Selection of Suitable Reference Genes for Quantitative Real-time Polymerase Chain Reaction in Prunus mume during Flowering Stages and under Different Abiotic Stress Conditions

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ABSTRACT. Mei (Prunus mume) is widely cultivated in eastern Asia owing to its favored ornamental characteristics and its tolerance for low temperatures. Reverse transcription quantitative real-time polymerase chain reaction (qRT-PCR) is a widely used method for gene expression analysis, requiring carefully selected reference genes to ensure data reliability. The aim of this study was to identify and evaluate reference genes for qRT-PCR in mei. Ten candidate reference genes were chosen, and their expression levels were assessed by qRT-PCR in four sample sets: 1) flowering mei; 2) mei undergoing abiotic stress; 3) different genotypes of Prunus species; and 4) all mei samples. The stability and suitability of the candidate reference genes were validated using commercially available software. We found that protein phosphatase 2A-1 (PP2A-1) and PP2A-2 were suitable reference genes for flowering with ubiquitin-conjugating enzyme E2 (UBC) also being suitable for different genotypes of Prunus species. UBC and actin (ACT) were most stably expressed under abiotic stress. Finally, the expression of an AGAMOUS homolog of Arabidopsis thaliana (PmAG) and a putative homolog of Group 2 late embryogenesis abundant protein gene in A. thaliana (PmLEA) were assessed to allow comparisons between selected candidate reference genes, highlighting the importance of careful reference gene selection.

Prunus mume belongs to the family Rosaceae, subfamily Prunoideae, and was cultivated in China more than 3000 years ago for its ornamental qualities and its fruit (Chen, 1996). As an early-blooming garden ornamental, mei is widely cultivated in eastern Asia owing to its favored ornamental characteristics, including colorful corollas, varying types of flowers, and pleasant fragrance. It is also favored because of its inherent tolerance to low temperatures, allowing it flowers in winter or early spring, while most other ornamental trees are still dormant (Chen, 1996; Sun et al., 2013). These unique properties give mei high cultural and commercial value in both China and elsewhere.

Previous research on mei has primarily focused on its physiological and biochemical characteristics (Chen, 2012; Li et al., 2011; Zhang, 2011; Zhao et al., 2010); however, relatively few studies have focused on the transcription and expression of genes in mei, and we have little knowledge on concerning the genetic mechanisms underlying the biological and ornamental traits of mei. Recently, the draft genome sequence of mei has been published (Zhang et al., 2012b), thus providing a strong foundation for gene profiling studies in mei and facilitating genome-level comparisons among Prunus species. We believe the availability of the mei genome will stimulate research in the functional genomics of the Prunus genus.

Real-time reverse transcription polymerase chain reaction is a sensitive method that is widely used to detect and verify changes in the mRNA expression levels of genes at different developmental stages (Koo et al., 2010; Vaucheret et al., 2004) and under various abiotic stress (Borges et al., 2012; Du et al., 2013). Normalized quantification of gene expression levels using this method depends on the stable expression of endogenous reference genes (Güenin et al., 2009). To date, stably expressed reference genes have been reported for more than 62 different plant species (summarized in Supplemental Table 1). Reference genes traditionally used in other species include ACT, elongation factor 1-alpha (EF1α), alpha tubulin (TU4), and ubiquitin (UBQ). Newer reference genes include PP2A and SAND family protein gene (SAND), which have been used in plants because of their stable expression under different experimental conditions (Chen et al., 2010; Chi et al., 2012; Goulao et al., 2012). However, the expression levels of these genes can be changed across different samples or by external factors. Moreover, a commonly used reference gene in an organism may not be appropriate in other organisms or under different conditions (Gao et al., 2012). These reference genes (ACT, EF1α, TU4, UBQ, PP2A, SAND) have been found to be not stably expressed in other plants such as Fagopyrum esculentum (Demidenko et al., 2011), Phyllostachys edulis (Fan et al., 2013), Platycladus orientalis (Chang et al., 2012), and Salvia miltiorrhiza (Yang et al., 2010). Furthermore, it has been shown that there are no genes that are constantly expressed throughout the different stages in the plant’s lifecycle (Fan et al., 2013; Hong et al., 2008); thus, it highlights the need to identify species- and stage-specific reference genes for qRT-PCR analyses.
In mei, translation elongation factor 2 (TEF2) has been used as a reference gene for a putative homolog gene of Group 2 late embryogenesis abundant protein gene in Arabidopsis thaliana (PmLEA) expression analyses under abscisic acid (ABA) treatment (Du et al., 2013), and UBQ has been used as a reference gene for dormancy-associated MADS-box transcription factors (PmDAM) gene expression analyses associated with endodormancy (Sasaki et al., 2011). However, TEF2 and UBQ were found to be unsuitable reference genes in Phaseolus vulgaris and Ammopiptanthus mongolicus (Borges et al., 2012; Shi et al., 2012). There has been no systematic analysis of suitable reference genes for development stages, different abiotic stress conditions, and different genotypes of mei. Therefore, it is necessary to carry out validation studies of reference genes used for gene expression analyses in mei.

The goal of this study was to identify suitable reference genes for conducting qRT-PCR studies in mei. We assessed the expression of 10 candidate reference genes, including ACT, EF1α, PP2A-A1, PP2A-A2, RNA polymerase II (RPRI), SAND, TEF2, TUA, UBQ, and UBC, which have been previously reported to be stably expressed in A. thaliana (Czechowski et al., 2005), Prunus persica (Tong et al., 2009), and Rosa hybrida (Klie and Debener, 2011). The variation in expression level of these candidates was assessed by qRT-PCR in four sample sets: 1) flowering mei; 2) mei undergoing abiotic stress; 3) different genotypes of Prunus species; and 4) all mei samples. To verify the usefulness of the selected reference genes, the expression pattern of PmAG (an AGAMOUS homolog gene of A. thaliana) and PmLEA19 was analyzed in different sample sets, respectively. We believe this work will facilitate future studies on gene expression in mei and among the other Prunus species.

Materials and Methods

Plant material and treatments. All the mei samples were taken from Beijing Jufeng International Plum Blossom Garden, Beijing, China, between July 2012 and Feb. 2013. Among them, flower samples at different developmental stages were collected from 5-year-old plants of P. mume ‘Changrui Lve’, including differentiating and developing buds and fully opened flowers. The seedlings used in the abiotic stress experiments were grown in a greenhouse at 16 to 25 °C at 60% relative humidity under a 12-h light/dark cycle. For salt, osmotic, and ABA stress treatments, 6-month-old seedlings were carefully removed from the soil, their roots were washed with tap water, and they were placed in solutions containing 150 mM NaCl, 10% polyethylene glycol 6000 (PEG6000), or 150 mM ABA for 12 h, respectively. For the cold and heat stress treatments, the seedlings were grown at 4 or 40 °C for 12 h, respectively. Leaves were collected from all control and experimental seedlings for further analysis.

The different genotypes tested included fully flowering samples from three botanical cultivar groups of P. mume: True Mei Group (P. mume ‘Changrui Lve’, ‘Danfen Chuizhi’, ‘Wuzhu Sha’, ‘Chaotang Gongfen’, ‘Sanlun Yudie’, ‘Subai Taige’), Apricot Mei Group (P. mume ‘Fenghou’), Bliireiana Group (P. mume ‘Meiren’), and some other Prunus species (P. davidiana, P. armeniaca, P. cerasifera, P. triloba, P. tomentosa, P. yedoensis). In all cases, samples were collected and immediately frozen in liquid nitrogen and stored at –80 °C until RNA extraction. Samples were collected from three plants to provide three biological replicates.

Total RNA isolation and cDNA synthesis. Total RNA was extracted from samples using TRIZol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Potentially contaminating genomic DNA was eliminated by treatment with RNase-free DNase I according to the manufacturer’s instructions (Promega, Madison, WI). RNA concentration and purity were determined using a spectrophotometer (Nanodrop ND-1000; Thermo Fisher Scientific, Wilmington, DE). RNA samples with an absorbance ratio at OD 260/280 between 1.9 and 2.2 and OD 260/230 less than 2.0 were used for further analysis. RNA integrity was verified by 1.5% agarose gel electrophoresis. Samples with a 28S/18S ribosomal RNA ratio between 1.5 and 2.0 and without smears on the agarose gel were used for subsequent experiments. For each sample, 2 μg of total RNA was used as a template for reverse transcription using the TIANScript cDNA First Strand cDNA Synthesis Kit (Tiangen, Beijing, China) according to the manufacturer’s instructions. The cDNAs were diluted 1:10 with nuclease-free water before qRT-PCR analyses.

Selection of potential reference genes. The 10 candidate genes evaluated in this experiment were based on the mei transcriptome data (Zhang et al., 2012b) according to meeting one or more of the following criteria: 1) reference genes traditionally used in mei for transcript normalization (Du et al., 2013; Sasaki et al., 2011); 2) reference genes described in the literature for qRT-PCR normalization in P. persica (Tong et al., 2009) or R. hybrida (Klie and Debener, 2011); and 3) mei homologs of reference genes tested for transcript level normalization and quantification in A. thaliana (Czechowski et al., 2005). BLASTN with a default setting was used to search for mei coding sequences with high similarity (e-value 10^-6) to A. thaliana genes (Demidenko et al., 2011).

Design and detection of the specificity of primers. The primers for the 10 candidate reference genes and one functional gene from mei were designed using Primer Express 2.0 software (PE Applied Biosystems, Foster, CA) with the default parameters. To verify the amplification specificity of primer pairs, universal RT-PCR using the Premix Ex Taq (TaKaRa Biotechnology, Dalian, China) was performed, and only primer pairs that showed a single amplification product with the expected amplicon size verified by 2% agarose gel electrophoresis, and no product in the no template control group, were selected for further analysis (Supplemental Fig. 1). The amplification efficiency of primer pairs (E) and correlation coefficient (R²) estimates were derived from a standard curve generated from a serial dilution of the mixed cDNA from all tested samples as the template performed for each candidate reference gene in triplicate.

Reverse transcription quantitative real-time PCR. Quantitative RT-PCR reactions were carried out in 96-well blocks with a PikoReal Real-time PCR System (Thermo Fisher Scientific, Waltham, U.K.) using the SYBR Premix Ex Taq™ Kit (TaKaRa Biotechnology) according to the manufacturer’s instructions. The reactions were carried out in a 20-μL volume containing 2 μL of diluted cDNA, 200 nM of each primer, and 10 μL of 2× PCR Master Mix (TaKaRa Biotechnology) under the following conditions: 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Then, a thermal denaturing cycle of 60 to 95 °C with 0.5 °C increment for 1 s was applied to determine the dissociation curves. All qRT-PCR reactions were carried out in biological and technical triplicates. A non-template control was also included in each run for each gene. The final
## Table 1. Genes and primer sets used for real-time polymerase chain reaction.

| Gene symbol | Gene name | GenBank accession | homolog locus | Primer pair (F/R, 5'–3') | Product size (bp) | Tm (°C) | E (%) | R²* |
|-------------|-----------|-------------------|---------------|---------------------------|-------------------|---------|-------|-----|
| ACT         | Actin2/7  | Pm005252          | AT5G09810     | CCCTAAGGCTAACAGAGAAAGA    | 212               | 84.6    | 100   | 0.999 |
|             |           |                   |               | CAGCAAGGTCAGAGCAAGAAAT    |                   |         |       |     |
|             |           |                   |               | CATTITGATTTGAGGGTGAT      |                   |         |       |     |
|             |           |                   |               | TGAAGTGGGAGTCGGAGGG       |                   |         |       |     |
| EF1α        | Elongation factor –1α | Pm009018          | AT1G07940     | AGGGTTCCGTGCAATAATAGA     | 141               | 84.8    | 95.64 | 0.994 |
|             |           |                   |               | TGTAGCAGCAGATCAGAAT       |                   |         |       |     |
|             |           |                   |               | ATATAGCTGCTCGTTCAACC      |                   |         |       |     |
|             |           |                   |               | AAAAACAGTCAACCATTCTT      |                   |         |       |     |
| PP2A-1      | Protein phosphatase 2A | Pm029033          | AT1G13320     | AGGGTTCGGCTCGCAATAATAGA   | 169               | 80.4    | 98.40 | 0.999 |
|             |           |                   |               | TGTAGCAGCAGATCAGAAT       |                   |         |       |     |
|             |           |                   |               | ATATAGCTGCTCGTTCAACC      |                   |         |       |     |
|             |           |                   |               | AAAAACAGTCAACCATTCTT      |                   |         |       |     |
| PP2A-2      | Protein phosphatase 2A | Pm006362          | AT1G59830     | AGGGTTCGGCTCGCAATAATAGA   | 113               | 80.6    | 98.44 | 0.998 |
|             |           |                   |               | TGTAGCAGCAGATCAGAAT       |                   |         |       |     |
|             |           |                   |               | ATATAGCTGCTCGTTCAACC      |                   |         |       |     |
|             |           |                   |               | AAAAACAGTCAACCATTCTT      |                   |         |       |     |
| RPII        | RNA polymerase subunit | Pm021411          | AT2G15430     | TGAGCATACACCTATGATGATGAGAGTTGACAGCAGCACTGAGATCC  | 128               | 80.0    | 103.76 | 0.999 |
|             |           |                   |               | TGTAGCAGCAGATCAGAAT       |                   |         |       |     |
|             |           |                   |               | ATATAGCTGCTCGTTCAACC      |                   |         |       |     |
|             |           |                   |               | AAAAACAGTCAACCATTCTT      |                   |         |       |     |
| SAND        | SAND family protein | Pm001035          | AT2G28390     | GCGAGACCAATCACACATCACC    | 92                | 84.4    | 101.05 | 0.996 |
|             |           |                   |               | ACTTCTAACCTGCAACTAACCC    |                   |         |       |     |
|             |           |                   |               | ATATAGCTGCTCGTTCAACC      |                   |         |       |     |
|             |           |                   |               | AAAAACAGTCAACCATTCTT      |                   |         |       |     |
| TEF2        | Translation elongation factor 2 | Pm011035          | AT1G56070     | GGTGTGACGAGATGAGTGATG     | 129               | 84.5    | 98.31 | 0.998 |
|             |           |                   |               | TGTAGCAGCAGATCAGAAT       |                   |         |       |     |
|             |           |                   |               | ATATAGCTGCTCGTTCAACC      |                   |         |       |     |
|             |           |                   |               | AAAAACAGTCAACCATTCTT      |                   |         |       |     |
| TUA         | Alpha Tubulin-5 | Pm000088          | AT5G19780     | TACCGAACAATCCTTACCACC     | 197               | 82.6    | 98.64 | 0.998 |
|             |           |                   |               | CCGAACGATACACACACC       |                   |         |       |     |
|             |           |                   |               | ATATAGCTGCTCGTTCAACC      |                   |         |       |     |
|             |           |                   |               | AAAAACAGTCAACCATTCTT      |                   |         |       |     |
| UBQ         | Ubiquitin  | Pm009747          | AT4G02890     | AAGGCGAGATCCGAGACAGGAG    | 158               | 86.4    | 96.26 | 0.999 |
|             |           |                   |               | CACCGAGAGCGAAGCCACCAA     |                   |         |       |     |
|             |           |                   |               | ATATAGCTGCTCGTTCAACC      |                   |         |       |     |
|             |           |                   |               | AAAAACAGTCAACCATTCTT      |                   |         |       |     |
| UBC         | Ubiquitin-conjugating enzyme E2 | Pm024097          | AT5G53300     | GCAAGTGGGATGTTCTCTGTGAGAGTGGAGCTACCTCAGGTC       | 146               | 81.6    | 97.47 | 0.987 |
|             |           |                   |               | CACCGAGAGCGAAGCCACCAA     |                   |         |       |     |
|             |           |                   |               | ATATAGCTGCTCGTTCAACC      |                   |         |       |     |
|             |           |                   |               | AAAAACAGTCAACCATTCTT      |                   |         |       |     |
| PmAG        | AGAMOUS homologue | EU068730          | —             | TACCGAACAATCCTTACCACC     | 220               | 83.6    | 97.66 | 0.998 |
|             |           |                   |               | CCGAACGATACACACACC       |                   |         |       |     |
|             |           |                   |               | ATATAGCTGCTCGTTCAACC      |                   |         |       |     |
|             |           |                   |               | AAAAACAGTCAACCATTCTT      |                   |         |       |     |

*F/R* = forward primer/reverse primer.
*M*Melting temperature.
*E* Amplification efficiency of primer pairs.
*R²* Correlation coefficient.
quantification cycle (Cq) values were the means of nine values (biological triplicate, each in technical triplicate).

Statistical analyses. To select suitable reference genes, the stability of mRNA expression of each candidate gene was statistically analyzed with three publicly available Excel (Microsoft, Redmond, WA)-based software packages: geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004). All three software packages were used according to the manufacturer’s instructions.

Results

Reference gene amplicons, primer specificity, and PCR amplification efficiency. Specific amplification for each tested primer pair was confirmed by the presence of a single peak in melting curve analysis proceeding 40 cycles of amplification (Supplemental Fig. 2). Furthermore, each amplicon was cloned and sequenced to confirm that it matches the predicted target sequence. The qRT-PCR amplification efficiency was determined according to their Cq value with the formula (Et al., 2004), and BestKeeper (Pfaffl et al., 2004). All three software packages were used according to the manufacturer’s instructions.

Expression profiling of candidate reference genes. A real-time qRT-PCR assay was designed for transcript profiling of the 10 candidate reference genes in 30 diverse samples (Table 1). The expression levels of the candidate reference genes were determined according to their Cq value with the transcripts of these genes showing different levels of abundance (Fig. 1). The average Cq values mostly ranged from 17 to 24 cycles across all tested samples (Fig. 1). ACT and UBC had the lowest Cq (mean Cq of 18.58 and 19.10, respectively), indicating the highest level of expression. TEF2 and SAND were expressed at relatively low levels (mean Cq of 23.15 and 24.71, respectively). PP2A-1 and PP2A-2 showed the least gene expression variation (CV of 4.60% and 4.62%, respectively), whereas TEF2 and RPII were the most variable across all samples with a CV of 10.58% and 8.99%, respectively. Taken together, these results indicate that the expression level of none of the selected genes had a constant expression level and varied widely under different experimental conditions.

Expression stability of candidate reference genes. To find the most stably expressed candidate reference gene for use with qRT-PCR in the different mei sample sets, three widely used programs were used to evaluate candidate reference gene expression: geNorm, NormFinder, and BestKeeper. Cq data were collected from all samples and were used directly for stability calculations for BestKeeper analysis and were transformed to relative quantities for geNorm and NormFinder analysis (Andersen et al., 2004; Guénin et al., 2009; Vandesompele et al., 2002).

The geNorm program is a Visual Basic application tool for Excel and was used to assess the suitability of reference genes by calculating the gene expression stability value M (default value of M ≤ 1.5). This program relies on the principle that the expression ratio of two reference genes should be constant throughout different experimental conditions. The most stable reference gene has the lowest M value, whereas the least stable gene presents the highest M value (Vandesompele et al., 2002).

We analyzed four gene expression sample sets from mei using geNorm (Fig. 2). For the flowering developmental stages and different genotype samples, PP2A-2 and UBC were the most stably expressed genes with the lowest M values, ranking at the top position when all 21 mei samples were analyzed together (Figs. 2A, 2B, and 2D). Under these experimental conditions, the geNorm ranked TEF2 in the last position with the highest M value. When the samples under abiotic stress were analyzed, PP2A-2 and ACT showed the most stable expression and ranked top, whereas EF1α ranked at the bottom (Fig. 2C).

Evaluation of our expression data revealed that UBC and PP2A-2 ranked at the top at flower developmental stages samples in mei and for different genotypes of Prunus species, indicating that these genes were stably expressed and may be suitable reference genes for qRT-PCR normalization; ACT and PP2A-2 ranked at the top when evaluation of our expression data under abiotic stressed in mei, indicating that these genes may be suitable reference genes for qRT-PCR normalization for abiotic-stressed samples in mei. On the other hand, TEF2 and EF1α were the least suitable reference genes under our experimental conditions.

Through calculating the pairwise variation (Vn/Vn+1) between NFn and NFn+1, geNorm also determines the optimal number of reference genes required for effective normalization in qRT-PCR. Vandesompele et al. (2002) proposed Vn/Vn+1 less than 0.15, meaning that 0.15 is the cutoff value for V; if Vn/Vn+1 less than 0.15, it is not necessary to use n + 1 reference genes as internal controls (Vandesompele et al., 2002). As shown in Figure 3, for samples of abiotic stress in mei and genotypes of Prunus, V2/V3 value was more than 0.15, whereas the V3/V4 value was 0.110, suggesting that three reference genes, PP2A-2, UBC, and ACT genes, would be necessary for accurate normalization of qRT-PCR data. Similarly, when we calculated the Vn/Vn+1 values among all 21 tested samples of mei, V3/V4 (0.149) less than 0.15, meaning that at least three reference genes (PP2A-2, UBC, and PP2A-1) would be necessary for normalization.

![Fig. 1. Expression level of candidate reference genes across all samples. A box and whisker plot is shown for the expression level of our candidate reference genes. The line across each box depicts the median, the box depicts the interquartile range, the whiskers represent the 95% confidence intervals, and the black dots represent outliers; Cq = final quantification cycle values.](image-url)
The NormFinder program is another Excel-based application used to determine the expression stability of reference genes, which is based on a variance estimation approach that ranks all reference gene candidates based on intra- and intergroup variations and combines both results into a stability value for each candidate reference gene. In this program, stable gene expression is indicated by lower average expression stability values (Andersen et al., 2004).

As shown in Supplemental Table 2, the rankings of the top candidate reference genes determined by NormFinder were consistent with those determined by geNorm with some minor differences. For flower developmental stages samples, ACT showed the lowest expression stability value when determined by NormFinder followed by PP2A-1, whereas it ranked third with geNorm. When we evaluated the different genotypes, NormFinder determined PP2A-2 and PP2A-1 to be the top two most stably expressed genes, whereas PP2A-2 ranked first and PP2A-1 ranked fourth by geNorm. When we evaluated all the mei samples, PP2A-1 emerged as the most stably expressed, whereas it was ranked third by geNorm. When we considered the least stable genes, both methods showed the same results with the least stable genes being TEF2 and RPII across all samples and different genotypes, whereas TUA and TEF2 were bottom during the flower developmental process.

The BestKeeper program is another Excel-based program designed to analyze the stability of candidate reference genes based on the CV and the SD of the raw Cq values. Reference genes with the lowest CV ± SD values were identified as the most stable genes (Pfaffl et al., 2004). Many suitable reference genes for given experimental conditions in many plants have been successfully evaluated using this method (Chang et al., 2012; Cordoba et al., 2011; Fan et al., 2013; Zhu et al., 2013) with genes with a SD greater than 1 being considered unacceptable (Chang et al., 2012; Cordoba et al., 2011; Zhu et al., 2013).

As shown in Supplemental Table 2, during the flower developmental process, PP2A-2 ranked in the top position using BestKeeper and geNorm but ranked eighth using NormFinder. Under the abiotic stress conditions, UBC had the lowest CV value (0.76) with a SD (0.15) lower than 1, showing the most stable expression. This was in agreement with NormFinder, although it was ranked third by geNorm. Among the samples from different Prunus species, UBC and PP2A-2 ranked in the top two positions using BestKeeper, which was in agreement

Fig. 2. Average expression stability of candidate reference genes was evaluated by geNorm program (Vandesompele et al., 2002), which calculates an average expression stability values (M) based on the average pairwise variation existing between all pairs of the candidate genes for samples at flower development stages (A), different Prunus genotypes (B), under abiotic stress (C), and all mei samples (D). A lower M value indicates more stable expression.

Fig. 3. Pairwise variation (V) between NF_n and NF_{n+1} measurements of the candidate reference genes by geNorm program (Vandesompele et al., 2002). When V_n/V_{n+1} is less than 0.15, then the optimal number of reference genes is N.
with geNorm although the order was different, because PP2A-2 was ranked first and UBC ranked fourth according to NormFinder. When we evaluated gene stability across all the experimental samples of mei using BestKeeper, PP2A-1 emerged as the most stably expressed gene, which was also ranked first by NormFinder and third by geNorm. When we evaluated the least stable genes, BestKeeper showed similar results as the other two methods of analysis with TUA and TEF2 ranked bottom during the flower developmental process, EF1α ranked bottom among the abiotic-stressed samples, and TEF2 and RPII ranked bottom across the different genotypes.

**REFERENCE GENE VALIDATION.** The AGAMOUS gene plays an important role during the development of the reproductive organs in plants (Die et al., 2010; Hou et al., 2011; Ito et al., 2004). Dehydrins play important roles in plant desiccation tolerance. The expression of PmLEA19 was up-regulated under various abiotic stress treatments (Du, 2013). The use of different reference genes to calculate relative expression data of genes can have a significant influence on the final normalized results (Chang et al., 2012; Fan et al., 2013; Ito et al., 2004).

When normalized using the two most stable reference genes (PP2A-1 and PP2A-2) and several middle-ranked stable gene combinations (UBC + ACT and UBC + ACT + EF1α) calculated by three software programs (Table 2) as an internal control, the relative expression level of PmAG showed a similar trend with slightly variability during bud development process. However, these were observed to be completely different when the least stable reference genes, TEF2 and TUA, were used as the internal control (Fig. 4A).

Among the samples with different genotypes, PP2A-1, PP2A-2, and UBC were the top ranked references genes as determined by all three software programs. When using these genes as internal controls, PmAG expression showed a similar trend. When the bottom ranked RP II gene was used for normalization, the expression profile of PmAG showed obvious differences with overestimated expression in P. mume ‘Chaotang Gongfen’ and ‘Sanlun Yudie’ and under estimated expression in another five Prunus species (Fig. 4B).

Under the abiotic stress conditions, the expression profiles of PmLEA19 normalized using four different reference gene combinations (Fig. 4C) showed a similar trend. When UBQ and EF1α, unstably expressed genes (Table 2), were used as the reference for normalization, the expression profile of PmLEA19 changed (Fig. 4C).

**Discussion**

Gene expression studies can lead to a better understanding of the biological processes in many organisms. Specifically, reverse transcription followed by qPCR represents the most powerful technology for comparing the expression profiles of target genes (Grimplet et al., 2007; Ohdan et al., 2005). Recently, the mei genome has been made available, providing the foundation for gene expression profiling studies in mei and genome-level comparisons and analysis among different Prunus species. The use of reference genes as internal controls to normalize mRNA levels is a requirement of qRT-PCR (Wong and Medrano, 2005). To evaluate the best reference genes for different flower development stages, genotypes, and abiotic-stressed samples of mei, three different statistical approaches (geNorm, NormFinder, and BestKeeper) were used to identify the expression stability of 10 candidate reference genes. The results from these three different statistical approaches showed some differences, especially in the top ranked genes. Inconsistency among these methods is not unexpected given that they are based on distinct statistical algorithms (Andersen et al., 2004; Pfaffl et al., 2004; Vandesompele et al., 2002). Similar variability has also been observed when selecting reference genes in other plants under different conditions such as cold-stressed P. orientalis (Chang et al., 2012), developing Vernicia fordii seeds (Han et al., 2012),

### Table 2. The ranking of the expression stability of 10 reference genes according to geNorm, NormFinder, and Bestkeeper software for all mei samples, at flower development stages, under abiotic stress, and different Prunus genotypes samples.

| Ranking* | Software† | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|----------|-----------|---|---|---|---|---|---|---|---|---|---|
| Total    | S         | PP2A-1 | UBC | PP2A-2 | ACT | SAND | EF1α | TUA | UBQ | RPII | TEF2 |
|          | G         | PP2A-2 | UBC | PP2A-1 | ACT | SAND | TUA | EF1α | UBQ | RPII | TEF2 |
|          | N         | PP2A-1 | UBC | SAND | ACT | PP2A-2 | EF1α | TUA | UBQ | RPII | TEF2 |
|          | B         | PP2A-1 | RPII | PP2A-2 | SAND | UBC | EF1α | TEF2 | ACT | UBQ | TUA |
| Flower development | S         | PP2A-2 | PP2A-1 | ACT | UBC | EF1α | SAND | RPII | UBQ | TEF2 | TUA |
|          | G         | PP2A-2 | UBC | ACT | PP2A-1 | EF1α | UBQ | RPII | SAND | TEF2 | TUA |
|          | N         | ACT | PP2A-1 | EF1α | UBQ | SAND | UBC | RPII | PP2A-2 | TEF2 | TUA |
|          | B         | PP2A-2 | PP2A-1 | UBC | RPII | SAND | EF1α | ACT | UBQ | TEF2 | TUA |
| Abiotic stress | S         | UBC | ACT | SAND | PP2A-2 | PP2A-1 | RPII | TEF2 | TUA | UBQ | EF1α |
|          | G         | PP2A-2 | ACT | UBC | SAND | PP2A-1 | RPII | TEF2 | TUA | UBQ | EF1α |
|          | N         | UBC | ACT | SAND | PP2A-1 | RPII | PP2A-2 | TEF2 | UBQ | TUA | EF1α |
|          | B         | UBC | SAND | ACT | RPII | PP2A-2 | PP2A-1 | TEF2 | TUA | UBQ | EF1α |
| Different genotypes | S         | PP2A-2 | UBC | PP2A-1 | ACT | SAND | TUA | EF1α | UBQ | TEF2 | RPII |
|          | G         | PP2A-2 | UBC | ACT | PP2A-1 | SAND | TUA | EF1α | UBQ | TEF2 | RPII |
|          | N         | PP2A-2 | UBC | ACT | UBC | TUA | TEF2 | RPII | UBQ | TUA | EF1α |
|          | B         | UBQ | PP2A-2 | PP2A-1 | SAND | ACT | EF1α | TUA | UBQ | TEF2 | RPII |

*1 = most stable gene, 10 = least stable gene.
†S = summary of the consensus ranking based on the rankings provided by each software, G = geNorm (Vandesompele et al., 2002); N = NormFinder (Andersen et al., 2004); B = Bestkeeper (Pfaffl et al., 2004).
**Different developmental stages of Carica papaya** (Zhu et al., 2012), and stressed and developing Hedysarum coronarium (Cordoba et al., 2011).

In our study, the top ranked genes identified by the three different algorithms were occasionally different, but comprehensive comparison and analysis of the data showed that PP2A-1 and PP2A-2 were suitable as internal controls for gene expression normalization in sample sets of flower developmental stages and different genotypes of Prunus. The expression profiles of PrmAG when normalized to PP2A-1 and PP2A-2, as compared with the least stably expressed reference genes (TUA and RPII), provided superior data demonstrating that PP2A-1 and PP2A-2 were suitable reference genes for gene expression normalization. In R. hybrida, different tissues from three different genotypes (Klie and Debener, 2011), PP2A-4 was ranked higher in comparison with other traditional reference genes. PP2A-4 has also been demonstrated to be the most stable reference gene for different genotypes and different flower and fruit developmental stages in Pisum sativum (Die et al., 2010), Gossypium hirsutum (Artico et al., 2010), Cucurbita pepo (Obrero et al., 2011), and A. thaliana (Czechowski et al., 2005).

Previously, ACT has been considered to be one of the most stably expressed genes and is widely used as a reference gene for expression normalization in many plants (Fan et al., 2013; Luo et al., 2010). In the present study, ACT showed the most stable expression determined by three algorithms for the abiotic-stressed samples of mei, whereas during the flowering process, ACT was ranked middle by geNorm and BestKeeper, although it was still ranked top by NormFinder. The main reasons for this discrepancy may be that the ACT product not only acts as a form of filament providing cells with mechanical support and driving forces for movement, but also contributes to biological processes such as sensing environmental stimuli (Fan et al., 2013; Pollard and Cooper, 2009). UBC has also been demonstrated to be stably expressed in many plants. It was identified as one of the top ranked reference genes in different floral development phases of Lycoris longituba (Cui et al., 2012); in male and female reproductive tissues, spikelets, roots, and leaves of Brachiarhia brizantha (Silveira et al., 2009); and across acclimation and de-acclimation treatments of Eucalyptus globules (Fernández et al., 2010). Similarly, UBC also showed the most stable expression when we evaluated abiotic-stressed samples of mei and flowering samples from different genotypes of Prunus species. When all the mei samples were analyzed together, UBC was the most stably expressed reference gene we studied. In this study, UBQ generally ranked fourth, sixth, or eighth when analyzed using the three algorithms, indicating that it may be unsuitable as a reference gene for gene expression normalization during the whole process of bud development, dormancy and dormancy release, and flowering, although it showed stable expression associated with endodormancy in mei (Sasaki et al., 2011), highlighting the importance of careful reference gene selection.

To our knowledge, this study is the first systematic analysis for the selection of superior reference genes for qRT-PCR in mei, for samples of flower developmental stages in mei, different genotypes of Prunus, and under different abiotic (ABA, osmotic, salt, cold, and heat) stress conditions. Analysis of expression stability using geNorm, NormFinder, and BestKeeper revealed that PP2A-1 and PP2A-2 could be considered to be appropriate reference genes for gene expression analysis for flowering mei with UBC also suitable for different genotypes of Prunus species and UBC and ACT for samples under abiotic stress. Moreover, our results also identified the least stable reference genes, which should be avoided when analyzing gene expression levels under the given experimental conditions. This study also highlights the importance of identifying suitable reference genes for different plant species and for different experimental conditions.
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Supplemental Table 1. Reported stable reference genes of quantitative reverse transcription polymerase chain reaction in plants.

| No. | Plant species | Reported stable genes | Experiment conditions | Literature cited |
|-----|---------------|-----------------------|-----------------------|------------------|
| 1   | *Alopecurus myosuroides* | UBQ, TUB and GADPH | Under herbicide stress | Petit et al., 2012 |
| 2   | *Ammopiptanthus mongolicus* | EIF1 and EIF3 | 22 experimental samples covering the control and different time points under cold, dry, salt, and heat stresses | Shi et al., 2012 |
| 3   | *Anoectochilus roxburghii* | EIF1β and ACT2 | Leaf, stem, root, flower, and peduncle tissues | Zhang et al., 2012a |
| 4   | *Aquilaria sinensis* | TUA, RPL, and GAPDH | Stress | Gao et al., 2012 |
| 5   | *Arabidopsis* | PP2A and UBC | Development and a range of environmental conditions | Czechowski et al., 2005 |
| 6   | *Arachis hypogaea* | ACT11, TUAS, UKN2, PEPKR1, and TIP41 | Various tissue types, seed developmental stages, salt and cold treatments | Chi et al., 2012 |
|     |               | ACT1, UBI1, and GAPDH | Seed developmental stages | Patricia et al., 2011 |
|     |               | ACT1, UBI1, and GAPDH | Cold treatments | Jose et al., 2011 |
|     |               | ACT1, UBI1, and GAPDH | Salt-treated leaves | Jiang et al., 2011 |
|     |               | ACT1, UBI1, and GAPDH | Salt-treated roots |  |
|     |               | ACT1, UBI1, and GAPDH | Across all four species, organs, and treatments studied |  |
|     |               | TBP2, RPL8C | NaOAc and MeJA stress-stimulated hairy roots |  |
|     |               | PLD and ACT | Two tissues of the seed: embryo and cotyledon |  |
|     |               | ACT | Mature peanut seed |  |
| 7   | *Brachiaria brizantha* | UBC, EIF4A, and EF1α | Ovary tissues | Silveira et al., 2009 |
|     |               | EF1 and UBC | Male and female reproductive tissues, spikelets, roots, and leaves |  |
| 8   | *Brachypodium distachyon* | UBC18 | Different plant tissues and grown under various growth conditions | Hong et al., 2008 |
|     |               | UBQ4 and UBQ10 | Different plant tissues and growth hormone-treated plant samples |  |
|     |               | SamDC | Various environmental stresses |  |
| 9   | *Brassica juncea* | ELFA, ACT2, CAC, and TIPS-41 | Vegetative stages | Chandna et al., 2012 |
|     |               | TIPS-41 and CAC | Reproductive stages |  |
|     |               | GAPDH, TUA, TIPS-41, and CAC | Total developmental stages |  |
|     |               | UBQ9 and TIPS-41 | Various stress and hormone treated samples |  |
|     |               | CAC and TIPS-41 | Five cultivars |  |
| 10  | *Brassica napus* | UP1, UBC9, UBC21, and TIP41 | Vegetative tissues | Chen et al., 2010 |
|     |               | ACT7, UBC21, TIP41, and PP2A | Maturing embryos |  |
| 11  | *Brassica rapa ssp. chinensis* | ACT and CYP | Developmental stages | Xiao et al., 2012 |
|     |               | CYP, TUB, and UBC30 | Different tissues (from flowering to seed set) |  |
|     |               | CYP and TUB | Biotic stress |  |
|     |               | UBC30, EF1α, and ACT | Abiotic stress |  |
|     |               | ACT, CYP, UBC30, EF1α, and UBQ | All tested samples |  |

*Continued next page*
| No. | Plant species          | Reported stable genes | Experiment conditions                                                                 | Literature cited |
|-----|------------------------|-----------------------|---------------------------------------------------------------------------------------|------------------|
| 12  | *Capsicum annuum*      | EF1α and UEP          | Different plant tissues (root, stem, leaf, and flower) and from plants treated with    | Bin et al., 2012 |
|     |                        |                       | hormones (salicylic acid and gibberellic acid) and abiotic stresses (cold, heat, salt, and drought) |                  |
|     |                        | BTUB and UBQ3         | Abiotic stress and hormonal treatments                                               | Wan et al., 2011 |
|     |                        | GAPDH and UBQ3        | Different tissues                                                                    |                  |
| 13  | *Carica papaya*        | EIF, TBP1, and TBP2   | Various tissues, different storage temperatures, different cultivars, developmental  | Zhu et al., 2012 |
|     |                        |                       | stages, postharvest ripening, modified atmosphere packaging, 1-MCP treatment, hot water treatment, biotic stress, and hormone treatment |                  |
| 14  | *Chrysanthemum*        | MTP, SKIP16, and PGK  | Different tissues under various developmental stages and leaves with varied photoperiodic treatments | Fu et al., 2013  |
| 15  | *Chrysanthemum*        | EF1α, PP2A            | Aphid-infested plants                                                                | Gu et al., 2011  |
| 16  | *Cichorium intybus*    | ACT, EF, and rRNA     | Leaf and root tissues                                                                | Maroufi et al., 2010 |
| 17  | *Citrus*               | FBOX and GAPDH        | Under the different conditions and subsets tested                                     | Mafra et al., 2012|
|     |                        | UBQ1                  | Flower developmental stages and somatic embryogenesis process                        | Liu et al., 2013 |
|     |                        | 18SrRNA, ACT, and RPII| Diverse plant organs and floral tissues                                             | Yan et al., 2012 |
|     |                        | GAPDH, UBQ10, AP47, and EF1α| Genotype                                                         | Goulao et al., 2012 |
|     |                        | UBQ10, GAPDH, ACT, and EF1α| Cold stress                                                        | Barsalobres-Cavallari et al., 2009 |
|     |                        | GAPDH, ACT, EF1α, and APT| Drought stress                                                   |                  |
|     |                        | UBQ10, GAPDH, ACT, and ELF4A| Multiple stress                                           |                  |
|     |                        | GAPDH                  | Five tissue/organ samples (root, stem, leaf, flower, and fruits)                  |                  |
| 18  | *Coffea* spp.          | CACS, F-box, TIP41, and EF| Different cucumber tissues and under various stresses and growth regulators            | Migocka and Papierniak, 2011 |
| 19  | *Cucumis sativus*      | UFP, EF1α, KPI36αA, PP2A, and CAC| Different stresses and at different developmental stages                      | Obrezo et al., 2011 |
| 20  | *Cucurbita pepo*       |                       | Various stress conditions                                                          | Li et al., 2012b |
| 21  | *Eremosparton songoricum* | EF and α-TUB, EF and ACT| Differing germination stages                                                       |                  |
|     |                        |                       | Multiple adult tissue samples                                                       |                  |
| 22  | *Eucalyptus*           | Cdk8, TEF2, and aspartyl-tRNA synthetase | Six Eucalyptus species and three different organs/tissues | de Oliveira et al., 2012 |
| 23  | *Eucalyptus globulus*  | UBC, αTUB, and EF1α   | Across acclimation and deacclimation treatments                                      | Fernández et al., 2010 |
| 24  | *Euphorbia esula*      | SAND, PTB, ORE9, and ARF2| Various experimental conditions for seed, adventitious underground bud, and other organs of leafy spurge | Chao et al., 2012 |
| 25  | *Fagopyrum esculentum* | Expressed1, SAND, and CACS| Different plant structures (leaves and inflorescences at two stages of development and fruits) | Demidenko et al., 2011 |
| No. | Plant species         | Reported stable genes | Experiment conditions                                                                 | Literature cited |
|-----|-----------------------|-----------------------|----------------------------------------------------------------------------------------|------------------|
| 26  | *Fraxinus* spp.       | *EF1α*                | At least five different tissues (phloem, roots, shoots, immature leaves, and mature leaves), and two developmental stages (young and old) among three ash species | Rivera-Vega et al., 2012 |
| 27  | *Gossypium hirsutum*  | *PP2A1* and *UBQ14*   | Six distinct plant organs, eight stages of flower development, four stages of fruit development and in flower verticils | Artico et al., 2010 |
| 28  | *Glycine max*         | *β-ACT* and *18S rRNA*| Drought stress                                                                         | Stolf-Moreira et al., 2011 |
| 29  | *Hedysarum coronarium*| *TUA1*, *TUA2*, and *UBQ*| Sulla tissues under two conditions of abiotic stress and at various stages of development | Cordoba et al., 2011 |
| 30  | *Helianthus annuus*   | *αTUB*, *βTUB*, and *EF1α*| Leaves of different ages and exposed to different treatments                           | Fernandez et al., 2011 |
| 31  | *Ipomoea batatas*     | *ARF*, *UBI*, *COX*, *GAP*, and *RPL*| For every cultivar across total tested samples                                           | Park et al., 2012  |
| 32  | *Lens culinaris*      | *TIF*, *ACT*, and *18S rRNA*| During cold stress and inoculation with *Aphanomyces euteiches* | Saha and Vandemark, 2013 |
| 33  | *Linum usitatissimum* | *TEF*, *UBQ*, and *GADPH*| Flax roots, internal and external stem tissues, leaves and flowers at different developmental stages | Huis et al., 2010 |
| 34  | *Litchi chinensis*    | *GADPH*               | Total samples, including different varieties, tissues, organs, developmental stages and NAA treatment | Zhong et al., 2011 |
|     |                       | *ACT*, *RPII* and *UBQ*| Varieties and fruit developmental stages                                                  |                  |
|     |                       | *EF1α*                | Organs                                                                                 |                  |
| 35  | *Lolium perenne*      | *EF1α* and *1T521-B*  | Different defoliation management in the field                                             | Lee et al., 2010  |
| 36  | *Lycoris longituba*   | *EIF* and *HIS*       | Root, stem, leaf, sepal, petal, stamen, carpel, fruit, and six phases of floral development | Cui et al., 2012  |
| 37  | *Musa acuminata*      | *UBC*                 | Different phases of floral development                                                   | Podevin et al., 2012 |
| 38  | *Nicotiana tabacum*   | Plastid-encoded: *RPS3*, *NDHI*, and *INI*; nuclear-encoded: *ACT9*, *aTUB*, and *SSU* *L25*, *EF1α*, and *UBC2*| Transgenic *Nicotiana* tabacum plants with elevated or diminished cytokinin content        | Cortleven et al., 2009 |
|     |                       |                       | Developmentally distinct tissues and from plants exposed to several abiotic stresses    | Schmidt and Delaney, 2010 |
| 39  | *Nymphaea tetragona*  | *AP47* and *ACT11*    | Roots subjected to various treatments                                                    | Luo et al., 2010 |
|     |                       | *ACT11* and *EF1α*    | Different tissues                                                                       |                  |
|     |                       | *UBC16* and *ACT11*   | Leaves subjected to various treatments                                                   |                  |
| 40  | *Oryza sativa*        | *EF1* and *ACT1*      | During seed development                                                                  | Li et al., 2010 |
| No. | Plant species | Reported stable genes | Experiment conditions | Literature cited |
|-----|---------------|------------------------|-----------------------|-----------------|
| 41  | *Phaseolus vulgaris* | IDE and ACT11, Skip16 and ACT11 | Biotic stress | Borges et al., 2012 |
| 42  | *Phyllostachys edulis* | NTB, TIP41, and UBQ | Six tissue samples (root, stem, mature stem, leaf, flower, and leaf sheath) | Fan et al., 2013 |
| 43  | *Pinus pinaster* | TIP41, NTB, and CAC | Two developmental stages (before and after flowering) | de Vega-Bartol et al., 2011 |
| 44  | *Pismum sativum* | PP2A, TUB, ACT, TFE2, and HIS3 | Different tissues, treatments and genotypes | Die et al., 2010 |
| 45  | *Platycladus orientalis* | UBC and aTUB | Developmental stages and under all stress conditions across different tissues and cold treated samples | Chang et al., 2012 |
| 46  | *Populus* | EF1α and 18S rRNA, TIP4-like and PT1 or CDC2 and ACT2 | Adventitious rooting of hardwood cuttings | Xu et al., 2011 |
| 47  | *Prunus persica* | TEF2, UBQ10, and RP II | Root, leaf, stem, flowers, and different treated fruit | Tong et al., 2009 |
| 48  | *Quercus suber* | ACT and CACS | Leaves, reproduction cork, and periderm from branches at different developmental stages or collected in different dates | Marum et al., 2012 |
| 49  | *Raphanus sativus* | TEF2, RPII, and ACT | Across 27 radish samples, representing a range of tissue types, cultivars, photoperiodic and vernalization treatments, and developmental stages | Xu et al., 2012 |
| 50  | *Rhododendron micranthum* | EF1α and UBQ, EF1α and 18S rRNA, CYP and EF1α | Different tissues (leaf, root, stem, and flower) at the same developmental stage | Yi et al., 2012 |
| 51  | *Rosa hybrida* | PP2A, SAND, and UBC, PP2A, SAND, and UBC | Different tissues from three different genotypes and in leaves treated with various stress factors | Klie and Debener, 2011 |
| 52  | *Saccharum sp.* | GAPDH | Different tissues | Iskandar et al., 2004 |
| 53  | *Salvia miltiorrhiza* | ACT and UBQ | Roots, stems, leaves, sepals, petals, stamens, and pistils | Yang et al., 2010 |
| 54  | *Senecio cruentus* | SAND, ACT, and PP2A | Different color lines of cineraria during their flower developmental stages | Jin et al., 2013 |
| 55  | *Sesamum indicum* | UBQ6 and APT, TUB, DNAJ, Histone, UBQ6, ACT | Plant development | Wei et al., 2013 |
| 56  | *Solanum lycopersicon* | CAC, TIP41, expressed and SAND, GAPDH and PGK, EF1 | Different tomato developmental stages | Expósito-Rodríguez et al., 2008 |
| 57  | *Solanum phureja* | EF1α | During biotic (late blight) and abiotic stresses (cold and salt stress) | Nicot et al., 2005 |

*Continued next page*
| No. | Plant species                  | Reported stable genes                  | Experiment conditions                                                                 | Literature cited |
|-----|-------------------------------|----------------------------------------|---------------------------------------------------------------------------------------|------------------|
| 58  | *Triticum aestivum*           | *TaFNRII, ACT2, rm26, CYP18-2, and TaWINI* | Winter wheat flag leaves                                                              | Gabriela et al. 2011 |
|     |                               | *TaFNRII, ACT2, and CYP18-2*           | Two winter wheat varieties (Tommi and Centenaire) grown under three treatments (organic, conventional, and no nitrogen) |                  |
| 59  | *Trifolium pratense*          | *UBC2 and UBQ10*                       | Leaf tissue                                                                           | Mehdi Khanlou and Van Bockstaele, 2012 |
|     |                               | *UBC2 and YLS8*                        | Stem tissue                                                                           |                  |
|     |                               | *EF4a and UBC2*                        | Root tissue                                                                           |                  |
|     |                               | *YLS8 and UBC2*                       | Across tissues                                                                         |                  |
| 60  | *Vernicia fordii*             | *ACT7, UBQ, GAPDH, and EF1a*           | Different tissues/organs and developing seeds from four cultivars                     | Han et al., 2012 |
| 61  | *Vitis vinifera*              | *ACT7, EF1, GAPDH, and TEF1*           | Different tissues/organs                                                                | Reid et al., 2006 |
|     |                               | *GAPDH, ACT, EF1a, and SAND*           | Grape berry development                                                                | Gamm et al., 2011 |
|     |                               | *VATP16 and 60SRP*                     | Leaves and berries infected by *Plasmopara viticola* and *Botrytis cinerea*, respectively |                  |
| 62  | *Zea mays*                    | *18S rRNA and GAPDH*                   | Osmoprimed and germinated seeds                                                       | Chen et al., 2012 |
|     |                               | *CUL, FPGS, LUG, MEP, and UBCP*        | Different tissues of plants grown at various experimental conditions                  | Manoli et al., 2012 |

*Detailed in the Literature Cited section of the text.*

MeJA = jasmonic acid methyl ester; 1-MCP = 1-methylcyclopropene; NAA = 1-naphthaleneacetic acid; ABA = abscisic acid; N = nitrogen.
### Supplemental Table 2A. Expression stability of the reference genes calculated by NormFinder (Andersen et al., 2004).  

| Ranking | Gene (Stability) | Flower development Gene (Stability) | Abiotic stress Gene (Stability) | Different genotypes Gene (Stability) |
|---------|------------------|-----------------------------------|--------------------------------|-----------------------------------|
| 1       | PP2A-1 (0.08)    | ACT (0.05)                        | UBC (0.18)                     | PP2A-2 (0.06)                     |
| 2       | UBC (0.35)       | PP2A-1 (0.10)                     | ACT (0.21)                     | PP2A-1 (0.11)                     |
| 3       | SAND (0.37)      | EF1α (0.14)                       | SAND (0.23)                    | ACT (0.11)                        |
| 4       | ACT (0.37)       | UBQ (0.36)                        | PP2A-1 (0.30)                  | UBC (0.31)                        |
| 5       | PP2A-2 (0.37)    | SAND (0.41)                       | RPII (0.33)                    | SAND (0.32)                       |
| 6       | EF1α (0.67)      | UBC (0.46)                        | PP2A-2 (0.33)                  | TUA (0.39)                        |
| 7       | TUA (0.71)       | RPII (0.50)                       | TEF2 (0.64)                    | EF1α (0.46)                       |
| 8       | UBQ (1.01)       | PP2A-2 (0.53)                     | UBQ (0.65)                     | UBQ (0.67)                        |
| 9       | RPII (1.27)      | TEF2 (0.70)                       | TUA (0.75)                     | TEF2 (1.76)                       |
| 10      | TEF2 (1.29)      | TUA (0.78)                        | EF1α (0.85)                    | RPII (1.80)                       |

*Reference genes with the lowest stability values were identified as the most stable genes by NormFinder (Andersen et al., 2004).*

### Supplemental Table 2B. Expression stability of the reference genes calculated by BestKeeper (Pfaffl et al., 2004).  

| Ranking | Gene (CV ± SD*) | Flower development Gene (CV ± SD*) | Abiotic stress Gene (CV ± SD*) | Different genotypes Gene (CV ± SD*) |
|---------|-----------------|-----------------------------------|--------------------------------|-----------------------------------|
| 1       | PP2A-1 (3.42 ± 0.76) | PP2A-2 (3.60 ± 0.78) | UBC (0.76 ± 0.15) | UBC (1.17 ± 0.22) |
| 2       | RPII (3.49 ± 0.82) | PP2A-1 (4.18 ± 0.93) | SAND (0.86 ± 0.21) | PP2A-2 (1.45 ± 0.30) |
| 3       | PP2A-2 (3.68 ± 0.78) | UBC (4.44 ± 0.87) | ACT (1.29 ± 0.25) | PP2A-1 (1.64 ± 0.36) |
| 4       | SAND (3.86 ± 0.95) | RPII (4.52 ± 1.04) | RPII (1.77 ± 0.41) | SAND (1.98 ± 0.48) |
| 5       | UBC (3.98 ± 0.76) | SAND (5.06 ± 1.27) | PP2A-2 (1.94 ± 0.40) | ACT (2.34 ± 0.41) |
| 6       | EF1α (4.16 ± 0.82) | EF1α (5.44 ± 1.07) | PP2A-1 (2.07 ± 0.47) | EF1α (3.03 ± 0.61) |
| 7       | TEF2 (5.74 ± 1.34) | ACT (5.74 ± 1.10) | TEF2 (2.48 ± 0.58) | TUA (3.04 ± 0.60) |
| 8       | ACT (5.77 ± 1.08) | UBQ (6.05 ± 1.28) | TUA (2.99 ± 0.62) | UBQ (3.56 ± 0.82) |
| 9       | UBQ (5.94 ± 1.33) | TEF2 (7.63 ± 1.81) | UBQ (3.84 ± 0.86) | TEF2 (6.83 ± 1.53) |
| 10      | TUA (6.02 ± 1.23) | TUA (8.50 ± 1.82) | EF1α (5.26 ± 1.03) | RPII (10.19 ± 2.22) |

*Reference genes with the lowest CV ± SD values were identified as the most stable genes by BestKeeper (Pfaffl et al., 2004).*

*V = Coefficient of variation of the raw Cq values.*

*SD = The standard deviation of the raw Cq values.*

### Supplemental Table 2C. Pairwise variation (V).  

| V2/3 | V3/4 | V4/5 | V5/6 | V6/7 | V7/8 | V8/9 | V9/10 |
|------|------|------|------|------|------|------|-------|
| Total | 0.189 | 0.149 | 0.14 | 0.157 | 0.159 | 0.182 | 0.202 | 0.189 |
| Flower development | 0.19 | 0.11 | 0.112 | 0.112 | 0.092 | 0.09 | 0.122 | 0.114 |
| Abiotic stress | 0.127 | 0.121 | 0.134 | 0.095 | 0.126 | 0.11 | 0.122 | 0.125 |
| Different genotypes | 0.122 | 0.079 | 0.1 | 0.095 | 0.108 | 0.126 | 0.278 | 0.262 |

### Supplemental Fig. 1. Polymerase chain reaction amplification specificity of the 10 reference genes and PmAG gene. Only a single amplification product for each primer pair with the expected amplicon size was verified by 2% agarose gel electrophoresis. M = Marker D2000; ACT = actin gene; EF1α = elongation factor 1-alpha gene; PP2A = protein phosphatase 2A gene; RPII = RNA polymerase II gene; SAND = SAND family protein gene; TEF2 = translation elongation factor 2 gene; TUA = alpha tubulin gene; UBQ = ubiquitin gene; UBC = ubiquitin-conjugating enzyme E2 gene; PmAG = an AGAMOUS homolog gene of A. thaliana.
Supplemental Fig. 2. Melting curves of the 10 reference genes and PmAG gene. ACT = actin gene; EF1α = elongation factor 1-alpha gene; PP2A = protein phosphatase 2A gene; RPII = RNA polymerase II gene; SAND = SAND family protein gene; TEF2 = translation elongation factor 2 gene; TUA = alpha tubulin gene; UBG = ubiquitin gene; UBC = ubiquitin-conjugating enzyme E2 gene; PmAG = an AGAMOUS homolog gene of A. thaliana; Temperature (°C) = the amplification temperature; –d(RFU)/dT = the rate of change of the relative fluorescence units (RFU) with time (T).
Supplemental Fig. 2. Continued.
Supplemental Fig. 2. Continued.