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GENETIC TRANSFORMATION OF AVOCADO WITH S-ADENOSYLMETHIONINE HYDROLASE (SAMASE) AND EVALUATION OF TRANSFORMANTS AFTER THREE YEARS

R.Litz[1], D.Efendi[1,2], S.H.T.Raharjo[1,3], G.Padilla[1] and P.A.Moon[1]
[1]University of Florida TREC, 18905 SW 280 Street, Homestead FL USA 33031-3314. email: relitz@ufl.edu
[2]Department of Agronomy, Bogor Agricultural University, Bogor 12280, Indonesia.
[3]Faculty of Agriculture, University of Pattimura, Poka, Ambon 97233, Indonesia

Avocado was genetically transformed with the gene SAMase that degrades S-adenosylmethionine, a precursor of ethylene. SAMASE is in pAG4092 under the control of an avocado fruit promoter with *nptII*, which confers resistance to kanamycin sulfate. Embryogenic avocado suspension cultures were cocultured with log phase acetosyringone-activated *Agrobacterium tumefaciens* strain EHA 105 containing pAG4092 in 50 ml of liquid medium for three days at 125 rpm in darkness at 25^{o}C, and then transferred into fresh MSP3:1 medium supplemented with 200 mg litre^{-1} cefotaxime and 500 mg litre^{-1} carbenicillin for eight days with a change of medium after two days. Embryogenic cultures were then transferred into fresh MS3:1P medium supplemented with 50 mg litre^{-1} kanamycin sulfate and then four days later to fresh medium with 100 mg litre^{-1} kanamycin sulfate. Somatic embryo development occurred on semi solid MS medium supplemented with 30 g litre^{-1} sucrose, 20% (v/v) filter-sterile coconut water and 100-300 mg litre^{-1} kanamycin sulfate. The shoots from somatic embryos transformed with *samase* were excised and micrografted *in vitro* on decapitated 'Peterson' seedling rootstocks. Rapidly growing transgenic shoots were excised and grafted on 'Peterson' rootstocks in the nursery. In order to expedite flowering in 'Suardia' shoots containing *samase*, bud wood was grafted on 'Hass' and 'Lula' interstocks. Although flowering has not yet occurred, alterations in morphology, probably due to gene insertions at different loci, have been observed that appear to be stable. This strategy is intended to extend avocado shelf life and to enable on-tree storage of Antillean avocados.

Key Words: genetic transformation, ethylene, ripening, somatic embryo

TRANSFORMACIÓN GENÉTICA DE AGUACATE CON S-ADENOSILMETIONINA HIDROLASA (SAMASA) Y EVALUACIÓN DE LOS TRANSFORMANTES DESPUÉS DE TRES AÑOS

R.Litz[1], D.Efendi[1,2], S.H.T.Raharjo[1,3], G.Padilla[1] y P.A.Moon[1]
[1]University of Florida TREC, 18905 SW 280 Street, Homestead FL USA 33031-3314. email: relitz@ufl.edu
[2]Department of Agronomy, Bogor Agricultural University, Bogor 12280, Indonesia.
[3]Faculty of Agriculture, University of Pattimura, Poka, Ambon 97233, Indonesia

La palta fue transformada de manera genética por el gen SAMASA que degrada el compuesto S-adenosilmetionina, precursor del etileno. El gen SAMASA se incorporó al vector pAG4092 bajo el control del promotor de una celulasa

específica del fruto, junto con *nptII*, que otorga resistencia a sulfato de kanamicina. Se depositaron masas embriogénicas sobre papel de filtro y se dañaron ligeramente con un pincel. A continuación fueron co-cultivadas con la cepa EHA 105 de *Agrobacterium tumefaciens* con el vector pAG4092 en 50 ml de medio líquido. A los tres días se transfirieron a medio nuevo MS3:1P suplementado con 200 y 500 mg litro^{-1} de cefotaxima y de carbenicilina durante ocho días, cambiando de medio a los dos días. Los cultivos embriogénicos fueron entonces transferidos a medio MS3:1P con 50 mg litro^{-1} de kanamicina, cambiándolo a los cuatro días e incrementando el contenido en kanamicina a 100 mg litro^{-1}. El desarrollo de los embriones somáticos tuvo lugar en medio MS con 30 g litro^{-1} de sucrosa, 20% (v/v) de agua de coco y 100-300 mg litro^{-1} de sulfato de kanamicina. Los brotes de los embriones somáticos transformados se microinjertaron sobre plántulas 'Peterson' germinadas *in vitro* y se injertaron en vivero sobre patrones 'Peterson'. Con el fin de acelerar la floración de los 'Suardia' transformados se injertaron púas sobre patrones intermedios 'Hass' y 'Lula'. Aunque todavía no florecieron, se han observado alteraciones estables en la morfología debidas, probablemente, a inserciones en distintos loci. Con esta estrategia se pretende alargar la vida de almacenamiento del aguacate y permitir la conservación en el árbol de las variedades Antillanas.

INTRODUCTION

Most of the avocado production in North American is accounted for by cultivars of the semi tropical Mexican race or hybrids of the Mexican x Guatemalan races, e.g., 'Hass'. Fruit of the Mexican and Mexican x Guatemalan cultivars do not ripen while the fruit are still attached to the tree and remain on the trees accumulating oil for 2-4 months after reaching maturity (Tingwa and Young, 1975; Sitrit *et al*., 1986; Whiley, 1992). By careful selection of microclimate and on-tree storage, it has been possible for California growers to supply fruit of a single cultivar 'Hass' (Mexican x Guatemalan) year-round. This has been variously attributed to 1) the presence of an ethylene inhibitor in the fruit stem (Tingwa and Young, 1975; Morton, 1987); 2) translocation of an ethylene inhibitor into the fruit from the tree (Adato and Gazit, 1974; Whiley, 1992; Kays, 1997); and 3) the emission of trace amounts of ethylene from avocado fruit that are attached to the tree (Sitrit *et al.,* 1986).

The fruit of West Indian and West Indian X Guatemalan hybrids mature and ripen on-the-tree, and drop if not harvested at maturity. The fruit cannot be stored on the trees (Whiley, 1992). Consequently, to ensure availability of tropical fruit year-round, several avocado cultivars, each with a different harvesting season, must be grown. For example, Crane *et al.* (1998) noted that in Florida, approximately 30 avocado cultivars are grown commercially in order to ensure fruit availability from the end of May through the beginning of March. There is therefore no uniform standard for appearance and quality for tropical avocados. Extending the on-tree storage of West Indian and Guatemalan X West Indian avocado types could address this problem so that fewer cultivars could provide fruit year-round, and storage costs could be reduced.

Avocado fruit are strongly climacteric (Adato and Gazit, 1974; Morton, 1987; Kays, 1997), and ripening is mediated by ethylene. The ripening phase is biphasic; the first phase is a lag phase or preclimateric and the second phase is the climacteric peak (Sitrit *et al*., 1986; Starret and Laties, 1991, 1993; Kays, 1997). Starret and Laties (1991) referred to the lag phase as System I Ethylene, where endogenous ethylene is low, and the second phase as System II Ethylene that causes and accompanies a respiration climax attended by ripening phenomena. Starret and Laties (1993) found that cellulase, polygalacturonase, and ACC oxidase are not involved in the initiation of the climacteric, because none of them is induced during the lag period in intact fruit. Ethylene acts as a natural triggering mechanism for the induction of the respiration climacteric (Kays, 1997). Ethylene also regulates fruit ripening by coordinating the expression of genes that are responsible for a variety of processes, including enhancement of the rate of respiration, autocatalytic ethylene production, chlorophyll degradation, carotenoid synthesis, conversion of starch to sugars and increased activity of cell wall degrading enzymes (Gray *et al*., 1992).

Various methods for prolonging fruit shelf-life and preventing senescence have been employed, e.g., application of aminoethoxyvinylglycine (AVG) as an ethylene synthesis inhibitor and silver ions (Ag^{+}) and 2,5-norbornadiene (NBD) as ethylene action inhibitors, carbon dioxide, controlled atmosphere, and low-temperature storage. These techniques are expensive and fail to prevent fruit senescence satisfactorily. Storage at low temperature is a problem for tropical fruits like avocado, because the fruit is subject to chilling injury (Morton, 1987; Kays, 1997). Low-temperature storage can delay ripening and retard the appearance of mRNAs for cellulase, polygalacturonase, ethylene forming enzyme and other mRNAs of unknown function (Dopico et al., 1993). Exposure of mature Mexican and Mexican x Guatemalan avocado fruit to the ethylene inhibitor 1-methylcyclopropene (1-MCP) have demonstrated that fruit ripening can be delayed by 10-25 days (depending on the cultivar) (Lemmer & Kruger, 2003; Lemmer et al., 2003).

By blocking ethylene production in developing avocado fruit of the West Indian and West Indian x Guatemalan types using a genetic engineering strategy, we are developing a new strategy to enable on-tree storage of tropical avocado fruit and extend the shelf life of what is now a highly perishable commodity. This innovation will allow the producers of tropical avocados to 1) rationalize their production by growing only a few superior cultivars; 2) market a uniform product; and 3) lower the post harvest costs of storage and handling.

MATERIALS AND METHODS

Somatic embryogenesis. The genetic transformation of avocado has been based upon highly embryogenic suspension cultures (Witjaksono and Litz, 1999a,b; Witjaksono et al., 1999), Cruz Hernandez et al., 1998; Litz et al., 2005; Efendi, 2003). Embryogenic cultures (PEMs) of West Indian and West Indian x Guatemalan avocados were induced on induction medium consisting of B5 major salts (Gamborg and Miller, 1968), supplemented with MS minor salts (Murashige and Skoog, 1962), 4.14 µM picloram and (in mg l^{-1}) thiamine HCl (4), myo-inositol

(100), sucrose (30,000) and 8 g l^{-1} TC agar. The plant growth medium (10 ml aliquots) was in sterile disposable Petri dishes, and cultures were maintained in darkness at 25°C.

Embryogenic cultures were transferred onto fresh semisolid maintenance medium (complete MS basal medium with picloram) for 1–2 subcultures, and then subcultured at 2–4 week intervals. Embryogenic suspension cultures were established by inoculating 100–300 mg of 8–10-day-old PEMs from semisolid maintenance medium into filter-sterilized 40 ml liquid maintenance medium modified to contain 12 mg $litre^{-1}$ NH_4NO_3 and 30.3 mg $litre^{-1}$ KNO_3 in 125 ml Erlenmeyer flasks. Embryogenic suspension cultures were subcultured at biweekly intervals into filter-sterilized medium, and were maintained on a rotary shaker at 120 rpm at 25°C in darkness (Witjaksono and Litz, 1999b).

Plant recovery from somatic embryos. In order to initiate somatic embryo development from embryogenic cultures, PEMs were subcultured onto semisolid MS medium which was supplemented with 30 g $litre^{-1}$ sucrose, 4 mg $litre^{-1}$ thiamine HCl, 100 mg $litre^{-1}$ myo inositol, 20% (v/v) filter-sterilized coconut water and 6.0 g $litre^{-1}$gellan gum in darkness at 25°C (Witjaksono and Litz, 2002) with subculture onto fresh medium at 2-3 month intervals. Mature somatic embryos germinated after embryo enlargement has ceased, usually 4-5 months after transfer onto maturation medium. When root or shoot growth was apparent, the cultures were transferred to 16h light provided by cool white fluorescent tubes (approx. 60 µmol m^{-2} s^{-1}). The recovery of plants from somatic embryos was low. Therefore, shoots that developed from regenerating transformed somatic embryos were micrografted onto in vitro-grown seedlings and later grafted ex vitro onto one-month-old seedling rootstocks (Raharjo and Litz, 2005). The transformed plants are currently being maintained in a secure greenhouse. The first grafts of transformed avocado onto mature grafted plants have been performed to reduce the juvenile period of woody trees (Pena et al., 2000).

Genetic transformation. Embryogenic cultures derived from West Indian and West Indian x Guatemalan cultivars were genetically transformed with binary vector pAG4092 in *Agrobacterium tumefaciens* strain EHA101. The pAG4092 has the *nptII* gene that encodes resistance to the antibiotic kanamycin under the AGT01 promoter located near the left border and the *samK* gene driven by an avocado fruit-specific cellulase promoter located near the right border. The *samK* gene is a modified *samase* and encodes for SAM hydrolase that catalyzes the conversion of SAM to methylthioadenosine (Good et al., 1994). Since SAM is the metabolic precursor of ACC, the proximal precursor of ethylene, the depleted SAM pool will inhibit ethylene biosynthesis (Good et al., 1994; Kramer et al., 1997). Embryogenic avocado suspension cultures in their logarithmic phase of growth were subcultured onto sterile filter paper and abraded with a sterile camel hair brush. They were then cocultured with log phase acetosyringone-activated *Agrobacterium tumefaciens* strain EHA105 containing vector pAG4092 in 50 ml of liquid medium for three days at 125 rpm in darkness at 25°C, and then transferred into fresh MSP3:1 medium supplemented with 200 mg $litre^{-1}$ cefotaxime and 500 mg $litre^{-1}$ carbenicillin for eight days with a change of medium after two days. As reported by Cruz-Hernandez *et al.* (1998), growth of

embryogenic suspensions was suppressed by 50% with 50 mg liter^{-1} kanamycin sulfate; 50% growth suppression on semi solid medium requires 100 mg liter^{-1} kanamycin sulfate. Complete suppression of growth of embryogenic cultures occurred on semi solid medium containing 200 mg liter^{-1} kanamycin sulfate. Integration of *nptII* into the avocado genome was confirmed by PCR and southern hybridization (Doyle and Doyle, 1990; Miller, 1972).

RESULTS

Embryogenic cultures were induced from 'Suardia', 'Booth 7', 'Waldin' and 'Donny'. Maintenance cultures were co-cultured with genetically engineered *Agrobacterium tumefaciens* strain EHA101 containing pAG4092. Following the elimination of A. tumefaciens, the cultures were selected for resistance to 200 mg liter^{-1} Kanamycin sulfate for three months with weekly subculture to fresh medium. More than 200 independently transformed lines of West Indian and West Indian x Guatemalan avocados containing the SAMase gene were recovered. Putatively transformed lines were transferred to maturation medium containing 400 mg liter^{-1} kanamycin sulfate. After seven months, mature somatic embryos began to germinate, and their shoots were either micrografted or grafted ex vitro on seedling rootstocks. Plants have been recovered from approx. 100 of these lines. The transformation of embryogenic cultures was confirmed by growth of cultures in 400 mg/liter kanamycin sulfate and by Southern blot hybridization. Transformed plants are currently being maintained in a secure greenhouse.

Transformed plants are being assessed for several growth and development parameters relative to the control, non transformed trees: growth rate; ethylene emission from vegetative, floral tissue and developing fruit; leaf, floral and tree form and architecture; flower abscission; immature fruit abscission. Transformed embryogenic cultures and leaves from transformed plants were sampled for ethylene emission. The ethylene data for control and experimental tissues have been approximately the same, which one would expect since the samase gene is under the control of a fruit-specific promoter.

Among regenerated transgenic 'Suardia' plants there have been three distinct plant morphologies, one of which has affected general growth and vigor (Figure 1a). This morphotype grows much more slowly than plants from the other transgenic lines and the nontransformed controls, and shows symptoms of nutrient deficiency. Plants of the two other transgenic lines are growing vigorously; however, the leaf shape and size have both been affected (Figure 1b and c): Figure 1b has narrow lanceolate leaves whereas, the plant in Figure 1c has broad oblanceolate leaves relative to the control, nontransformed plants.

Figure 1. Recovery of transgenic avocado plants containing the samase gene. (a). Regenerant showing slow growth, weak stem and nutritional deficiency. (b). Regenerant showing lanceolate leaves and a rigid stem. (c). Regenerant showing oblanceolate leaves and a rigid stem.

Since the SAMase gene is under the control of an avocado fruit-specific promoter, we will be looking for and selecting plants in which there is no ethylene emission during fruit maturation. Fruit maturation will be compared with the parental clones, and over successive fruiting seasons, flowering, fruit set and fruit maturation and ripening will be characterized for the various transformed lines. Transgenic material with superior phenotype will be identified.

DISCUSSION

This study is contingent upon the following: 1) successful recovery of avocado plants that have been transformed with the SAMase gene under the control of an avocado fruit ripening-specific promoter; 2) efficient establishment of transformed avocado plants ex vitro; 3) circumventing the juvenile period of genetically transformed plants derived from embryogenic culture by sequential micrografting. Since the SAMase gene is under the control of an avocado fruit-specific promoter, we anticipate that we can enable on-tree storage of tropical avocado fruit and extend the shelf life of what is now a highly perishable commodity. This innovation will allow the producers of tropical avocados to 1) rationalize their production by growing only a few superior cultivars; 2) market a uniform product; and 3) lower the post harvest costs of storage and handling.

Fruit ripening is under genetic control, e.g., genes encoding β-1,4-glucanase (avocado), polygalacturonase (tomato) and trypsin inhibitor (tomato) show increased expression during ripening. During ripening of mature avocado fruit, a number of mRNAs increase, i.e., the messages for cellulase (Christoffersen et al., 1984; Dopico et. al., 1993); cytochrome P-450 oxidase (Bozak et al., 1990); polygalacturonase and ACC oxidase (Dopico et al., 1993). Different cDNAs associated with avocado fruit ripening have been reported: polygalacturonase cDNA, referred to as pAVOpg (Kutsunai et al., 1993), and pAVOe3 (McGarvey et al., 1990; 1992). McGarvey et al. demonstrated that pAVOe3 is similar (76%) to pTOM13, an ACC oxidase gene from tomato but is also weakly similar to E8 protein of tomato (31%). A high degree of conservation between pTOM13 and pAVOe3 implies a conservation of function (McGarvey et al., 1992).

During ethylene biosynthesis, ATP-methionine-S-adenosyltransferase converts methionine to SAM (S-adenosylmethionine). ACC synthase (S-adenosyl-L-methionine methylthioadenosine-lyase) converts SAM to ACC (1-aminocyclopropane-1-carboxylic acid). ACC is converted to ethylene by ACC oxidase (Kionka and Armhein, 1984; Kende, 1989; Penarruba et al., 1992; McKeon et al., 1995; Kende and Zeevaart, 1997). Kionka and Armhein (1984) and Penarruba et al. (1992) also noted that malonyl transferase can irreversibly conjugate the ACC to N-malonyl-ACC (MACC), thereby removing ACC from ethylene production.

The production of ethylene during fruit ripening depends on activating some genes that encode enzymes in the ethylene biosynthesis pathway. Conversely, ethylene production can be reduced by blocking specific gene activity, e.g., methionine-S-adenosyltransferase, the genes encoding ACC synthase, ACC oxidase, and by overexpressing the genes encoding malonyl transferase, SAM hydrolase and ACC deaminase. Suppressing ethylene biosynthesis can be achieved by eliminating the substrate for ACC synthase by means of SAM hydrolase (SAMase) which converts SAM to a non-toxic by-product, 5'-methylthioadenosine and homoserine, which are recycled within the plant cell, so that SAM is not available for conversion to ACC. The gene is used behind a specific fruit-ripening promoter so that SAMase would be expressed in mature green fruit before or just as it would normally start to ripen. This approach has been utilized with tobacco (Bestwick et al., 1991), tomatoes (Good et al., 1994; Kramer et al., 1997), raspberry (*Rubus ideaus* L.) and strawberry (*Fragaria x ananassa*) (Mathews et al., 1995a, b) and cantaloupe (Clendennen et al., 1999).

We will select plants in which there is no ethylene emission during fruit maturation. Selections that show outstanding on-the-tree-fruit storage will be identified and cloned.

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