academicJournals

Vol. 12(18), pp. 2427-2436, 1 May, 2013
DOI: 10.5897/AJB12.2939
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Identification of a β-galactosidase fruit pulp-specific promoter and its use in silencing constructs to reduce fruit softening in papaya

Wei-yi He[1,2], Xiao-jing Chen[1]*, Liette Vasseur[2,3], Yan-hong Shen[1], Bing-guo Lu[1] and Dong Liang[1]

[1]Institute of Genetics and Breeding in Horticultural Plants, College of Horticulture, Fujian Agriculture and Forestry University, Fuzhou 350002, China.
[2]Institute of Applied Ecology and Research Centre for Biodiversity and Eco-Safety, Fujian Agriculture and Forestry University, Fuzhou 350002, China.
[3]Department of Biological Sciences, Brock University, 500 Glenridge Avenue, St. Catharines, L2S 3A1, Ontario, Canada.

Accepted 18 April, 2013

***β*-Galactosidases have been proposed as key hydrolytic enzymes involved in cell wall degradation and rapid softening during postharvest processing and storage of papaya. To genetically improve the shelf life of papaya, we identified a softening-related *β-Gal* gene in fruit pulp at the 50% pericarp yellowing stage and isolated a novel *β-Gal* promoter region. An online database search predicted that the core promoter motifs and *cis*-acting elements in the isolated sequence were related to phytohormones, particularly to ethylene and stress responsiveness. GUS staining revealed different patterns of transient *GUS* expression in papaya organs driven by the putative promoter, with the highest level in the fruit pulp followed by the embryo and the root. Further, such expression was wound inducible in vascular tissues. Co-transformation of the two T-DNAs was performed, mediated by *Agrobacterium tumefaciens* harboring a plant expression vector with the fruit pulp-specific promoter and an inverted repeat *β-Gal* cassette in one T-DNA and marker genes in the other T-DNA. A total of 24 regenerated plantlets were obtained, one of which was identified to be co-transformed using GUS staining, PCR assay and Southern blotting.**

Key words: *β-Gal*, *Carica papaya*, fruit softening, gene silencing, marker-free transgenic plants, two-T-DNA transformation.

INTRODUCTION

Papaya (*Carica papaya* L.) is a popular fruit in the world due to its unique taste and nutritional value (Chandrika et al.,2003). While papaya has a huge market potential, as a climacteric fruit (Bapat et al., 2010), producers are challenged by its rapid postharvest ripening and softening, which are triggered by ethylene biosynthesis.

*Corresponding author. Email: xjchen804@sina.com. Tel: 86-591-83789167. Fax: 86-591-83789450.

Abbreviations: ACS, 1-Aminocyclopropane-1-carboxylate synthase; **ACO,** -aminocyclopropane-1-carboxylate oxidase; ***β*-GAL,** *β*-galactosidase; **EST,** expressed sequence tags; **IRB,** inverted repeat *β-Gal*; **IPCR,** inverse PCR; **CTAB,** cetyltrimethylammonium bromide; **MS,** Murashige and Skoog.

The physiological process of ripening is complex, under the influence of gene expressions linked to various factors such as cell wall hydrolases (Paull and Chen, 1983; Thumdee et al., 2010), contents of sugars, pigments and volatile compounds (Nogueira et al., 2012). Two characterized genes, *ACS* and *ACO*, encoding 1-aminocyclopropane-1-carboxylate synthase and 1-aminocyclopropane-1-carboxylate oxidase, respectively, are involved in ethylene biosynthesis (Neupane et al., 1998; Chen et al., 2003; López-Gómez et al., 2004). Cosuppression (López-Gómez et al., 2009) and antisense (Neupane et al., 1998) transformation of these genes have shown a potential for delaying rapid ripening in papaya. However, ripening of the papaya fruits transformed with antisense *ACS* and *ACO* require exogenous ethylene (Brummell and Harpster, 2001). Fresh-cut processing often requires the fruits to be at a certain ripening stage (Paull and Chen, 1997), and the cut fruits soften and deteriorate more rapidly than their intact counterparts due to the wound-induced activities of hydrolytic enzymes (Karakurt and Huber, 2003). The development of a novel variety of papaya fruit with a reduced rate of softening, without hampering other ripening-related traits, is highly favorable for the industry.

Papaya *β*-galactosidase (*β*-GAL, EC 3.2.1.23) is up-regulated during ripening and can catalyze the release of galactose from cell wall polymers composed of *β*-(1→4)-D-galactan (Smith et al., 1998). It is reported to be closely associated with fruit softening (Lazan et al., 1995, 2004; Ali et al., 1998; Karakurt and Huber, 2003; Sañudo-Barajas et al., 2009). The modified capacity of *β*-GAL in the cell wall of fruits (Ali et al., 1998) can lead to cell wall degradation, and is independent of polygalacturonase (Fabi et al., 2009), *α*-galactosidase (Soh et al., 2006) and xylanase (Chen and Paull, 2003; Manenoi and Paull, 2007). Further, the role of *β-Gal* in fruit softening of papaya has been validated by the investigation of expressed sequence tags (ESTs) (Devitt et al., 2006), mRNA differential display RT-PCR analysis (Karakurt and Huber, 2007) and Real-time PCR (Othman et al., 2011), although other cell wall hydrolases were identified during fruit ripening by using cDNA-AFLP (Fabi et al., 2010) and proteomic (Nogueira et al., 2012) approaches.

In this study, we used a *β-Gal* closely related to cell wall softening to examine its potential use in transgenic fruits to combat the issue of softening. The corresponding promoter region was first isolated and characterized regarding its organ expression specificity *in vitro*. Genetic transformation mediated by *Agrobacterium tumefaciens* was then conducted based on a two-T-DNA plant expression vectors (Komari et al., 1996) containing a self-promoter-driven inverted repeat *β-Gal* (IRB) cassette (Wesley et al., 2001). Our results confirmed the importance of *β-Gal* in the softening process of papaya and the potential of *β-Gal* gene silencing to slow down this process. Our study indicates the need to search for and develop novel marker-free transgenic varieties with more increased resistance to postharvest ripening and softening which will have huge economic impact on the papaya industry.

MATERIALS AND METHODS

The papaya cultivar, 'Zhanghong', used in this study was provided by the Zhangzhou Institute of Agricultural Sciences, Fujian Province, China. Fruits were harvested at four stages of pericarp development (immature green, 25% yellowing of the pericarp (25%-Y), 50%-Y and 100%-Y). Pulps were pretreated in liquid nitrogen, and stored at -80°C until used.

Nucleic acid extraction and PCR template preparation

RNA was isolated from the papaya pulp using the TRIZOL® reagent (Invitrogen, Shanghai) following the manufacturer's instructions. The first-strand cDNA was synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA). Genomic DNA was extracted from tissues of *in vitro*-propagated papayas using a cetyltrimethylammonium bromide (CTAB)-based method. Approximately 1 µg of genomic DNA was digested with *EcoR*I or *Hind*III (Takara, Dalian) at 37°C for 10 h. The purified digestion product was performed self-ligation with T4-DNA ligase (Takara, Dalian) at 16°C for 12 h. Additional purification for the ligated product was performed for the preparation of the sample to be used in inverse PCR (IPCR).

Gene cloning and promoter isolation

Primer sets (Table 1) were designed from reported *β-Gal* genes from the papaya fruit available in the GenBank database of the National Center for Biotechnology Information (NCBI) (GenBank accession nos. AF064786, AF079874, AF136187, AJ012578, AJ505945, DQ003060, and DT527740). Semi-quantitative RT-PCR assays targeting *β-Gal* sequences were carried out in three replicates from different papaya fruits at four stages. *Actin* was used as an internal standard. Quantitation of *β-Gal* expression based on the agarose gel electrophoresis result was performed by using Quantity One software (Bio-rad, USA). According to the restriction sites presented in the genomic sequence of the candidate *β-Gal* gene, three IPCR primer sets were designed for amplification of its promoter region, each of which involved two rounds of nested PCR (Figure 1).

All PCR assays were carried out in a 25 µL volume containing 5 mmol/L per dNTP, 5 pg per primer, 1.25 unit of *Ex Tag* (Takara), 2.5 µL of 10× *Ex Tag* buffer (Mg^{2+} Plus) and 1 µL of the sample DNA. The semi-quantitative PCR program consists of 3 min at 94°C followed by 30 cycles of 40 s at 94°C, 40 s at 52°C and 50 s at 72°C. Each round of the IPCR program began with 3 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 56°C and 3 min at 72°C. The program for the amplification of the *β-Gal* promoter region consists of 3 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C.

The amplified DNA fragments were sub-cloned into the pMD-18-T-vector (Takara, Dalian) and transformed into *E. coli* DH5α. Single colonies were selected and sequenced by the Invitrogen™Biotechnological Company (Shanghai). For the *β-Gal* fragments from the four developmental stages, five random colonies from each stage were sequenced to determine the most abundant *β-Gal* transcript. The gene coding and promoter sequences of the *β-Gal* gene (GenBank Accession No. AF064786) were submitted to GenBank (EU650664).

Table 1. The primer sequences used in the experiments.

DNA fragment amplified	Name: Primer sequence (from 5' terminus to 3')
β-Gal consensus sequence	BCP1: GAACGGACAATGAGCCTTTCAAGGC BCP2: CGGAAGAATGCTGATGGACCATG
Actin	ActP1: CACTGCTGAGCGGGAAATTGT ActP2: GATCCTCCAATCCAGACACTGT
First step of nested IPCR	1-IP11: GCTTCTGGGATAATGAATGGAGCCAG 1-IP12: GGTGCATCAAGCTGGTCTGTATG 1-IP21: GACCAGGTTTGTCTTCAACATGATAGC 1-IP22: GTGCGATAG AGAATGAGTATGGACC
Second step of nested IPCR	2-IP11: TAGGATTGAAAACGAAGGACCCAGT 2-IP12: TGCTACTAGCTATGATTACGATGCC 2-IP21: GAAAACGAAGGACCCAGTATTCCATAT 2-IP22: GAGTATGGTATGCATAGTGATCAGG
Third step of nested IPCR	3-IP11: CTAATTAGGAAAGTGGCGATAGCAACATG 3-IP12: the same as 1-IP12 3-IP21: GTCTTTGACCTCCACATCATCTTCT 3-IP22: the same as 1-IP22
β-Gal promoter region[a]	BPP1: *CGC*GTCGACAGCTTGTTCAAAGATAAAAG (*Sal*I) BPP2: *CTG*CCATGGGGTTGAGATCATGGTTGAAG (*Nco*I)
Spanning partial fragment of one *β-Gal* and PDK intron	BKP1: GAGAATGAGTATGGACCTATTGAGTGG BKP2: CTTCTTCGTCTTACACATCACTTGTCA
PDK intron	PDKP1: TGACAAGTGATGTGTAAGACGAAG PDKP2: GTTACCTT GTTTATTCATGTTCGACT

[a]The restriction sites are underlined and the protective bases are in italics.

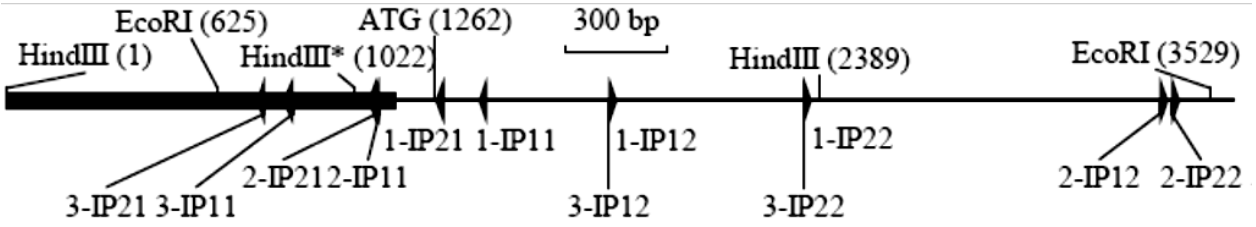

Figure 1. Isolation of the *β-Gal* promoter region using IPCR. The distribution of the *Eco*RI and *Hind*III restriction sites (the putative star activity site is indicated with an asterisk), start codon (ATG), primer sets for three steps of IPCR (indicated with arrows) and the isolated *β-Gal* promoter region (black bar) are shown.

Online promoter prediction

Three online databases, Plantcare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002), PLACE (http://www.dna.affrc.go.jp/PLACE/) (Higo et al., 1999) and the Neural Network Promoter Prediction database (http://fruitfly.org:9005/seq_tools/promoter.html) (Reese, 2001), were used for prediction of the *cis*-acting elements and the transcriptional start site.

Tissue specificity of the *β-Gal* promoter

The previously obtained *β-Gal* promoter was amplified using the BPP1 and BPP2 primers to replace CaMV 35S promoter upstream of *GUS* in the pCAMBIA 1301 plasmid (Table 1). The resulting recombinant plasmid was named as p1301/BP, and introduced into *A. tumefaciens* EHA 105. The engineered strain of EHA 105 was prepared for co-cultivation with either fruit pulp, pericarp, stem, leaf blade, petioles, sarcotesta, inner seed coat, endosperm, immature zygotic embryos, cotyledon, hypocotyl or root. Transient GUS activity in these organs was detected by histochemical staining using the method described by Jefferson (1987). Each treatment included 30 replicates with one additional negative control co-cultivated in sterilized water.

Preparation of the engineered EHA 105

A single colony of EHA 105 harboring the plant expression vector p2301/BPTTRG, consisting of two independent T-DNA regions (Figure 2), was inoculated into liquid YEB medium with 100 mg/L of

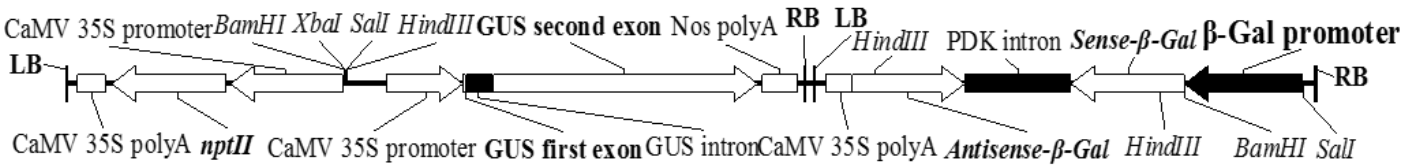

Figure 2. Two T-DNA regions in the binary plant expression vector p2301/BPTTRG. One region contains an IRB expression cassette driven by the *β-Gal* promoter, and the other contains *GUS* and the *npt*II gene for plant selection. The restriction sites are indicated by enzyme names with pointed bars.

kanamycin sulfate, 100 mg/L of streptomycin and 10 µmol/L of acetosyringone, and then cultured at 28°C with 225 rpm for 12 h. The bacterial culture was centrifuged at 28°C with 5000 rpm for 10 min, and re-suspended using the same volume of liquid MS medium containing 100 µmol/L of acetosyringone. The inocula were maintained in the liquid MS medium for additional 3 h and the final concentration was diluted to 0.6 at $OD_{600\ nm}$ prior to transformation.

Co-cultivation and recovery of transformed calli

About 500 g embryogenic calli (corresponding to ~800 in number) were used and soaked in the engineered EHA 105 at 28°C for 30 min. They were then dried on filter paper for 30 min and co-cultivated with the engineered EHA 105 in induction medium in the dark at 28°C for 3 days.

After co-cultivation, the cultures were cleaned in sterilized water six times and then dried. Calli were selected *via* subculturing every 20 days using embryogenesis medium containing Murashige and Skoog (MS) modified basal salt mixture (with half-strength MS inorganic salts and full-strength organic salts), 2.0 mg/L 2, 4-D, 400 mg/L glutamine, 1.0 g/L activated charcoal, 60 g/L sucrose and 7 g/L agar supplemented with 100 mg/L kanamycin sulfate and 300 mg/L carbenicillin. Subsequently, the calli were transferred to MS medium containing 50 mg/L of kanamycin sulfate and 300 mg/L of carbenicillin until somatic embryos were formed. Fully developed embryos were cultured in propagation medium containing MS salts, 0.2 mg/L BA, 0.02 mg/L NAA, 20 mg/L adenine sulfate, 30 g/L sucrose and 7 g/L agar supplemented with 50 mg/L kanamycin sulfate and 200 mg/L carbenicillin for germination. The shoot tips were removed and grown in the same medium.

Identification of co-transformants

Leaves of potential transgenic and non-transgenic papayas were used to perform GUS staining using the method described by Jefferson (1987).

A PCR assay was conducted to detect T-DNA carrying the IRB construct in GUS-positive transformants, with non-transgenic papaya as a negative control and p2301/BPTTRG as a positive control. Specific primer sets (Table 1, BKP1 and BKP2) spanning a fragment of one *β-Gal* gene and one PDK intron were designed to amplify a predicted 833 bp fragment. The PCR were carried out using the following conditions: 3 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C.

To carry out Southern blotting, 15 µg of DNA from a PCR-positive plantlet was digested with *Eco*RI at 37°C for 12 h. We chosed *Eco*RI recognition site because we found it was not on the construct (vector p2301/BPTTRG) used for genetic transformation. The same digestion was performed on p2301/BPTTRG as a positive control and on DNA from a non-transgenic papaya as the negative control. The products were size fractionated by electrophoresis through a 1.0% (w/v) agarose gel at 70 V for 1 h, then transferred to a nylon membrane (Amersham, Sweden) using the capillary method. A 561 bp probe derived from the PDK intron was synthesized using the PDKP1 and PDKP2 primers (Table 1) and the p2301/BPTTRG plasmid as a template. The probe was labeled using a DIG DNA labeling and detection kit (Roche, Switzerland). Prehybridization, hybridization and immunological detection were performed according to the kit manual at 68°C for hybridization and 65°C for stringent washing.

RESULTS

Identification of a softening-related *β-Gal* in the fruit pulp

Expression of *β-Gal* genes at different stages of fruit ripening were at a high level during ripening with greater accumulation of transcripts at the 50%-Y stage (Figure 3) when reportedly, the fruit pulp begins to soften rapidly and the activity of *β*-GALs increases markedly (Ali et al., 1998). Sequencing of the most abundant transcripts identified at each stage produced sequences corresponding to a reported fruit softening-related *β-Gal* gene, *pPBG*II (Othman et al., 2011), suggesting that suggesting that this β-Gal member actively participates in the papaya fruit softening.

Isolation and analysis of the *β*-Gal promoter region

Three flanking fragments of 121, 397 and 625 bp (after removing overlapped known sequences), resulting in a total promoter region of 1143 bp, were obtained from the IPCR (Figure 4) and validated using PCR amplification. Several core promoter motifs and *cis*-acting elements associated with phytohormones and stress responsiveness were found (Figure 5). The predicted transcription start site and TATA-box were located at -133 and -144 upstream of the start codon, respectively. Motifs for ethylene responsiveness (Montgomery et al., 1993) corresponding to the previously identified GCC (López-Gómez et al., 2004) and ERE (Chen et al., 2003) boxes in the papaya *ACO* promoter region and motifs for embryo and root specificity were predicted in the putative *β-Gal* promoter.

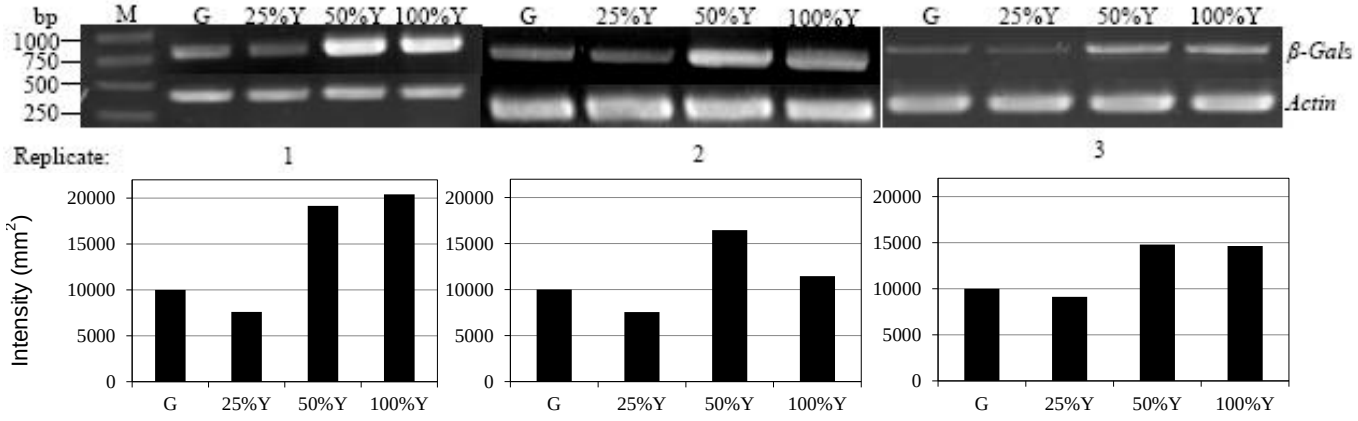

Figure 3. Semi-quantitative RT-PCR analysis of *β-Gal* genes from papaya pulp at different ripening stages. G: Immature green; 25%Y: 25% yellowing; 50%Y: 50% yellowing; 100%Y: 100% yellowing. The expressions of *β-Gal*s were quantified based on agarose gel electrophoresis results. The expression value of each replicate at immature green stage (G) was normalized into 10,000.

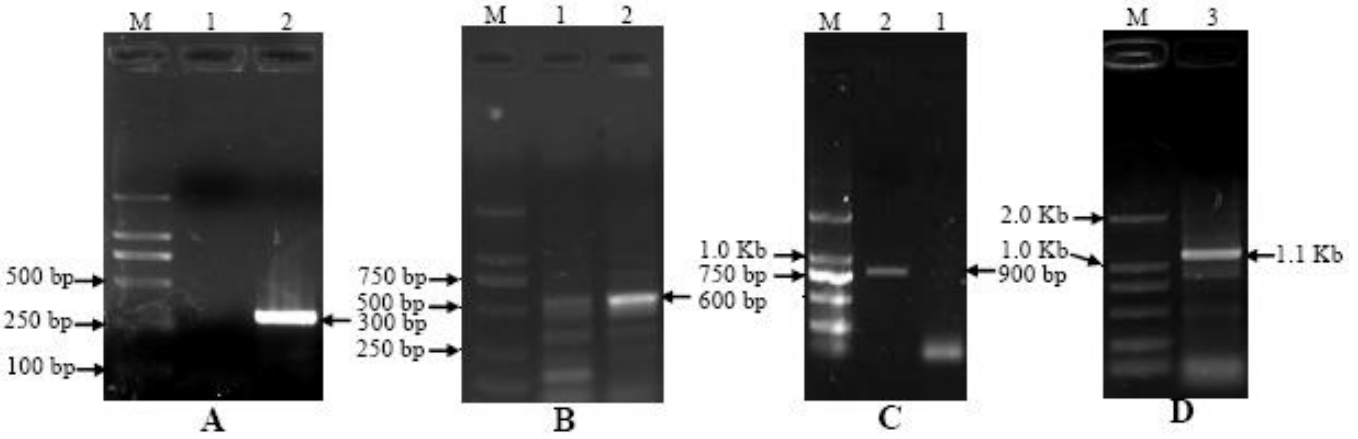

Figure 4. Isolation of the *β-Gal* promoter region using IPCR. **A,** The fragment generated in the first step of IPCR. **B,**The fragment generated in the second step of IPCR. **C,** The fragment generated in the third step of IPCR. **D,** Confirmation of the IPCR results. 1, The first round of each step of IPCR; 2, the second round of each step of IPCR; **3**, *β-Gal* promoter region; M, molecular weight marker.

Organ-specific expression of the *β-Gal* promoter driving a GUS reporter

Among the tested organs, fruit pulp showed the strongest GUS staining (Figure 6A), indicating that the isolated 5' flanking sequence exhibited promoter activity with specificity primarily in the fruit pulp. In addition, the moderate staining detected in the zygotic embryo (Figure 6B) and roots (Figure 6C) confirmed our bioinformatic prediction and suggested the possible involvement of *β-Gal* in the physiological metabolism of the two organs. Staining was not visible in the cotyledon and hypocotyl, except for a light staining at the junction between the two organs (Figure 6D, indicated by arrows). The promoter appeared to have no function in regulating organ-specific expression in sarcotesta, inner seed coat, endosperm, pericarp, leaf blade, petiole and stem (Figure 6E, F, G, H and J). Cutting the surface of vascular tissue however might induce *GUS* expression (Figure 6I and K, indicated by arrows).

Regeneration and identification of co-transformed plantlets

Differentiation of somatic embryos in embryogenesis medium containing kanamycin sulfate was observed within 3 to 4 months (Figure 7A). Two months after embryogenesis, well-developed somatic embryos germinated (Figure 7B). However, abnormal development of the roots was observed due to the formation of calli on the root and hypocotyl. A total of 24 shoot tips were

```
1     AGCTTGTTCA AAGATAAAAG TGAGATTCTT TTTAAAAGGG TTTAACTTAA AATGGTACAA
61    GTATTATTCC TCATAATATT TACATACGTA TGTATATACT GGGGAGGTGG GGTTCTTATT
121   GTCTACTTAG GAGTTATAAA ATTAGAATAA TATAGTTGAG TAAAATTGGT GATCAATTAT
181   TGAATTGGAT GAAACTAATT TTGATTAGTT AAAACTTGAT TTGAGTGGCA GGTCTTACGT
241   CAACTAATTA TAACTACCAC TCAACAAATT TTTTTTTTTT TTTTTTTTTT TTAAATTGAT
301   TTGTTAATCA TATATATCAA TAAAATTAAT TTACATAGTT TAGTTAACAC TAGTATTTTG
361   CATCTTCATA CGATCATGTT ATAGTCCTTT CCTATGACAA CAAGCAAATT AATCATTCTT
421   GGTTGCTTGG AAAAATAATA TATTGGACAC GTGGCAGTCT GTGAGTGGCC ATCAAGAGAT
481   TCGAAATACC CTTTAAGTGA AAGAAACCAT CTTCTCTCTT CCTTTAATAA TTTATTTATT
541   CTACACGTTT TTTTCTTCTC TAAAATTCAA AGTAATTGGC AAAAGAAATT AAAATTTTAT
601   TTAATACCTG TCTTCGTTGC CTTGGAATTC TAATAAATTA TCATTTTTCT TTAATTAAAA
661   AATATTGAGA GGTTAATTGC ATTGGAACAA TTTTTTTTAT GCATAAAAAA CTAAAAAGGG
721   GAAGTAGCAA AAGAGAAGAT GATGTGGAGG TCAAAGACAA ATGCAATTTC GCCGGTTTAA
781   ATTTTATCCA CTTCTCCTAA TTAGTTTATT TCATGTTGCT ATCGCCACTT TCCTAATTAG
841   TTTTTGATAT ATATATATAT ATATATACAT ACATATTATT ATATTTCTAT TAATAGTGTA
901   ATAAAAATTA TATTAATCAT CTTTTTTTAA AAATTAGATA ATTGGTGCTT TATATTTTTC
961   TCTATAGAAT ATGGATTATG GCTTGAAAAA ATTCAAAAAA TTTTAAACAA ATTATTAATT
1021  TAGAAGATGA TACAAAAATA AAAGGTAATA TATTTATTTA TATGGAATAC TGGGTCCTTC
1081  GTTTTCAATC CTATATATAT ATATACGAAG CAGTTTTGGT CTTCTTCAAC CATGATCTCA
1141  ACC
```

Figure 5. *β-Gal* promoter region. The colored regions indicate the following predicted elements: transcription start site (T, site 1115, in red); core promoter elements (TATA-box, 1092 to 1105, background in red); organ specificity in the embryo (347 to 353 and 447to 453, in pink) and root (76 to 80, 440 to 444, 662 to 666, 873 to 877, 881 to 885, 910 to 914 and 952 to 956, background in blue); responsiveness to ethylene (564 to 571 and 990 to 997, background in green), abscisic acid (445 to 455, background in yellow), gibberellin (738 to 747 and 785 to 791, in brown), methyl jasmonate (238 to 242, background in pink), salicylic acid (917 to 926, in green), heat stress (995 to 1004, in gray), and defense and stress (781 to 790, background in gray); enhancer region of the CAAT-box (174 to 178, 265 to 269, 318 to 321, 405 to 409, 688 to 692, 758 to 762, 764 to 768, 1008 to 1012 and 1086 to 1089, in blue), TA-rich region (844 to 865, in light blue) and 5' UTR Py-rich region (552 to 561, background in brown); matrix attachment region (950 to 959, in orange); poly T(24) stretch (269 to 292, background in dark yellow).

removed and cultured in propagation medium containing kanamycin sulfate for further development (Figure 7C).

The leaves from the regenerated plantlets were all GUS positive; however, the non-transgenic papaya plantlets could not be stained (Figure 8A-C). Only one plantlet was found to contain the T-DNA of interest based on a PCR assay (Figure 8D), indicating that the co-transformation of the two T-DNAs was not as efficient as expected. A single hybridized band was observed in the digested genomic DNA from the co-transformed papaya (Figure 8E), indicating the presence of the transforming construct.

DISCUSSION

Fruit softening during the postharvest storage and processing has become a critical limitation for papaya commercialization. In this study, rather than genetically inhibiting ethylene biosynthesis, we examined the use of a softening-related *β-Gal* gene as a candidate for genetic transformation aimed at reducing the postharvest softening of papaya fruit. In addition to silencing the softening-related *β-Gal* gene, we have provided a possibility that the IRB transgene could extend the silencing effect to other members of this multigene family, and overcome the functional complementation of hydrolyase isoforms (Brummell and Harpster, 2001). This approach might provide the possibility of breeding a novel transgenic cultivar suitable for fresh-cut processing, with a reduced rate of the fruit softening while maintaining other ripening-related traits independent of exogenous ethylene.

As previously shown, the expression of *β-Gal* gene is

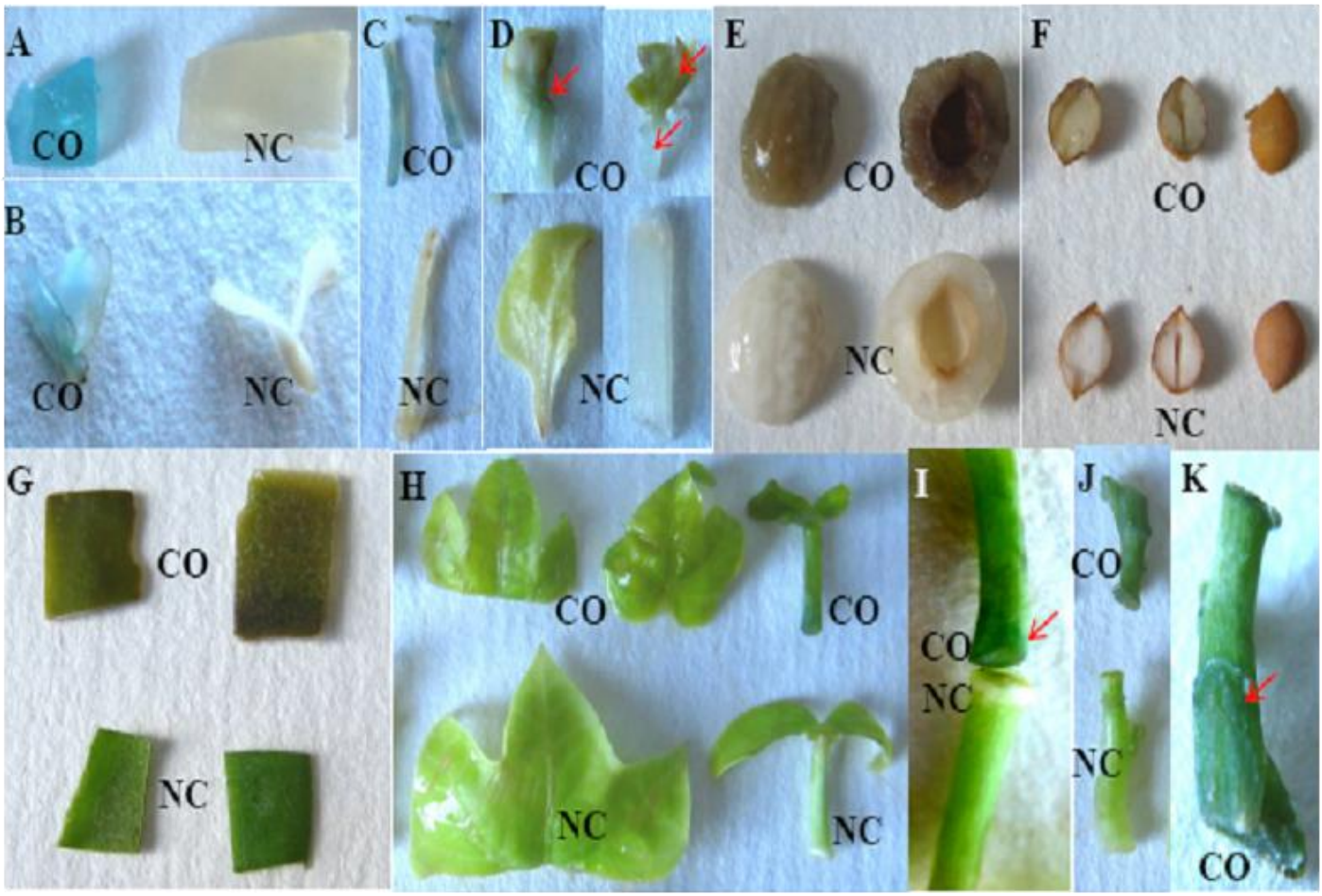

Figure 6. Characterization of the organ specificity of the expression of the putative *β-Gal* promoter transiently expressing GUS using a histochemical assay. Different papaya organs, including the fruit pulp (A), immature zygotic embryo (B), root (C), cotyledon (D), hypocotyl (D), sarcotesta (E), inner seed coat (F), endosperm (F), pericarp (G), leaf blade (H), petiole (H, I) and stem (J, K), were co-cultivated (CO) with *A. tumefaciens* EHA 105 harboring p1301/BP and carrying a chimeric form of *GUS*. The same organs cultivated in sterilized water were used as negative control (NC).

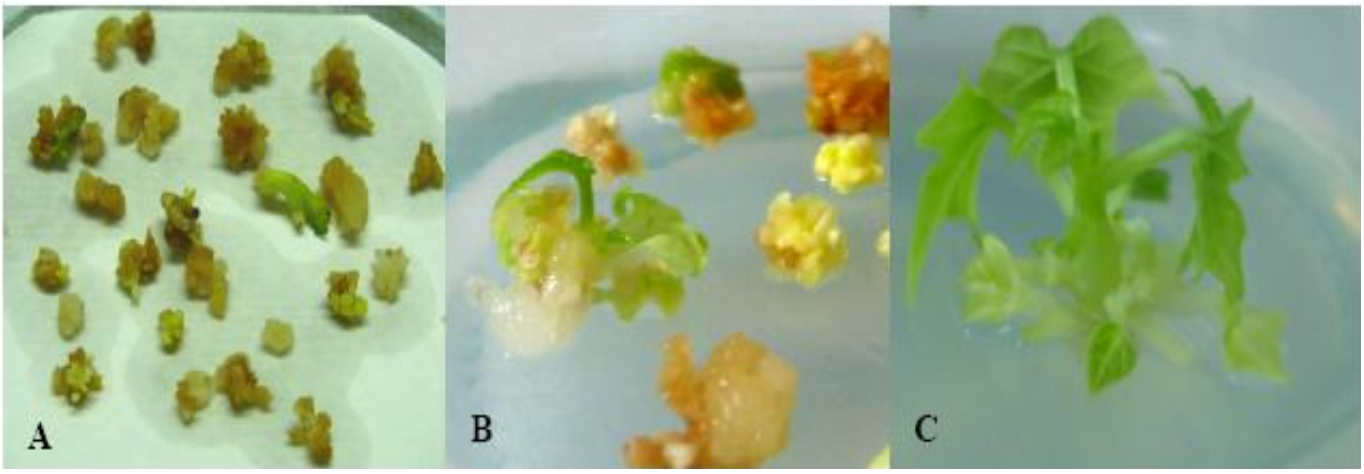

Figure 7. Genetic transformation. Papaya somatic embryogenesis (A), germination of the somatic embryo (B) and plant regeneration (C) in medium containing kanamycin sulfate approximately 8 months after co-cultivation of friable embryogeniccalli with *Agrobacterium* EHA 105 harboring p2301/BPTTRG.

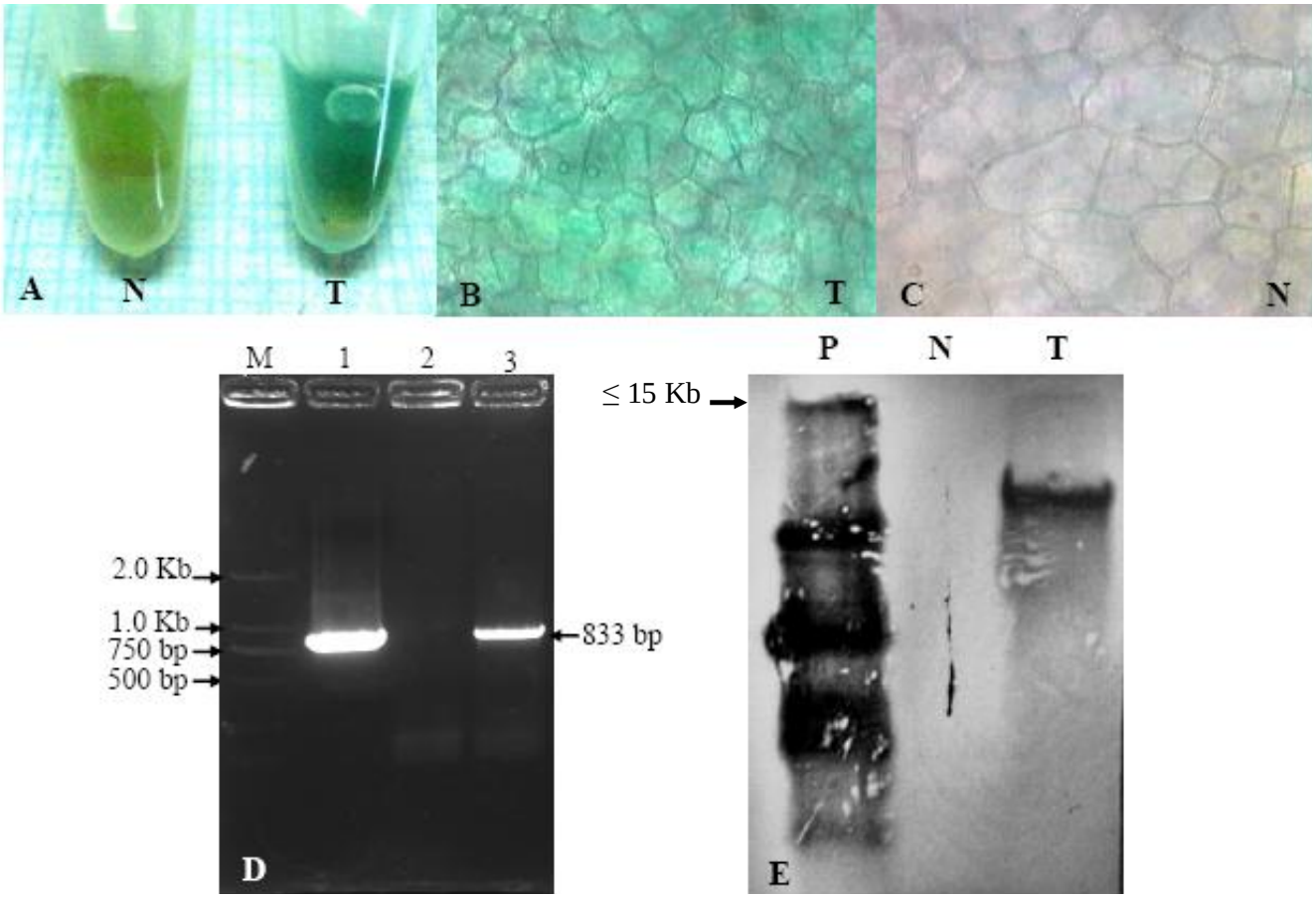

Figure 8. Identification of co-transformed papaya *via* GUS staining, PCR and Southern blotting assays. **A,** GUS staining of leaves from putatively transgenic (T) and non-transformed (N) papaya. **B and C**, Leaf cells of transgenic (T) and non-transformed (N) papaya after staining. D: Detection of IRB construct *via* a PCR assay. 1, Positive control (p2301/BPTTRG); 2, negative control (DNA of non-transformed papaya); 3, DNA of GUS-positive papaya. **E,** DNA from PCR-positive (T), non-transformed papaya (N) and plasmid p2301/BPTTRG (P).

up-regulated during papaya fruit ripening (Soh et al., 2006). The staining observed at the cut surface of petioles and stems suggested the existence of wound-responsive elements, as previously reported in the papaya *ACO* gene promoter (Chen et al., 2003) and further confirms the key role of this *β-Gal* in papaya softening under normal and stressed conditions. These findings may explain the differences in *β-Gal* expression, enzyme activities and changes in the cell wall matrix between intact and fresh-cut papayas during storage (Karakurt and Huber, 2003, 2007). The specificity of the *β-Gal* promoter allows the silencing effect to be primarily limited to the papaya pulp, thus avoiding the potential physiological disorders caused by constitutive suppression of endogenous genes.

One of the issues concerning the safety of transgenic crops is the potential risk of selectable markers that might escape and enter ecosystems to generate unexpected impacts on human life after release in the fields (Iwaki and Arakawa, 2006; Wei et al., 2006). The *Cre*/*lox* recombination system has been applied to papaya transformation for the perturbation of ethylene biosynthesis, and the selectable markers can be removed, through the subsequent hybridization between two types of transgenic plants: one contains *lox* recognition sites between the selection marker gene and the other one expresses *Cre* gene (Neupane et al., 1998). In the present study, the co-transformation mediated by *A. tumefaciens* with the two-T-DNA/one plasmid system can also segregate for the makers, which depends on incorporation of the T-DNAs into differentchromosomes of the plant genome. If the two T-DNAs are genetically unlinked, they can segregate in the sexually hybridized progeny. While the rate of transformation of single T-DNA in papaya can be ~20% (Dhekney et al., 2007), we obtained, by using a similar transforming protocol, a low co-transformation rate of the two T-DNAs (0.125%, 1/800). This indicates that the integration of two independent T-DNAs into genome during transformation may be complex. Indeed, the two-

T-DNA co-transformation rate in papaya was found to be much lower than that in the rice (*Oryza sativa*), in which the transforming system has been well established and the co-transformation rate reaches ~8% (Rao et al., 2011; Wakasa et al., 2012). Despite these results, we believed that this approach could be useful in the future for breeding a marker-free transgenic papaya in the favor of food security and environmental safety, and warrants further research.

ACKNOWLEDGEMENTS

We are grateful to Dr. Gui-xin Chen from College of Horticulture, Fujian Agriculture and Forestry University (FAFU) for providing pKANNIBAL vector. We thank Professor Minsheng You from Institute of Applied Ecology, FAFU and Professor Jianjun Chen from Institute of Food and Agricultural Sciences, University of Florida for critical comments. This work was supported by the major project of Department of Science and Technology in Fujian Province (No. 2008N0008).

REFERENCES

Ali ZM, Ng S., Othman R, Goh LY, Lazan H (1998). Isolation, characterization and significance of papaya *β*-galactanases to cell wall modification and fruit softening during ripening. Physiol. Plant. 104:105-115.

Bapat VA, Trivedi PK, Ghosh A, Sane VA, Ganapathi TR, Nath P (2010). Ripening of fleshy fruit: Molecular insight and the role of ethylene. Biotechnol. Adv. 28:94-107.

Brummell DA, Harpster MH (2001). Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. Plant Mol. Biol. 47:311-340.

Chandrika UG, Jansz ER, Wickramasinghe SMDN, Warnasuriya ND (2003). Carotenoids in yellow- and red-fleshed papaya (*Carica papaya* L). J. Sci. Food Agric. 83:1279-1282.

Chen NJ, Paull RE (2003). Endoxylanase expressed during papaya fruit ripening: purification, cloning and characterization. Funct. Plant Biol. 30:433-441.

Chen YT, Lee YR, Yang CY, Wang YT, Yang SF, Shaw JF (2003). A novel papaya ACC oxidase gene (*CP-ACO2*) associated with late stage fruit ripening and leaf senescence. Plant Sci. 164:531-540.

Devitt LC, Sawbridge T, Holton TA, Mitchelson K, Dietzgen RG (2006). Discovery of genes associated with fruit ripening in *Carica papaya* using expressed sequence tags. Plant Sci. 170:356-363.

Dhekney SA, Litz RE, Moraga Amador DA, Yadav AK (2007). Potential for introducing cold tolerance into papaya by transformation with C-repeat binding factor (CBF) genes. In Vitro Cell. Dev. Biol. Plant 43:195-202.

Fabi JP, Lajolo FM, Oliveira do Nascimento JR (2009). Cloning and characterization of transcripts differentially expressed in the pulp of ripening papaya. Sci. Hortic. 121:159-165.

Fabi JP, Mendes LRBC, Lajolo FM, Oliveira do Nascimento JR (2010). Transcript profiling of papaya fruit reveals differentially expressed genes associated with fruit ripening. Plant Sci. 179:225-233.

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999). Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res. 27:297-300.

Iwaki M, Arakawa Y (2006). Transformation of *Acinetobacter* sp. BD413 with DNA from commercially available genetically modified potato and papaya. Lett. Appl. Microbiol. 43:215-221.

Jefferson RA (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5:387-405.

Karakurt Y, Huber DJ (2003). Activities of several membrane and cell-wall hydrolases, ethylene biosynthetic enzymes, and cell wall polyuronide degradation during low-temperature storage of intact and fresh-cut papaya (*Carica papaya*) fruit. Postharvest Biol. Technol. 28:219-229.

Karakurt Y, Huber DJ (2007). Characterization of wound-regulated cDNAs and their expression in fresh-cut and intact papaya fruit during low-temperature storage. Postharvest Biol. Technol. 44:179-183.

Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996). Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J. 10:165-174.

Lazan H, Ng SY, Goh LY, Ali ZM (2004). Papaya *β*-galactosidase/galactanase isoforms in differential cell wall hydrolysis and fruit softening during ripening. Plant Physiol. Bioch. 42:847-853.

Lazan H, Selamat MK, Ali ZM (1995). *β*-Galactosidase, polygalacturonase and pectin esterase in differential softening and cell wall modification during papaya fruit ripening. Physiol. Plant. 95:106-112.

Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouzé P, Rombauts S (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. Nucleic Acids Res. 30:325-327.

López-Gómez R, Cabrera-Ponce JL, Saucedo-Arias LJ, Carreto-Montoya L, Villanueva-Arce R, Díaz-Perez JC, Gómez-Lim MA, Herrera-Estrella L (2009). Ripening in papaya fruit is altered by ACC oxidase cosuppression. Transgenic Res. 18:89-97.

López-Gómez R, Morales-Domínguez F, Mendoza Alcázar O, Gómez-Lim MA (2004). Identification of a genomic clone to ACC oxidase from papaya (*Carica papaya* L.) and expression studies. J. Agric. Food Chem. 52: 794-800.

Manenoi A, Paull RE (2007). Papaya fruit softening, endoxylanase gene expression, protein and activity. Physiol. Plant. 131:470-480.

Montgomery J, Goldman S, Deikman J, Margossian L, Fischer RL (1993). Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. Proc. Natl. Acad. Sci. U.S.A. 90:5939-5943.

Neupane KR, Mukatira UT, Kato C, Stiles JI (1998). Cloning and characterization of fruit-expressed ACC synthase and ACC oxidase from papaya (*Carica papaya* L.). Acta Hortic. 461:329-338.

Nogueira SB, Labate CA, Gozzo FC, Pilau EJ, Lajolo FM, Oliveira do Nascimento JR. (2012). Proteomic analysis of papaya fruit ripening using 2DE-DIGE. J. Proteomics 75:1428-1439.

Othman R, Chong H, Choo T, Ali Z (2011). Three *β*-galactosidase cDNA clones related to fruit ripening in papaya (*Carica papaya*). Acta Physiol. Plant 33:2301-2310.

Paull RE, Chen NJ (1983). Postharvest variation in cell wall-degrading enzymes of papaya (*Carica papaya* L.) during fruit ripening. Plant Physiol. 72:382-385.

Paull RE, Chen W (1997). Minimal processing of papaya (*Carica papaya* L.) and the physiology of halved fruit. Postharvest Biol. Technol. 12:93-99.

Rao MVR, Parameswari C, Sripriya R, Veluthambi K (2011). Transgene stacking and marker elimination in transgenic rice by sequential *Agrobacterium*-mediated co-transformation with the same selectable marker gene. Plant Cell Rep. 30:1241-1252.

Reese MG (2001). Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. Comput. Chem. 26:51-56.

Sañudo-Barajas JA, Labavitch J, Greve C, Osuna-Enciso T, Muy-Rangel D, Siller-Cepeda J (2009). Cell wall disassembly during papaya softening: Role of ethylene in changes in composition, pectin-derived oligomers (PDOs) production and wall hydrolases. Postharvest Biol. Technol. 51:158-167.

Smith DL, Starrett DA, Gross KC (1998). A gene coding for tomato fruit *β*-galactosidase II is expressed during fruit ripening. Plant Physiol. 117:417-423.

Soh C, Ali ZM, Lazan H (2006). Characterisation of an *α*-galactosidase with potential relevance to ripening related texture changes. Phytochemistry 67:242-254.

Thumdee S, Manenoi A, Chen N, Paull R (2010). Papaya fruit softening: Role of hydrolases. Trop. Plant Biol. 3:98-109.

Wakasa Y, Ozawa K, Takaiwa, F (2012). *Agrobacterium*-mediated co-transformation of rice using two selectable marker genes derived from rice genome components. Plant Cell Rep. 31:2075-2084.

Wei XD, Zou HL, Chu LM, Liao B, Ye CM, Lan CY (2006). Field released transgenic papaya affects microbial communities and enzyme activities in soil. Plant Soil 18:734-740.

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. 27:581-590.