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Gel express: a novel frugal method quantifies gene relative expression in conventional RT-PCR

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Abstract

Background: Real-time PCR system is a valuable scientific mainstream needed for quantifying specific gene expression. Nevertheless, compared with conventional PCR, the real-time PCR system is extremely expensive and not affordable for limited or mid-budget research laboratories. Here, a novel, doable and low-cost recipe (referred to as gel express) is developed to quantify gene expression using conventional RT-PCR assay. The novelty of the gel express method is based on replacing crossing point (CP) values with integrated density (IntDen) values of PCR amplicon bands in real-time PCR regular mathematical formulas.

Results: In this work, gene expression profiles of two different rice stress-marker genes (OsCYP94C2a and OsLOX8) were quantified in response to mechanical wounding at different time points (0, 30, 60, and 150 min). In the gel express method, the free software ImageJ was employed to measure integrated density (IntDen) values of PCR amplicon bands in agarose gel images. IntDen values were then used instead of crossing point (CP) values according to the following modified formula: $[E^{\text{IntDen(ref)}}/E^{\text{IntDen(target)}}]_{\text{sample}} \div [E^{\text{IntDen(ref)}}/E^{\text{IntDen(target)}}]_{\text{control}}$. Gene relative expression profiles (dynamic expression pattern) quantified by gel express method in both genes were highly comparable with real-time RT-PCR. R^2 values were 0.9976 and 0.9975 in OsCYP94C2a and OsLOX, respectively. PCR amplification efficiency (*E*) for all studied genes could be calculated depending on IntDen values through experimentally designed calibration curves. PCR amplification efficiencies with all studied genes obtained by gel express were all in the accepted range. For better-visualized PCR amplicons thus detectable biological effects between treatments, the number of PCR cycles applied in gel express method (IntCyc) was experimentally estimated to be 29 cycles.

Conclusions: Gel express is a novel, cost-effective and feasible recipe for quantifying gene relative expression in conventional RT-PCR. The expression pattern quantified by gel express is highly comparable and fits the expression data revealed by the used real-time PCR system.

Keywords: Gel express, Integrated density, Crossing point, ImageJ, Conventional RT-PCR

1 Background

Quantifying gene expression is an important molecular parameter for understanding how living organisms respond to different environmental or biological stress forms. It is also required to monitor fine molecular changes during developmental growth and differentiation [1]. There are main four methods for quantifying specific gene expression: Northern blotting and in situ hybridization [2], RNase protection assays [3, 4], and the reverse transcription-polymerase chain reaction (RT-PCR) [5]. However, for transcriptomic broader scope, microarray and next-generation sequencing (RNA-seq) are the two most well-known adopted strategies [6]. The quantification of mRNA for a specific gene through conventional RT-PCR is the most popular gene expression method, particularly for low or rare expression level genes [7]. Nevertheless, the technique notoriously provides mere qualitative data and thus needs to be further quantified for more reliable conclusions [8].

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The emergence of the fluorescent-dependent PCR techniques (commonly known as real-time PCR or quantitative PCR) using SYBR green chemistry greatly enhanced the quantification of mRNA abundance of specific genes, i.e., genes with known sequences. Cleverly, real-time PCR could combine amplification and analysis steps, thus eliminating the need for post-PCR processing as agarose gel electrophoresis and subsequent imaging systems [9]. Importantly, it is required to confirm candidate gene expression to validate RNA-seq data and reduce errors of wide-range sequencing platforms [10]. Nevertheless, the real-time PCR system is extremely expensive and not accessible for a wide range of research laboratories. Most of the costs come from the real-time PCR instrument itself with a price possibly exceeding 10 times more than any conventional PCR machine. Furthermore, there are some additional essential running costs such as kits and special disposable (PCR tubes/plates with special lids) which, if not fairly provided, the system could be paused.

Four main tasks should be achieved by using real-time PCR systems: gene relative expression, DNA copy number, viral load, and allelic discrimination [11]. The scope of this work is to provide a frugal approach for molecular biology research laboratories interested in reliable gene relative expression quantitative studies but have no access to operative real-time PCR systems. Therefore, the "gel express" approach was developed to facilitate the quantification of mRNA abundance of specific genes using reverse transcription conventional PCR (RT-PCR). Compared with real-time RT-PCR, gel express showed highly comparable dynamic expression patterns. In this work, the adaptability of the new recipe (referred here as gel express) as a reliable method for quantifying gene relative expression using conventional RT-PCR was discussed.

2 Methods

2.1 Plant materials, growth and stress conditions

Seeds of Egyptian rice cultivar Giza 177 kernels (provided by Rice Research and Training Center (RRTC), Agricultural Research Center (ARC), Giza, Egypt)were manually de-husked and surface disinfected using 70% ethanol for 1 min then rinsed in 50% chlorox for 30 min with gentle shaking. The seeds were subsequently washed 5 times with sterile distilled water then air-dried under the hood on sterile filter paper for 10 min. The seeds were sowed on wet sterile cotton in sterile baby food jars and incubated at room temperature for 10 days till the second leaf emerged [12]. The stress of choice in this study was the mechanical wounding damage, which was applied using clean metal forceps on second leaves. Wounded leaves were harvested after 0 (control), 30, 60, and 150 min and immediately kept in liquid nitrogen then stored at − 80 °C for subsequent molecular analysis.

2.2 Total RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from the second leaf of control (0 min) and wounded rice plants (30, 60, and 150 min) using Direct-zol RNA Miniprep (Zymo Research, USA) according to manufacturer's instructions which included in-column DNase treatment. The concentration and purity of all RNA samples were quantified through NanoDrop® spectrophotometer (Thermo Fisher Scientific, USA) at 260 nm and 260/280 nm, respectively. The cDNA was synthesized from 2000 ng total RNA as a template using the RevertAid First-Strand cDNA synthesis kit according to manufacturer protocol (Thermo Scientific, Lithuania).

2.3 Monitoring PCR amplification steps using gel express and real-time PCR

20 ng cDNA for each sample (1 µl of 1:5 diluted cDNA) was used as the DNA template in either PCR method. For real-time PCR, HERA SYBR® Green RT-qPCR kit (Willofort, UK) was used according to manufacturer default protocol. The quantitative PCR system used in this study was QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, USA). The amplification program was as follows: 95 °C for 2 min, and 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 1 min.

In the case of gel express, conventional PCR reaction was performed using amar OnePCR master mix (GeneDirex, Taiwan) by applying manufacturer protocol. The thermal cycles program used with conventional PCR machine (T100™ Thermal Cycler, Biorad, USA) was as follows: 94 °C for 2 min, cycles# (individually adjusted, see below) of 94 °C 30 s, 60 °C for 30 s and 72 °C for 1 min, and the final extension was at 72 °C for 5 min. The experiment was designed that each conventional PCR reaction represents a certain desired number of cycles (cycles#). The investigated cycles# were 18, 20, 22, 24, 26, 28, 30, 32, and 34. Each number of cycles was independently represented by an individual PCR tube for each gene. When the PCR machine informed the end of each investigated number of cycles, the lid of the PCR machine was manually opened and the relevant tube was transferred from the PCR machine heated block into a pre-chilled metal rack for at least 10 min to stop the reaction. The investigated genes in either PCR system are OsCYP94C2a (jasmonates turnover), OsLOX8 that encodes lipoxygenase 8 (jasmonates biosynthesis), and rice β-actin as reference gene. The sequences of forward and reverse primers are listed in Table 1.

Table 1 The sequences of forward and reverse primers for investigated genes

Gene Name	Gene Function	Accession number (MSU)	Forward (5′ to 3′ prime)	Reverse (5′ to 3′ prime)
OsCYP94C2a	Jasmonates turnover	LOC_Os11g05380	GAAGACTTCACTTCTACTGCAACA	ACGAGGAAACCATGACGAAC
OsLOX8 (Jasmonates biosynthesis)	Lipoxygenase	LOC_Os08g39850	CGTTCATGTCATCATGTGTGT	GCACCATTCAATGTAAATGCTATC
Actin	Actin (ref)	LOC_Os10g36650	ATGCCATTCTTCTCCGTCTT	GCTCCTGCTCGTAGTC

The PCR product of each sample was electrophoresed through 1.5% agarose gel stained with a standard concentration of Ethidium Bromide (1 µg/ml) and visualized by UV transilluminator. The agarose gel was then photographed by the Gel Documentation system (Biorad, USA) and the raw agarose gel images (blackish background/whitish bands) were saved for subsequent image analysis steps. Gel express protocol was applied to measure the integrated density (IntDen) value of each PCR amplicon using ImageJ software but with raw agarose gel images. Subsequently, IntDen values were plotted against each cycle number for estimating IntCyc or integrated cycle. IntCyc value refers here to the number of cycles recommended as an end-point cycle in the gel express method. It is assumed that IntCyc value is preferred to represent the middle of the exponential phase in the PCR amplification steps plot.

2.4 Determining conventional and real-time PCR amplification efficiencies (*E*)

The cDNA amount in every PCR reaction was 2 µl representing the different quantities of cDNA serially diluted in 1:2 ratio with total starting cDNA quantity of 200, 100, 50, 25, 12.5, 6.125, and 3.0625 ng per PCR reaction. Real-time and conventional PCR master mixes were prepared as previously mentioned. In gel express, PCR reaction was programmed to end after 25 cycles in OsCYP94C2a, and 33 cycles in OsLOX8. The reference gene actin was independently examined with each target gene according to the number of cycles. The PCR product of each sample was electrophoresed through 1.5% agarose gel stained with a standard concentration of Ethidium Bromide (1 µg/ml) and visualized by UV transilluminator. The agarose gel was then photographed by the Gel Documentation system (Biorad, USA), and the color of the obtained raw agarose gel images was inverted from blackish background/whitish band to be whitish background/blackish bands. The IntDen value of each PCR amplicon band was determined using ImageJ (see detailed protocol below) then plotted against the $\log_2$ of each corresponding starting cDNA quantity. In real-time PCR, each crossing point (CP) value was plotted against the corresponding $\log_2$ starting cDNA quantity. PCR amplification efficiency (E) was calculated according to [13]: $E = d^{[-1/\text{slope}]}$, where d is the dilution factor of cDNA quantities (here equals 2), so the formula is $E = 2^{[-1/\text{slope}]}$. Efficiency percentage $= (E - 1)$ *100.

2.5 Relative gene expression pattern quantification by gel express and real-time PCR

Conventional and real-time PCR methods were performed using 20 ng cDNA for each sample as the DNA template. The setting up of both real-time and conventional PCR reactions was mentioned earlier in this chapter. The thermal cycler program for conventional PCR machine was as follows: 94 °C for 2 min, 29 cycles (IntCyc) of 94 °C 30 s, 60 °C for 30 s and 72 °C for 1 min, and the final extension was at 72 °C for 5 min. PCR reaction was stopped by holding the tubes at 4 °C for at least 10 min. The PCR product of each sample was electrophoresed through 1.5% agarose gel stained with a standard concentration of Ethidium Bromide (1 µg/ml) and visualized by UV transilluminator. The agarose gel was then photographed by the Gel Documentation system (Biorad, USA). Raw agarose gel images color pattern (blackish background/whitish bands) was directly inverted using Gel Doc system software to be whitish background/blackish bands, then saved for subsequent image processing work.

2.6 Measuring integrated density (IntDen) values in inverted agarose gel images

Using previously saved color-inverted agarose gel images, integrated density (IntDen) values were measured using ImageJ software as follows: 1) Converting image to grayscale: Image ► Type ► 8-bit. 2) Image scale: Straight line ► Mark the width of one agarose well ► Analyze ► Set Scale ► input the known length and unit (0.5 cm in this study). 3) Measuring Integrated Density (IntDen) values: Rectangle ► mark the desired band area ► Press "t" to add it to ROI ► click measure after adding all marked samples. Save the raw data table as CSV file and then re-save it as excel. It is important to make sure that the rectangle area is the same with all measured band samples. The measurements should

be taken for target and reference genes that should be loaded together within the same agarose gel. During documentation of the agarose gel for taking images, imaging conditions should be the same for all agarose gels, i.e., zoom degree, light, contrast, exposure, etc.

2.7 Calculating relative gene expression using gel express method and real-time PCR

Gene relative expression was quantified by real-time PCR according to a mathematical model developed by [13] as follows:

$$\left[E^{\mathrm{CP(ref)}}/E^{\mathrm{CP(target)}}\right]_{\mathrm{sample}} \div \left[E^{\mathrm{CP(ref)}}/E^{\mathrm{CP(target)}}\right]_{\mathrm{control}}$$

In gel express method, integrated densities (IntDen) replaced CP values in previous formula with IntDen values according to the following formula:

$$\left[E^{\mathrm{IntDen(ref)}}/E^{\mathrm{IntDen(target)}}\right]_{\mathrm{sample}} \div \left[E^{\mathrm{IntDen(ref)}}/E^{\mathrm{IntDen(target)}}\right]_{\mathrm{control}}$$

3 Results

3.1 Gel express monitors PCR amplification steps and estimates integrated cycle (IntCyc)

The integrated density of each PCR amplicon band visualized in raw agarose gel images (black background/white bands, Fig. 1b) was measured using ImageJ and then plotted against each corresponding cycle (Fig. 1a). The PCR amplicon band was initially detectable to eyes after 22 cycles in actin, 24 cycles in OsCYP94C2a, and 28 cycles in OsLOX8 (Fig. 1b). The integrated cycle (IntCyc) is estimated as 29 cycles (dashed red line, Fig. 1a); it should be applied for all investigated genes (target and reference). The term integrated cycle (IntCyc) in this study refers to the number of cycles that is recommended for the gel express method as an end-point PCR-dependent method. It is assumed to be at the middle of the exponential phase as shown by the red dashed line in Fig. 1a. The amplification plot of the three studied genes was revealed by QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, USA) and presented in Fig. 1c. The amplification plot of gel express

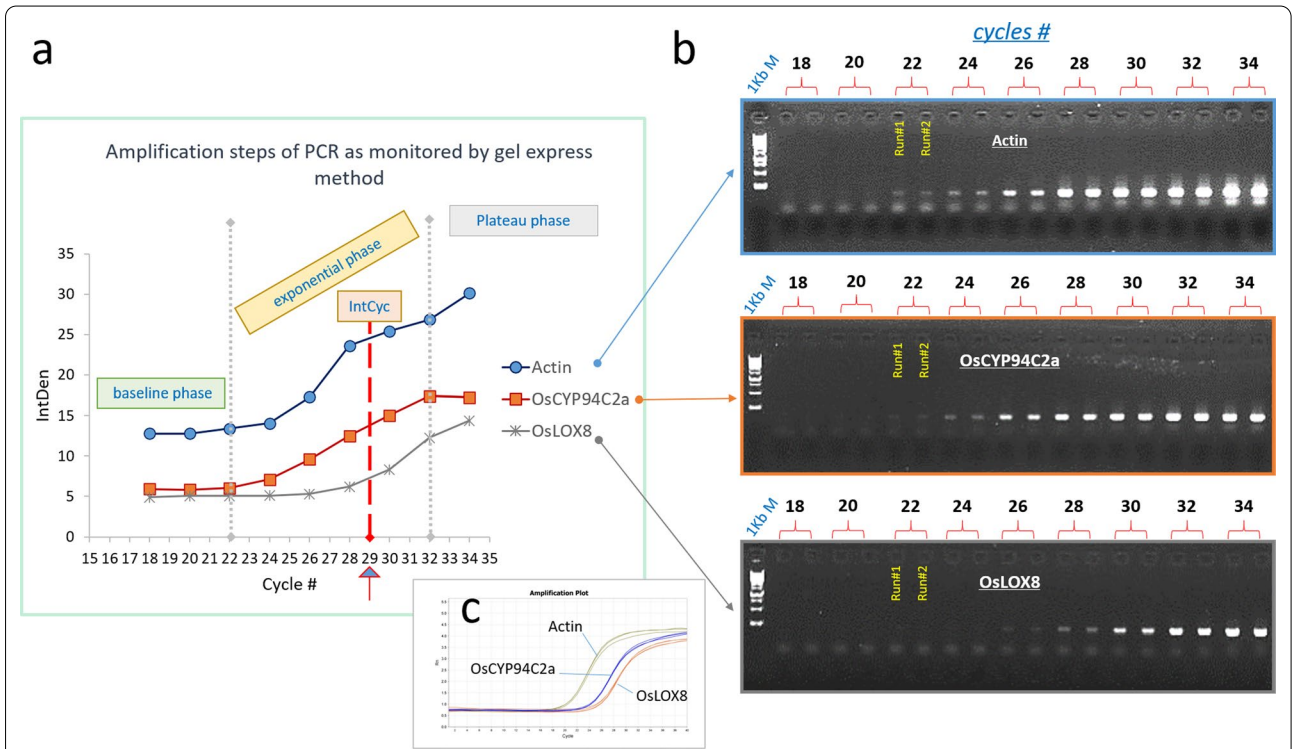

Fig. 1 Monitoring amplification steps of conventional PCR using gel express method and estimating integrated cycle (IntCyc) value for OsCYP94C2a, OsLOX8, and the reference gene actin. **a** plotting IntDen values of each PCR amplicon band against corresponding number of cycles. Integrated cycle (IntCyc) was assumed to be slightly beyond the middle of the exponential phase which is estimated to be 29 cycles. **b** Visualizing PCR amplicon bands for each studied gene where 5 ul aliquots were taken at 18, 20, 22, 24, 26, 28, 30, 32, and 34 cycles. **c** Amplification plot of the studied gene as directly viewed by QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, USA). Means represented in **a** were from at least 2 independent technical replicates presented as Run#1 and Run#2 in (**b**)

and real-time PCR were in accordance; both show actin as the most expressed gene followed by OsCYP94C2a then OsLOX8 (Fig. 1a, c).

3.2 Gel express could determine PCR amplification efficiency (*E*)

The PCR amplification efficiency (*E*) with each investigated gene was determined by both gel express method and real-time PCR (Fig. 2). The *E* values were calculated based on the equation: $E = d^{[-1/\text{slope}]}$, where d is the used dilution factor of cDNA amounts (here $d = 2$), and slope represents the coefficient of PCR efficiency plot for each used primer. IntDen and CP values were separately plotted against the logarithmic (base 2) values of the cDNA quantities. The real-time PCR efficiency plot revealed that *E* values were 2.3, 2.14, and 2.2 for actin, OsCYP94C2a, and OsLOX8, respectively (Fig. 2c). PCR amplification efficiency with the reference gene actin was independently calculated with each target gene under the applied number of cycles. PCR efficiency (*E*) in OsCYP94C2a and actin genes after 25 cycles were 1.73 and 1.89, respectively (Fig. 2a). OsLOX8 expression level was much lower than OsCYP94C2a after 25 cycles (data not shown). Therefore, *E* values for OsLOX8 and actin gene were calculated after 33 cycles and found to be1.7 and 2.17, respectively (Fig. 2b). The PCR amplicon bands processed by gel express method for producing corresponding calibration curves are presented in Fig. 2d.

3.3 Gene dynamic expression displayed by gel express is highly comparable to real-time PCR

The expression profiles of OsCYP94C2a and OsLOX8 were quantified in response to wounding stress after 0, 30, 60, and 150 min (Figs. 3 and 4). Real-time PCR and gel express methods showed that OsCYP94C2a was strongly upregulated in response to wounding stress

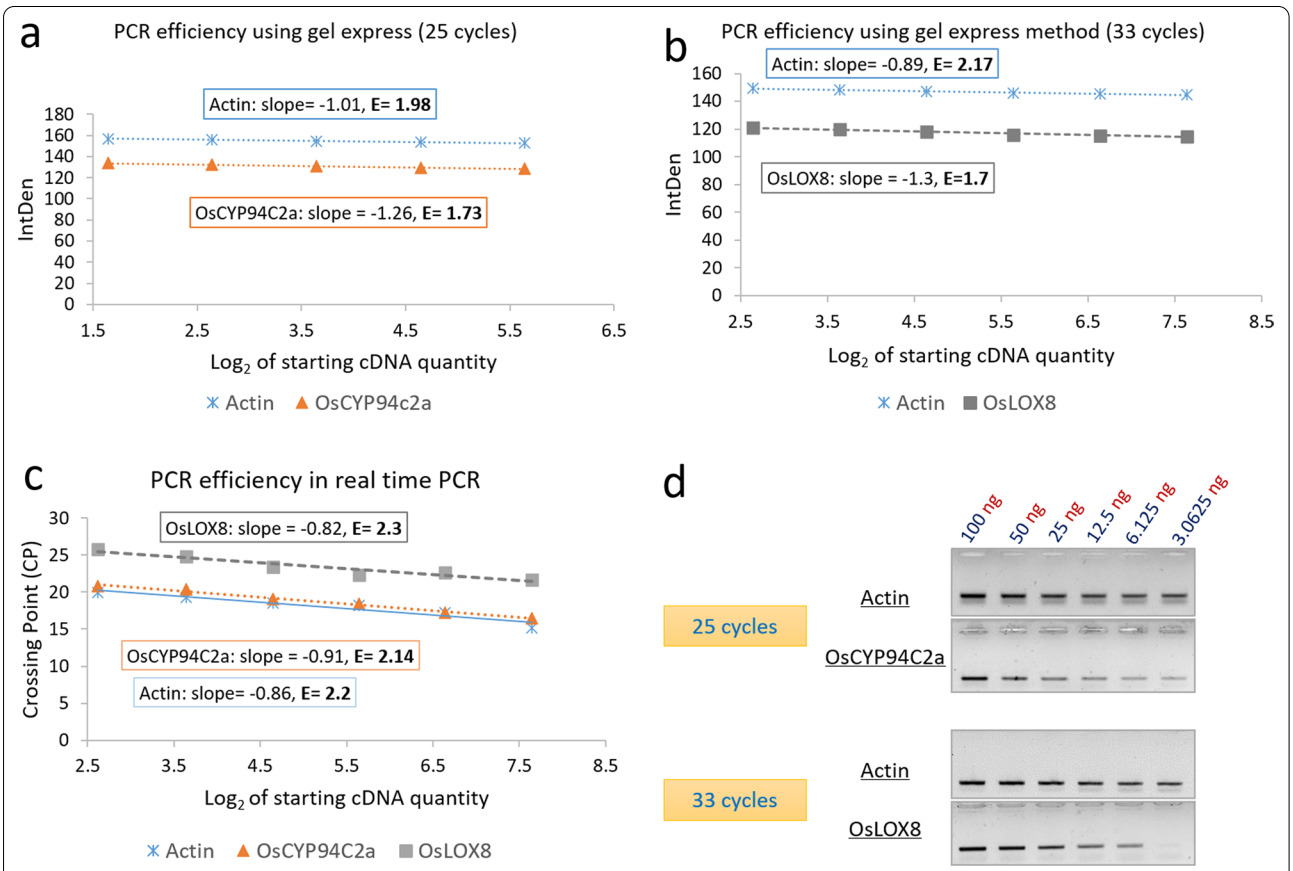

Fig. 2 PCR amplification efficiency determination using gel express and real-time PCR methods. **a** Integrated densities (IntDen) values were plotted against the $\log_2$ values of initial cDNA quantities for the target gene OsCYP94c2a and reference gene actin after 25 cycles of amplification. **b** Integrated densities (IntDen) values were plotted against the $\log_2$ values of initial cDNA quantities for the target gene OsLOX8 and its reference gene actin after 33 cycles of amplification. **c** crossing points (CP) values were plotted against $\log_2$ values of initial cDNA quantities for target genes OsCYP94C2a and OsLOX8, and the reference gene actin. For **a**, **b**, and **c**, slopes were determined then used to calculate PCR efficiency *E* values according to the formula $E = 2^{[-1/\text{slope}]}$ [13]. Means in **a**, **b** and **c** represented at least 3 independent technical replicates. **d** Representative agarose gel electrophoresis (image with inverted color) used to plot (**a**) and (**b**)

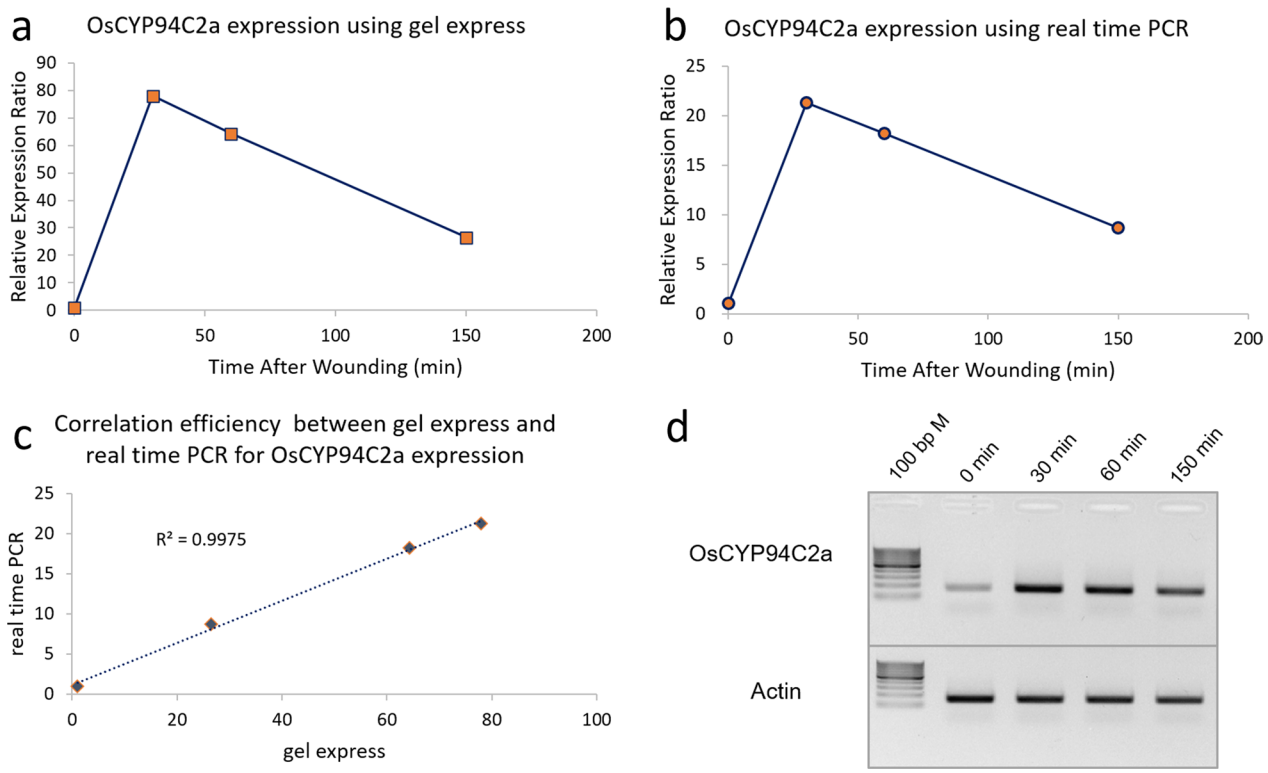

Fig. 3 Dynamic gene expression profile for the target gene OsCYP94C2a using gel express (**a**) and real-time PCR method (**b**) at four different time point after wounding: 0 (control), 30, 60 and 150 min. The expression pattern quantified by gel express method was associated with real-time PCR through regression analysis (**c**), R^2 equals to 0.9975. **d** Agarose gel electrophoresis (inverted color images) showing the migration of PCR product of OsCYP94C2a and actin fragments after 29 cycles of amplification used to plot (**a**)

after 30 min and then the expression ratio was gradually attenuated after 60 and 150 min from wounding (Fig. 3a, b). The calculated relative ratio of expression was 21.25, 18.23, and 8.66 in the real-time PCR method compared to 78, 64.3, and 26.5 with gel express. Although the values of relative gene expression were not the same in gel express compared to the real-time PCR method at each time point, the correlation between the two methods was high as indicated by R^2 that equals 0.9975 (Fig. 3c). Figure 3d illustrates the visualized OsCYP94c2a PCR amplicon bands in a representative agarose gel image with inverted colors (similar to a photographic negative) used by gel express method for gene expression quantification.

The expression level of OsLOX8 was also investigated in rice seedlings in response to wounding damage (Fig. 4). Unlike OsCYP94C2a that was expressed in a fluctuating style, OsLOX8 was expressed in an accumulative pattern, i.e., becomes higher over time following the wounding event. The expression ratios in real-time PCR were 4.45, 10.31, and 24.97 after 30, 60, and 150 min, respectively (Fig. 4b), while expression ratios produced by the gel express method were 2.13, 5.4, and 13.1 (Fig. 4a). Dynamic pattern expression of OsLOX8 gene was constant in both PCR methods where $R^2 = 0.9976$ (Fig. 4c). A representative agarose gel image for OsLOX8 PCR-specific amplicon bands that have been analyzed by gel express for determining relative gene expression is presented in Fig. 4d. The protocol of gel express method by describing photographed steps for measuring integrated density (IntDen) of each PCR amplicon band is illustrated in Fig. 5.

4 Discussion

In this work, gel express recipe was developed to quantify the relative expression ratios of specific genes using conventional RT-PCR, the most cost-effective universal system for amplifying even low amounts of mRNA [14]. Several methods were already developed to analyze agarose gel images for variable purposes as comparative molecular weight detection and drawing phylogenetic trees [15, 16]. Intensities of PCR amplicon bands visualized in raw agarose gel images (blackish background/whitish bands) were directly used to represent mere direct ratios of intensities [17, 18]. For doing so, intensities of target gene bands in raw agarose gel images were divided by reference gene bands intensities to calculate

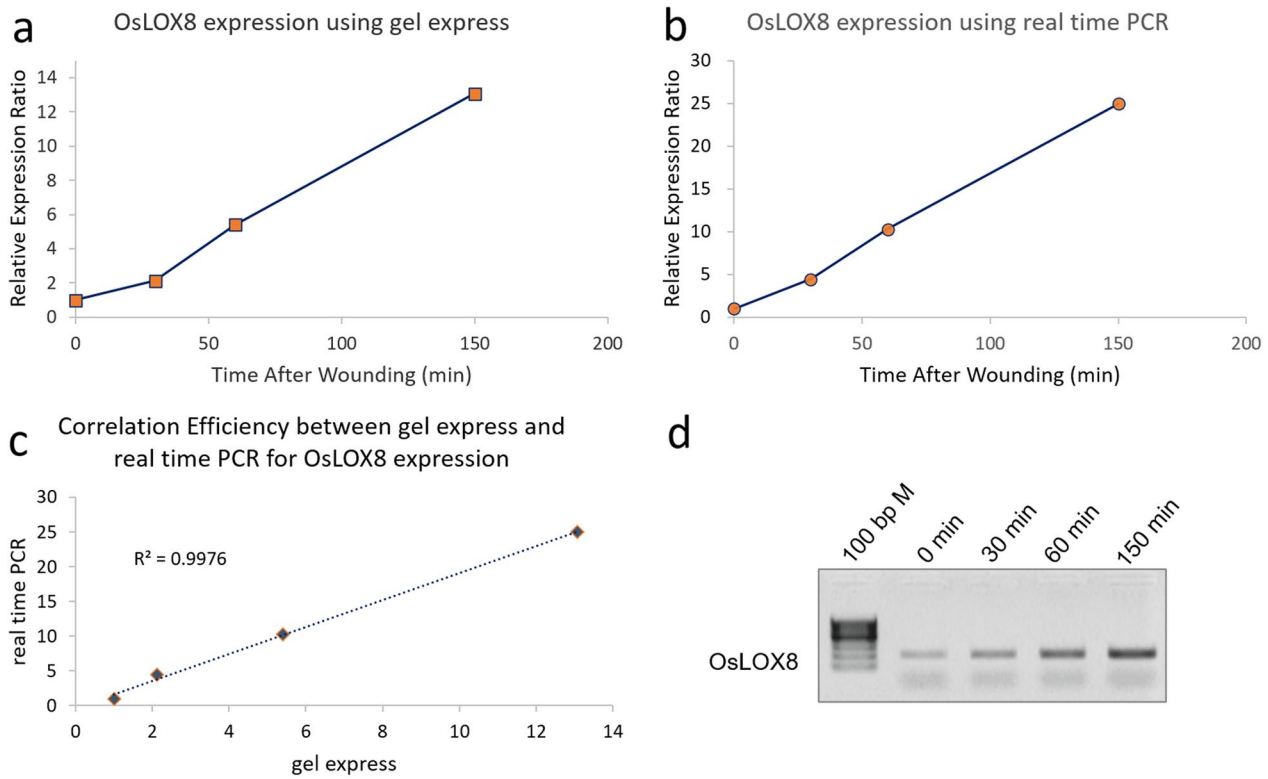

Fig. 4 Dynamic gene expression profile for the target gene OsLOX8 using gel express (**a**) and real-time method (**b**) at four different time point after wounding: 0 (control), 30, 60 and 150 min. The expression pattern quantified by gel express method was associated with time PCR output data through regression analysis (**c**), R^2 equal to 0.9976. **d** Agarose gel electrophoresis (inverted color images) showing the migration of RT-PCR product of actin and OsLOX8 fragments after 29 cycles of amplification

the semi-quantitative relative transcripts abundance [19, 20]. Gel express, on the other hand, is a quantification tool encompassing analyzing agarose gel images to determine PCR amplicon bands integrated density (IntDen). Subsequently, IntDen values are used directly instead of crossing point (CP) determinant in real-time PCR equations for quantifying gene relative expression as follows:

$$\left[E^{\mathrm{IntDen(ref)}}/E^{\mathrm{IntDen(target)}}\right]_{\mathrm{sample}} \div \left[E^{\mathrm{IntDen(ref)}}/E^{\mathrm{IntDen(target)}}\right]_{\mathrm{control}}$$

Integrated density (IntDen) is the sum of the values of the pixels in the whole image or selected part of it. When an agarose gel inverted-color image is converted to grayscale by 8-bite option using ImageJ software, this creates 256 values: 0 for pure black, 256 for pure white, and 254 Gray values steps in between (https://imagej.nih.gov/ij/docs/menus/analyze.html). Indeed, in inverted agarose gel images, IntDen of highly expressed OsCYP94C2a sample at 30 min after wounding was 69.3 arbitrary units (AU) while it was 76.4 AU with the low expressed control not-wounded sample (Fig. 5). Provided identical rectangle area for all selected bands using ImageJ (Fig. 5d), the higher expressed sample (69.3 AU) with more blackish gray pixels produced lower IntDen values. On the other side, the lower expressed control sample (76.4 AU) possessed more whitish-gray pixels thus higher IntDen values. This kind of variation in IntDen values between high and low gene expression samples is similar to CP values pattern in a real-time PCR system. It is well known that genes with upregulated expression levels (more abundant mRNA) have lower CP values compared to low-expressed samples that have higher CP values [13]. Therefore, the IntDen value is assumed to be a reliable substitute for the CP value in real-time PCR mathematical models. Gel express method facilitates quantifying gene relative expression using real-time PCR mathematical model yet with determinants derived from conventional PCR assay, i.e., IntDen values.

The mathematical model developed by [13] was adopted in this study for calculating the relative expression ratio. Equally important, $2^{-\Delta\Delta Ct}$ method is also a widely accepted mathematical strategy for analyzing quantitative gene expression data [21]. Nevertheless, it relied on the hypothetical assumption that the

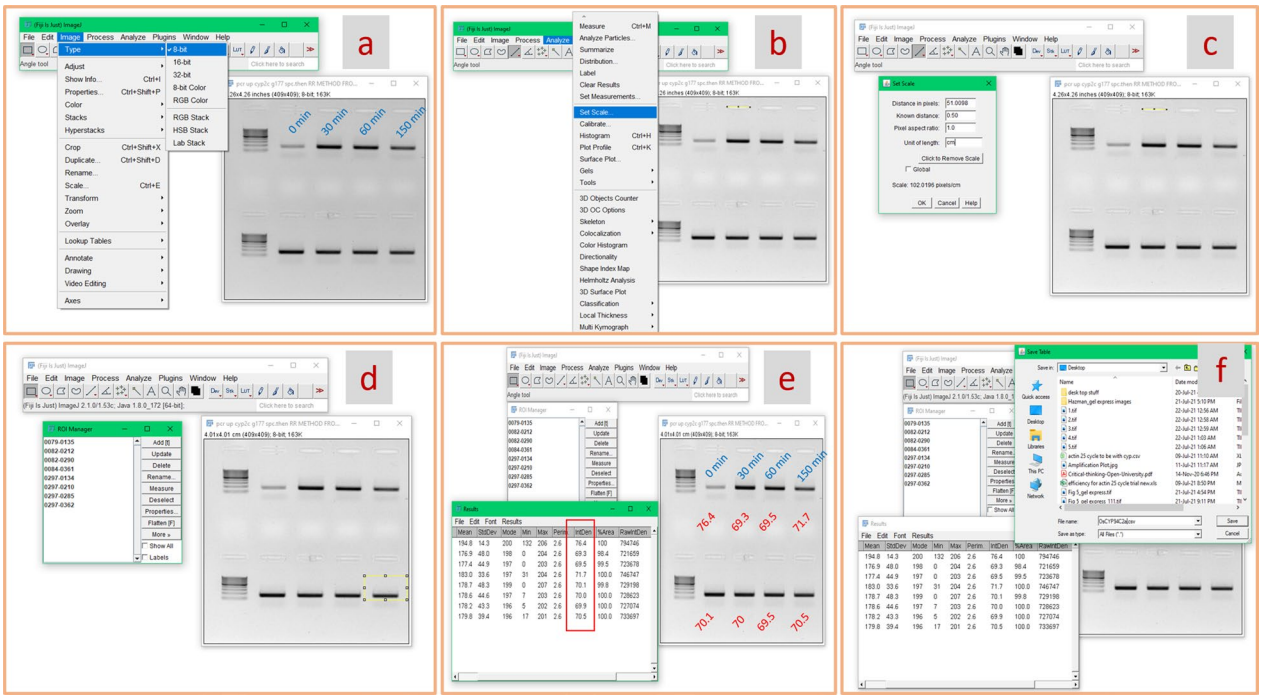

Fig. 5 Photographed-steps protocol of gel express method for attaining integrated densities (IntDen) values of PCR amplicon bands from agarose gel inverted-color image. The presented example illustrates quantifying OsCYP94C2a expression in response to mechanical wounding stress after 0, 30, 60 and 150 min in rice seedlings. The software used was the freeware ImageJ. **a** opening image by ImageJ software and changing its type to 8-bite grayscale, **b** and **c** illustrating scaling image depending on agarose gel well width (in this study it was 0.5 cm), **d** selecting the area around the band of interest, preferably as shown in the example, then press "t" to open ORI manager, **e** after selecting all bands then click "measure" button in ROI manager, and **f** illustrated saving raw data as CSV file

efficiency of a real-time PCR reaction E is 100% ($E=2$) for all examined samples and tested genes [22]. In this work, however, E values for OsCYP94C2a, OsLOX8, and actin genes were experimentally calculated by setting up suitable standard curves using the formulas $E=2^{[-1/\text{slope}]}$ and Efficiency percentage (%) $=(E-1)$ * 100. PCR amplification efficiency that is calculated with gel express method for all genes was in the accepted range ($\approx$ 60 to 110%) [23]. In OsCYP94C2a genes with a 25 cycle PCR program, E values were 1.73 (98% and 73%). While in the case of OsLOX8 genes with a 33 cycle PCR program, E values were 1.7 (117% and 70%). Interestingly, although the number of cycles was different, E values for conventional PCR reactions with both target genes were almost the same (1.73 and 1.7). Empirical data supported that most accepted E values should not be lower than 1.65 otherwise PCR reaction would be considered as failed [24]. The higher PCR efficiency (>100% or $E>2$) is an undesired parameter also; it is probably due to the presence of PCR inhibitors in highly concentrated samples [25].

OsCYP94c2a and OsLOX8 target genes represent two different responsive expression styles, fluctuating and accumulating, respectively. This allowed a satisfactory evaluation for gel express method under two different styles of gene expression patterns compared to real-time PCR assay. For actualizing gel express results, gene expression patterns were compared to real-time PCR at four different time points (0, 30, 60, and 150 min after wounding). OsCYP94c2a expression profile was previously studied in rice and found to fluctuate in response to wounding [26]. In case of OsCYP94C2a, regression analysis confirmed that gel express method is reliable and considered highly comparable to real-time PCR ($R^2=0.9975$, Fig. 3C). OsLOX8 (homolog to LOX2 in *Arabidopsis*) encodes the chloroplast precursor (EC 1.13.11.12) which is a member of the 13-lipoxygenases gene family in rice with herbivore-induced defense response [27, 28]. OsLOX8 expression pattern was linearly accumulative, i.e., the level of mRNA abundance is getting higher over time (Fig. 4b, d). Similar to OsCYP94C2a, OsLOX8 expression pattern calculated by gel express was highly associated with real-time PCR (R^2 equals 0.9976).

Obviously, the absolute numerical values of relative expression ratios for each time point were not identical in

both investigated PCR methods and in either target gene. Nevertheless, the high R^2 values in regression analysis (Figs. 3c and 4c) are referring to the high level of association between gel express and real-time PCR assay. Unsurprisingly, absolute values derived from the gel express method were different from the values revealed by used real-time PCR system. [29] reported that gene expression values can significantly vary even with different real-time PCR systems. Furthermore, some readily detected genes using a real-time PCR system were completely undetected using another different one. Clearly, both gel express and real-time PCR strategies adopted different detection parameters and thus produced different absolute values yet both provided highly comparable dynamic expression patterns. For accuracy, gel express method could detect an amplitude level of nearly two in OsLOX8 gene expression at 30 min after wounding (Fig. 4b). This implies a considerable sensitivity for integrated density as a reliable measuring parameter for the quantification of gene expression.

Optionally, integrated density (IntDen) could be also utilized with raw gel images (blackish background/whitish bands) to estimate integrated cycle (IntCyc). In this study, IntCyc refers to the number of cycles for gel express method (29 cycles, Fig. 1a). IntCyc improves the visualization differences between PCR amplicon bands under different treatments, thus better biological comparison. Therefore, it is recommended to consider 29 cycles as the initial default number of cycle in gel express method with target and reference genes. The relative expression ratio could be calculated easier with no need to prepare time-consuming and technically complicated efficiency (E) calibration curves in case E is hypothetically assumed to be 2 ($E=2$). This could be attained by either using gel express IntDen values in replace of Ct (cycle threshold) in delta-delta Ct formula developed by [21] using the following equation: $2^{\Delta\Delta \mathrm{IntDen}}$ or in the equation originally developed by [13]:

$$\left[2^{\mathrm{IntDen(ref)}}/2^{\mathrm{IntDen(target)}}\right]_{\mathrm{sample}} \div \left[2^{\mathrm{IntDen(ref)}}/2^{\mathrm{IntDen(target)}}\right]_{\mathrm{control}}.$$

5 Conclusions

The extremely high cost of the real-time PCR system, qPCR instrument in particular, is and will remain the most challenging factor that hinders analyzing quantitative gene expression data in limited budget research institutes. Therefore, the presented work addressed this problem by suggesting a novel, cost-effective and reliable method, named here gel express, to quantify gene relative expression in conventional PCR system yet using real-time PCR mathematical models. Gel express method encompasses two main novel steps: (1) measuring integrated density (IntDen) values of PCR amplicon bands in agarose gel images using the free software ImageJ (see Fig. 5 for detailed photographed-steps protocol) and (2) using IntDen value for each PCR band as an alternative to crossing point (CP) in equation developed by [13] to calculate gene relative expression ratios. Compared to data derived from the well-acknowledged real-time RT-PCR, gel express is thought to be a doable, fast and cost-effective method for achieving high-quality quantitative gene relative expression measurements using conventional RT-PCR.

Abbreviations

cDNA: Complementary DNA; CP: Crossing point; *E*: PCR amplification efficiency; IntCyc: Integrated cycle; IntDen: Integrated density; OsCYP94C2a: Cytochrome P450 of the subfamily CYP94 subclade C member 2a; OsLOX8: Lipoxygenase8; RT-PCR: Reverse transcription polymerase chain reaction.

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Authors' contributions

MH is the only author of this work. MH designed and conducted the experiments, performed the measurements, analyzed the data and wrote the manuscript. The author read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare there is no competing interests.

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