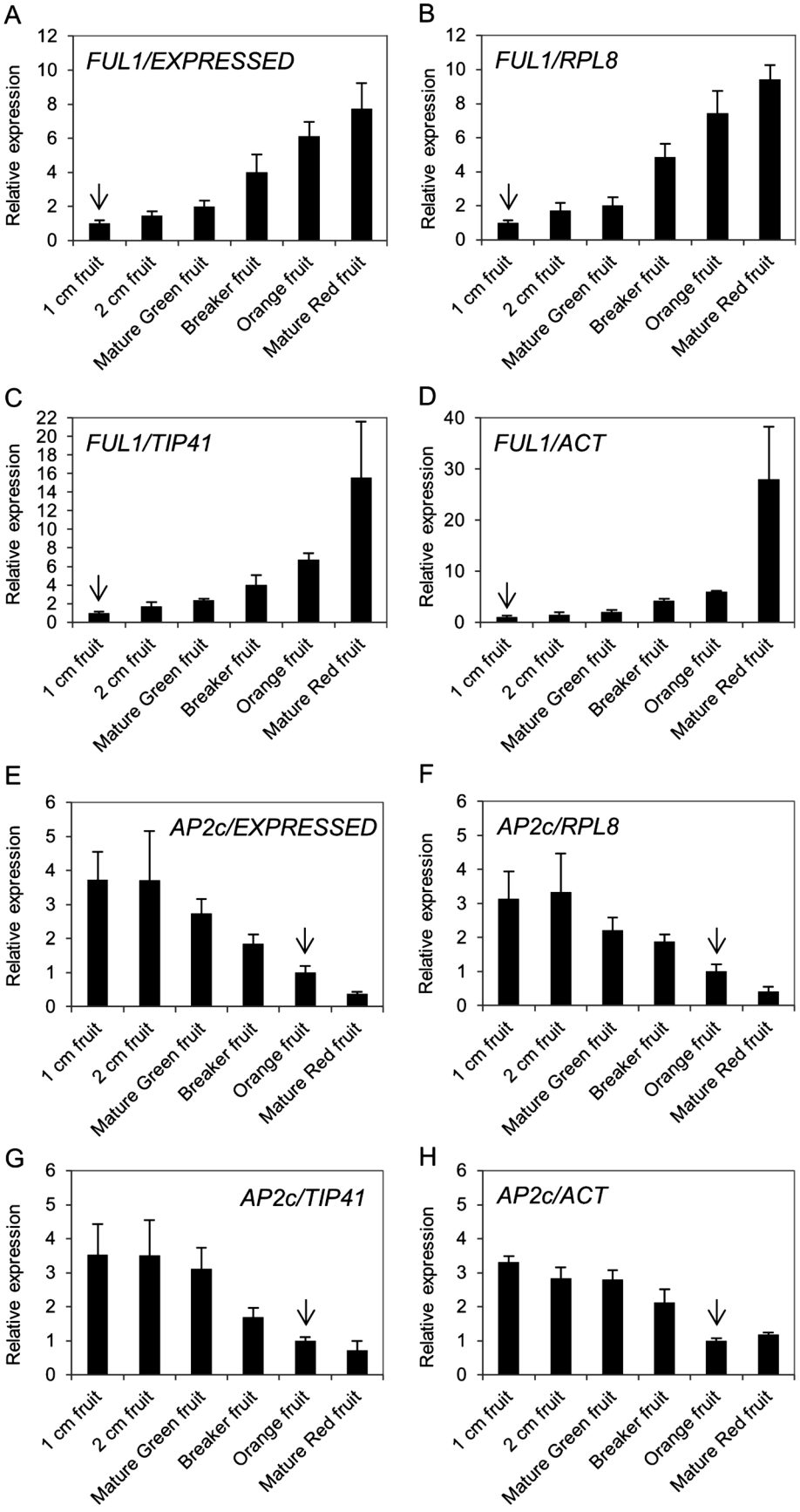

Figure 5. Expression profiles of *FUL1* (A to D) and *AP2c* (E to H) in developing fruits normalized against *EXPRESSED* (A and E), *RPL8* (B and F), *TIP41* (C and G) and *ACT* (D and H). For relative representations, the expression levels at "1 cm fruit" and "orange fruit" stages were adjusted to 1 for *FUL1* and *AP2c*, respectively (arrows). Error bars represent the standard error of the mean.

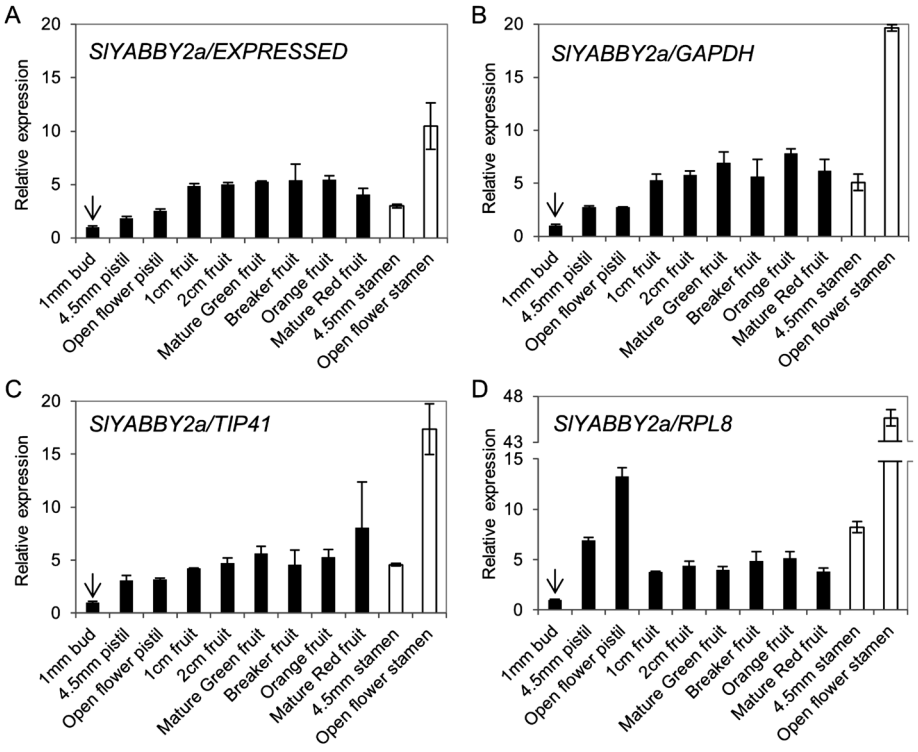

Figure 6. Expression profile of *SlYABBY2a* in flower and fruit datasets normalized against *EXPRESSED* (A), *GAPDH* (B), *TIP41* (C) and *RPL8* (D). For relative representations, the expression levels at "1 mm bud" (arrows) were adjusted to 1. White bars indicate the expression of *SlYABBY2a* in stamens. Error bars represent the standard error of the mean.

(Figure 6C; unstable candidate in fruit sample group) and *RPL8* (Figure 6D; unstable candidate in flower sample group), the expression level of *SlYABBY2a* was over-estimated in mature red fruit, and open flower stamen, respectively.

Conclusion

qRT-PCR is a simple and easy method to analyze expression of genes of interest in wide range of tissue samples collected from different developmental stages. In this study, we investigated and suggested the suitable internal control genes to analyze gene expressions in reproductive organs of dwarf tomato cultivar, Micro-Tom. To allow spatiotemporal profiling of gene expressions, we collected samples from 17 different reproductive tissues at different developmental stages, including leaf as a representative vegetative tissue. Among the eight commonly studied housekeeping genes, *EXPRESSED* was characterized as a reliable internal control gene for our sample groups. *EXPRESSED* was also reported as a remarkable internal control gene to analyze gene expressions in other tomato cultivars including Ciliegia, Santa Clara, Moneymaker, and MT-*Rg1* (Expósito-Rodríguez et al. 2008; González-Aguilera et al. 2016; Lacerda et al. 2015). We also suggested that *EXPRESSED* and *RPL8* were the optimal internal controls to analyze gene expression in developing fruits. However we must keep in mind that the expression of *RPL8* was unstable in tissues other than fruits. Thus, we also suggested that other internal controls such as *TIP41* or *GAPDH* should also be utilized to obtain reliable data from samples including floral tissues. Indeed, our evaluation indicated that *GAPDH* was also a usable internal control gene for monitoring the expression profiles through fruit developing starting from flowers. This is the first and sound report representing the credible internal control genes for qRT-PCR analysis in broad range of samples.

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