Transgenic Res (2009) 18:89–97
DOI 10.1007/s11248-008-9197-0

ORIGINAL PAPER

Ripening in papaya fruit is altered by ACC oxidase cosuppression

Rodolfo López-Gómez · Jose Luis Cabrera-Ponce · Luis Jorge Saucedo-Arias · Lorena Carreto-Montoya · Ramon Villanueva-Arce · Juan Carlos Díaz-Perez · Miguel Angel Gómez-Lim · Luis Herrera-Estrella

Received: 26 September 2007 / Accepted: 11 June 2008 / Published online: 9 July 2008

Abstract Papaya (*Carica papaya*) is a very important crop in many tropical countries but it is highly susceptible to parasitic diseases, physiological disorders, mechanical damage and fruit overripening. Here we report a study on ACC oxidase cosuppression and its effects on papaya fruit ripening. Papaya ACC oxidase was isolated using PCR and embriogenic cells transformed by biolistic using the CaMV 35S promoter to drive the expression of the PCR fragment in sense orientation. Fifty transgenic lines were recovered and 20 of those were grown under field conditions. Southern analysis showed incorporation of the transgene in different copy numbers in the papaya genome. Fruits were evaluated in terms of texture (firmness), colour development, respiration and ethylene production. A sharp reduction in ethylene and CO2 production was detected, whereas softening and colour development of the peel were also altered. Overall, transgenic fruits showed a delay in ripening rate. A reduction in mRNA level for ACC oxidase in transgenic fruit was clearly detectable by northern blot. More studies are necessary before this technology can be used to extend the shelf life of papaya fruit.

Keywords Climacteric · Ethylene · Fruit ripening · Papaya · ACC Oxidase · Cosuppression · Biolistic

Abbreviations
ACC 1 Aminocyclopropane-1-carboxilic acid
GC Gas chromatography

R. López-Gómez (✉) · L. Carreto-Montoya
Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edificio B1 Ciudad Universitaria, Morelia, MI CP 58060, Mexico
e-mail: lgomez@zeus.umich.mx

J. L. Cabrera-Ponce · L. J. Saucedo-Arias · M. A. Gómez-Lim · L. Herrera-Estrella
Departamento de Ingeniería Genética, CINVESTAV-Irapuato, Km 9.6 carretera Irapuato-León, Apartado Postal 629 Irapuato, GT, Mexico

R. Villanueva-Arce
Centro de Desarrollo de Productos Bioticos IPN, Km 8 Carr Yautepec-Jojutla, Yautepec, MO, Mexico

J. C. Díaz-Perez
Department of Horticulture, Tifton Campus, University of Georgia, Tifton, GA 31793, USA

Introduction

Fruit ripening is a complex, genetically programmed process that culminates in dramatic changes in colour, texture, flavour and aroma of the fruit. Based on their respiratory pattern, fruits can be divided into two groups: climacteric, in which ripening is accompanied by a peak in respiration and a concomitant burst of ethylene, and non-climacteric in which there is no change in respiration and ethylene production

remains at a very low level (Alexander and Grierson 2002). The sharp increase of ethylene production in climacteric fruits at the onset of ripening is thought to induce changes in biochemical and physiological attributes (Jiang and Fu 2000). Ethylene regulates fruit ripening by regulating the expression of specific genes (Barry and Giovannoni 2007). Ethylene is synthesized in higher plants from methionine via S-adenosyl methionine and 1-aminocyclopropane-1-carboxylic acid. The role of the two key enzymes of the pathway, ACC synthase and ACC oxidase, during fruit ripening has been the focus of research of many groups over several years.

Papaya, with a world production of 6.8 million Mt in 2005 (FAO 2006), is a major economic crop in many tropical countries. The fruit is a valuable export commodity, which generates hard currency earnings in many developing countries. Papaya shipments arriving at terminal markets have a range of disorders associated with over-ripeness, mechanical injury and parasitic diseases (Capellini et al. 1988).

Storage studies on papaya harvested at different harvest maturities have identified fruit softening as a key quality factor to be controlled (Paull 1993). In this respect, enzymes involved in ethylene synthesis represent key targets for research. In climacteric fruits, such as papaya, the rise in ethylene production parallels the respiration rate and peaks at the same time as the respiratory climacteric (Paull and Chen 1983). Studies on ethylene production in papaya fruit have focused on measurements of ACC oxidase activity. The highest level of activity has been found in the exocarp of 75% ripe fruit (Chan et al. 1990). Molecular studies have demonstrated expression of two ACC syntase genes during papaya fruit ripening (Mason and Botella 1997). Expression of ACC oxidase genes has also been examined during this process. In preclimacteric fruits, the ACC oxidase message is detectable at very low levels in the peel but it is highly abundant in the pulp. The message is also easily detectable in the peel of climacteric fruit which also contains high levels of ACC oxidase mRNA in the pulp. In postclimacteric fruit the message is detectable both in the peel and the pulp but the levels are lower than those in climacteric fruit (López-Gómez et al. 2004). Two different ACC oxidase genes participate in papaya fruit ripening (Chen et al. 2003).

By antisense inhibition of ACC oxidase (Picton et al. 1993, Schaffer et al. 2007), or RNAi (Xiong et al. 2005) or ACC synthase antisense (Oeller et al. 1991) it has been possible to delay tomatoe fruit ripening. Expression of antisense ACC oxidase in melon fruit also alters the normal ripening pattern (Ayub et al. 1996). Other studies have further demonstrated that introduction of truncated gene constructs in the sense orientation, can result in suppression of homologous host genes, a phenomenon called co-suppression (de Carbalho-Nievel et al. 1995; Napoli et al. 1990; Yu et al. 2003; Mahmoud et al. 2004). Papaya fruit is susceptible to overripening caused by ethylene and the technology in use today to extend the shelf life of papaya is based on the control of ethylene action and production. The availability of fruits with low endogenous ethylene production might substantially reduce losses caused by overripening. For all these reasons, this work was undertaken with the aim to generate transgenic papaya plants containing a fragment of the ACC oxidase gene in the sense orientation to block ethylene production and delay the ripening rate. This is the first report of the use of cosuppression technology in a tropical fruit (the papaya).

Material and methods

Plant material

Papaya fruits (*Carica papaya* cv Maradol) were obtained from a local market to isolate the ACC oxidase fragment. Ethylene and CO_2 production of individual fruits was monitored by GC (Hofman and Yang 1980).

PCR and sequence analysis

Total RNA was extracted from both peel and pulp at each ripening (preclimacteric and climateric) stage by the method described by López-Gómez and Gómez-Lim (1992). Complementary DNA was synthesized from pulp RNA using reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany). Sequences for papaya ACC oxidase were amplified from the cDNA by polymerase chain reaction (PCR), using degenerate primers for ACC oxidase. Forward primer: 5′ GC(A/T/G/C) TG(C/T) GA(A/G) AA(T/C) TGG GG(G/A/C/T) TT 3′. Reverse primer: 5′ AA (A/G) TT(C/T) CA(G/A) GC(A/C/G/T) AA(A/G)

GA(A/G) 3′ (López-Gómez et al. 1997). A PCR product of the appropriate length, 816 bp, was cloned into the PCRII vector (InvitroGen, Carsbald CA), and sequenced using the Sequenase enzyme according to the manufacturer′s instructions (United States Biochemical, Cleveland, OH), with deoxyadenosine 5′-[α-(^{35}S) thio]-triphosphate (Amersham Biosciences, Piscataway, NJ). The entire PCR product was sequenced on both strands. For transgene detection, we designed a set of primers derived from the CaMV 35S promoter (5′end) and from the ACC oxidase fragment (3′ end); reverse: 5′ GTCAGCGGCCTCCAACTCCTC 3′ and forward: 5′ CACTGACGTAAGGGATGACG 3′, to amplify a fragment of about 816 bp.

Vector construction

To prepare the papaya ACC oxidase sense construction, we used the pKYLX80 plant vector containing the CaMV 35S promoter and the transcriptional terminator of the RUBISCO gene (Schardl et al. 1987). The ACC oxidase insert (cCPACCO-1) was removed by HindIII/XbaI digestion from the PCRII vector and ligated into the same sites of PKYLX80. The resulting construct was named pKYCPACCO-1 and purified as described (Sambrook et al. 1989).

Generation of papaya transformants

Papaya embryogenic calli at the globular stage were transformed by particle bombardment according to Cabrera-Ponce et al. (1995, 1997). The papaya genotype used was Maradol obtained from the INIFAP Research Institute (Tabasco, México). An equimolar mixture of plasmid pKYCPACOO-1 and pGPTV-Bar (Becker et al. 1992) was precipitated onto tungsten microprojectiles M10 (0.73 μm diameter GTE Sylvania, Precision Materials Group, Towanda, Pa) and used for the bombardment. Tranformed embryogenic calli were selected on 3 mg/l PPT (PESTANAL®, Sigma-Aldrich, St. Louis, MO) and 100 mg/l G418 (Sigma-Aldrich). Transformed shoots were transferred to M3 medium (MS medium supplemented with 3 g/l activated charcoal, 2% sucrose, 2.5 g/l gelrite at pH 5.8) plus 3 mg/l PPT and 100 mg/l G418 to induce root elongation for one month. Rooted plants were placed in pots with peat most (Sunshine Mix 3, Fisons, Canada), applying a fungicidal solution (1 mg/ml benomyl) in the roots to avoid fungal contamination. Plants were covered with a plastic bag and grown in a Plant Tissue Culture Chamber model CU-36L (Percival Scientific, Perry, IA) at 25°C under a 16-h photoperiod provided by cool white fluorescent lamps (50 μM m^{-2} s^{-1}) for up to two months before transferring to the greenhouse. Using the same type of soil, plants were transferred to the greenhouse with day/night temperatures of 32°C ± 2/14°C ± 2, and a relative humidity varying between 60%–90% all year. Light fertilization was done using a 20:30:10 (nitrogen, phosphorous and potassium) fertilizer applying 100 ml of fertilizer solution (3 g/l) per pot, four times during plant development. Plants were kept in these conditions for about three months and then were transferred to the field, irrigated each other day and fertilized monthly (Triple 17 fertilizer 17:17:17 nitrogen, phosphorus and potassium).

RNA Isolation and RNA blots

Total RNA was extracted from papaya fruit tissue by the method described by López-Gómez and Gómez-Lim (1992). Samples of total RNA (10 μg) were subjected to electrophoresis in a formaldehyde-containing gel, followed by transfer to a Hybond-N membrane (Amersham) and hybridization to the ^{32}P-labeled cCPACCO-1 insert (Sambrook et al. 1989; Ausubel et al.1991). The insert was labelled to a specific activity of 10^8–10^9 cpm/μg by random priming (Invitrogen, Carlsbad, CA). Blots were washed at 65°C twice (15 min) at high stringency (1 mM Na_2 EDTA, 40 mM $NaHPO_4$, 1% SDS) and subsequently exposed to a Kodak XAR-5 film at −70°C.

DNA Hybridization and DNA blots

Papaya genomic DNA was isolated from young leaves as described by Dellaporta et al. (1983). Ten micrograms of DNA were digested with the appropriate restriction enzyme and electrophoresed on a 0.8% agarose gel. The gel was blotted to a Hybond-N membrane (Amersham) and hybridized to the labelled insert of cCPACCO-1 at 65°C by the phosphate buffer method (Ausubel et al. 1991). Blots were washed at 65°C twice (15 min) at high stringency (1 mM Na_2 EDTA, 40 mM $NaHPO_4$, 1% SDS). The blots were subsequently exposed to a

Kodak XAR-5 film at −70°C using intensifying screens.

Analysis of fruit

Skin colour was measured with a colorimeter (Color Mate Color Analyzer, Milton Roy, Rochester, N.Y.), using the colour system L*a*b*. The colorimeter was calibrated with a white reflective plate. Colour was measured at the equatorial level on two opposite sides of the fruit. Firmness was measured as resistance to penetration (3 mm) using a digital texturometer equipped with a cylindrical tip 7 mm in diameter.

Plant growth

Regenerated plants were grown in a greenhouse for 2 months and subsequently grown in the field at CEPROBI-IPN experimental station in Yautepec, Morelos, México.

Results

Cloning of papaya ACC oxidase fragment

PCR amplification of fruit pulp cDNA, with degenerate primers corresponding to conserved regions of other ACC oxidase genes, yielded a product of the appropriate length (Fig. 1a). Sequencing of the 816 bp product revealed (cCPACCO-1) high identity to a previously reported ACC oxidase gene (López-Gómez et al. 2004). The fragment was cloned between the CaMV 35S promoter and the RUBISCO terminator to yield construct pKYCPACOO-1 (Fig. 1b).

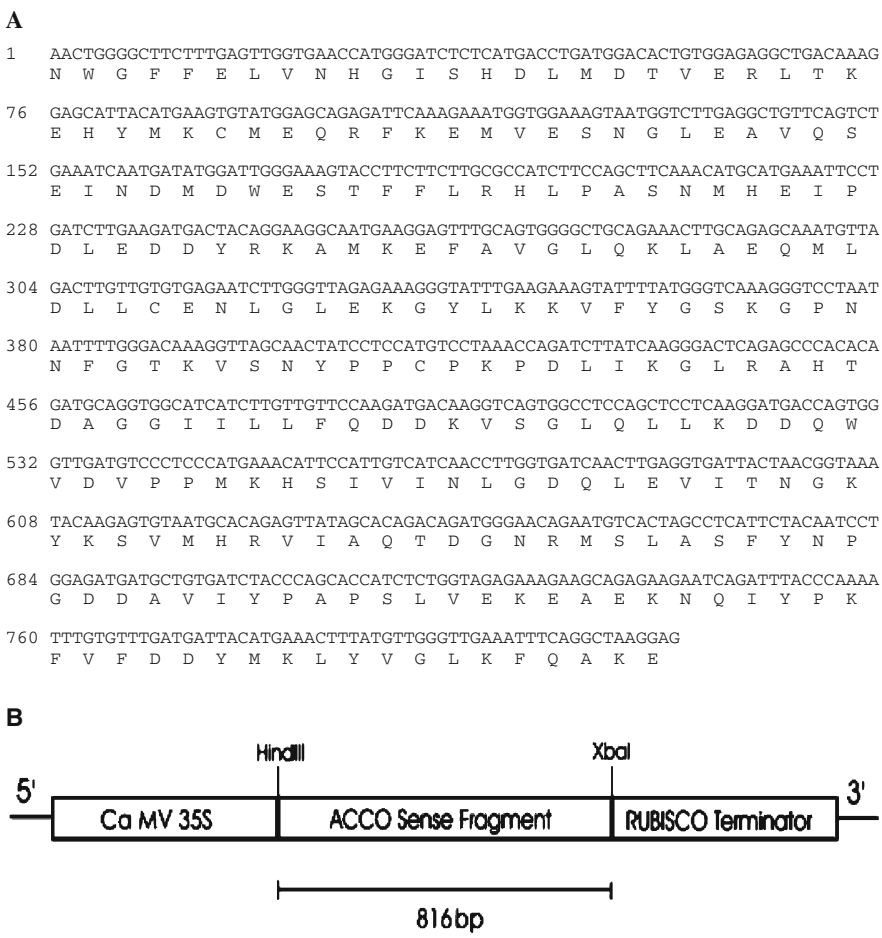

Fig. 1 (**a**) Nucleotide sequence and deduced amino acids of the ACC oxidase fragment obtained by RT-PCR from papaya pulp tissue. (**b**) Map of the construct pKYCPACOO-1 containing the ACC oxidase fragment cloned in PKYLX80 in the sense orientation. The ACC oxidase fragment is flanked by the CaMV 35S promoter and the RUBISCO terminator

Papaya transformation and recovery of transgenic plants

Fifty embryogenic calli resistant to the herbicide were obtained on selective medium, propagated and regenerated into plants. Transgenic shoots regenerated from embryogenic calli were able to grow in sealed, plastic containers where ethylene readily accumulates. Considering that non-transformed calli did not grow in these conditions, this result was a preliminary indication that inhibition of ethylene took place in the transformed shoots, enhancing the capability to grow better in such an environment. Another evidence was based on the fact that the functional unit for PPT resistance was encoded in the plasmid pGPTV-Bar in which the *bar* coding sequence is under the control of the nopaline synthase promoter but the β-glucuronidase gene did not have a promoter. Nevertheless, 40% of the regenerated papaya plants were GUS positive, indicating activity of endogenous promoters. Perhaps, it also could be due to the RUBISCO termination signal, which has been demonstrated to be leaky (Roy et al. 2006).

Analysis of transgenic papayas

Out of twenty papaya plants grown in the field, only 14 plants survived. We extracted DNA from 5 selected transgenic lines (CPACO 4, 5, 6, 12, 13) and used PCR to detect the sense construct. We obtained an amplified product of approximately 800 bp only in the transgenic lines, whereas the control plant did not show any band (Fig. 2).

A Southern blot experiment was performed to estimate the copy number of the trangene insertions in the genome of the recovered plants. Figure 3 illustrates the number of copies of the transgene in the different lines. Multiple insertions of the transgene were evident in most lines, which is a common result when using biolistic for transformation (Fladung 1999).

The transgene did not seem to cause any phenotypic abnormality. Transformed lines produced plants with similar height to the control plants (1.5–2.0 m) and some smaller individuals (0.5–1.0 m high). The weight of transgenic fruits were similar to that of control fruit with about 1.65 kg in average. In control plants, the fruit took between 20–23 weeks to reach the breaker state, however in transgenic fruits we could not observe during this time the typical yellowing of the breaker stage of control papayas.

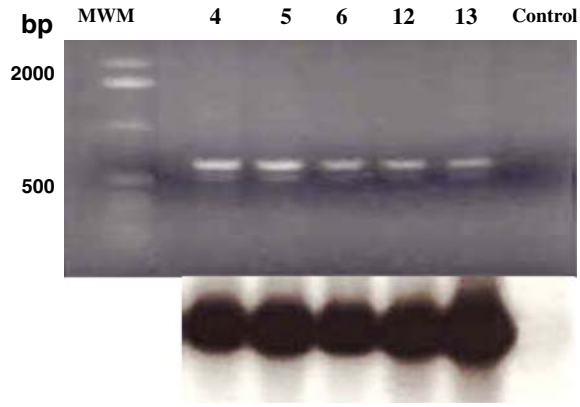

Fig. 2 Hybridization of the PCR product amplified from different papaya transgenic lines. Total DNA was extracted from different transgenic lines (CPACO4, CPACO5, CPACO6, CPACO12 and CPACO13) and analyzed by PCR using the set of primers described in the methods section. A 624 bp fragment was amplified from each line and separated on a 0.8% agarose gel (upper image). The fragments were then transfered to a nylon membrane and hybridized (at high astringency) with the papaya ACC oxidase fragment (CAPACCO-1) as probe (lower image). The numbers at the top refer to the lines examined. MWM, molecular weight markers are indicated on the left. Control, non-transformed plant

For purposes of reproducibility, we employed fruits of the same age for physicochemical analysis. We report analyses of two transgenic lines, CPACO5 and CPACO12. We selected these lines because they showed similar growing patterns to control plants in the field. Under the climate and agronomic practices of Yautepec, Morelos, the plants took about 8 months to grow and produce hermaphrodite flowers.

Etylene and CO_2 production

Ethylene and CO_2 production of transgenic fruits of lines CPACO 5 and 12 compared with non-transformed fruits is shown in Figs. 4 and 5. In transgenic fruits, ethylene production was reduced to about 60% of the normal level (Fig. 4). This result suggests that ethylene production during papaya fruit ripening is a consequence of the expression of more than one gene (Chen et al. 2003), and that CO_2 production is associated to the ethylene production in papaya fruit. On the other hand, control fruits showed the normal climacteric pattern, whereas transgenic

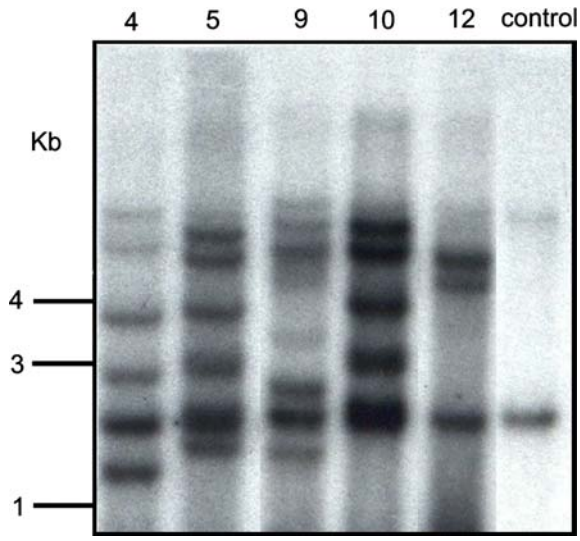

Fig. 3 Southern blot of papaya transgenic lines. Five micrograms of genomic DNA purified from the leaves of transgenic lines 4, 5, 9, 10 and 12 (numbers at the top) and from a non-transformed plant, were digested with Xba-HindIII, fractionated on a 0.8% agarose gel and blotted to a nylon membrane. The membrane was hybridized to the labeled ACC oxidase fragment (CAPACCO-1) at 65°C. Blots were washed at 65°C at high-astringency, and subsequently autoradiographed at −70°C. The numbers on the left indicate molecular weight markers. MWM, molecular weight markers are indicated on the left. Control, non-transformed plant

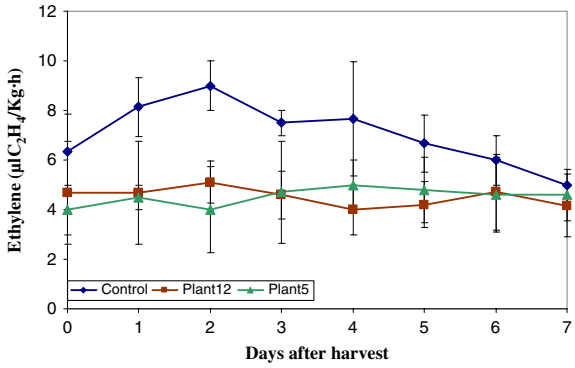

Fig. 4 Ethylene production in papaya transgenic fruits. Papaya transgenic fruits (lines 5 and 12) of the same age as control fruits were collected, weighted daily and placed in closed containers. After 60 min a 1 ml sample was removed from the headspace and analyzed by gas chromatography. The fruits were then removed from the container and incubated at 25°C. The procedure was repeated for 7 days. Each point represents the average of ethylene produced by three fruits

fruits showed no climacteric pattern (Fig. 5). Clearly, ethylene is somehow involved in the climacteric peak in papaya.

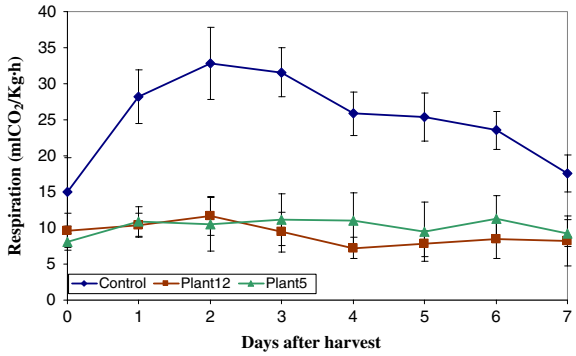

Fig. 5 CO_2 production in papaya transgenic papaya fruits. Fruits from lines 5 and 12 were collected and processed as described in the legend to Fig. 4. CO_2 production was determined as described in the legend of Fig. 4. Each point represents the average of CO_2 produced by three fruits

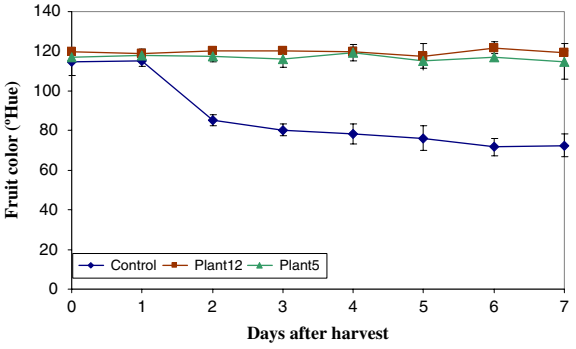

Fig. 6 Changes in peel color of transgenic fruit. The color of the peel was measured with a colorimeter (Color Mate Color Analyzer, Milton Roy, Rochester, N.Y.), using the colour system L*a*b*. Each point represents the average of three fruits

Analysis of fruit

The colour of the skin in transgenic fruits remained green longer than in control fruits (Fig. 6). In addition, transgenic fruits stayed firm much longer than control fruits (Fig. 7). By 15 days post harvest, the colour of the peel was still green and the fruits were still firm. Because of this delayed ripening, fruits were allowed to ripen on the tree for seven additional days but the fruits never seemed to reach the typical yellow color. The peel looked yellowish and thinner than normal but it never reached the full yellow/orange color. The mesocarp stayed firm during all this time and by day 15 the fruits were already being infected by a variety of pathogens and

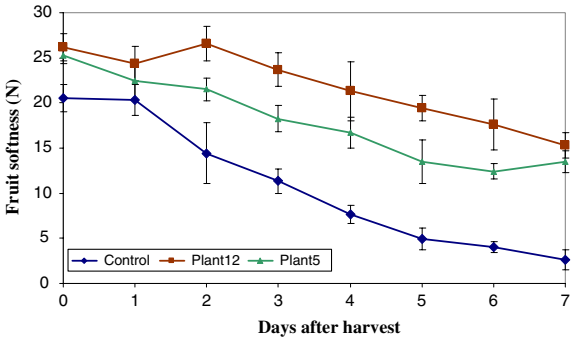

Fig. 7 Changes in flesh firmness of transgenic fruit. Firmness was measured as resistance to penetration (Newton) using a digital texturometer Each point represents the average of firmness of three fruits

the experiment had to be terminated. Overall, the fruits did not ripe after 21 days on the three. Consistent with these results, a drastic reduction in the expression of the ACC oxidase mRNA was detected by northern blot analysis (Fig. 8).

Discussion

In this work, we have generated transgenic papaya plants that constitutively express the ACC oxidase fragment. This fragment reduced ethylene production in fruit. Shoots regenerated from transformed

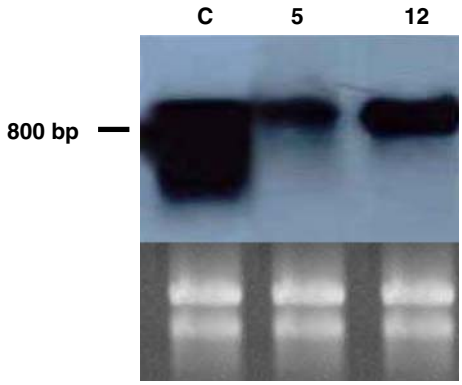

Fig. 8 Expression of ACC oxidase in transgenic fruits. Samples of pulp were taken at the climacteric stage from a non-transformed fruit (C) and from transgenic fruits (5 and 12) and total RNA was extracted. Ten micrograms from each sample were separated on a denaturing gel (bottom panel), and blotted onto nylon membrane and hybridized at 65°C using CAPACCO-1 as probe. Blots were washed at 65°C at high-astringency, and subsequently autoradiographed at −70°C (Top panel). The number on the left indicates the approximate size of the transcript

embryogenic calli were able to grow in sealed containers whereas non-transgenic shoots showed no growth, probably because of the ethylene accumulated inside the container. Transgenic papaya plantlets were able to grow for at least two months in such conditions. When papaya is cultured in vitro for large-scale production, because of the senescence induced by ethylene accumulated in enclosed containers, regeneration and rooting are critical. For several papaya genotypes, it may take from 3–4 months for healthy plants to develop. In our system, well-established transgenic plants were obtained in only two months. Transgenic plants were quite robust in comparison to non transformed plants, and this is a system that probably could be used for large-scale micropropagation of papayas.

Using Southern blot we were able to determine that the transgenic plants contained multiple copies of the transgene, which is an expected result from using biolistics, making the transgenic plants unstable (Fladung 1999). The DNA from a non-transformed plant also presented two hybridizing bands, a faint and a strong one. We performed RT-PCR using papaya RNA as template and the amplified product (ACC oxidase) is present in the papaya genome. We had reported earlier that ACC oxidase may be encoded by more than one gene (López-Gómez et al. 2004). Chen et al. 2003 demonstrated the participation of two ACC oxidase genes during papaya fruit ripening. This may explain the presence of the two bands in our study. We also observed a non climacteric-like behavior in the transgenic fruit and a delayed ripening of the fruit, but we did not detect complete inhibition of the ripening process. Since genetic manipulation does not block ethylene production altogether, it is possible that the remaining ethylene produced by the fruit was enough to induce ripening albeit at a lower rate. Our results suggest that the silenced gene could be associated with the climacteric peaks of ethylene and CO_2. Development of color of the peel and changes in firmess of the pulp were delayed in transgenic fruits and these results suggest that the activity of enzymes involved in these processes are somehow associated with ethylene. Papaya fruits of the cultivars Golden, Gold and Rainbow treated with 1-methylcyclopropene (1-MCP) showed a reduction in the ethylene production and a delayed fruit softness (Fabi et al. 2007, Manenoi et al. 2006). 1-MCP binds to ethylene receptors and strongly inhibits ethylene-mediated fruit ripening in

climacteric fruit (Blankenship and Dole 2003). Our results are in total agreement with those reports. The reduction of ethylene levels obtained by cosupression of ACC oxidase possibly implies that less receptors are binding to ethylene so that fruit ripening is altered. In the case of the 1-MCP effect on papayas although ethylene is present, the number of receptors binding is lower as a consequence of the 1-MCP union to the receptors therefore altering fruit ripening. Our experiments suggest the possible existence of a minimal ethylene concentration necessary for the development of the "normal" papaya ripening program (sensitivity to ethylene). These levels are probably in relation to the abundance of the ethylene receptors in the different cultivars of apple fruit as has been suggested by Tatsuki and Endo (2006). We observed a marked wrinkling of the peel of transgenic fruits with time, probably related with a loss of turgor of the cells and after 15 days after harvest, even though the pulp still presented some firmness, the peel was thinner than the nontransformed fruits. This observation suggests that the process of ripening probably involves different mechanisms in pulp and peel and that ethylene could affect each tissue differently. Alternatively, it could be that there are processes in the papaya ripening program that are independent of ethylene as has been shown in tomato, melon and apple (Jeffery et al. 1984; Hadfield et al. 2000; Pech et al. 2002; Schaffer et al. 2007). We also noticed that senescence of leaves of transgenic plants was altered. Leaves of transgenic papaya plants did not abscise as early as leaves from non-transformed plants (data no shown). This was similar to what occurs in transgenic tomato (Picton et al. 1993). The papaya plants were grown in the field and stayed healthy all the time. This was important to establish, since ethylene plays a central role in plant metabolism and reduction of its levels might produce deleterious effects in the plants. Papaya is a fruit that normally ripens either on or off the tree. Our transgenic fruits did not reach full maturity even after being 15 days off the tree. By then, the fruits looked dehydrated and the experiment was discontinued. Leaving the fruits on the tree for up to 21 days had no apparent effect on fruit ripening. Fruit were still unripe and showed significant decay.

Taken together, these results suggest that papaya fruit is very sensitive to ethylene and that a reduction in ethylene levels could dramatically affect the progress of ripening. The northern blot results suggests that cosuppression of ACC oxidase does not inhibit totally the mRNA for these genes. This result could be consequence of the participation of the two genes in the papaya ripening or to PTGS mechanisms (Hammond et al. 2001). Our work suggests that ethylene is a critical regulator of papaya fruit ripening and that expression of more than one member of ACC oxidase is necessary for fruit ripening. This is the first report of papaya delayed fruit ripening by cosuppression. In spite of the reduction of ethylene production in transgenic papayas, the fruit did not stay firm as long as the transgenic tomatoes (4 to 5 months after harvest) produced using a similar approach (Klee et al. 1991; Xiong et al. 2005; Oeller et al. 1991; Picton et al. 1993). This suggests that the ripening program in papaya may be different to that in tomato and that that role of ethylene in the ripening of these two fruit may not be quite the same. Although the inhibition of fruit ripening in papaya may have potential commercial application, more studies are necessary to make sure that the inhibition of ethylene evolution does not impair papaya fruit quality.

References

Alexander L, Grierson D (2002) Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. J Exp Bot 53(277):2039–2055

Ausubel FM, Brent R, Kingston RE, More DD, Seidman JG, Smith JA, Sthul K (1991) Current protocols in molecular biology. Greene Publishing Associates Inc. and Willey and Sons Inc, USA, pp 5.3.2–5.3.8

Ayub R, Guis M, Ben Amor M, Guillot L, Roustan JP, Latché Bouzayen AM, Pech JC (1996) Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. Nat Biotechnol 14:862–866

Barry CS, Giovannoni JJ (2007) Ethylene and fruit ripening. J Plant Growth Regul 26:143–159

Becker D, Kemper E, Schell J, Materson R (1992) New plant binary vectors with selectable markers located proximal to the left T-DNA border. Plant Mol Biol 20(6):1195–1197

Blankenship SM, Dole JM (2003) 1-Methylcyclopropane: a review. Postharvest Biol Technol 28:1–25

Cabrera-Ponce JL, Vegas A, Herrera-Estrella L (1995) Herbicide resistant trangenic papaya plants produced by and efficient particle bombardement transformation methods. Plant Cell Rep 15:1–7

Cabrera-Ponce JL, López L, Assad-García N, Medina-Arevalo C, Bailey AM, Herrera-Estrella L (1997) An efficient particle bombardment system for the genetic transformation of asparagus (*Asparagus officinalis* L.). Plant Cell Rep 16:255–260

Capellini RA, Ceponis MJ, Lighter GW (1998) Disorders in apricot and papaya shipment to New York market 1972–1985. Plant Dis 72:366–368

Chan HT, Couey HM, Sakay WS (1990) Distribution of the ethylene forming enzyme in ripening *Carica papaya.* Proceedings of the 7th World Congress of Food Science & Technology, Singapore, September 29, 1987

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

de Carbalho- Nievel F, Frendo P, Inzé D, Cornelissen M, Van Montagu M (1995) Co-suppression of β-1, 3-glucanase genes in *Nicotiana tabacum*. Curr Top Microbiol Immunol 197:91–103

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparations: version II. Plant Mol Biol 1:19–21

Fabi JP, Cordenunsi BR, s Barreto GP, Mercadante AZ, Lajolo FM, Nascimento OliveiraDo (2007) Papaya fruti ripening: Response to ethylene and 1-Methylcyclopropene (1-MCP). J Agric Food Chem 55:6118–6123

FAO (2006) http://faostat.fao.org/site/408/DesktopDefault.aspx?PageID = 408

Fladung M (1999) Gene stability in transgenic aspen (Populus). I. Flanking DNA sequences and T-DNA structure. Mol Gen Genet 260:574–581

Hadfield KA, Dang T, Guis M, Pech JC, Bouzayen M, Bennett AB (2000) Characterization of ripening-regulated cDNAS and their expression in ethylene-suppresed Charentais melon fruit. Plant Physiol 122:977–983

Hammond SM, Caudy AA, Hannon GJ (2001) Post-transcriptional gene silencing by double-stranded RNA. Nat Rev Genet 2:110–119

Hofman NE, Yang SF (1980) Changes of 1-amino-cyclopropane-1-carboxylic acid content in ripening fruits in relation to their ethylene production rates. J Am Soc Hortic Sci 105:492–495

Jeffery D, Smith C, Goodenough P, Prosser I, Grierson D (1984) Ethylene-independent and ethylene-dependent biochemical changes in ripening tomatoes. Plant Physiol 74:32–38

Jiang Y, Fu J (2000) Ethylene regulation of fruit ripening: molecular aspects. Plant Growth Regul 30:193–200

Klee HJ, Hayford MB, Kretzmer KA, Barry GF, Kishore GM (1991) Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. Plant Cell 3:1187–1193

López-Gómez R, Gómez-Lim MA (1992) A method for extracting intact RNA from fruits rich in polysaccharides using ripe mango mesocarp. Hort Science 27(5):440–442

López-Gómez R, Campbell A, Dong JG, Yang SF, Gómez-Lim MA (1997) Ethylene biosythesis in banana fruit: isolation of a genomic clone to ACC Oxidase and expression studies. Plant Sci 123:123–131

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(4):794–800

Mahmoud SS, Williams M, Croteau R (2004) Cosuppression of limonene–3-hydroxylase in peppermint promotes accumulation of limonene in the essential oil. Phytochemistry 65:547–554

Manenoi A, Bayogan ERV, Thumdee S, Paull RE (2006) Utility of 1-methylcyclopropene as papaya postharvest treatment. Postharvest Biol Technol 44:55–62

Mason MG, Botella JR (1997) Identification and characterisation of two 1-Aminocyclopropane-1-carboxylate (ACC) synthase cDNAs expressed during papaya (*Carica papaya*) fruit ripening. Aust J Plant Physiol 24:239–244

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of chimeric Chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. Plant Cell 2:279–289

Oeller PW, Min-Wong L, Taylor LP, Pike DA, Theologis A (1991) Reversible inhibition of tomato fruit senescence by antisense RNA. Science 5(254):437–439

Paull RE (1993) Pineaple and papaya. In: Seymour G, Taylor J, Tucker G (eds) Biochemistry of fruit ripening. Chapman and Hall, London, pp 291–323

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

Pech JC, El-Yahyaoui F, Bernadac A, Flores B, Romojaro F, Latché A, De Billerbeck G, Ambid C, Bower J, Holford P (2002) Role of ethylene on various ripening pathways and on the development of sensory quality of charentais Cantaloupe melons. In: Nishimura S et al (ed) Proceedings of 2nd IS on Cucurbits. Acta Hort (ISHS) 588:303–307

Picton S, Barton SL, Bouzayen M, Hamilton AJ, Grierson D (1993) Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. Plant J 3(3):469–481

Roy R, Purty RS, Agrawal V, Gupta SC (2006) Promotorless *gus* gene shows leaky β-glucuronidase activity during transformation of tomato with *bspA* gene for drought tolerance. Biol Plant 50(3):352–358

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning a laboratory manual*, Second Edition edn. Cold Spring Harbor laboratory press, USA, pp 7.43–7.45

Schaffer RJ, Friel EN, Souleyre EJF, Bolitho K, Thodey K, Ledger S, Bowen JH, Ma JH, Nain B, Cohen D, Gleave AP, Crowhurst RN, Janssen BJ, Yao JL, Newcomb RD (2007) A genomic approach reveals that aroma production in apple is controlled by ethylene predominantly at the final step in each biosynthetic pathway. Plant Physiol 144:1899–1912

Schardl CL, Byrd AD, Benzion G, Altschuler MA, Hildebrand DF, Hunt AG (1987) Design and construction of a versatile system for the expression of foreign genes in plants. Gene 61:1–11

Tatsuki M, Endo A (2006) Analyses of expression patterns of ethylene receptor genes in apple (*Malus domestica* Borkh.) fruits treated with or without 1-Methylcyclopropene (1-MCP). J Japan Soc Hort Sci 75(6):481–487

Xiong AS, Yao QH, Peng RH, Li X, Han PL, Fan HQ (2005) Different effects on ACC oxidase gene silencing triggered by RNA interference in transgenic tomato. Plant Cell Rep 23:639–646

Yu O, Shi J, Hession AO, Maxwell CA, McGonigle B, Odell JT (2003) Metabolic engineering to increase isoflavone biosynthesis in soybean seed. Phytochemistry 63:753–763