The effects of ACS (1-aminocyclopropane-1-carboxylate synthase) gene down regulation on ethylene production and fruit softening in transgenic apple

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Summary: A detailed examination of the production of ethylene and other ripening parameters during storage period has been undertaken in transgenic apple fruits, where the ethylene biosynthesis was inhibited by antisense ACS (1-aminocyclopropane-1-carboxylate synthase) gene. Data indicate down regulation of ethylene production, softening and spoilage in some transgenic lines. In some cases ethylene production was inhibited for over 90 percent, considerable reduction of softening and spoilage was observed probably due to the reduced activity of cell wall degradable enzymes. ACS activity was also monitored during ripening. The fruits of the best transgenic lines could be stored for minimum 4–5 months longer under 5 °C cold room storage conditions and one month longer at normal room temperature. This molecular approach can provide an alternative way to replace the commonly used and costly atmospheric storage of fruits.

Key words: transgenic apple, ethylene production, softening, ACS expression, storage

Introduction

Ethylene plays an important role in plant development including fruit ripening. Climacteric fruits such as apples, tomatoes, bananas, pears, peaches, cucurbits undergo a burst of respiration accompanied by marked changes in composition and texture. In this group of fruits a sharp increase in ethylene production is observed and ripening can also be induced by the treatment with exogenous ethylene. The clarification of this autocatalytic mechanism and the biosynthesis of ethylene *(Yang & Hoffman,* 1984) and the possibility of inhibition of gene expression by antisense RNA *(Ecker & Davis,* 1986) offer a unique opportunity in higher plants: to block the steps of ethylene biosynthesis by transformation of the appropriate antisense gene(s). Using of antisense RNA and DNA the physiological processes in plants can be deliberately manipulated. This technique uses natural genes or gene-segments of plants but in antisense orientation, which consequently blocks the protein production of this gene. As a result, we can control and modify the known metabolic pathways. The two key enzymes in the ethylene biosynthetic pathway are ACS (1-aminocyclopropane-1-carboxylate-synthase),which catalyzes the synthesis of ACC (aminocyclopropane-carboxylate) from S-adenosyl methionine, and ACC-oxidase, which catalyzes the conversion of ACC to ethylene *(Yang & Hoffman,* 1984). Genes coding for ACS have been cloned and sequenced from more than 40 species including apple *(Dong* et al., 1991; *Lay-Yee & Knighton,* 1995; *Rosenfield* et al., 1996; *Harada* et al., 1997; *Sunako* et al., 2000). The first successful cloning of genes involved in ethylene biosynthesis occured in tomato *(Oeller* et al., 1991) and made possible the construction of ripening mutants using reverse genetics for experimentally testing the various theories of fruit ripening *(Gray* et al., 1992; *Theologis,* 1992; *Theologis* et al., 1993). Beside their scientific value, these results have great practical importance as they both permit the extension of the storage life of fruits and decrease the cost of storage. Based on the results with tomatoes, research was initiated with apples in order to delay post-harvest ripening and extend storage life of fruits through transformation with the antisense ACS gene *(Hrazdina,* 1993, *Hrazdina* et al., 2000).

Ripening specific ACS gene (MdACS2-acc. No.: U73815) was isolated from McIntosh apple *(Rosenfield* et al., 1996) and was introduced in antisense orientation into the pBI 121 binary vector replaced the *gusA* gene. This construct was inserted into the T-DNA of *Agrobacterium tumefaciens* strain LBA 4404 then Royal Gala and McIntosh leaf segments were transformed in 1995 (in H.S. Aldwinckle's laboratory – Cornell University, Geneva Campus) using *Agrobacterium*-mediated transformation system *(Norelli* et al., 1994). Transgenic plants were propagated on kanamycin containing agar medium, transferred to the greenhouse for conditioning and then to the field in 1996 and 1997.

The goal of our research was to investigate the effect of ethylene down regulation on the process of post-harvest ripening in the fruits of transgenic trees.

Material and method

Plant material: Fruit samples were collected in the transgenic orchard (Cornell University, Geneva Campus) at the beginning of September 2001 from 90 Royal Gala (RG) and 58 McIntosh transgenic and several control trees. These samples were stored at normal room temperature (22 °C) and cold room (5 °C), respectively. Three fruits from each trees (lines) were placed in plastic jars (in the case of cold storage jars were allowed to come to room temperature), then jars were sealed for 24 hours before ethylene measurement. Ethylene production was at least monthly measured in each transgenic and control line during the whole storing period, over 7 months.

Detailed experiments were carried out with TG 196, TG 197, TG 198 selected transgenic- and two non-transformed control RG trees. Fifteen apples (3–3 fruits in 5 replicates) from each tree were stored at room temperature and ethylene production was monitored for 8 weeks on every second day after harvest. One apple was removed from each line after every measurement for 26 days and other ripening parameters were measured, than cortical tissues were stored at –80 °C for further experiments. Measurement of ethylene production was followed with the remaining 2–2 apples for 8 weeks after harvest.

Ethylene production: After 24 hours storing at room temperature 1 ml gas were removed from the sealed jars by syringe and ethylene growth was measured by gas chromatography (SRI 8610 equipped with 2 mm X 90 cm silica gel column) calibrated with external ethylene standards. The temperature of the column was maintained at 80 °C and nitrogen was used as carrier gas. Analysis of variance was performed and the least significant differences calculated, using the general linear model procedure in SPSS statistical software version 8.0.

Brix and fruit firmness: Brix was determined by AO ABBE refractometer. The Brix refractometer value shows the concentration percentage of the total soluble solids content as total of all the solids dissolved in the fruit juice (sugar, salts, protein, acids, etc.). Fruit firmness was measured in Newton with QA FT327 hand penetrometer then cortical tissue samples were frozen in liquid N_2 and stored in deepfreezer until RNA extraction.

RNA isolation: A combination of *Lopez-Gomez* et al., 1992 and *Franke* et al. 1995 methods was used with minor modifications. Total RNA was isolated from frozen cortical tissues (7.5 g) by ground to fine powder in liquid N_2 and suspended in 15 ml RNA extraction buffer (150 mM Tris, 50 mM EDTA, 2% SDS, pH was adjusted to 7.5 with 1 M boric acid) containing 1% of 2-mercaptoethanol. The homogenate was incubated at 65 °C for 5 min, then vortexed with 0.25 volumes of 100% ethanol and 0.11 volume 5 M potassium acetate. It was extracted with the same volume of chloroform/isoamylalcohol (49:1) and centrifuged at 15000 g for 15 min at 4 °C. The aqueous phase was extracted with the same volume of phenol/chloroform (1:1) and then again chloroform/isoamylalcohol (49:1). RNA was precipitated in 0.6 volumes 8 M LiCl at –20 °C overnight and was collected by centrifugation at 15000 g for 90 min at 4 °C and washed with 75% ethanol. The RNA was resuspended in 400 µl sterile water and precipitated in 25.2 µl 5 M potassium acetate and 1 ml 100% ethanol at –20 °C overnight. The RNA was collected by centrifugation at 15000 g for 15 min at 4 °C and washed with 75% ethanol. RNA was resuspended in 300 µl DEPC-treated water and incubated with 150 µl 100% ethanol on ice for 30 min then centrifuged at 15000 g for 10 min at 4 °C. Finally, the RNA in the supernate was precipitated in 0.1 volume 3 M sodium acetate (pH: 5.2) and 2.5 volume 100% ethanol. The pellet was air-dried and resuspended in 200 µl DEPC-treated water.

Northern analysis: Samples (15 µg RNA) from each transgenic line were denatured and fractionated on 1% agarose gel containing 2.2 M formaldehyde and transferred to Hybond N^+ membrane (Amersham) by capillary transfer in 20X SSC overnight. Prehybridization was carried out in ∪ 2 ml/10 cm^2 of membrane of 5X SSC, 5X Denhardt solution, 1% SDS, 50% formamide, 10% dextran-sulfate, 100 µg/µl sonicated and denatured salmon sperm DNA solution for 3.5 hours at 42 °C. The membrane was hybridized with (^{32}P)-labelled cDNA probes encoding of 1094 bp MdACS2 cDNA at 42 °C overnight. Probes were random-prime labelled using the Prime-a-Gene System (Promega) labelling kit. After hybridization the blot was washed twice at room temperature with 0.2X SSC/0.1% SDS for 5 min, twice at 42 °C 0.2 X SSC/0.1% SDS for 15 min and twice at 50 °C with 0.1X SSC/0.1% SDS for 15 min. Membranes were exposed to X-ray film (Kodak) at –80 °C for 5 days.

Southern analysis: DNA was extracted from young leaf tissues by the method of *Dellaporta* et al. (1983) and Southern analysis was performed using standard procedures (*Sambrook* at al. 1989). A fragment containing sequences from the CaMV35S promoter and the MdACS2 gene was used as a probe, labelled with ^{32}P using random primers.

ACS Extraction and Activity assay: A combination of *Lizada & Yang* (1979) and *Yip* et al. (1991) methods was used with minor modifications. The fruits were transferred to 0 °C and stored for three days before extraction. 25 g of cortical tissues were homogenized with equal volume of homogenizing buffer: 400 mM potassium phosphate buffer (pH:8.5), 1 mM EDTA, 10 µM pyridoxal phosphate and 0.5% 2-mercaptoethanol then squeezed through of two layers of Miracle cloths. The pellet fraction was collected by 15000 g centrifugation for 30 min and resuspended in 5 ml of 20 mM potassium phosphate buffer (pH:8.5), 1 mM EDTA, 10 µM pyridoxal phosphate, 30% glycerol and 1 mM 2-mercaptoethanol. The same volume of solubilization buffer was added with stirring, which contains the same buffer supplemented with 0.2% Triton X-100.

After incubation for 30 min it was centrifuged at 15000g for 20 min and the resulting supernatant contained ACS.

ACS extracts 0.2 ml from each sample were assayed in 12X75 mm test tubes, added 200 μM S-adenosyl-methionine, 10 μM pyridoxal 5`-phosphate, 50 mM Hepes-KOH buffer (pH:8.5), 1 μM $HgCl_2$ and the volume was brought up to 900 μl with water. The vessels were sealed with rubber serum stoppers and kept on ice. 100 μl of cold mixture of 5% NaOCl and saturated NaOH (2:1) was injected through the stoppers then the flasks were vortexed for 5 sec and kept on ice for 2.5 min. The mixtures were then agitated for another 5 sec prior to gas sampling (1ml). Ethylene was assayed on gas chromatograph in which the column was maintained at 80 °C.

Results and discussion

General evaluation of transgenic trees was carried out in 90 Royal Gala and 58 McIntosh lines. Among McIntosh lines some fruits showed lower ethylene production with 30–40% inhibition, but there was no significant difference in ACS activity as compared to controls (data not shown). In case of Royal Gala eight trees, were found where 60–97% inhibition was observed accompanied with low ACS activity values (*Table 1*). Most of the lines showed no differences compared to controls while in some lines higher ethylene production was observed. The variance between the transgenic lines probably due to the different copy numbers of integrated transgene, positional effect or different regulation and inhibition of individual ripening specific genes.

Table 1 Ripening parameters of the best transgenic lines at cold storage on 37th day in 2001

	Transgenic line	C_2H_2 (nmol/100g/h)	Soluble solids (°Brix)	Firmness (Newton)	ACS activity (nmol/mg prot/15min)
2001	Control Royal Gala	230.3	16.4	86.2	24.5
	TG 196	70.21	16.3	95.3	11.1
	TG 197	8.11	15.0	98.7	2.9
	TG 198	91.37	16.2	90.72	13.1
	TG 233	95.75	16.5	69.2	17.9
	TG 236	15.37	17.0	94.1	9.8
	TG 507	34.74	20.0	72.6	4.3
	TG 508	11.52	17.8	105.5	0.9
	TG 508	6.64	22.8	111.1	1.3

Ethylene production during storage at room temperature

Sharp increase of ethylene production was observed in control fruits. The initial period (2 weeks) was rather exponential than linear (*Figure 1/a*). At the peak of ethylene production exceed 200 nmol/100g/h. Ethylene production began to decline after 3 weeks in a prolonged silencing way with small recrudescences. Similar ethylene curve was published by *Song* et al. 1996, where internal ethylene level of apples was followed in room temperature after harvest. In their work waving of internal ethylene level after the peak period was more conspicouos probably due to the activity of different ripening specific ACS genes.

The delay of onset of ethylene synthesis was 1–4 weeks in transgenic lines. Exponential then linear raise was also observed, however the peak of ethylene level remained lower (*Figure 1/a*). (Parameters of TG 198 tree were absolutely the same as TG 196 in this experiment therefore its data not shown.) Those fruits which were harvested about 4 weeks ahead of their optimal harvest time *(Song* et al.) showed a very similar pattern of ethylene production to our TG 197 transgenic line. Antisense fruits of TG 197 tree did not show the respiratory burst even when they were stored for 1 month at room temperature. Later sharp increase of ethylene production was observed in these fruits too, but the peak was only 1/3 of the controls. Since our fruits were harvested from the same orchard at the optimal time shows that the initiation of autocatalytic ethylene production was delayed in our best transgenic lines.

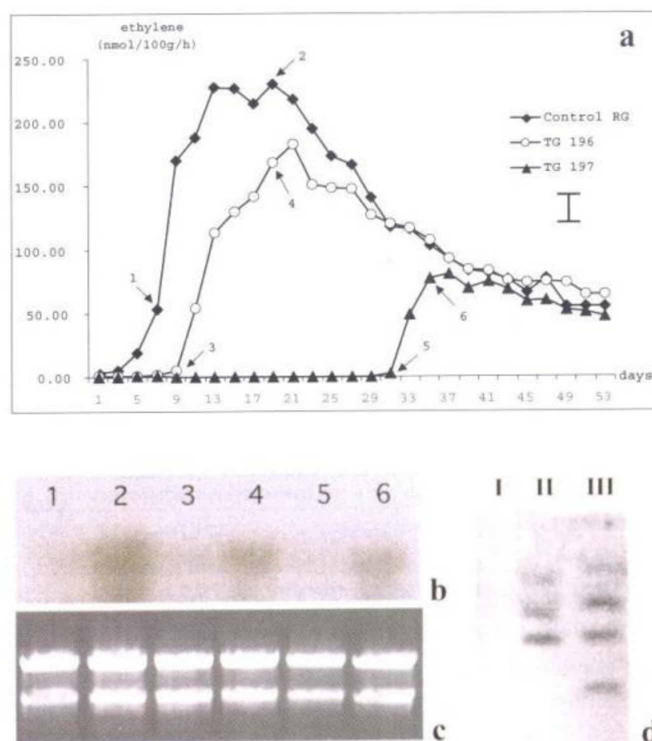

Figure 1 ***(a)*** Ethylene production of selected transgenic and control fruits stored at room temperature. Treatment means (n = 5) and the least significant difference (5%) are shown. Cortical tissue from fruits at indicated stages (1–6) was taken for RNA extraction ***(b):*** Expression of MdACS2 transgene in genotypes tested. Total RNA (15 μg) was hybridized with P^{32} labelled DNA probe specific for MdACS2 (U73815). Numbers 1–6 mean the samples taken from transgenic and control fruits during storage indicated by arrows on *Fig. 1/a*. ***(c):*** rRNA loading control. ***(d):*** Copy number of antisense gene integrated in transgenic lines tested. Southern blot of DNA isolated from control (I), TG 196 (II) and TG 197 (III), probed with a labelled fragment of CaMV 35S promoter and MdACS2 gene.

Expression of MdACS2

Expression of MdACS2 gene was followed from early stage of fruit development to ripening by Northern analysis using ^{32}P labelled MdACS2 probe. There were not visible hybridization patterns during fruit development till the harvest. The first positive reaction was observed at the second day of storage in control fruits shown the initial accumulation of ACS mRNA and the ascent of ethylene production.

Levels of ACS mRNA's characterized by Northern analysis in 2 stages of ripening were presented in TG 196, TG 197 transgenic and control lines (*Figure 1/b*). Samples were taken from the beginning and near to the peak of ethylene production from each line. Maximum expression of ACS in control fruits (lane2) was significantly higher than in TG 196 (lane4) and TG 197 (lane 6) transgenic trees. This observation absolutely coincides with the measured ethylene production of apples at these times (*Figure 1/a*).

Copy number of integrated transgenes

Getting wider spectrum of gene inhibition and reduction of ethylene production higher numbers of independetly transformed antisense plants must be used (*Picton* et al., 1993). It has also been reported previously that increasing the antisense gene dosage enhances the severity of the antisense phenotype (*Hamilton* et al., 1990) and position of integration may be critically important (*Stockhaus* et al., 1990). To check the gene dosage in our best transgenic lines Southern analysis was performed using a segment of the CaMV 35S promoter and MdACS2 gene as probe (*Figure 1/d*). Suprisingly 3–4 copies of antisense genes per homozygote were introduced. In tomato where, ethylene synthesis were inhibited to 5% of normal by antisense ACC-oxidase gene 2 antisense genes were estimated (*Hamilton* et al., 1990) while using antisense ACC-synthase where the reduction in ethylene production was 99.5%, 10 copies of antisense genes were estimated per homozygote. Presumably both the gene dosage and the good position of transgene in the genome play an important role in forming of antisense phenotype.

Ethylene production during storage in cold room

In case of cold storage fruits of transgenic trees showed low ethylene level approximately for 4 months. Following measurements revealed growth in ethylene production (except of TG 508) and after 5 months approached, then exceed of the control, where descending tendency was observed by this time (*Figure 2*). There was no fruit production of TG 508 transgenic line in 2000 that is why it is not presented in the more detailed experiments in room temperature.

One month later remarkable production of ethylene was traceable only in transgenic fruits while the controls had already 'burnt out'. Presumably the accumulation of ACS mRNA starts up the autocatalytic mechanism of ethylene biosynthesis in TG 196 and TG 197 transgenic lines.

There was no remarkable ethylene production observed in TG 508 during the whole storage period. After 6 months of storage all fruits were placed to room temperature and ethylene production was followed every second day (*Figure 2*). Ethylene production was dropped drastically in each tested lines except in TG 508.

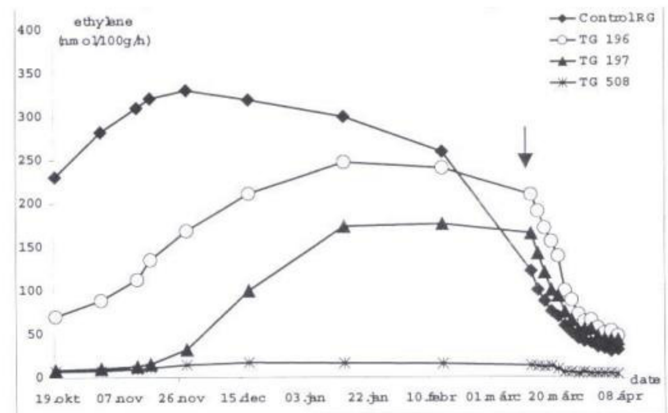

Figure 2 Ethylene production in fruits of selected transgenic and control lines at 5 °C for 6 months. Arrow indicates time of transfer to room temperature

Changing of firmness and total soluble solids during ripening

In control fruits ethylene curve was risen only for three weeks at room temperature then ethylene production was dropped in a prolonged way. At the peak time the firmness of fruits had already been reduced by more than 50% and contents of soluble solids increased (*Figure 3 a/b*).

Antisense fruits of TG 197 tree did not soften at all. Nevertheless the Brix values approached the level of controls at the end of this period indicating not too much difference between their sugar contents. Dynamics of ripening and softening of TG 196 fruits was similar to the control with moderate ethylene peak. There was only a slight difference between their soluble solids, but the reduction of fruit-flesh firmness was delayed in TG 196 transgenic fruits. The variance among fruits from the same tree is conspicuously high since it is naturally hard to find apples in the same ripening stage even on a single tree. That is why only the main trends were signed by linear regression (*Figure 3*).

Antisense fruits showed a marked increase in resistance to shrivelling, splitting and post-harvest spoilage following extended storage at room temperature (*Figure 4*).

This can be explained by the reduced activity of cell wall degradable and ethylene controlled enzymes such as polygalacturonase, pectin methyl esterase, cellulase and hemicellulase due to the lower level of ripening induced ethylene.

'Knock out' transgenic genotypes

Despite of low ethylene production, TG 507 and TG 508 transgenic lines showed the highest Brix and firmness

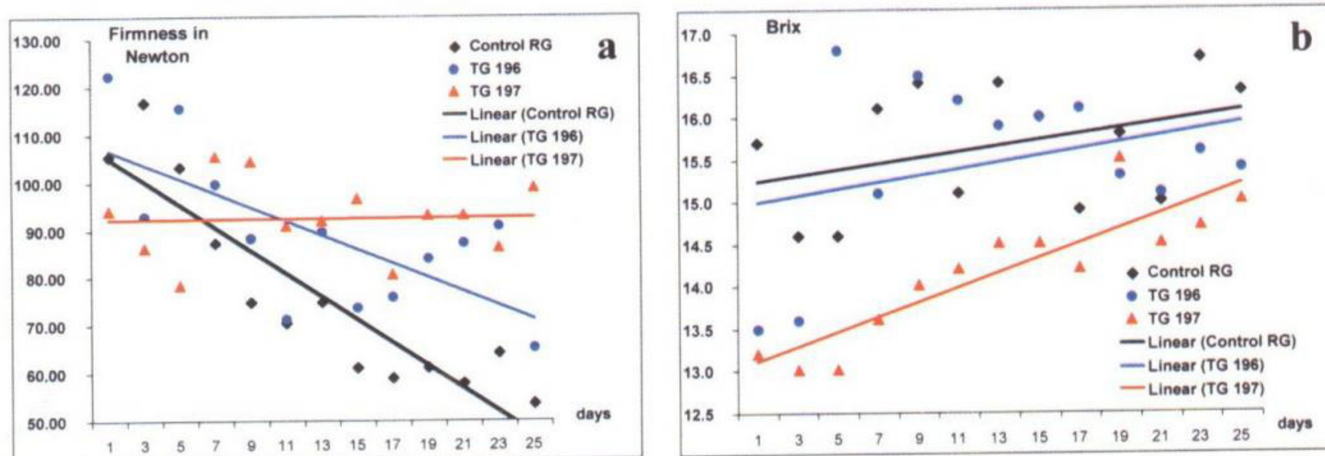

Figure 3 Firmness (**a**) and Brix (**b**) values in selected transgenic and control lines stored at room temperature

Figure 4 Transgenic and control apples stored 90 days at room temperature

values. Changes in morphology were also observed in these fruits (*Figure 5*). Fruits remained smaller with a berry shape, the stem became smaller and thicker and it was hard to separate from fruits. The fruitflesh was still extremely hard but the most delicious with high sugar content and concentrate flavour. Supposedly the lack of ethylene production caused also inhibition in expansion of cells during fruit development. Alternatively, the integration of transgene into the genome ruined gene(s) plays important role in cell expansion. Following examinations of TG 508 transgenic line may provide additional information of physiology and biology of fruit ripening since this line should be considered as 'knock out' genotype for ripening specific ethylene production.

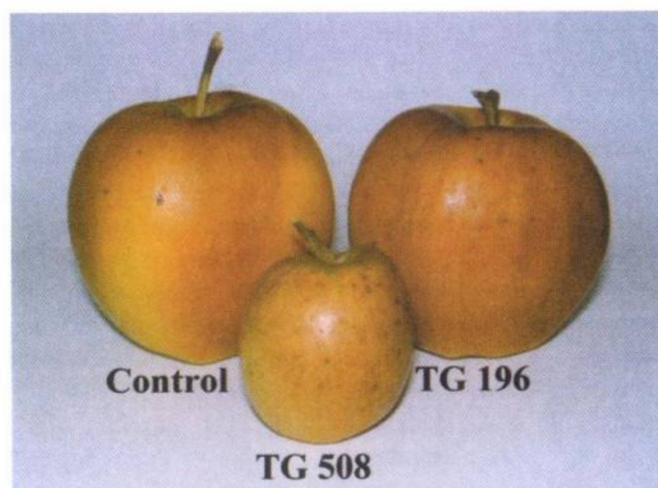

Figure 5 Fruit morphology of TG 508 'knock out' transgenic genotype

The presented data shows down regulation of ethylene production, softening and spoilage in some transgenic Royal Gala lines. The MdACS2 gene as a member of the ACS gene family shows ripening specific expression as it has been proven by Northern analysis. Its mRNA activity has close correlation with the ethylene production of apples. In those transgenic lines, where the greatest inhibition of ethylene production was achieved, the transgene was introduced in 3-4 copies at least, indicating a possible correlation between the gene dosage and antisense phenotype. Upon the detailed examinations at room temperature and cold room support our conception is, that ethylene down-regulated fruits can be stored for few months longer in the same quality without using the costly controlled atmospheric storage.

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References

Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983): A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter 1: 19–21

Dong, J. G., Kim, W. T., Jip, W. K., Thompson, G. A., Li, L., Bennett, A. B. & Yang S. F. (1991): Cloning of a cDNA encoding 1-aminocyclopropane-1-carboxylate synthase and expression of its mRNA in ripening apple fruit. Planta 183: 38–45.

Ecker, J. R. & Davis R. W. (1986): Inhibition of gene expression in plant cells by expression of antisense RNA. Proc Natl Acad Sci USA 83: 5372–5376.

Franke, K. E., Liu Y. & Adams D. O. (1995): Yield and quality of RNA from grape berries at different developmental stages. Am. J. Enol. Vitic. 46: 315–318

Gray, J., Picton, S., Shabbeer, J., Schuch, W. & Grierson, D. (1992): Molecular biology of fruit ripening and its manipulation with antisense genes. Plant Mol Biol 19:69-87.

Hamilton, A. J., Lycett, G. W. & Grierson D. (1990): Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature 346: 284–287

Harada,T., Sunako,T., Sakuraba, W., Goto, S., Akada, S., Senda, M., Ishikawa, R. & Niizeki, M. (1997): Genomic nucleotide sequence of a ripening-related 1-aminocyclopropane-1-carboxylate synthase gene (MdACS-1). Plant Physiology 113: 1465

Hrazdina, G. (1993): Time for new hard apples. Hung. Agr. Res. 2: 4–7.

Hrazdina, G., Kiss, E., Rosenfield, C., Norelli, J. & Aldwinckle, H. (2000): Down regulation of ethylene production in apples. In: Use of Agriculturally Important Genes in Biotechnology, G. Hrazdina ed. NATO Advanced Research Workshop, IOS Press Amsterdam, pp. 26–31

Lay-Yee, M. & Knighton, M. (1995): A full-length cDNA encoding 1-aminocyclopropane-1-carboxylate synthase from apple. Plant Physiol 107: 1017–1018.

Lizada, M. C. C. & Yang, S. F. (1979): A simple and sensitive assay for 1-aminocyclopropane-1-carboxilic acid. Analytical Biochemistry 100: 140–145

Lopez-Gomez, R. & Gomez-Lim, M. A. (1992): A method for extracting intact RNA from fruits rich in polysaccharides using ripe mango mesocarp. Hortscience 27: 440–442

Norelli, J. L., Aldwinckle, H. S., Beltran, L. D. & Jaynes, J. M. (1994): Transgenic 'Malling 26' apple expressing the attacin E gene has increased resistance to *Erwinia amylovora*. Euphytica 77: 123–128

Oeller, P. W., Wong, L. M., Taylor, L. P., Pike, D. A. & Theologis, A. (1991): Reversible inhibition of tomato fruit senescence by antisense RNA. Science 254:437–439.

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

Rosenfield, C. L., Kiss, E. & Hrazdina, G. (1996): Md-ACS2 and Md-ACS3: Two new 1-aminocyclopropane-1-carboxylate synthases in ripening apple fruit. Plant Gene Register PGR96-122. Plant Physiology 112: 1735

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989): Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory press, Cold Spring Harbor, NY

Song, J. & Bangerth, F. (1996): The effect of harvest date on aroma compound production from 'Golden Delicious' apple fruit and relationship to respiration and ethylene production. Postharvest Biology and Technology 8: 259–269

Stockhaus, J., Höfer, M., Renger, G., Westoff, P., Wydrzynski, T. & Willmitzer, L. (1990): Anti-sense RNA efficiently inhibits formation of the 10 kd polypeptide of photosystem II in transgenic potato plants: analysis of the role of the 10 kd protein. EMBO J. 9: 3013–3021

Sunako, T., Ishikawa, R., Senda, M., Akada, S., Niizeki, M. & Harada, T. (2000): MdACS-5A and 5B, two wound-responsive genes encoding 1-aminocyclopropane-1-carboxylate synthase in apple. Plant Physiology 122: 620

Theologis, A. (1992): One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. Cell 70: 181–184.

Theologis, A., Oeller, P. W., Wong, L. M., Rottmann, W. H. & Gantz, D. M. (1993): Use of a tomato mutant constructed with reverse genetics to study fruit ripening, a complex developmental process. Developmental Genetics 14: 282–295.

Yang, S. F., Hoffman, N. E. (1984): Ethylene biosynthesis and its regulation in higher plants. Ann Rev Plant Physiol 35: 155–189.

Yip, W. K., Dong, J. G. & Yang, S. F. (1991): Purification and characterization of 1-aminocyclopropane-1-carboxylate synthase from apple fruits. Plant Physiology 95: 251–257