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Full Length Research Paper

Evaluation of the expression of internal control transcripts by real-time RT-PCR analysis during tomato flower abscission

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Many investigations of the abscission mechanisms in plants are based on gene expression studies. Quantitative real-time RT-PCR (qRT-PCR) is widely and increasingly used for mRNA quantification, and the results are typically referenced to an internal control gene to avoid bias. We investigated the suitability of twelve tomato (*Lycopersicon esculentum* Mill. var. *Liaoyuanduoli*) housekeeping genes during hormone, high salt and temperature-induced abscission. The free software-based applications NormFinder and qBase PLUS were used to statistically identify the best internal controls for a given set of biological samples. The expression stability of a number of housekeeping genes were validated during tomato abscission. The two most suitable reference genes for the commonly used treatments of the major hormones related to abscission are *TBP* and *RPL8*. In some cases, more than three reference genes may be required, depending on the type of samples being compared. Four suitable reference genes (*TBP/DNAj/RPL8/EXPRESSION*) are recommended for more complex analysis, such as hormone and bio-stress induced abscission samples.

Key words: Abscission, housekeeping gene, Tomato, *Lycopersicon esculentum, TBP, DNAj, RPL8, EXPRESSION.*

INTRODUCTION

Abscission commonly refers to the process by which a plant (http://en.wikipedia.org/wiki/Plant) drops one or more parts. Abscission mainly occurs in a predicted region called the abscission zone (AZ) which is composed of six to eight layers of small flat cells which cut across the pedicel. Distinct patterns of cell and tissue coordination occur in a small area during pedicel abscission, zone differentiation and separation.

The response of Type II cells, which comprise the AZ in higher plants, to the hormones ethylene and auxin has been well characterized. The time-course of abscission can be conveniently divided into two stages, based on the response of cells to hormones: a first stage during which addition of auxin retards abscission, and a second stage during which addition of auxin accelerates abscission (Addicott, 1970). Meir et al. (2010) precisely described the tomato pedicel flower abscission stage, and documented that different sensitivities to auxin and ethylene exist during each stage of tomato flower abscission. Ethylene significantly accelerates abscission; however, the role of ethylene is recognized to regulate the timing of abscission, rather than act as an initiator of abscission. Studies of floral organ abscission and ethylene response mutants of the model plant species *Arabidopsis thaliana* suggest that both ethylene dependent abscission and ethylene independent abscission occur (Fernandez et al., 2000; Jinn et al., 2000; Butenko et al., 2003; Patterson and Bleecker, 2004). Other factors can modulate abscission, including bio-stress factors,

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Abbreviations: 1-MCP, 1-Methylcyclopropene; **AR,** abscission rate; **AZ,** abscission zone; **CV,** coefficient of variation; **IAA,** indole-3-acetic acid; **M,** expression stability value; **qRT-PCR,** quantitative real-time RT-PCR; **RQ,** relative quantities; **NF,** normalization factor; **TBP,** TATA binding protein; **RPL8,** ribosomal protein l8.

low and high temperature and high salinity, which disrupts cellular component homeostasis through super-oxide production, leading to metabolic dysfunction and expression of cell wall degrading enzymes (Sakamoto et al., 2008).

Many investigations of the abscission mechanism in plants are based on gene expression studies, through which numerous novel stress-responsive genes have been discovered. Quantitative real-time RT-PCR (qRT-PCR) is widely and increasingly used for mRNA quantification (Bustin, 2000). To avoid bias, qRT-PCR is typically referenced to an internal control gene, and ideally, the experimental conditions should not influence expression of the internal control gene (Schmittgen and Zakrajsek, 2000). However, many studies have shown that internal standards, including the housekeeping genes used to quantify mRNA expression, can vary with the experimental conditions (Thellin et al., 1999; Warrington et al., 2000; Stürzenbaum and Kille, 2001; Radonic et al., 2004). In a study involving cardiac stem cells, *ACTB* and *GAPDH* were found to be the most consistent, while β2M, HPRT-1 and RPLP-1 varied significantly between neonatal and adult cardiac cells. In this regard, several free software-based applications such as NormFinder (Andersen et al., 2004) or qBase PLUS (Hellemans et al., 2007) permit a statistical identification of the best internal controls from a group of candidate normalization genes, for a given set of biological samples.

The tomato is an important model for genetic and molecular studies, and tomato abscission is a topic of scientific curiosity. Literature search revealed a single report in which several classical housekeeping genes are proposed as internal controls for tomato, based on the relative abundance of tomato expressed sequence tags. Marino et al. (2008) observed that, 3 of 11 references genes were suitable for transcript normalization in a series of tomato developmental samples.

In this study, we validated the expression stability of a number of housekeeping genes, and selected the most suitable reference genes (*TBP* and *RPL8*) for two hormone treatments which are commonly used experi-mentally, and are the major causes of abscission. In some cases, more than three reference genes may be required, depending on the type of samples compared. A combination of four suitable reference genes (*TBP/DNAj/ RPL8/EXPRESSION*) is recommend for complex analysis, such as, the analysis of hormone and bio-stress induced abscission.

MATERIALS AND METHODS

Plant Material

L. esculentum Mill. var. *Liaoyuanduoli*, an indeterminate and popular variety in Northeast China, were planted in September 2008 at Shenyang Agriculture University, Shenyang, China. Plants were grown in soil for 45 days in a greenhouse (25 ± 3°C day, 15 ± 3°C night) with natural light. To study pedicel abscission, pedicels with open flowers, with fresh yellow petals at approximately a 90° opening angle, which exclude fertilization and small bud, were immediately trimmed into 20 mm explants under water to reduce the risk of xylem embolism and dehydration. Pedicel explants were generated by removing the flower from the floral part to leave a 2 cm explants. These explants were used for subsequent experiments.

Explant treatments and abscission rate assessment

The proximal ends of 50 explants were inserted into 1% (w/v) agar medium in 9 cm Petri dishes with four dishes placed within each 40 × 25 × 20 cm glass container at 25°C. In order to avoid wound induced ethylene production, the container was connected with air-condition to maintain constant air flow through them. The agar medium contained deionized water (CK) or was supplemented with 10 mM $AgNO_3$, 200 mM NaCl (high salinity) or 20 $\mu g \cdot g^{-1}$ indole-3-acetic acid (IAA). Ethylene treatment was performed by placing explants into the glass container and injecting ethylene at a final concentration of 20 $\mu l\ l^{-1}$. 1-Methylcyclopropene (1-MCP) pretreatment was performed for 1 h at 20 $\mu g \cdot g^{-1}$, and then the explants were placed in the glass container. The 4°C and 37°C treatments were performed by placing the glass containers in a SANYO MLR-351 incubator. Sampling of the experimental series was performed over a 32 h period and comprised a total of 27 different samples.

Pedicel accumulative abscission rate (AR) was recorded every 8 h until 56 h. Ethylene treatment was performed and accumulative AR calculated as described in Wang et al. (2005). For qRT-PCR, pedicel explants were sampled at 2 and 4 h, after which they were sampled at every 8 h until 56 h, by cutting the pedicel into 2 mm AZ segments of the joint position. Samples were frozen in liquid N_2 and stored at -80°C before RNA isolation.

RNA isolation and real-time PCR analysis

Total RNA was extracted using the RNAprep Pure Plant Total RNA Extraction Kit (Qiagen, Germany), followed by DNase I treatment to remove genomic DNA. Purified total RNAs had a mean 260/280 nm ratio of 1.90 to 2.01 (SD = 0.12) and RNA concentration was normalized after Nanodrop 2000 quantification (Thermo, USA). RNA integrity was evaluated by using agarose gel electrophoresis stained with ethidium bromide. The qRT-PCR analysis was performed as described by Jain et al. (2006). Briefly, cDNA was synthesized from 2 µg total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, USA). 1 µl cDNA was used as template and mixed with 200 nM of each primer and SYBR Green PCR Master Mix (Qiagen) for 35 cycles' qRT-PCR analysis using the ABI Prism 7500 Sequence Detection System (Applied Biosystems). All primers pair-designed by Marino et al. (2008) amplified the desired unique cDNA segment, and the efficiencies in this article are recorded in Table 1. The specificity of the qRT-PCR reactions was verified by melting curve analysis. Baseline and threshold cycles (C_t) were automatically determined using the ABI 7500 Software v2.0.3. The relative mRNA levels for each of the *TAPG4* or *CHI9* genes in RNA isolated from the various samples were quantified with respect to the internal standard *ACTIN*. At least two independent RNA isolations of 30 pedicel samples were used for cDNA synthesis with three biological replicates and three technical qRT-PCR replicates for each cDNA sample.

Statistical analysis of gene expression stability

The suitability of the candidate control genes was evaluated by

Table 1. Details of primers used in the study.

Gene symbol	Tomato accession number	Real-time PCR primers		Amplicon length (pb/Tm)	Efficiency
GAPDH	U97257	F: GGCTGCAATCAAGGAGGAA	R:AAATCAATCACACGGGAACTG	207/78.1	0.921 ± 0.021
EFA1	X53043	F: TACTGGTGGTTTTGAAGCTG	R:AACTTCCTTCACGATTTCATCATA	166/79.2	0.946 ± 0.089
TBP	SGN-U329249	F:GCTAAGAACGCTGGACCTAATG	R:TGGGTGTGCCTTTCTGA	184/76.1	0.936 ± 0.036
RPL8	X64562	F:CCGAAGGAGCTGTTGTTTGTA	R:ACCTGACCAATCATAGCACGA	184/79.3	0.896 ± 0.022
APT	BT012816	F:CCATGAGGAAACCCAAGAAGT	R:CCTCCAGTCGCAATTAGATCAT	143/78.5	0.892 ± 0.067
DNAJ	AF124139	F:GAGCACACATTGAGCCTTGAC	R:CTTTGGTACATCGGCATTCC	158/79.6	0.885 ± 0.051
TUA	AC122540	F:AGCTCATTAGCGGCAAAGAA	R:AGTACCCCCACCAACAGCA	163/77.0	0.962 ± 0.021
TIP41	SGN-U321250	F:ATGGAGTTTTTGAGTCTTCTGC	R:GCTGCGTTTCTGGCTTAGG	235/78.3	0.932 ± 0.029
SAND	SGN-U316474	F:TTGCTTGGAGGAACAGACG	R:GCAAACAGAACCCCTGAATC	164/78.2	0.933 ± 0.032
CAC	SGN-U314153	F:CCTCCGTTGTGATGTAACTGG	R:ATTGGTGGAAAGTAACATCATCG	173/76.4	0.925 ± 0.014
EXPRESSED	SGN-U346908	F:GCTAAGAACGCTGGACCTAATG	R:TGGGTGTGCCTTTCTGAATG	183/76.0	0.872 ± 0.035
ACTIN	Q96483	F:TGTCCCTATTTACGAGGGTTATGC	R:AGTTAAATCACGACCAGCAAGAT	189/78.6	0.962 ± 0.022
TAPG4	U70481.1	F:GGGAATCCCAAGAGCAAGGAGTACA	R: CCATTGCTAGGTCTTGCCCATGTT	106/58.1	0.922 ± 0.018
CHI9	NM_001247474.1	F: TCACAGGCCGGAGGAGCACTT	R: GACCGCCAGGACACTGGCTC	118/58.0	0.945± 0.021

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; *EFα1*, elongation factor alpha 1; *BP*, TATA binding protein; *RPL8*, ribosomal protein l8; APT, adenine phosphoribosyltransferase; *DNAJ*,DnaJ-like protein; *TUA*, alpha-tubulin; TIP41,TIP41-like protein ; *SAND*, SAND family protein; *CAC*, SGN-U314153; *Expressed*, SGN-U346908; *ACTIN*, beta-*ACTIN*; *TAPG4*, tomato abscission polygalacturonase 4; *CHI9*, *S. lycopersicum* chitinase NM_001247474.

applying two different statistical approaches to the C_t values expression data, to provide complementary measures of gene expression stability. In the first approach, C_t values were converted into relative quantities (RQs) using the sample with the lowest C_t as a calibrator, taking into account the calculated amplification efficiencies for each primer-pair (Table 2). The qBase PLUS algorithm (Hellemans et al., 2007) enables normalization to more than one reference gene and is able to take into account gene-specific E, which distinguishes it from the ΔΔCt method (Van desompele et al., 2002). The RQs were imported into the qBase PLUS software (http://www.biogazelle.com/products) to obtain an expression stability value (M) and accurate normalization factor (NF). A pairwise variation of 0.15 was accepted as the cutoff value.

In the second approach, C_t values were log-transformed and imported into the NormFinder software (Andersen et al., 2004) (http://www.mdl.dk/publicationsnorm finder.htm), which is based on a mathematical model of gene expression to enable an estimation of the intra- and intergroup variation to produce a combined stability value. Candidate control genes with the lowest intra and intergroup variation have the highest stability value and will be ranked top, and thus, subdivision of the sample set in at least two coherent groups is required for the correct application of this approach. In this sense, we initially established the following sample-groups: abscission(n = 11), non-abscission (n = 37), CK (control) (n = 8), ethylene treatment (n = 7), NaCl treatment (n = 8), 4°C treatment (n = 5), 37°C treatment (n = 5), $AgNO_3$ treatment (n = 5), 1-MCP treatment (n = 5) and IAA treatment (n = 5). The third statistical approach determines the expression stability for each control gene as the coefficient of variation (CV) of the relative expression levels after normalization. This evaluation strategy has been incorporated in the qBase PLUS program.

In the third evaluation approach, the coefficient of variation of normalized relative expression levels was calculated for candidate genes throughout all treatment samples. This statistical approach, proposed by Hellemans et al. (2007) has been implemented in the qBase PLUS software (http://www.biogazelle.com/products). First, mean C_t values were transformed into RQs using the specific amplification efficiency of each primer-pair and the sample with the lowest C_t as calibrator (formula 11). Then, a sample-specific NF was calculated as the geometric mean of the RQs estimate for all candidate genes (formula 13). Finally, NRQs were calculated as the ratio of the RQ estimated for a gene/sample pair and the corresponding sample NF (formula 15).

The tomato pedicel abscission related gene *TAPG4* (*L. esculentum* abscission polygalacturonase 4 U70481.1) and *CHI9* (*Solanum lycopersicum* chitinase NM_001247474.) expression was analyzed with the best and worst reference gene in the control and ethylene treatment.

RESULTS

Hormone and bio-stress effects on abscission

The AR of tomato pedicel explants increased after

Table 2. AR of control tomato floral pedicel explants or after treatment with IAA, ethylene, 1-MCP or $AgNO_3$, or incubated at 4 or 37°C (n = 9).

Treatment	Time (h)				
	0	8	16	24	32
CK (control)	0	0	36.8 ± 5.5	58.2 ± 3.6	79.6 ± 7.3
Ethylene	0	16.8 ± 2.3	62.3 ± 6.2	100	100
NaCl	0	9.8 ± 1.2	45.2 ± 3.3	79.8 ± 6.5	100
4°C	0	0	0	0	0
37°C	0	0	0	0	0
$AgNO_3$	0	0	0	0	0
1-MCP	0	0	0	0	0
IAA	0	0	0	0	0

8 h and reached 100% at 48 h in the control. IAA, 1-MCP, $AgNO_3$, 4°C and 37°C treatment delayed abscission and inhibited relative AR prior to 32 h, while NaCl treatment significantly accelerated relative AR (P<0.05). Ethylene increased and enhanced the abscission peak (44% from 8 to 16 h) and completed abscission at 24 h (Table 2).

Housekeeping gene variations in the experimental abscission series

The C_t dataset was analyzed by using qBase PLUS and NormFinder to identify the most stably expressed reference gene during tomato flower abscission. The sample set was subdivided into four groups for both qBase PLUS and NormFinder analyses, according to the different treatment, or two groups based on abscission or non-abscission. Variations in the RQ of each gene-pair in the sample series was evaluated by qBase PLUS, and the CV was analyzed by NormFinder.

The results from both software analyses are shown in Table 3. Notably, the NormFinder output rank of stable genes varied between the abscission or non-abscission analysis group (n = 2) and different treatment analysis groups (n = 8). The statistical analyses indicated that *DNAj, TBP, RPL8* and *EXPRESSED* ranked as the most stably expressed housekeeping genes, *ACTIN, CAC* and *APT* had a relatively stable expression level, while *SAND, TIP41, GADPH, TUA* and *EFα1* were the least reliable control genes with the most unstable expression patterns. As described by Marino et al. (2008), the average of the two NormFinder results for each analysis group was calculated for each housekeeping gene, and ranked from 1 (most stable) to 12 (least stable). When two or three candidate genes were co-localized within a particular ranking (that is, the CV of the corresponding expression stability values was 15%), both were scored using the average CV.

According to the consensus ranking, the best genes for normalization during different methods of treatment-induced abscission were *DNAj, TBP, RPL8* and *EXPRESSED*. Analysis of the pairwise variation between two sequential NF revealed that three genes are sufficient to calculate an accurate sample-specific NF, based on the geometric mean of their RQs, with a variation value NF4/NF5 (V4/5 = 0.142) lower than the default cut-off value of 0.15. The mean M and CV values for the *DNAj, TBP*, *RPL8* and *EXPRESSED* genes in all experimental series were 0.509 and 0.388 respectively. In short, the *DNAj, TBP, RPL8* and *EXPRESSED* gene quadruple is recommended for the accurate normalization of gene expression measures encompassing the complete abscission process in tomato.

The control gene combinations recommended for the different treatment sample subsets (Table 4) were constructed from the genes which were ranked among the top six housekeeping genes. It was clear that normalization of expression within a single treatment has a different requirement to normalization of expression between different treatments. Two control genes are sufficient for accurate normalization in hormone treatments, indicated by the V2/3 values lower than 0.15. In salt and temperature treatments, three control genes are required for accurate normalization. When the sample subsets were comprised of two or three different treatments, the evaluation procedure indicated that four control genes are necessary for reliable normalization (Table 4). The control genes recommended for the normalization in the complete tomato developmental series (*TBP/DNAj/RPL8/EXPRESSION*) were also suitable for normalization of combinations of samples from two different types of treatment. The only exception was the subset of hormone and salt treated samples, which are suitably normalized with the *DNAj/RPL8/TBP/APT* genes.

TAPG4 and *CHI9* were assumed as the abscission marker for abscission procession (Patterson and Bleecker, 2004), and we analyzed their expression patterns used to elucidate the great effect of internal reference genes on exactly profiling the interesting gene expression. The results show that there is difference in the whole abscission process and even in the earlier

Table 3. Ranking of the candidate control genes according to their expression stability in the different treatment series.

qBase PLUS	NormFinder		Coefficient of variation	Consensus	Rank
	2 groups	8 groups			
RPL8/TBP	*DNAj/TBP*	*TBP/RPL8*	*DNAj*	*DNAj/TBP/RPL8/ EXPRESSED*	1
			RPL8		2
DNAJ	*RPL8*	*DNAj*	*TBP*		3
CAC	*ACTIN*	*EXPRESSED*	*ACTIN*		4
EXPRESSED	*EXPRESSED*	*CAC*	*EXPRESSED*	*ACTIN*	5
APT	*CAC*	*ACTIN*	*CAC*	*CAC*	6
ACTIN	*TUA*	*APT*	*APT*	*APT*	7
SAND	*TIP41*	*SAND*	*SAND*	*SAND*	8
TIP41	*APT*	*TUA*	*TIP41*	*TIP41*	9
TUA	*SAND*	*GAPDH*	*GAPDH*	*GADPH*	10
GAPDH	*EFα1*	*TIP41*	*TUA*	*TUA*	11
EFα1	*GAPDH*	*EFα1*	*EFα1*	*EFα1*	12

Expression data were evaluated with three different statistical approaches and their outcomes were summarized in a consensus ranking. Expression stability decreases from top to bottom.

stage (before 8 h), as the timing of separating this difference was greater. Furthermore, in the worst reference analyzed, the gene expression trends was greatly changed during 16 to 32 h in CK while 8 to 24 h in ethylene.

DISCUSSION

Although, microarray data has been analyzed to determine the most stably-expressed genes in *Arabidopsis* (Czechowski et al., 2005) and rice (Jain and Khurana, 2009), several works have been published where genes from *Arabidopsis* could be used as bait to find reference genes in other species such as cotton and *Brachiaria* (Artico et al., 2010). However, it is not possible to directly transpose suitable housekeeping genes from one species to another. For example, *UBQ10* is very stable in *Arabidopsis* (Czechowski et al., 2005), but not in rice (Jain et al., 2006) nor in soybean nor in *Brachypodium*.

Reasons for the changes of expression level in the housekeeping genes are that most of them can vary significantly across tissue types. Additionally, there are numerous reports showing that their expression is often affected by experimental treatments, stage of development and cell type. Therefore, as there are no universally-suitable reference genes, it is necessary to verify the expression levels of candidate reference genes under the same experimental conditions used for the gene of interest. Ideal reference genes have a constant level of expression, which does not vary in the organs or tissues studied, and is not influenced by the experimental treatment. It is better to use as many housekeeping genes as possible and that there is no "standard" housekeeping gene, which you can use for every cell line/cell type.

In tomato, screening of the most stable multiple control genes is becoming a standard. An outstanding work was performed during its development by Marino et al. (2008), and it was observed that little is known about the effects of hormones and bio-stress factors, such as, temperature and salt on gene expression during tomato abscission. Tomato flower explants abscission is a dramatic physiological change, occurring in relatively short period and requiring cooperation between the entire AZ, composed of layer cells, and the neighboring cells which form the protected layer. During abscission, the expression of housekeeping genes varies greatly in both the AZ and neighboring cells. Additionally, the different experimental factors used to induce abscission may invoke different mechanisms, which could affect the stability of housekeeping gene expression. Many of the comparisons (that is, leaves, fruit and inflorescences) are tissues

Table 4. Housekeeping gene combinations recommended for samples of the different treatments.

Sample	Recommended housekeeping genes	Mean stability value		
		M	CV	Vn/n+1$_z$
H	*TBP/RPL8(DNAj)*	0.301	0.269	0.141
S	*RPL8/DNAj/TBP*	0.398	0.276	0.137
T	*SAND/TBP/EXPRESSION*	0.312	0.296	0.11
H+S	*DNAj/RPL8/TBP/APT*	0.596	0.336	0.146
H+T	*TBP/EXPRESSION/DNAj/RPL8*	0.512	0.324	0.146
S+T	*DNAj/RPL8/TBP/EXPRESSION*	0.576	0.352	0.143
H+S+T	*TBP/DNAj/RPL8/EXPRESSION*	0.509	0.388	0.142

For each samples of each treatment, the optimal housekeeping genes are displayed in the corresponding consensus ranking. The optional control genes for individual treatments are shown in the bracket with the resulting stability values. [Z]Pairwise variation of NFn/NFn+1 ratios, n being the number of recommended control genes; H, hormone treatment; S, high salt treatment; T, temperature treatment.

that proceed through senescence at late stages of development. The abscission process occurs independently of the senescence, and thus, it is possible that the results obtained from these comparisons are difficult to comprehend. To obtain a solid basis for the normalization of gene expression data during abscission, we evaluated the expression stability of 12 candidate reference genes in both the abscission and non-abscission explants throughout whole abscission stage to minimal the senescence effect.

The *DNAJ, EXPRESSED* and *RPL8* genes have been previously described as "candidate controls" in tomato inflorescences, leaves and fruit samples respectively. Meir et al. (2010) has established a well frame to study tomato abscission by comparing the gene expression between AZ and non-AZ part (distal side). In this study and according to the minimum information for publication of quantitative real-Time PCR (MIQE) guidelines, we compared gene expression in tomato flower abscission induced by different treatment which included at least three control genes for accurate results.

GAPDH was revealed to be the most stably expressed gene in a systematic expression stability study of different flax tissues, including stems, flowers, roots and leaves (Huis et al., 2010), consistent with recent observations in *Brachypodium distachyon* during cold/heat stress (Hong et al., 2008) and in *Coffea arabica* L. organs and tissues (Barsalobres-Cavallari et al., 2009). Cruz et al. (2009) has shown that *GAPDH* is also a good reference gene for different tissue samples in coffee but fails when samples were under distinct stresses; however, this study indicates that *GAPDH* and *TUA* should be avoided as control genes in tomato abscission, as their expression stability is far from acceptable. Although, *ACTIN* is a suitable normalization gene for developmental studies (Jian et al., 2008), but it appears to be unstable in many other biological processes (Guenin et al., 2009). In the same way, *tubulin* is stable during orobranche development (Gonzaìlez-Verdejo., 2008), but is apparently unstable during development according to geNorm analyses (Hong et al., 2008) and abiotic stress (Martin et al., 2008).

Hormone treatment can directly induce or delay abscission, mainly via the ethylene-dependent process, while high salt and temperature bio-stress treatment mediates abscission via indirect ROS-induced stress. Biotic stress often results in lipid peroxidation of polyunsaturated fatty acids, involving an enzymatic mechanism driven by lipoxygenase (LOX) and a non-enzymatic mechanism driven by direct reactive oxygen species (ROS) attack. Accumulation of ROS may act as intermediate signaling molecules to regulate the expression level of genes involved in abscission. Our analysis indicates that the combination of *TBP/DNAj/RPL8/EXPRESSION* are suitable to normalize hormone and temperature treatment, or temperature and salt treatment, but are not suitable for hormone and salt treated samples. The separate analyses showed that hormone and salt treatment share the same three optimal reference genes; however, the difference in the optimal reference genes suitable for temperature treatment imply that temperature has a significant impact on the expression of *TBP* and *RPL8*. The *TAPG4* and *CHI9* analyzed by the best and worst internal reference gene show a great conflict in control and ethylene treatment. The wrong reference gene might confuse our analyses and changed the expression trends of our interesting gene. This might be the key for abscission researcher to find the important role by using qRT-PCR.

Organ abscission is a ubiquitous, complex highly programmed and predictable process, which provides an accepted tool to research plant development and hormone signaling mechanisms. In *Arabidopsis*, bean and tomato, the molecular investigation of abscission zone development and organ shedding has yielded many advances. An accurate and detailed expression analysis of abscission related genes could reveal more information on the transcriptional networks which govern the molecular and physiological processes of abscission. Our study validated the expression stability of a number of housekeeping genes, and selected the two most suitable reference genes (*TBP* and *RPL8*) for the

common experiments involving application of the hormones involved in abscission. In some cases, more than three reference genes may be required, depending on the types of samples that are compared. Four suitable reference genes (*TBP/DNAj/RPL8/EXPRESSION*) are recommended for more complex analysis, including analysis of abscission in samples induced by hormones and bio-stress.

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