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A quick protocol to facilitate the selection of putative delayed ripening transgenic papaya lines for field evaluation

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Abstract

A quick protocol is needed to determine transgene, *ACC oxidase* 2 expression in putative transgenic Eksotika papaya lines targeting at selecting potential lines for field evaluation. In this study, three housekeeping genes, 18S ribosomal RNA, 40S ribosomal protein and actin, were used for normalisation of the *ACC oxidase* 2 gene expression. Comparison with a non-transformed seedling-derived plants revealed that 42 putative transgenic lines exhibited between 2- and 5-fold reduction of *ACC oxidase* 2 expression level. Based on the gene expression data of the in vitro putative transgenic papaya plants obtained, 24 independent potential lines were selected for field evaluation. Gene expression analysis on field grown papaya plants showed similar profile of *ACC oxidase* 2 expression levels as compared to in vitro papaya plants. Increase in storage shelf life was also examined in the transgenic lines grown in the field with the most potential transgenic line was 27-3, which required 14 days to achieve full yellow colour index. Overall, the findings in this study revealed that reduction of ethylene was successful in the Eksotika papaya by manipulating the *ACC oxidase* 2 using the antisense technique. This reflects that future production of longer shelf life Eksotika papaya fruits is possible through antisense technique and it will help boost the papaya industry further by opening up new export markets in distant destinations.

Keywords: antisense, transgenic, real-time PCR, housekeeping gene, gene expression

Introduction

Genetic engineering has become an important tool for improvement of crop quality. Although the process of generating transgenic papaya plant appears simple and straightforward, but the interpretation of the results could be complicated due to the large number of putative transgenic plants that are needed to be screened. Previously, the transgene expression in transgenic plant were determined using Northern analysis. Polymerase chain reaction (PCR) results can determine the presence of transgene, but is not sufficient enough to determine the potential line that should be targeted for field evaluation. Thus, the selection of PCR positive transgenic plants that are suitable for further evaluation needs

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careful consideration. Currently, with the advancement of technology, the expression profile of transgene in PCR positive plants can be estimated using real-time PCR, and the gene expression results can be used to assist in the selection of the transgenic lines. Real-time PCR is a popular high-throughput technique for gene expression analysis in plants. The most commonly used technique for quantitative analysis of gene expression is relative quantification, in which the target concentration in each sample is calculated relative to the housekeeping gene, and the transcript expression level is determined as a target/reference ratio. The main purpose of using a housekeeping gene is for normalisation of the non-biological variation in the amount of input material. The most commonly used housekeeping genes in real-time PCR include ribosomal small subunit (18S) ribosomal RNA, beta-actin (*ACTB*), glyceraldeyde-3-phosphate dehydrogenase (*GAPDH*) and beta glucuronidase (*GUS*) (Radonic et al. 2004).

Normalisation using only a single housekeeping gene can produce biased results, and therefore, in this study normalisation of three housekeeping genes was used and the normalisation was calculated based on the geometric mean of their expression levels (Vandesompele et al. 2002; Pfaffl et al. 2004). For gene expression analysis, two-step PCR protocol and DNA binding SYBR Green I were used. Vandesompele (2002) reported that using SYBR Green with two steps PCR would ease the elimination of primer-dimers through manipulation of the melting temperature. However, this two-step protocol has the disadvantage of possible DNA contamination during the real-time PCR process (Marisa et al. 2005).

In this study, the embryogenic Eksotika papaya calli were transformed with 1-aminocyclopropane-1-carboxylic oxidase 2 (*ACO*2) gene. This gene is involved in fruit ripening in Eksotika (Sew et al. 2011). Ripening is an important process in climacteric fruit like papaya, but natural ripening in fruit can cost large postharvest losses. In papaya fruit ripening, one way to increase the shelf life is by reducing the activities of enzymes involved in the ripening process. Therefore in this study, *ACO*2 gene was transformed into Eksotika papaya with the final aim to prolong the shelf life of the papaya fruit. The study reported here is the results of gene expression profile of transgenic papaya lines produced from transformation using *ACO*2 gene compared to non-transformed seedling-derived control plant. The results obtained would facilitate the selection of the potential transgenic papaya lines that should proceed to further field evaluation. Further field trial analysis of these positive transgenic lines is important to confirm the expression of the trait characteristic required.

Materials and methods

Embryogenic callus of Eksotika papaya (*Carica papaya* L.) cultures were initiated from immature zygotic embryos obtained from Malaysian Eksotika papaya fruit of 90 days after pollination. After harvesting, the fruits were washed with tap water and air-dried for 5 min before storing at 4 °C until subsequently used in the isolation of zygotic embryo. The immature embryos were cultured on solid callus induction medium. This induction medium consisted of half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962), 50 mg/litre myo-inositol, full strength MS vitamin (thiamine-HCL, phyridoxine, glycine and nicotinic acid), 60 g sucrose, 45.2 µm 2,4-D, 0.14 g/litre adenine hemisulfate, 400 mg/litre glutamine and 4 g phytagel. The pH of the medium was adjusted to 5.8 prior autoclaving for 15 min at 121 °C and 15 psi. All cultures were incubated at 25 ± 2 °C in the dark. After 4 weeks, the cotyledons were removed and the callus was used for *Agrobacterium tumefaciens* transformation.

The full length of *ACC Oxidase* 2 gene (1441 bp) isolated from Eksotika papaya was inserted in an antisense orientation

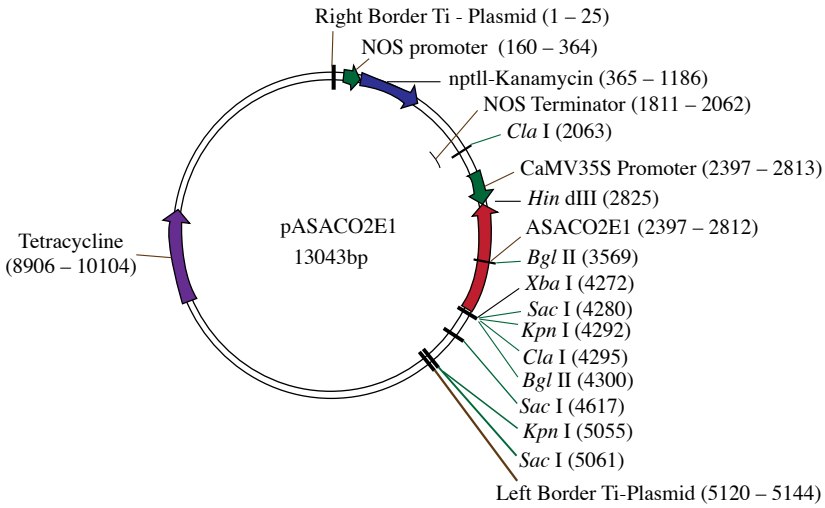

Figure 1. Schematic diagram of pASACO2E1 gene construct with full length of antisense ACO2. The sequence base pair length for ACO2 gene (1441 bp) as indicated in red. RB:T-DNA right border, LB:T-DNA left border, 35S:cauliflower mosaic virus 35S RNA promoter, nptII:kanamycin resistance gene, nos promoter: nopaline synthase promoter, nos terminator: nopaline synthase terminator

in plant transformation vector, pGA643 plasmid that is driven by *CaMV35S* promoter, harbours an *npt*II selectable marker gene, and a *nos* terminator. This antisense gene cassette was designated as pASACO2E1 (*Figure 1*).

One-month-old embryogenic cultures of Eksotika papaya were transformed with the pASACO2E1 gene construct using a previously established *Agrobacterium*-mediated transformation method for Eksotika (Vilasini et al. 2000). Selection of the transformed calli was carried out on half-strength Murashigee and Skoog (1962) medium supplemented with kanamycin. The selection process was carried out for a total of 4 months. The first selection was carried out with 75 mg/litre kanamycin for 1 month, followed by 150 mg/litre kanamycin for the remaining 3 months.

The resistant calli that proliferated from the selection media were transferred to De Fossard (De Fossard et al. 1974) maturation medium (De Fossard medium without plant growth regulator) for 1 month. Proliferating calli with green shoot were cultured on De Fossard regeneration medium supplemented with 0.89 µm 6-benzyladenine (BA), 1.1 µm 1-napthaleneacetic acids (NAA) and 150 ml coconut water for shoot regeneration. The putative transgenic plantlets with approximately 4 cm in height were cultured on half-strength liquid MS medium supplemented with vermiculite at the ratio of 1:1 for rooting. The good rooted plantlets were transferred into the soil mixture (consisted of soil:sand:vermiculite with a ratio 1:1:1) for acclimatisation. Regenerated transgenic papaya plants were first hardened and acclimatised in contained environment, transgenic glasshouse before planting under nethouse condition. A total of 24 independent antisense transgenic plants were grown in soil under insect proof netting enclosure. The planting distance of the plants was 2.2 m between rows and 2 m within row.

The putative transgenic plants were further analysed using PCR to examine the presence of transgene(s) in the plant genome. Genomic DNA was extracted from papaya leaves using the Qiagen kit (Qiagen, Hilden, Germany) with 100 mg starting material for each sample. Approximately

Table 1. List of primers used for analysis of antisense transgenic papaya plants

Name	Sequence (5'-3')	Length (bp)	Amplicon size (bp)
*npt*IIF	CCTTATCCGCAACTTCTTTACC	22	610
*npt*IIR	CACCATGATATTCGGCAAGCAG	22	
*ACO*2F	ACTGACGTAAGGGATGA	17	472
*ACO*2R	TACATTGCCGTAGATGA	17	

Table 2. List of primer pairs used for real-time PCR

Name	Sequence (5'-3')	Length (bp)	Amplicon size (bp)
*ACO*2F	GCTGGGTTTTACTCTTTTATGTG	23	140
*ACO*2R	ACTTCCAAACACCATGATTAGGG	23	
ActinF	TTCCACTATGTTCCCTGGTATT	22	119
ActinR	TCCTATCCAGACGCTGTATTTC	22	
18sRF	TTGTTTGATGGTATTTGCTACTCGG	25	136
18sRR	TGAATCATCAGAGCAACGGGCAGAG	25	
40sRPF	TGGCAAAGCCTACAAAGACTATCA	24	77
40sRPR	AGGAATGGGAAGGGAGGGAGAT	22	
CyCloF	CAAGCAGTTTGTTCTCCCTCAC	22	81
CyCloR	TGGACTTCTTTGCCTGGATTAG	21	

50 ng of each extracted genomic DNA was used for PCR analysis. PCR analyses were carried out using the primer pair flanking the 35S promoter and the *ACO*2 gene, and also a primer pair for the *npt*II gene. The list of primers used is as shown in *Table 1*. The following thermal cycling conditions were used: 2 min at 94 °C; 35 cycles of 30 sec at 94 °C, 45 sec at 62 °C and 1 min 30 sec at 72 °C; and finally at 72 °C for 10 min. A volume 20µl of each PCR amplified products were subjected to 1.0% (w/v) agarose gel electrophoresis.

To determine the suitability of the housekeeping genes as internal controls in the real-time PCR assay for quantification of the target mRNAs, the expression of four different housekeeping genes were studied. The housekeeping genes used in this study were 18S ribosomal RNA, 40S ribosomal, actin and cyclophillin genes *(Table 2)*. The primers were first validated using the normal PCR analysis to determine the specificity of primers used. Only the primers which produced single amplicon were used for further validation using real-time PCR. Real-time PCR was performed using the ABI PRISM 7700 system (Applied Biosystem, California, USA).

The standard curve for all housekeeping genes and the *ACO*2 were first developed. Total RNA of control of Eksotika papaya seedling was extracted from approximately 100 mg of young leaves and 1 µg of total RNA was converted into cDNA (Qiagen, Hilden, Germany). The quantity and quality of the total RNA obtained were determined using a Nano drop spectrophotometer (Life Technologies, Wilmington, DE, USA) by measuring the absorbances at 230, 260 and 280 nm.

To validate the primer specificity and PCR efficiency of the targeted gene and the housekeeping gene, 10x serial dilutions were carried out starting from 1 µg of the total RNA that was converted to first strand cDNA. Real-time PCR was performed with a final volume of 20 µl. The reaction consisted of 2 µl of diluted cDNA, 10 µl (1 X) sybr green master mix, 200 nm each sense and antisense primers and the final volume was adjusted to 20 µl using

sterile distilled water. The PCR cycling conditions comprised a pre-denaturation step at 94 °C for 2 min, followed by 40 cycles repetition of the following steps: 94 °C denaturation for 15 s, 55 °C annealing for 30 s, 72 °C extension for 30 s and ending at 95 °C for 1 min. The slope obtained from the standard curve was used to calculate the PCR efficiencies using Equation 1. The acceptable PCR efficiencies for real-time PCR must range between 90 – 110%. The melting curve generated by real-time PCR was used to determine primer specificity and only primers which show a single peak of melting curve will be used for analysis of gene expression.

$$\text{Eff} = 10^{(-1/\text{slope})} - 1 \qquad \text{Equation 1}$$

Analysis of relative gene expression

Once the primer and the PCR efficiencies were validated, the analyses of gene expression can be determined. The same PCR mixture which contained 100 ng of first strand cDNA in the same PCR condition was used for gene expression analysis. The quantity of gene expression in every experimental tissue was expressed relative to the seedling-derived plant as a calibrator. Gene expression analysis was carried out on leaf samples of in vitro and field-grown putative transgenic plantlets. The C_t value was determined using the real-time PCR instrument software (ABI PRISM 7700, Applied Biosystem, California, USA). Each DNA template was analysed three times and the experiment was repeated twice. The average of two biological replicates was used for correlation analysis.

Shelf life analysis of planted transgenic lines

Regenerated transgenic papaya R_0 plants were hardened and acclimatised in contained environment, transgenic glasshouse before planting under nethouse condition. Both transgenic and non-transgenic plants were planted in completely randomised design. The planting distance of the plants was 2.2 m between rows and 2 m within rows. The fruits were placed on a working bench and allowed to ripen naturally at ambient temperature (25 ± 2 °C), and daily scoring of the colour index was determined by visual estimation until the fruit achieved the last stage of ripening, index 6 (fully yellow).

Results and discussion

Generating transgenic antisense Eksotika papaya plants

*ACO*2 is one of the most important enzymes involved in papaya fruit ripening (Sew et al. 2011). Fast ripening process in papaya can contribute to poor appearance, texture, flavour and overall fruit quality, and this can contribute to short storage life (Gonzalez-Aguilar et al. 2009). It is hypothesised that increasing the shelf life of papaya fruit through genetic engineering might be a promising option in improving the quality of the fruit. Therefore, in this study, the gene responsible for fruit ripening, *ACO*2 was transformed into embryogenic calli of Eksotika.

Transformation of pASACO2E1 construct which contained the *ACO*2 gene into 6,000 embryogenic calli successfully generated 60 putative transformed papaya lines after 4-month selection in kanamycin medium. The kanamycin in the selection medium will inhibit the growth of plant cells in non-transformed plant due to the lack of phosphotransferase to detoxify the kanamycin and this phenomenon will result in the failure of survival for the non-transformed plants. The transgenic plants containing the *npt*II gene are able to detoxify this antibiotic and will survive in the selection medium (Bao et al. 2001). Many researchers have reported the use of *npt*II as a marker gene for generating transgenic papaya plants and the selection period ranging from 4 months to a year (Cai et al. 1999; Chen et al. 2001; Laurena et al. 2002; Zimmerman and St Brice 2003). However, the growth of the putative transgenic plants in the selective medium is not a conclusive evidence for

successful production of transgenic plants. Therefore, molecular analysis using PCR need to be carried out to confirm the integration of transgenes in the genome. The PCR results showed that out of 60 putative calli transformed with pASACO2E1, 46 were positive for *npt*II and *ACO*2 genes. All these positive transgenic antisense lines were successfully regenerated into whole complete plantlets and were successfully transferred into soil with high survival rate.

Validation of housekeeping gene as internal control in real-time PCR

Real-time PCR can be used to measure the levels of gene expression of transgene in putative transgenic plants. Currently, this real-time RT-PCR has been rapidly used to replace Northern to analyse the levels of transgene expression in putative transgenic plants as it can speed up the molecular analysis process and is more convenient. In real-time PCR analysis, normalisation of the gene expression is important to obtain an accurate gene expression level. The use of more than one housekeeping gene is recommended for accurate normalisation and also to get reliable gene expression level (Vandesompele et al. 2002). Internal control gene or housekeeping gene is frequently used to normalise mRNA fraction during real-time PCR. However, the stability of a housekeeping gene is of utmost important. Thus in this study, four housekeeping genes were studied to determine the suitability of the housekeeping gene as an internal control in the real-time PCR analysis. Three housekeeping genes: 40S ribosomal protein, actin and cyclophillin were chosen based on the stability of expression of these genes in almost all Eksotika papaya tissue as reported by Waznul Adly et al. (2010). Meanwhile 18S was chosen because it is a common housekeeping gene used in gene expression study (Radonic et al. 2004).

All primer pairs were initially tested by using standard PCR with the same PCR conditions as for real-time PCR. Only the primers, which gave single amplicon that indicated the specificity of the primers, were used for the development of the standard curve and also for gene expression analysis. The amplification of single amplicon is very important to determine the real-time PCR accuracy. The results obtained indicated that all the primers tested were specific and had no false priming to the selected genes. The efficiencies of amplifying these 4 housekeeping genes were calculated and the housekeeping genes which gave correlation coefficient value of (R^2) of 0.99, slope of more than –3.4 and PCR efficiency of more than 99% were chosen for normalisation of the target gene expression. If the standard curve has a R^2 value of less than 0.99, it will affect the accuracy of the gene expression data which may result in poor quantification. PCR efficiency of 100% is shown by the slope of –3.32. The appropriate PCR efficiency is between 90 – 110% with the slope between –3.1 to –3.6 (Rasmussen et al. 2001; Tichopad et al 2003). The slope value must not be more than the recommended (–3.1 to –3.6), because it indicates problem with the samples or could be due to technical pipetting error.

Standard curve developed for housekeeping genes and the transgene must be within the acceptable range. Obtaining a good standard curve depends mainly on the suitability of the primers used. Based on the results obtained, only three housekeeping genes (18S ribosomal RNA, 40S ribosomal protein and actin) were finally chosen for normalisation of the transcripts. The primer specificity for *ACO*2 gene was also examined to analyse the amplification efficiency to avoid any bias due to non-specific PCR amplification, and a good standard curve was also successfully developed (*Figure 2*).

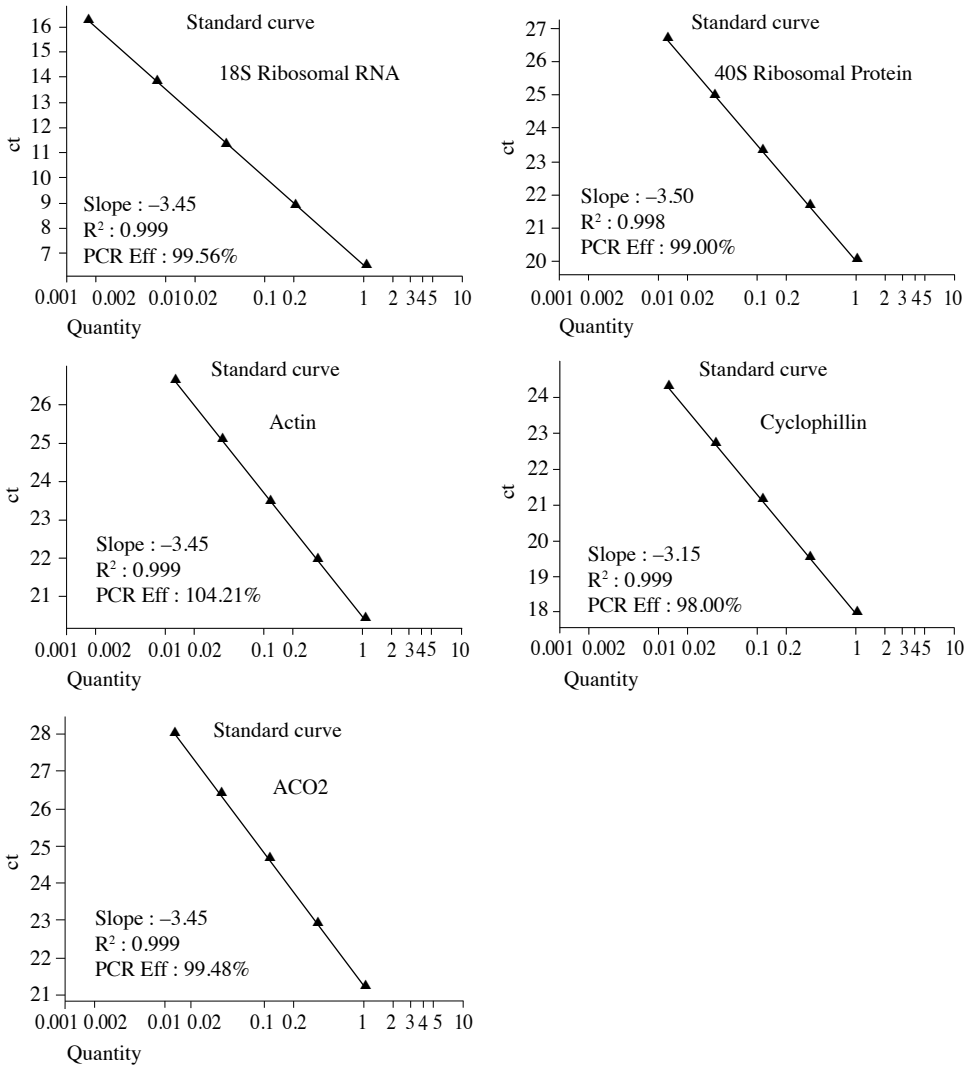

Figure 2. Real-time PCR efficiency plot of ΔCt versus log 5 fold dilution of the cDNA seedling control of Eksotika papaya

Gene expression analysis of in vitro putative transgenic papaya plants

Expression study of each putative transgenic papaya line produced was conducted using mRNA extracted from young leaves. The analysis of gene expression data was carried out following Pfaffl's method (2001) which was based on relative expressions of the target gene expressed in the sample and a control in comparison to an internal control gene expression. Gene expression level of the *ACO*2 gene in all 46 putative transgenic lines were compared to the seedling-derived plants (*Figure 3*). Out of 46 transgenic lines analysed, 42 transgenic lines showed reduction in expression levels of *ACO*2 transgene. The highest reduction expressions level of the target gene were shown in Line 3-1, Line 20 and Line 27-3 with 5-fold reduction. Other transgenic lines showed reduction of expression level of *ACO*2 between 1- and 3-fold. The expression of *ACO*2 in non-transformed tissue culture and vector transformed plants analysed

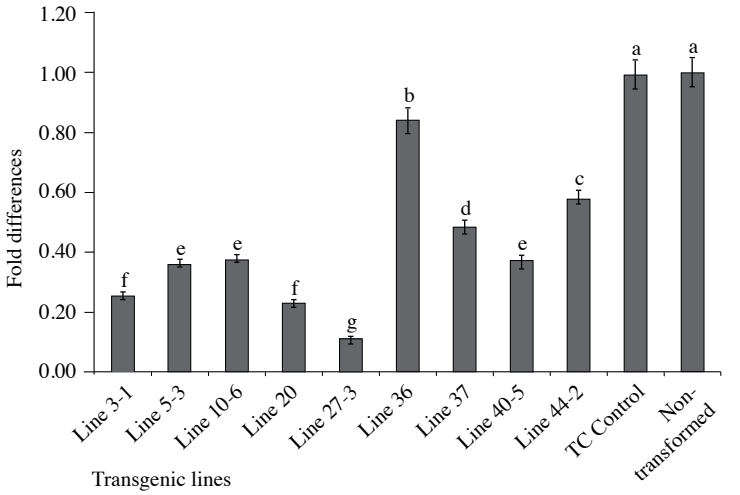

Figure 3. Gene expression analysis of putative transgenic plant harbouring pASACO2E1 construct. TC = Non-transformed plants; Non-transformed = Seedling-derived non-transformed plants

showed similar expression levels of *ACO*2 expression with seedling-derived control plant. This indicated that non-transformed tissue culture and vector transformed plant without gene of interest had no significant effect on the expression of *ACO*2 gene. These findings proved that the antisense of *ACO*2 gene is effective in reducing the expression of *ACO*2 gene in the transgenic papaya plants.

Effect on shelf life on reduction of ACO2 expression in transgenic plants

Further field trial of the transgenic papaya was carried out to evaluate the effectiveness of antisense technology in knocking down the *ACO*2 gene expression. To facilitate this field evaluation, selection of potential independent transgenic lines was done based on the results of gene expression study. Since the main aim of the transformation process was to reduce the gene expression of internal *ACO*2, therefore the selected transgenic line for further field evaluation was focused on positive lines that produced low levels of *ACO*2 expression. A total of 24 independent transgenic papaya plants were transferred into soil in nethouse. When compared to seedling-derived control fruit, 11 transgenic lines showed delayed in skin colour development. Based on the results obtained, it showed that the shelf life of the fruit was closely related with the level of *ACO*2 expression produced in each transgenic line *(Figure 4)*. A total of five transgenic lines (Line 1-8, Line 3-1, Line 20-2, Line 27-3 and Line 40-5) which showed the level of *ACO*2 expression less than 0.4 fold differences required more than 9 days to reach full yellow colour. Seedling-derived control fruit required 4 days to develop into full yellow colour index (Index 6). The lowest level of *ACO*2 gene expression in transgenic Line 27-3 of 10 fold lower than seedling-derived control resulted in the longest shelf life of fruit which required 14 days to achieve full yellow colour. The reduction of ethylene level may imply a hiccup in the ethylene-signaling pathway and an alteration of the fruit ripening process causing delayed ripening. The results obtained highlighted that ethylene production was successfully reduced in some transgenic papaya R_0 plants by transforming *ACC oxidase* gene into the genome of Eksotika papaya via the antisense technique. These shelf life analysis results suggested that the levels of

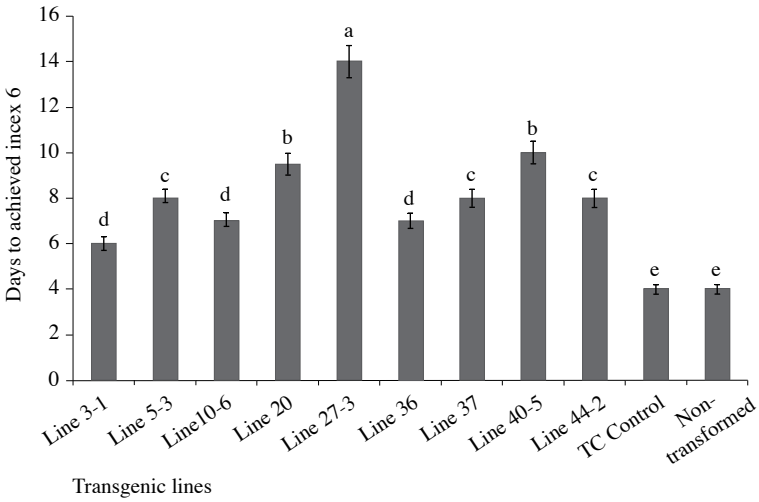

Figure 4. Shelf life analysis of selected transgenic plants grown in the nethouse. TC = Non-transformed plants; Non-transformed = Seedling-derived non-transformed fruit

ACO2 gene expression in transgenic lines is somehow associated with the shelf life of the transgenic produced.

Conclusion

Gene expression study using real-time RT-PCR is useful for precise and fast track selection of potential transgenic Eksotika papaya lines targeting for field evaluation. Eventhough the shelf life results are not 100% aligned with the gene expression data, but expected results can be predicted. Therefore, the expression level of transgene of in vitro putative transgenic plants can be used to select potential transgenic plants to be planted for further evaluation. It also helps to minimise the large number of positive transgenic lines to be evaluated. The results obtained proved that the expression of *ACO2* gene in transformed papaya could be knocked down using this antisense technique.

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Abstrak

Satu kaedah yang cepat diperlukan untuk menentukan pengekspresan transgene, *ACC oxidase* 2 dalam betik transgenik Eksotika bagi menyasarkan pemilihan barisan transgenik yang berpotensi untuk kajian lapangan. Dalam kajian ini, tiga gen perujuk, 18S ribosomal RNA, 40S ribosomal protein dan actin digunakan untuk menormalisasi pengekpresan gen *ACC Oxidase* 2. Perbandingan dengan pokok yang tidak ditransformasi, 42 barisan transgenik menunjukkan penurunan kadar pengekspresan gen *ACC Oxidase* 2 antara 2 – 5 kali ganda. Berdasarkan ekspresi gen *in vitro* ini, 24 barisan transgenik yang berpotensi dipilih untuk kajian lapangan. Analisis pengekspresan gen untuk pokok yang ditanam di ladang juga menunjukkan profil pengekspresan yang serupa dengan pokok *in vitro*. Peningkatan jangka hayat penyimpanan diperhatikan dalam kebanyakan barisan transgenik yang dipilih dengan yang paling berpotensi ialah barisan transgenik 27-3 yang memerlukan 14 hari untuk mencapai indeks kemasakan penuh. Ini mencerminkan bahawa penghasilan betik Eksotika yang mempunyai jangka hayat buah yang panjang boleh diperoleh dengan teknik ini dan boleh membantu bagi merangsang industri betik dengan membuka pasaran eksport baru ke destinasi yang lebih jauh.