Identification of reference genes for normalization of gene expression during flowering in Isabgol (Plantago ovata Forsk.)

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ABSTRACT

Understanding the genetic control of flowering is crucial in the development of early maturing Isabgol varieties and requires expression analysis of flowering genes. Quantitative real-time polymerase chain reaction (RT-qPCR) is one of the most common methods used for quantification of gene expression depending on the stability of reference genes for normalization. Selection of stable reference gene(s), is an essential step to avoid ambiguous expression results during RT-qPCR. In the present study, eight housekeeping genes (Actin, 18s, GAPDH, ADH, TUB, UBQ, ELF1 and ELF2) were evaluated for their expression stability in stem elongation stage, booting and anthesis stage in early (RIL18) and late (RIL55) genotypes of Isabgol. The genes Actin, GAPDH, ADH, UBQ, ELF1 and ELF2 were most stable genes across developmental stages and genotypes by geNorm, Normfinder, BestKeeper, and the comparative Delta-Ct method. However, the expression of 18s and TUB was stage specific. Overall expression stability rank of genes based on RefFinder was poGAPDH > poELF1 > poADH > poActin > poUBQ > poELF2 > poTUB > po18s across developmental stages and genotypes. Finally, we verified the reliability of most stable and unstable reference genes for quantification of phytochrome-A (poPHYA-2) gene involved in flowering pathway of plants. The study provides the foundation for reference gene(s) selection and will contribute towards more accurate target gene expression analysis of Isabgol during flowering.

Key words: Isabgol, Plantago ovata, reference genes, gene expression, realtime PCR.

INTRODUCTION

Isabgol (Plantago ovata Forsk.) is an important export oriented medicinal plant of India. Seed husk is the economic part used as a laxative and natural fiber. The seed husk is hydrophilic and has a swelling property. Consequently, it is a very good dietary fiber, which stimulates peristalsis and helps in bowel clearance. The husk is used as an anti-diarrheal drug. The husk is also used in calico printing, dyeing, agar-agar media preparation, gum and jelly making, as binder in tablets, as thicker and a fixative in ice-cream, confectionary and in cosmetics industries (Dhar et al., 2011). It has been used as a deflocculant in paper and textile manufacturing, as an emulsifying agent, as binder or lubricant in meat products, and as a replacement of fat in low-calorie foods (Dhar et al., 2011). Besides its economic importance, Isabgol is model plant to study mucilage biosynthesis and hetrochromatination of chromosomes.

Flowering and maturity are the major developmental processes that governs economic yield of crop plants. However, little is known about the molecular mechanisms underlying flowering in Isabgol. Gene expression of important flowering genes is needed to understand molecular mechanisms of flowering. Photoperiod and vernalization are the important physiological characteristic which play a crucial role in onset of flowering in Isabgol...
Long day conditions and low temperatures favour early onset of flowering. Cool dry weather favours seed maturity and mucilage biosynthesis (Janakiram et al., 2019). Understanding the molecular mechanisms of flowering and maturity forms the basis for targeted breeding of Isabgol. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a rapid and reliable method for the detection and quantification of changes in gene expression under different biological processes. However, uncontrolled errors between samples during the RT-qPCR those caused by RNA quantification and integrity, differences in pipetting, and reverse transcriptase efficiency largely affects detection and quantification gene expression (Gutierrez et al., 2008). Such uncontrolled variations between samples during the RT-qPCR can be curtailed through normalization of expression data using genes with stable expression independent of the sample physiological condition and the applied treatment, such genes are also referred to as reference genes. Not all reference genes are ideal to the physiological condition and the applied treatment hence, identification of appropriate reference genes for target gene expression detection and quantification is vital to enhance the reliability and reproducibility of RT-qPCR (Li et al., 2016; Mafra et al., 2012).

Housekeeping genes in plants, such as those encoding Actin (Qi et al., 2016; Hou et al., 2018; Reddy and Manivel, 2019), 18srRNA (Reddy and Manivel, 2019), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Reddy and Manivel, 2019), elongation factor (ELF) (Qi et al., 2016; Reddy et al., 2019), α-tubulin (Qi et al., 2016), and ubiquitin (Qi et al., 2016; Reddy and Manivel, 2019) have been used as reference genes for normalization of expression data from the RT-qPCR. However, expression of the housekeeping genes might vary for genotypes (Li et al., 2016), developmental stages (Chao et al., 2019) or environmental conditions, leading to erroneous interpretation of target gene expression. Many studies have shown that reference genes are not sufficiently stable under some treatments (Thellin et al., 1999; Barsalobres-Cavallari et al., 2009), during development (Bustin, 2002), or even in different tissues (Czechowski et al., 2005; Mallona et al., 2010) due to the variable nature of plant gene regulation and functions. Selection of such biased reference genes can result in misinterpretation of the qPCR data and consequently, output of misleading expression data. Therefore, it is important to identify and validate stable reference genes according to the genotype and to the experimental conditions prior to use for normalization. The statistical models, comparative ΔCq method (Silver et al., 2006), geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) are used for estimating variance reference genes for normalization (Vandesompele et al., 2002; Pfaffl et al., 2004). The models, geNorm and NormFinder used raw data as input and allows easy comparison, whereas, Bestkeeper calculates the geometric mean of the best suited genes by raw Cq values of each gene and employs a pairwise correlation analysis to determine the optimal reference genes (Pfaffl et al., 2004). Here, the study aims to identify suitable reference genes to develop an accurate, reliable and reproducible RT-qPCR method used for expression analysis of flowering genes in Isabgol. The stability and performance of eight housekeeping genes viz., Ubiquitin, Actin, 18rRNA, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-tubulin Alcohol dehydrogenase (ADH), Eukaryotic elongation factor-1 (ELF-1) and Eukaryotic elongation factor-2 (ELF-2) were evaluated in Isabgol under developmental condition (stem elongation, booting and anthesis) in early and late flowering genotypes. The study provided reference genes for the accurate quantification of gene expression of flowering genes.

MATERIALS AND METHODS

Plant materials and tissues

Two genotypes, RIL18 (early; 40-50 days to flowering) and RIL55 (late; 70-80 days to flowering) were used to identify stable reference genes under stem elongation, booting and anthesis flowering stages. The genotypes were developed from ICAR-Directorate of Medicinal and Aromatic Plants Research (DMAPR), Boravi, Anand, India. Pure seeds of all the genotypes were raised in the research block of ICAR-DMAPR, Anand, Gujarat, India during 2018-19. Different tissues of different stages (that is, leaves at stem elongation stage, flower bud at booting stage and mature flower at anthesis stage) were collected. The tissues were dipped in RNA later (Sigma-Aldrich, St. Louis, Missouri, USA) and were immediately frozen in liquid nitrogen, and then stored at -80°C for isolation of the total RNAs.

RNA extraction and cDNA synthesis

Total RNA was isolated using Trizol (Invitrogen) as per manufacturer’s instruction by using the standard protocol described by the manufacturer (Thermo Fisher Scientific 168 Third Avenue Waltham, MA, USA). The quality of the isolated RNA was checked on 1% denatured Agarose gel for the presence of 28S and 18S bands. cDNA was synthesized using oligo (dT) as primers by M-MLV reverse transcriptase according to the manufacturer’s instructions (Thermo scientific, Waltham, Massachusetts, USA). After the reverse transcription, synthesized cDNA was stored at -20°C until use.

Primers and real-time PCR

Eight constitutively expressing genes in plants were selected based on previous studies in senna (Reddy and
Table 1: Reference genes studied in Isabgol (*Plantago ovata* Forsk.).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Expected Tm (°C)</th>
<th>Annealing Temp (°C)</th>
<th>Expected size (bp)</th>
<th>Observed Size (bp)</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin (poActin)</td>
<td>XDAPOActin</td>
<td>CACCGAGTCTGGTGTTGAGGGTTG</td>
<td>TAGGGAAGGACACGACTTGA</td>
<td>59.4</td>
<td>58</td>
<td>114</td>
<td>112</td>
<td>0.91</td>
</tr>
<tr>
<td>18S rRNA (po18s)</td>
<td>XDAPO18S</td>
<td>AAAACGGCTACCCACATCAGA</td>
<td>CAGACTCAAAAGGCGG</td>
<td>58.3</td>
<td>58</td>
<td>118</td>
<td>118</td>
<td>0.99</td>
</tr>
<tr>
<td>Alcohol dehydrogenase (poADH)</td>
<td>XDAPOADH</td>
<td>CTTCAAATGCCCTTGCCA</td>
<td>TCTCTAGCTCTCAGTG</td>
<td>58.3</td>
<td>55</td>
<td>114</td>
<td>110</td>
<td>0.57</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate</td>
<td>XDAPOGAPDH</td>
<td>AGAATTGGCATGTTCAAAGG</td>
<td>GTTGCGAATGGTCAATCG</td>
<td>55.3</td>
<td>58</td>
<td>126</td>
<td>182</td>
<td>0.90</td>
</tr>
<tr>
<td>dehydrogenase (poGAPDH)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tubulin (poTUB)</td>
<td>XDAPOATUBULIN</td>
<td>GGGATATGTTTCACCAAGGA</td>
<td>TCCACACTTGAAGGCAGTTTG</td>
<td>57.3</td>
<td>55</td>
<td>102</td>
<td>100</td>
<td>0.90</td>
</tr>
<tr>
<td>Ubiquitin (poUBQ)</td>
<td>XDAPOUBQ</td>
<td>CCAAGATTGTTTCCACCAAGGA</td>
<td>TGGATTTGAGATCAGGAGGA</td>
<td>56.3</td>
<td>55</td>
<td>134</td>
<td>180</td>
<td>0.90</td>
</tr>
<tr>
<td>Eukaryotic elongation factor-1 (poELF-1)</td>
<td>XDAPOELF-1</td>
<td>GGGGTGAGATTTCTAGGG</td>
<td>ACCCGTGGCTGTATACCTTG</td>
<td>57.3</td>
<td>48</td>
<td>148</td>
<td>200</td>
<td>0.95</td>
</tr>
<tr>
<td>Eukaryotic elongation factor-2 (poELF-2)</td>
<td>XDAPOELF-2</td>
<td>AAGAGGCTTGGTGCACTTGG</td>
<td>CAAAGGTCATTTCCACATG</td>
<td>57.3</td>
<td>48</td>
<td>119</td>
<td>120</td>
<td>0.94</td>
</tr>
<tr>
<td>Phytochrome-A (poPHYA-2)</td>
<td>XDAPOPHYA-2</td>
<td>GGAATCGAGCAGTTAAA</td>
<td>CAAAGTCCTCAGGTAAAA</td>
<td>60.0</td>
<td>55</td>
<td>196</td>
<td>196</td>
<td>-</td>
</tr>
</tbody>
</table>

* Flowering gene

Manivel, 2019) and other plants (Chao et al., 2019; Mallona et al., 2010; Sgamma et al., 2016). Orthologs of the reference genes were picked from the leaf transcriptome library (Patel et al., 2020). The primers were designed using primer3 with default parameters (http://frodo.wi.mit.edu/primer3). The expected allele size range given was 100-200 bp while picking the primers as shown in Table 1. The specificity of the primers was further confirmed by BLAST search. Annealing temperatures were optimized according to individual primers by testing several annealing temperatures ranging from 50 °C to 65 °C around the respective primer Tm, and the annealing temperature with the best efficiency was chosen. Real-time PCR was performed in a 25 μl volume using SYBR premix (Genetix, Delhi, India) on the CFX Connect Real-time PCR detection system (Bio-Rad, Hercules, USA) by following a program: 5 min at 95 °C; followed by 45 cycles of amplification with denaturation for 5 s at 95 °C, annealing for 30 s at 55 °C, and extension for 20 s at 72 °C. A melting step was performed to confirm a single gene-specific peak by a stepwise temperature increase ranging from 60 °C to 95 °C at ramp rate 1 °C/s with continuous monitoring of fluorescence. No-template controls (NTCs) were included for each primer pair. Further analysis of amplicon specificity and size were evaluated running qPCR products in a standard 2% agarose gel electrophoresis. Triplicate under identical conditions were synchronously performed for all genes. Standard curves were made to calculate the amplification efficiency during real-time PCR using five-fold serial dilutions of cDNA for each tissue and each reference gene. The quantification cycle (Cq) was automatically determined for each reaction by the CFX Maestro™ Software (Bio-Rad, Hercules, USA). Finally, the specificity of the qRT-PCR reactions was determined by melt curve analysis of the amplified products.

**Data analysis**

We calculated the cycle threshold (Cq) values and real-time PCR efficiencies (E) for each reference gene to know the gene expression pattern. Relative standard curve was constructed using a 10-fold dilution series of cDNA from each tissue. PCR efficiency was determined by converting quantification cycles (Cq values) into raw data (relative quantities). The real-time E value was calculated from the given slopes in the equation: $E = \left[10^{(-1/slope)} - 1\right]$ (Hellemans et al., 2007). Only Cq values <35 were used for calculation of E values. Stability of Cq values were performed using RefFinder (https://www.heartcure.com.au/reffinder/?type=rreference) (Xie et al., 2012). RefFinder integrates comparative ΔCq method (Silver et al., 2006), geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) method to compare and rank the tested candidate reference genes. A one-way ANOVA was performed on Cq values obtained from the expression in genotypes and tissues. We used Microsoft Excel 2010 to calculate other statistical parameters.

**RESULTS**

**Selection of reference genes**

Housekeeping genes are largely used as reference genes for gene expression in plants and animal systems. However, the expression of housekeeping genes is regulated by the tissue, stage and genotype and other conditions. Eight housekeeping genes viz., Actin, 18srRNA, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Alcohol dehydrogenase
Table 2: Cycle threshold (Cq) values and reaction efficiency (E) for the reference genes studied in Isabgol (Plantago ovata Forsk.).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Genotype</th>
<th>poUBQ</th>
<th>po18s</th>
<th>poActin</th>
<th>poADH</th>
<th>poTUB</th>
<th>poGAPDH</th>
<th>poELF1</th>
<th>poELF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem elongation</td>
<td>RIL1B</td>
<td>26.93</td>
<td>20.53</td>
<td>30.81</td>
<td>34.29</td>
<td>35.28</td>
<td>29.70</td>
<td>24.45</td>
<td>27.45</td>
</tr>
<tr>
<td></td>
<td>RIL55</td>
<td>24.65</td>
<td>16.84</td>
<td>31.08</td>
<td>33.14</td>
<td>30.76</td>
<td>29.08</td>
<td>23.77</td>
<td>27.43</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>25.79</td>
<td>18.68</td>
<td>30.95</td>
<td>33.72</td>
<td>33.02</td>
<td>29.39</td>
<td>24.11</td>
<td>27.44</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.57</td>
<td>1.04</td>
<td>0.81</td>
<td>1.47</td>
<td>1.65</td>
<td>1.98</td>
<td>0.19</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>2.20</td>
<td>5.57</td>
<td>2.61</td>
<td>4.37</td>
<td>5.00</td>
<td>6.75</td>
<td>0.77</td>
<td>6.37</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>0.90</td>
<td>0.92</td>
<td>0.90</td>
<td>1.45</td>
<td>0.90</td>
<td>0.99</td>
<td>0.95</td>
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</tr>
<tr>
<td>Booting</td>
<td>RIL1B</td>
<td>24.69</td>
<td>11.61</td>
<td>30.58</td>
<td>32.67</td>
<td>29.80</td>
<td>30.19</td>
<td>24.90</td>
<td>28.23</td>
</tr>
<tr>
<td></td>
<td>RIL55</td>
<td>22.39</td>
<td>13.46</td>
<td>30.71</td>
<td>32.67</td>
<td>27.46</td>
<td>29.17</td>
<td>24.23</td>
<td>26.87</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>23.54</td>
<td>12.53</td>
<td>30.64</td>
<td>32.67</td>
<td>28.63</td>
<td>29.68</td>
<td>24.57</td>
<td>27.55</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>2.13</td>
<td>1.90</td>
<td>2.61</td>
<td>0.47</td>
<td>2.23</td>
<td>1.63</td>
<td>0.51</td>
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<tr>
<td></td>
<td>CV</td>
<td>9.03</td>
<td>15.16</td>
<td>8.52</td>
<td>1.45</td>
<td>7.78</td>
<td>5.48</td>
<td>2.08</td>
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</tr>
<tr>
<td></td>
<td>E</td>
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<td>0.99</td>
<td>0.93</td>
<td>0.43</td>
<td>0.90</td>
<td>1.00</td>
<td>0.95</td>
<td>0.94</td>
</tr>
<tr>
<td>Anthesis</td>
<td>RIL1B</td>
<td>20.64</td>
<td>7.43</td>
<td>31.04</td>
<td>32.98</td>
<td>25.42</td>
<td>28.50</td>
<td>23.38</td>
<td>26.79</td>
</tr>
<tr>
<td></td>
<td>RIL55</td>
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<td>12.62</td>
<td>30.99</td>
<td>32.70</td>
<td>27.17</td>
<td>29.14</td>
<td>24.33</td>
<td>28.52</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>21.32</td>
<td>10.02</td>
<td>31.02</td>
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<td>28.82</td>
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<tr>
<td></td>
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<td>2.33</td>
<td>0.89</td>
<td>1.24</td>
<td>0.63</td>
<td>1.29</td>
<td>1.15</td>
<td>2.09</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>2.03</td>
<td>23.26</td>
<td>2.87</td>
<td>3.77</td>
<td>2.41</td>
<td>4.48</td>
<td>4.82</td>
<td>7.57</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>0.90</td>
<td>1.00</td>
<td>0.90</td>
<td>0.33</td>
<td>0.90</td>
<td>0.90</td>
<td>0.95</td>
<td>0.94</td>
</tr>
<tr>
<td>Overall</td>
<td>RIL1B</td>
<td>24.09</td>
<td>13.19</td>
<td>30.81</td>
<td>33.31</td>
<td>30.17</td>
<td>29.46</td>
<td>24.25</td>
<td>27.49</td>
</tr>
<tr>
<td></td>
<td>RIL55</td>
<td>23.01</td>
<td>14.30</td>
<td>30.93</td>
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<td>28.46</td>
<td>29.13</td>
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<tr>
<td></td>
<td>Mean</td>
<td>23.55</td>
<td>13.75</td>
<td>30.87</td>
<td>33.08</td>
<td>29.31</td>
<td>29.30</td>
<td>24.18</td>
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</tr>
<tr>
<td></td>
<td>SD</td>
<td>2.95</td>
<td>5.74</td>
<td>1.59</td>
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<td>1.57</td>
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</tr>
<tr>
<td></td>
<td>CV</td>
<td>12.51</td>
<td>41.78</td>
<td>5.16</td>
<td>3.31</td>
<td>14.07</td>
<td>5.36</td>
<td>2.74</td>
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<tr>
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<td>0.91</td>
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<td>0.90</td>
<td>0.90</td>
<td>0.95</td>
<td>0.94</td>
</tr>
</tbody>
</table>

(ADH), α-Tubulin, Ubiquitin Eukaryotic elongation factor-1 (ELF-1) and Eukaryotic elongation factor-2 (ELF-2) (Table 1) were selected for this study based on reference gene studies in plants (Reddy and Manivel, 2019). Candidate reference gene sequences were identified from the transcriptomic data of Isabgol (Patel et al., 2020). The putative reference genes encode proteins with a wide variety of biological functions such as transcription factors, ubiquitous enzymes or cytoskeleton elements in plants.

Expression profile of reference genes

The housekeeping genes were PCR amplified using cDNA from stem elongation, booting and anthesis stages of RIL18 (early flowering) and RIL55 (late flowering) genotypes of Isabgol in a Real-time PCR detection system. Further, the specificity of primers designed were checked fragment assay using agarose gel electrophoresis and melting curve analyses following the RT-qPCR assays. The primer pairs amplified a single band of the expected size of 112 to 200 bp in agarose gel electrophoresis and formed no primer dimers or other non-specific amplification products and produced single peak on the melting curve analysis indicating the specificity of amplification (Supplementary Figure S1 and S2). The PCR efficiency of the eight candidate reference genes was high (> 0.90) for poUBQ, po18s, poActin, poTUB and poGAPDH as shown in Table 2. The sequences of the amplified PCR products confirmed the specificity of the primers.

Expression stability of reference genes

The mean Cq value of reference genes was below 33.08 cycles across developmental stages and genotypes indicating abundance gene expression in the samples. The gene, po18s was the most abundantly expressed with a mean Cq value of 13.75 developmental stages and genotypes which was followed by poUBQ (Cq of 22.55) and poELF1 (Cq of 24.18), whereas the gene poADH was least abundantly expressed with a mean Cq value of 33.08 (Table 2 and Figure 1) indicating variation in expression profile of reference genes. There was no significant variation in Cq
values of RIL18 and RIL55 genotypes of Isabgol (Supplementary Table 2). Expression stability of reference genes varied with developmental stages in the present study. Stem elongation stage is the most important developmental stage involved in flowering. All methods showed similar performance of reference genes. The gene, poELF1 (DeltaCq and geNorm), poADH (NormFinder), poELF2 (BestKeeper) and poGAPDH (geNorm) were consistently identified as most stable genes at stem elongation stage as shown in Table 3 and Figure 2. While the gene poTUB was identified as most unstable gene according to all the analysis which was followed by po18s at stem elongation stage. According to RefFinder, the stable of reference genes from low to high is as follows: poELF1 > poGAPDH > poELF2 > poActin > poADH > poUBQ > poTUB > po18s (Supplementary Table 2). Expression stability of reference genes on the normalization factor. A cut-off value of 0.15 that measures the effect of measuring additional reference genes on the normalization factor. A cut-off value of 0.15 was found lowest stable gene by all the methods of stability analysis. According to RefFinder, the stability of reference genes from low to high is as follows: poELF1 > poGAPDH > poELF2 > poActin > poADH > poUBQ > poTUB > po18s (Table 3). Similarly, in RIL55 genotype which is late flowering, the genes poADH (DeltaCq, NormFinder and GeNorm), poGAPDH (BestKeeper) and poActin (GeNorm) were consistently identified as most stable genes while po18s was found least stable gene by all the methods of stability analysis. The ranking of reference genes based on stability by the RefFinder is as follows: poADH > poActin > poGAPDH > poELF1 > poUBQ > poELF2 > poTUB > po18s. Overall, across developmental stages and genotypes, the genes poGAPDH (DeltaCq, and GeNorm), poActin (BestKeeper), poUBQ (NormFinder) and poELF1 (GeNorm) were consistently identified as most stable genes while po18s and poTUB were found least stable gene by all the methods of stability analysis (Table 3). According to RefFinder, the stability of reference genes from low to high is as follows: poGAPDH > poELF1 > poADH > poActin > poUBQ > poELF2 > poTUB > po18s.

Optimization of reference genes for normalization

For accurate normalization optimal number of reference genes were required which were calculated by using geNorm. geNorm determines pairwise variation (Vn/Vn+1) that measures the effect of measuring additional reference genes on the normalization factor. A cut-off value of 0.15 has been widely accepted as the criterion for selecting a suitable number of reference genes, below which the inclusion of additional reference genes are not needed (Vandesompele, 2002). The pair-wise variation V2/3, V3/4, V4/5, V5/6, V6/7, V7/8 were found lower than 0.15 in all stages and genotypes (Overall, stem elongation, booting and
Table 3: Stability of reference genes under different experimental conditions in Isabgol (*Plantago ovata* Forsk.).

<table>
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Validation of reference genes

The expression of Phytochrome-A (poPHYA-2) gene was used to evaluate reference gene under various developmental stages and genotypes. PHYA-2 encodes for Phytochrome-A of circadian pathway of flowering in plants. The relative expression of poPHYA-2 gene was determined by normalization using stable (poGAPDH, poELF1, poADH, poActin, poUBQ and poELF2) and unstable (poTUB and po18s) reference genes as determined by RefFinder (Table 3). The relative expression of poPHYA-2 in fold change ranged from 3.50 to 35153.29 (in RIL18) and 1.90 to 74.57 (in RIL55) at stem elongation compared to anthesis when normalized with stable and unstable reference genes as shown in Figure 4 and Supplementary Table 1. Likewise, the relative expression of poPHYA-2 gene expressed was 1.5 to 41.7 (in RIL18) and 0.6 to 3.6 (in RIL55)-fold in booting compared to anthesis indicating over estimated gene expression when normalized using unstable (poTUB and po18s) reference genes.

DISCUSSION

Understanding the genetic regulation of flowering will help manipulate grain yield and quality of Isabgol. Gene
expression analysis is an important tool to decipher the genetic regulation of flowering in plants (Patel et al., 2020). Realtime PCR has become a routine technique for detection and quantification of gene expression in molecular biology due to its sensitivity, accuracy, and high throughput nature (Bustin, 2000). Appropriate normalization factor is a key step to obtain accurate interpretation of the gene expression data from RT-qPCR. Reference genes with high stability in expression under similar experimental conditions were used for normalization. However, there is no single gene known that remains stable in diverse experimental conditions and genotypes (Gutierrez et al., 2008). Reference genes to study flowering genes were identified in Chionochloa and Celmisia (Jameson, 2019), Petunia (Mallona et al., 2010), Jatropha (Karuppaiya et al., 2017), Lagerstroemia (Zheng et al., 2018), Lettuce (Sgamma et al., 2016), Paonia suffruticosa (Li et al., 2016), Rhododendron (Xiao et al., 2016), Tomato (Choi et al., 2018), Artemisia (Ahmad et al., 2019) and Tea (Hao et al., 2014). The majority of the traditionally used reference genes are housekeeping genes which involve metabolism, cellular activities, cellular structure maintenance and protein translation. Nonetheless, information of stable reference genes to study flowering were scarce in Isabgol. This study represents the first effort to identify reference genes with highly uniform gene expression in different flowering stages in Isabgol. Flowering genes are expressed differently in stem elongation, booting and anthesis.
developmental stages (Patel et al., 2020) as well as early and late genotypes (Hou et al., 2018; Patel et al., 2020). In this study, the stability of eight reference genes various developmental stages and genotypes was analyzed. This is the first referenced gene validation reported for Isabgol. Most stable reference genes are used for accurate quantification of targets gene expression using RT-qPCR. Potential reference genes should have stable expression across developmental stages and genotypes. The expression stability of potential reference genes was evaluated using comparative ΔCq method, geNorm, NormFinder and BestKeeper to get best estimate of gene stability and in particular to avoid selection of co-regulated genes. In the end, we used RefFinder to combine and to validate the results for all the methods of stability.

Overall expression stability rank of genes based on RefFinder was poGAPDH > poELF1 > poADH > poActin > poUBQ > poELF2 > poTUB > po18s across developmental stages and genotypes. The results suggest that, poGAPDH was the most stable gene across developmental (Stem elongation, booting and anthesis) stages and genotypes (RIL18 and RIL55). The GAPDH gene encodes an enzyme essential for glycolysis and glycogenesis pathways, among others functions in several cellular processes (Guo et al., 2012). Earlier, GAPDH was found stable under stem elongation of flowering in plants (Jia et al., 2020). The GAPDH was the stable gene in the leaf tissue of senna (Reddy and Manivel, 2019). In this study, poELF1 gene was found to be the next in the rank after poGAPDH in the gene stability by the RefFinder. Eukaryotic Elongation factor 1α (ELF1) is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome during protein synthesis (Héricourt et al., 1999). ELF1α gene was previously identified as the most stable reference gene in flowering (Qi et al., 2016; Reddy and Manivel, 2019; Ahmad et al., 2019). The reference genes, Alcohol dehydrogenase (poADH), Actin (poActin), Ubiquitin (poUBQ) and Eukaryotic Elongation factor 2 (poELF2) are the stable reference genes after poGAPDH and poELF1. Alcohol dehydrogenase (ADH) facilitate the interconversion between alcohols and aldehydes or ketones with the reduction of nicotinamide adenine dinucleotide (NAD+) to NADH. Earlier, ADH identified as the most stable reference gene in plants (Cheng et al., 2013). Actin participates in many important cellular processes. Earlier, Actin also found stable under stem elongation of flowering in plants (Zheng et al., 2018; Ahmad et al., 2019). The main function of ubiquitin proteins is the selective degradation of short-lived protein in eukaryotic cells, performing a housekeeping role in the control in numerous cellular process (Kandasamy et al., 2001). UBQ gene was previously identified as the most stable reference gene (Qi et al., 2016, Reddy and Manivel, 2019). Eukaryotic elongation factor 1β (ELF2) is involved in translation elongation. Earlier, ELF2 was also used for normalization of gene expression (Héricourt and Jupin, 1999). Both 18S rRNA (18s) and Tubulin (TUB) have long been considered suitable reference genes plants (Li et al., 2016; Reddy and Manivel, 2019; Zhang et al., 2015). However, in the present study, 18s and TUB were identified as least stable across developmental stages and genotypes. TUB and 18s were involved basic cellular processes in plants. Earlier, Tubulin (TUB) in Tree Peony (Paeonia suffruticosa Andr.) (Qi et al., 2016, Li et al., 2016) and 18srRNA (18s) in Radish (Raphanus sativus L) (Duan et al., 2017) were reported as most unstable genes in the early and late flowering genotypes. We identified different sets of genes as the most stably expressed, reinforcing the idea that there is no universal reference gene for every situation and suggesting the use of the best gene set at each condition.

In our study, we found that the expression stability of reference genes could vary among stem elongation, booting and anthesis developmental stages. For instance, the most stable reference genes were poELF1 and poADH at stem elongation, poADH and poELF1 at booting and poTUB and poELF1 at anthesis. The difference in expression stability of candidate reference genes selected for the study may be due to differential gene regulation in developmental stages. Earlier the differences in the expression stability of reference genes in developmental stages were reported in Artemisia annua (Ahmad et al., 2019) and Linum usitatissimum (Hujs et al., 2010).

The reference genes, poELF1 and poGAPDH were most stable in RIL18 (early flowering) while the genes poADH and poActin were most stable in RIL55 (late flowering) indicating genotypic variation in the expression stability of reference genes. The genetic background of RIL18 and RIL55 genotypes and biological processes probably affected the expression stability of reference genes. Earlier, workers also reported the cultivar specific stability of reference genes (Mallona et al., 2010; Galli et al., 2015; Wang and Lu, 2016; Li et al., 2016) suggests that selection and validation of reliable reference genes for quantitative gene expression analysis was necessary not only for different species but also for different cultivars.

Phytochrome-A is a circadian control gene of flowering pathway (Somers et al., 1998). The relative expression of poPHYA-2 gene was normalized using stable (poGAPDH, poELF1, poADH, poActin, poUBQ and poELF2) and unstable (poTUB and po18s) reference genes as determined by RefFinder. The expression patterns of poPHYA-2 gene were similar, difference in the fold change values was observed when normalized with different reference genes. The expression of poPHYA-2 in the fold change was least affected by normalization using stable genes. In contrast, it was highly affected when normalized using unstable genes. These results indicate that the use of unstable reference genes might mask or introduce artificial changes in gene expression data, leading to misinterpretations of biological phenomena. Similar results have been found in RT-qPCR studies from Chionochloa pallens and Celmisia lyallii (Asteraceae) (Samarth and Jameson, 2019). Across
different growth stages and genotypes, optimal number of
reference genes determined by geNorm is presented in
Figure 3. Veazey and Golding (2011) used multiple
reference genes for normalizing functional gene expression,
while Ling and Salvaterra (2011) indicated that an
overabundance of reference genes may reduce data-
normalization robustness. From the study, it has become
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CONCLUSION

Normalization using stable reference gene is one
prerequisite for accurate quantification of the gene
expression using RT-qPCR. We identified for the first time
stable reference gene for normalization of gene expression
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Supplementary Figure 1: Specific amplification with different primers: L-(Ladder), 1-ADH (XDAPOADH), 2-α tubulin (XDAPOATUBULIN), 3-Ubiquitin (XDAPOUBIQUITIN), 4-18s RNA (XDAPO18s), 5-Actin (XDAPOACTIN), 6-GAPDH (XDAPOGAPDH), 7-ELF1 (XDAPOELF1), 8-ELF2 (XDAPOELF2).

Supplementary Figure 2: Expression profile of reference genes in Isabgol (*Plantago ovata* Forsk.). (A) po18s (XDAPO18s), (B) poUBQ (XDAPOUBIQUITIN) and flowering gene (C) poPHYA-2 (XDAPOPHYA-2) in the RIL18 (1: Stem elongation stage, 2: Booting stage and 3: anthesis) and RIL55 (4: Stem elongation stage, 5: Booting stage and 6: anthesis).