

**RECURSO DE REPOSICIÓN CONTRA LA RESOLUCIÓN NO. 11333 DEL 13 DE MARZO DE 2015 DESFIJADO EL EDICTO EL 17 DE ABRIL DE 2015.**

Señores

**Superintendencia de Industria y Comercio**

Dirección de Nuevas Creaciones

E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 13-116147-00013-0000

Fecha: 2015-04-29 14:13:25 Dep. 2020 DIR.NUEVASCR  
Tra. 2 PATENTES Eve: 1 REGDEPOSITO  
Act. 412 REPOSPRESRECU Folios: 74

**Ref: Expediente No. 13-116147**

**Solicitud de registro de patente de invención denominada  
"PROCESO PARA EL DESARROLLO DE LA PLANTA DE  
PIÑA LLAMADA ROSÉ"**

**Solicitante: DEL MONTE FRESH PRODUCE.**

Ana María Ruiz Rueda, identificada como aparece al pie de mi firma, y obrando en mi condición de apoderada de **DEL MONTE FRESH PRODUCE**, domiciliada en 241 Sevilla Ave, Coral Gables, FL 33134, Estados Unidos de América, atentamente manifiesto a usted que interpongo recurso de reposición contra la Resolución **No. 11333**, dictada por el señor Superintendente de Industria y Comercio, mediante la cual se niega la patentabilidad de la única reivindicación de la solicitud de patente de la referencia.

A continuación me permito fundamentar el recurso en el siguiente hecho:

- Mediante Resolución **No. 11333**, se niega la patentabilidad de la única reivindicación 1 de la presente invención basándose en la alegada falta de nivel inventivo de las mismas.

En vista de lo que establece la Resolución Impugnada, se puede apreciar que la reivindicación fue negada debido a que no describe características que le proporcione nivel inventivo frente a la combinación de los documentos **WO2006113709 (D1)** y **GenBank; AB037975.1 (D2)**.

Por esta razón, mediante el presente escrito se pretende ilustrar de una manera clara las razones por las cuales la presente solicitud contiene características diferenciadoras con respecto al estado de la técnica presentado.

A continuación se presenta la siguiente argumentación que justifica lo previamente enunciado:

1. Inicialmente, y para mayor claridad se presenta a continuación la reivindicación 1 de la solicitud:

*"1. Un proceso para desarrollar la piña Rosé (EF2-114) que comprende los siguientes pasos:*

*a. La piña (Ananas comosus) es transformada mediante una transformación de los tejidos organogénicos mediada por Agrobacterium tumefaciens;*

*b. Las cepas de Agrobacterium se cocultivan con tejidos receptores de piña;*

*c. Se insertan diferentes genes en el ADN-T en un vector binario para la introducción a la piña; en donde el vector binario es creado por medio de un plásmido pCW1; y*

*donde el vector binario está compuesto por los plásmidos pHCW.T-7 y pHCWflACC3'-2;*

*d. Se transfiriere los vectores binarios a la cepa GV3101 desarmada de A. tumefaciens, el GV3101 con el vector pHCW.T-7 es nombrado AG76 y el del vector pHCWflACC3'-2 es nombrado AG62;*

*e. Se mezclaron AG76 y AG62 para la transformación de la piña;*

*f. Se seleccionan los tejidos transformados en medios que contienen closulfuron y posteriormente se revisan para detectar la presencia de genes diana mediante PCR;*

*En donde los Elementos Genéticos del plásmido pHCW1 son: RB, pVSI, TetRA, pACYC, LB, MCS; y*

*En donde los Elementos Genéticos del ADN -T en el plásmido pHCW.T-7 en AG76 son: BRlp2.5, Psy, Ubpter, BRlp2.5, eLyc fragmento con sentido, ST-LS1, ST-LS1, eLyc fragmento antisentido, Ubpter, BRlp2.5, b-Lyc fragmento con sentido, ST-LS1, b-Lyc fragmento anti-sentido, Ubpter, y Marcador Seleccionable: EHS1.7-Ubp1.5, EHS1.7, Ubp1.5, ALS, ALS 3'; y*

*En donde los Elementos Genéticos del ADN -T en el plásmido pHCWflACC3'-2 en AG62 son los mismos elementos ya mencionados en el plásmido pHCW.T-7 en AG76 con la excepción de: flACS(+)-sentido, flACS(-) antisentido."*



2. En la reivindicación 1 modificada, se revela precisamente la configuración genética del plásmido inicial para generar el vector binario, y los Elementos Genéticos del ADN -T de los plásmidos utilizados para obtener las características fenotípicas deseadas, incluyendo los promotores.
3. A diferencia de la reivindicación 1, en D1 no sugiere una configuración tan precisa, sino un método general que no sólo sugiere la síntesis de carotenoides en frutas, sino muchas otras aplicaciones.
4. En el caso de D2, éste sólo revela el Gen de la fitoeno simasa de la mandarina, por lo que no entrega tampoco un método y configuración precisa del plásmido original inventado por el solicitante para la creación de la piña llamada Rosé.
5. Es importante resaltar que D1 explica el uso de los genes ISO y bLyc así como del gen PSY de mandarina (Documento D2) para manipular la biosíntesis de carotenoides, pero no hay mención del color rosa / rojo de la pulpa. Se entiende que el Gen PSY se introdujo en la Piña Rose, pero no presentó ninguna manifestación. Bien se sabe que el gen PSY ya ha sido publicado (Documento 2) por lo que no se está reclamando PSY en absoluto.
6. En cuanto a la invención, lo que funcionó para crear la Piña Rose fue la supresión de genes UT1 y UT2 utilizando genes bLyc y eLyc.
7. Se señala también que el gen eLyc no fue divulgado en D1.
8. Tal y como lo estableció la oficina la diferencia de la invención consiste en que el gen PSY insertado en el vector binario es derivado de Citrus Reticulata, y lo que se está reclamando es la supresión de los genes UT1 y UT2 de la secuencia del RNAi en el cual se utilizaron pequeños trozos de genes bLyc y eLyc para producir color rosa en Rose. Lo anterior no es deducible fácilmente por una persona normalmente versada en la materia.
9. Para obtener el color rosa/ roja en la pulpa el gen PSY no podría ser reemplazado nunca como lo sugiere el D1 por otra planta con mayor contenido de carotenoides, ya que lo que pretende proteger la presente solicitar de forma específica en la que se logra tal color rosa con el gen PSY.

10. En relación a la claridad, se resalta que la descripción de la materia objeto de patentabilidad es suficientemente obvia para una persona versada en la materia replique la invención.
11. Como prueba del proceso inventivo, se adjunta un documento que muestra más claramente al Examinador el proceso de creación de la planta, y sustenta la invención y desarrollo inventivo del solicitante.
12. Como conclusión se reitera que lo reclamado es la supresión de los genes UT1 y UT2 de la secuencia del RNAi en el cual se utilizaron pequeños trozos de genes bLyc y eLyc para producir color rosa en Rose, lo cual no es evidente para una persona versada en la materia. Por lo anterior se entiende que el método propuesto en la reivindicación 1 posee restricciones técnicas diferenciadores de lo propuesto en D1, y no es evidente para una persona versada en la materia llegar a la fruta presentada, al realizar la simple combinación entre D1 y D2.

### Conclusión

En consecuencia, atentamente me permito solicitar a ese Despacho, se sirva **tener en cuenta los argumentos aquí expuestos** y con base en ellos, revocar la decisión contenida en la Resolución impugnada y conceder el privilegio de patente solicitado para las reivindicaciones de la presente invención, toda vez que **ya se ha demostrado que las mismas cumplen con los requisitos de claridad, novedad, nivel inventivo y aplicación industrial** que exige la normativa Andina en materia de patentes.

### Anexos

- Capítulo reivindicatorio de la solicitud.
- Documento que muestra más claramente al Examinador el proceso de creación de la planta, y sustenta la invención y desarrollo inventivo del solicitante.



Atentamente,

**Ana María Ruiz**

C.C 39.790.288 de Bogotá.

T.P. 84909 C.S.J.

ABM/P-713 *LHA*



## CAPÍTULO REIVINDICATORIO

1. Un proceso para desarrollar la piña Rosé (EF2-114) que comprende los siguientes pasos:
  - a. La piña (*Ananas comosus*) es transformada mediante una transformación de los tejidos organogénicos mediada por *Agrobacterium tumefaciens*;
  - b. Las cepas de *Agrobacterium* se cocultivan con tejidos receptores de piña;
  - c. Se insertan diferentes genes en el ADN-T en un vector binario para la introducción a la piña; en donde el vector binario es creado por medio de un plásmido pCW1; y  
donde el vector binario está compuesto por los plásmidos pHCW.T-7 y pHCWfIACC3'-2;
  - d. Se transfiriere los vectores binarios a la cepa GV3101 desarmada de *A. tumefaciens*, el GV3101 con el vector pHCW.T-7 es nombrado AG76 y el del vector pHCWfIACC3'-2 es nombrado AG62;
  - e. Se mezclaron AG76 y AG62 para la transformación de la piña;
  - f. Se seleccionan los tejidos transformados en medios que contienen closulfuron y posteriormente se revisan para detectar la presencia de genes diana mediante PCR;

En donde los Elementos Genéticos del plásmido pHCW1 son: RB, pVSI, TetRA, pACYC, LB, MCS; y

En donde los Elementos Genéticos del ADN -T en el plásmido pHCW.T-7 en AG76 son: BRlp2.5, Psy, Ubpter, BRlp2.5, eLyc fragmento con sentido, ST-LS1, ST-LS1, eLyc fragmento antisentido, Ubpter, BRlp2.5, b-Lyc fragmento con sentido, ST-LS1, b-Lyc fragmento anti-sentido, Ubpter, y Marcador Seleccionable: EHS1.7-Ubp1.5, EHS1.7, Ubp1.5, ALS, ALS 3'; y

En donde los Elementos Genéticos del ADN -T en el plásmido pHCWfIACC3'-2 en AG62 son los mismos elementos ya mencionados en el plásmido pHCW.T-7 en AG76 con la excepción de: fIACS(+sentido, fIACS(-) antisentido.

## Molecular Characterization of Event EF2-114 Verifies the Integrity and Stability of the Inserted DNA

Event EF2-114 was transformed by co-cultivation with two disarmed *Agrobacterium tumefaciens* GV3101(pMP90) strains containing binary vectors, pHCW.T-7 and pHCWfIACC3'-2. Plasmid pHCW.T-7 contains three transgenes involved in carotenoid biosynthesis, which produce pink/red colored fruit due to accumulation of endogenous lycopene. *PSY* encodes the enzyme phytoene synthase, which catalyzes the conversion of geranylgeranyl pyrophosphate to phytoene, an intermediate in lycopene biosynthesis. The *bLcy* and *eLcy* transgenes suppress endogenous lycopene  $\beta$ -cyclase and  $\epsilon$ -cyclase via an RNAi-mediated mechanism resulting in the accumulation of lycopene in edible fruit tissues. Plasmid pHCWfIACC3'-2 contains a *fIACC3'* RNAi transgene to suppress endogenous ACC synthase (ACS) involved in ethylene biosynthesis. ACS encodes the enzyme aminocyclopropane-1-carboxylic acid synthase, which catalyzes the rate-limiting step in ethylene biosynthesis. Suppression of endogenous ethylene is intended to promote uniform flowering and fruit production. Both plasmids also contain the *SuRBHra* transgene, which encodes a modified acetolactate synthase used solely as a selectable marker during development.

Molecular analyses demonstrated that Event EF2-114 has a complex T-DNA(s) integration pattern with an intact copy of pHCW.T-7 T-DNA and a truncated copy of the pHCWfIACC3'-2 T-DNA inserted into the pineapple genome. No vector backbone sequences from pHCW.T-7 or pHCWfIACC3'-2 were detected in the genome of Event EF2-114.

### I. DESCRIPTION OF EVENT EF2-114

This section provides a description of Event EF2-114 being presented for food safety and nutritional assessment. The description identifies the crop, the transformation event to be reviewed, and the type and purpose of the modification, which will aid in understanding the nature of the food products that may be developed from Event EF2-114. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraph 22.

#### I.A. DESIGNATION OF THE TRANSFORMATION EVENT

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants," Event EF2-114 has been assigned the unique identifier FDP-00114-5.

#### I.B. TYPE AND PURPOSE OF GENETIC MODIFICATION



Event EF2-114 contains three transgenes involved in carotenoid biosynthesis, which produce pink/red colored fruit due to accumulation of endogenous lycopene. *PSY* encodes the enzyme phytoene synthase (EC 2.5.1.32), which catalyzes the conversion of geranylgeranyl pyrophosphate to phytoene, an intermediate in lycopene biosynthesis. The *bLcy* and *eLcy* transgenes suppress endogenous lycopene  $\beta$ -cyclase (EC 5.5.1.19) and  $\epsilon$ -cyclase (EC 5.5.1.18) using RNA interference (RNAi) technology in order to accumulate lycopene in edible fruit tissues.

Event EF2-114 also contains a *flACC3'* RNAi transgene to suppress endogenous ACS involved in ethylene biosynthesis. ACS (EC 4.4.1.14) catalyzes the rate-limiting step in ethylene biosynthesis. Suppression of endogenous ethylene is intended to promote uniform flowering and fruit production.

Event EF2-114 also contains the *SuRBHra* transgene, which encodes a modified acetolactate synthase (EC 2.2.1.6) used solely as a selectable marker during development.

#### I.C. DESCRIPTION OF THE GENETIC MATERIALS INTRODUCED INTO EVENT EF2-114

The genetic material introduced into Event EF2-114 is further described in Section IV.

##### I.C.1. CHARACTERISTICS OF THE RECIPIENT PLANT

The pineapple variety used as a recipient for the DNA insertion to create Event EF2-114 is MD2, a non-transgenic conventional variety developed by the Pineapple Research Institute (PRI) and released to the market by Del Monte Fresh Produce in the 1980s under the trade name Del Monte Gold® Extra Sweet Pineapple.

MD2 was developed for the fresh fruit market by conventional breeding from a cross between PRI hybrids 58-1184 and 59-443. MD2 produces a medium to large (1.3-2.5 kg) cylindrical square shouldered fruit with large flat eyes and an intense orange-yellow color. The clear yellow pulp is sweet, compact and fibrous. It is high in sugar (14-17% Brix) and ascorbic acid, but lower in total acid than the Smooth Cayenne pineapple variety. The core is tender, edible and thinner than Smooth Cayenne. However, the crown can be larger and occasionally the distal end is removed at harvest. The leaves are green and mostly spineless. MD2 is more productive than Smooth Cayenne. It is more resistant to internal browning, but susceptible to fruitlet core rot and more sensitive to *Phytophthora* than Smooth Cayenne. The morphology of MD2 pineapple is described in Appendix I.

## I.C.2. ANANAS COMOSUS

The *bLcy*, *eLcy*, and *flACC3*' partial coding sequences are derived from pineapple (*A. comosus*) grown in Costa Rica and were isolated by Wintz and Firoozabady (2011). The taxonomy of pineapple is (USDA NRCS, 2012):

|               |                                              |
|---------------|----------------------------------------------|
| Kingdom       | Plantae – Plants                             |
| Subkingdom    | Tracheobionta – Vascular plants              |
| Superdivision | Spermatophyta – Seed plants                  |
| Division      | Magnoliophyta – Flowering plants             |
| Class         | Liliopsida – Monocotyledons                  |
| Subclass      | Zingiberidae                                 |
| Order         | Bromeliales                                  |
| Family        | Bromeliaceae – Bromeliad family              |
| Genus         | <i>Ananas</i> Mill. – pineapple              |
| Species       | <i>Ananas comosus</i> (L.) Merr. – pineapple |

Pineapple has a long history of safe use as human food and is not a major food allergen or source of dietary toxins (see section II.C.2).

The full-length *bLcy* and *eLcy* genes encode lycopene  $\beta$ -cyclase and lycopene  $\epsilon$ -cyclase, which catalyze the cyclization of lycopene into  $\beta$ -carotene and lutein, respectively (Hirschberg, 2001). Only partial coding sequences of *bLcy* and *eLcy* were introduced into Event EF2-114 as a hairpin RNAi construct to achieve endogenous gene suppression. The full-length ACS gene encodes the enzyme aminocyclopropane-1-carboxylic acid synthase (EC 4.4.1.14), which catalyzes the rate-limiting step in ethylene biosynthesis. Only a portion of the *flACC3*' was introduced into Event EF2-114 as a hairpin RNAi construct to achieve endogenous gene suppression. Accordingly, no expression of these introduced pineapple genes is predicted.

## I.D. IDENTITY AND SOURCE OF GENETIC MATERIAL INTRODUCED INTO EVENT EF2-114

Event EF2-114 was produced by *Agrobacterium tumefaciens*-mediated transformation using two binary plasmids pHCW.T-7 and pHCWflACC3'-2. Except for the right border (RB) and the left border (LB) sequences, all of the genetic elements present in both T-DNAs<sup>1</sup> (both coding and

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<sup>1</sup> T-DNA refers to the DNA that is transferred to the pineapple nuclear genome during transformation.



regulatory sequences) are of plant origin: tangerine, pineapple or tobacco. Cloning of the individual genetic elements within each cassette and assembling of the cassettes were performed using standard recombinant molecular biology techniques (Maniatis et al., 1982; Sambrook and Russell, 2001). The nucleotide sequence of the T-DNA in pHCW.T-7 and pHCWflACC3'-2 was confirmed by DNA sequencing (Elim Biopharmaceuticals, Inc., Hayward, CA; data not shown).

There is no evidence of any safety issues related to the use of Event EF2-114 and there is no evidence of human or animal toxicity for any of the coding and non-coding DNA sequences present in Event EF2-114. DNA has always been present in food and, upon consumption, is quickly degraded by restriction nucleases present in the gastrointestinal tract of humans and animals to nucleic acids. According to the U.S. FDA (U.S. FDA, 1992), nucleic acids are present in the cells of every living organism, do not raise concerns as a component of food, and are generally recognized as safe. Results from an International Life Sciences Institute (ILSI) workshop on safety considerations of DNA in food were reported (Jonas, et al., 2001) and confirmed that: 1) all DNA, including recombinant DNA, is composed of the same four nucleotides; 2) there are no changes to the chemical characteristics or the susceptibility to degradation by chemical or enzymatic hydrolysis of recombinant DNA as compared to non-recombinant DNA; and 3) there is no evidence that DNA from dietary sources has ever been incorporated into the mammalian genome. Additionally, the European Food Safety Authority (EFSA) has reported that a large number of experimental studies have shown that recombinant DNA consumed by livestock has not been subsequently detected in tissues, fluids, or edible products of these farm animals (EFSA, 2007).

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## II. DESCRIPTION OF THE GENETIC MODIFICATION

This section provides a description of the transformation process and plasmid vector used in the development of Event EF2-114. Molecular analyses are an integral part of the characterization of crop products with new traits introduced by methods of biotechnology. Vectors and methods are selected for transformation to achieve high probability of obtaining the trait of interest and integration of the introduced DNA into the plant genome. This helps ensure that only the intended DNA encoding the desired trait(s) is integrated into the plant genome and facilitates the molecular characterization of the product. Information provided here allows for the identification of the genetic material present in the transformation vector delivered to the host plant and for an analysis of the data supporting the characterization of the DNA inserted in the plant found in Section V. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraphs 27, 28, and 29.

Event EF2-114 was developed through *Agrobacterium tumefaciens*-mediated transformation of conventional pineapple MD2 leaf base tissue utilizing transformation plasmids pHCW.T-7 and pHCWflACC3'-2. This section describes the intended technical effects of the genetic modification. It also describes the plasmid vectors, the donor genes, and the regulatory elements used in the development of Event EF2-114 and the deduced amino acid sequence of the expressed proteins in Event EF2-114. In this section, transfer DNA (T-DNA) refers to DNA that is transferred to the plant during transformation. An expression cassette is comprised of sequences to be transcribed and the regulatory elements necessary for the expression of those sequences.

### II.A. INTENDED TECHNICAL EFFECTS IN EVENT EF2-114

#### II.A.1. CAROTENOID BIOSYNTHESIS

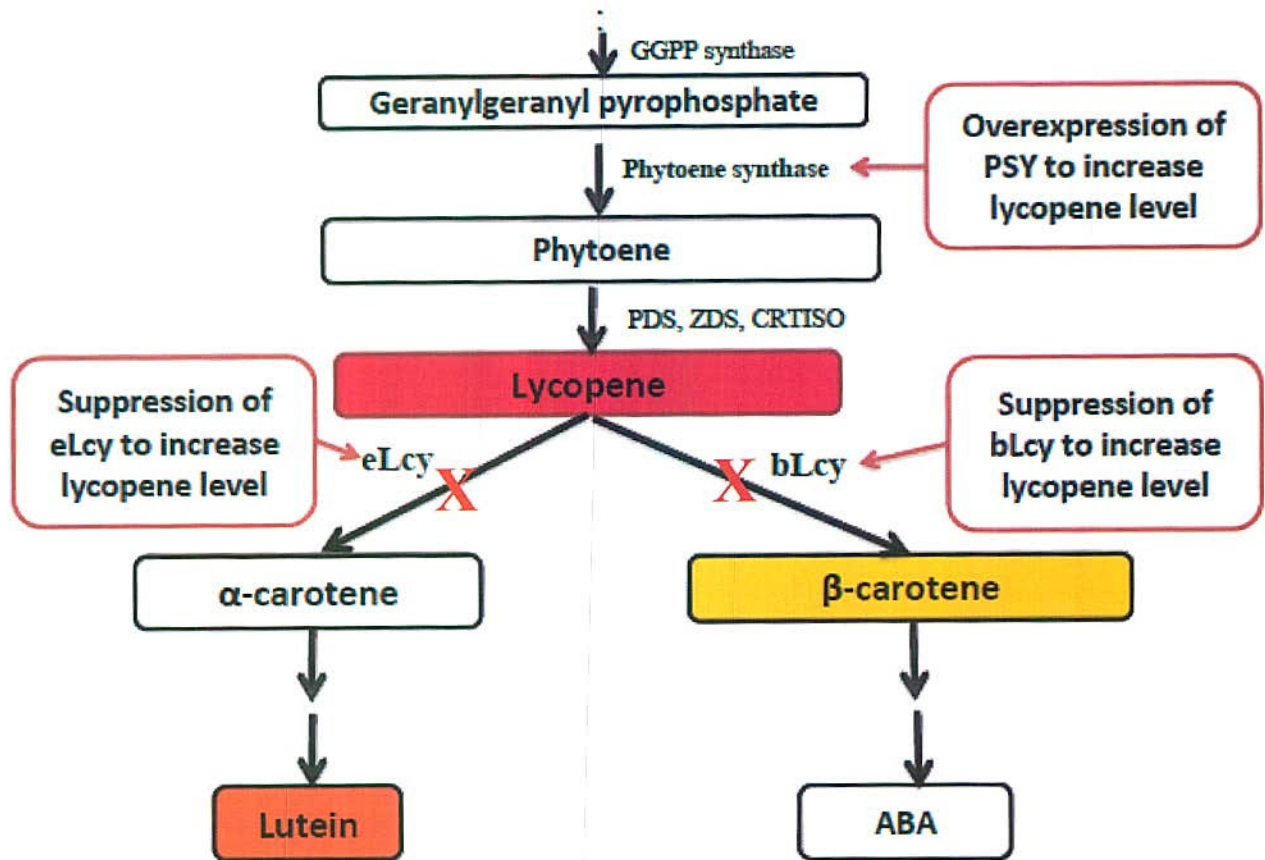
In plants, carotenoids are essential components of the photosynthetic apparatus and are responsible for the red, orange, and yellow color of many flowers and fruits. Understanding of carotenoid biosynthesis has advanced dramatically in recent years (Hirschberg, 2001; Fraser and Bramley, 2009) and their genetic manipulation and body of evidence for their health benefits have recently been reviewed (Fraser and Bramley, 2009). The pathway involves a series of desaturations, cyclizations, hydroxylations, and epoxidations commencing with the formation of phytoene (Ma et al., 2011). A subsequent series of desaturations is responsible for lycopene synthesis. After the desaturation reactions, the cyclization of lycopene is catalyzed by two enzymes, lycopene  $\beta$ -cyclase and  $\epsilon$ -cyclase, leading to the formation of  $\beta$ -carotene and  $\alpha$ -carotene, respectively (Cunningham et al., 1996).

Figure IV-1 illustrates the strategy used for the manipulation of carotenoid biosynthesis toward the development of Event EF2-114. Del Monte Fresh Produce introduced a phytoene synthase gene (*PSY*) in a fruit-specific manner to increase lycopene production. Del Monte Fresh Produce



also suppressed endogenous pineapple lycopene  $\beta$ -cyclase (*bLcy*) and  $\epsilon$ -cyclase (*eLcy*) genes in a fruit-specific manner by RNAi technology (Ma et al., 2011; Watanabe, 2011; Hammond et al., 2001) in order to increase accumulation of lycopene in pineapple fruit. The source, identity and function of each of the genetic elements are described in Table IV-1.

Figure II-1. Carotenoid biosynthesis pathway



Solid arrows represent reaction steps. The following enzymes and intermediate compounds are represented: GGPP synthase: geranylgeranyl pyrophosphate synthase; PSY: phytoene synthase; PDS: phytoene desaturase; ZDS:  $\zeta$ -carotene desaturase; CRTISO: carotenoid isomerase; eLcy: lycopene  $\epsilon$ -cyclase; bLcy: lycopene  $\beta$ -cyclase; ABA: abscisic acid.

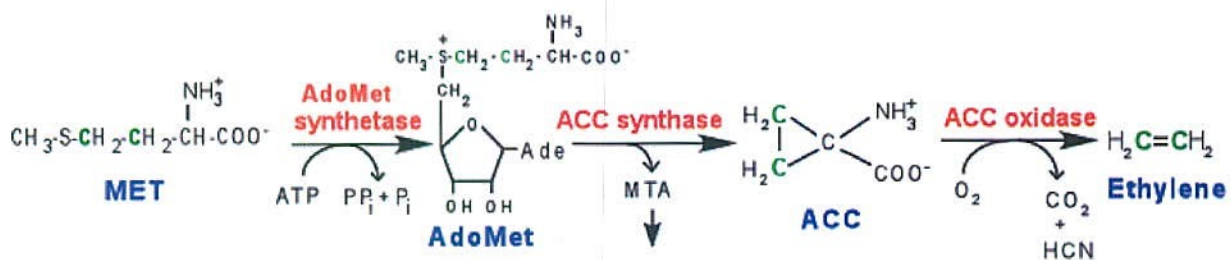
## II.A.2. FLOWERING CONTROL

The reproductive phase of pineapple begins in response to natural flowering or forced induction of reproductive development by application of a growth regulator (forcing). Natural flowering is induced by both cultural (size, weight and type of transplant material, planting time, nutrients) and environmental (short day length, moderate temperatures, low irradiance) factors (da Cunha, 2005), and can occur over many months. Synchronicity of flowering has been improved dramatically through forced induction using plant growth regulators such as ethylene producing chemicals (e.g., ethephon, auxin). Despite these production practices, a fraction of the crop (5% to up to 70% under certain conditions) flowers early, leading to disruption in harvest scheduling and market supply, increased harvest costs, increased pesticide use, and significant harvest losses.

Ethylene is a plant hormone that plays an important role in every phase of plant development, including seed germination, fruit ripening, leaf and flower senescence, and abscission. Unlike most other crops, pineapple flower initiation is induced by ethylene (Salisbury and Ross, 1992; Turnbull et al., 1993) and, therefore, in order to reduce natural, precocious flowering, Del Monte Fresh Produce introduced a transgene intended to suppress ethylene biosynthesis.

In plants, ethylene is derived from the amino acid methionine, which is converted by AdoMet synthetase to S-adenosyl-methionine (AdoMet) (Figure IV-2). AdoMet serves as a precursor in a number of biosynthetic pathways, including the production of polyamines. ACS catalyzes the conversion of AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC), which is the first committed and, in most instances, the rate limiting step in ethylene biosynthesis (Adams and Yang, 1979; Wang et al., 2002).

**Figure II-2. Ethylene biosynthesis pathway**



Enzymes catalyzing each step are shown in red, substrates are shown in blue. MET: methionine; AdoMet: S-adenosyl-methionine; MTA: methylthioadenine; ACC: 1-aminocyclopropane-1-carboxylic acid (Wang et al., 2002).

Del Monte Fresh Produce isolated a meristem ACS (*flACC3'*) gene from pineapple and constructed sense (*flACC+*) and antisense (*flACC-*) oriented sequences of the ACS gene (*flACC3'*), which are

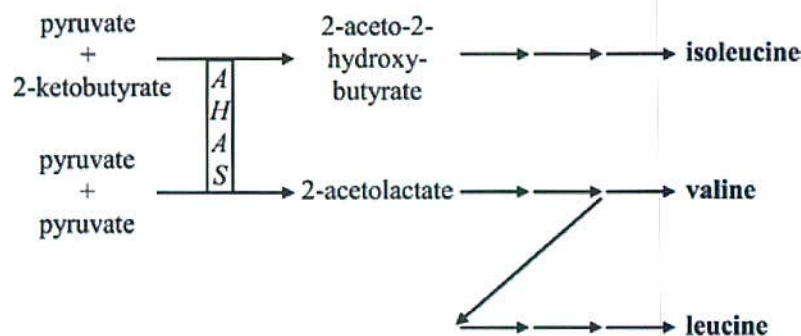


separated by an intron of the LS1 gene from potato (*Solanum tuberosum*) to form a hairpin structure. The RNAi construct is under the transcriptional control of the ubiquitin promoter (Ubpp) from pineapple (Wintz and Firoozabady, 2011). The source, identity and function of each of the genetic elements are described in Table IV-2.

### II.A.3. PLANT SELECTABLE MARKER

The transformation plasmids also contain a plant selectable marker gene cassette consisting of a mutant acetolactate synthase gene (*SuRBHra*) derived from tobacco (*Nicotiana tabacum*) under transcriptional control of the EHS-Ubpp fusion promoter. This promoter provides low expression of the *SuRBHra* gene in a constitutive manner to allow selection of transformed cells from tissue culture. Expression of the *SuRBHra* gene renders transformed cells resistant to sulfonyleureas. Sulfonyleureas are a group of compounds that inhibit ALS (Ray, 1984), the enzyme that catalyzes the first common step in the biosynthesis of the essential branched-chain amino acids isoleucine, leucine, and valine (Figure IV-3) (Lee and Duggleby, 2006).

**Figure II-3. Biosynthesis of branched-chain amino acids isoleucine, leucine and valine**



ALS (also called acetohydroxyacid synthase, AHAS) catalyzes the decarboxylation of pyruvate followed by a series of reactions resulting in production of the amino acids.

ALS genes are widely distributed in nature and are found in bacteria, fungi, and all plants. Various ALS genes have been used for production of herbicide resistant crops (Mazur and Falco, 1989). *SuRBHra* is an ALS enzyme in which two amino acids substitutions from wildtype render the enzyme insensitive to inhibition by chlorsulfuron (Lee et al., 1988). Plant cells that express the *SuRBHra* gene are capable of synthesizing the essential amino acids even in the presence of chlorsulfuron. *SuRBHra* was utilized as a selectable marker during the development of Event EF2-114 and serves no agronomic or other purpose in the plant.

## II.B. PLASMID VECTOR pHCW.T-7

Event EF2-114 was produced by *Agrobacterium tumefaciens*-mediated transformation using two plasmids pHCW.T-7 (Figure IV-4) and pHCWflACC3'-2 (Figure IV-6). The size of the pHCW.T-7 expression plasmid is approximately 36.6 kb.

Figure II-4. Circular map of pHCW.T-7

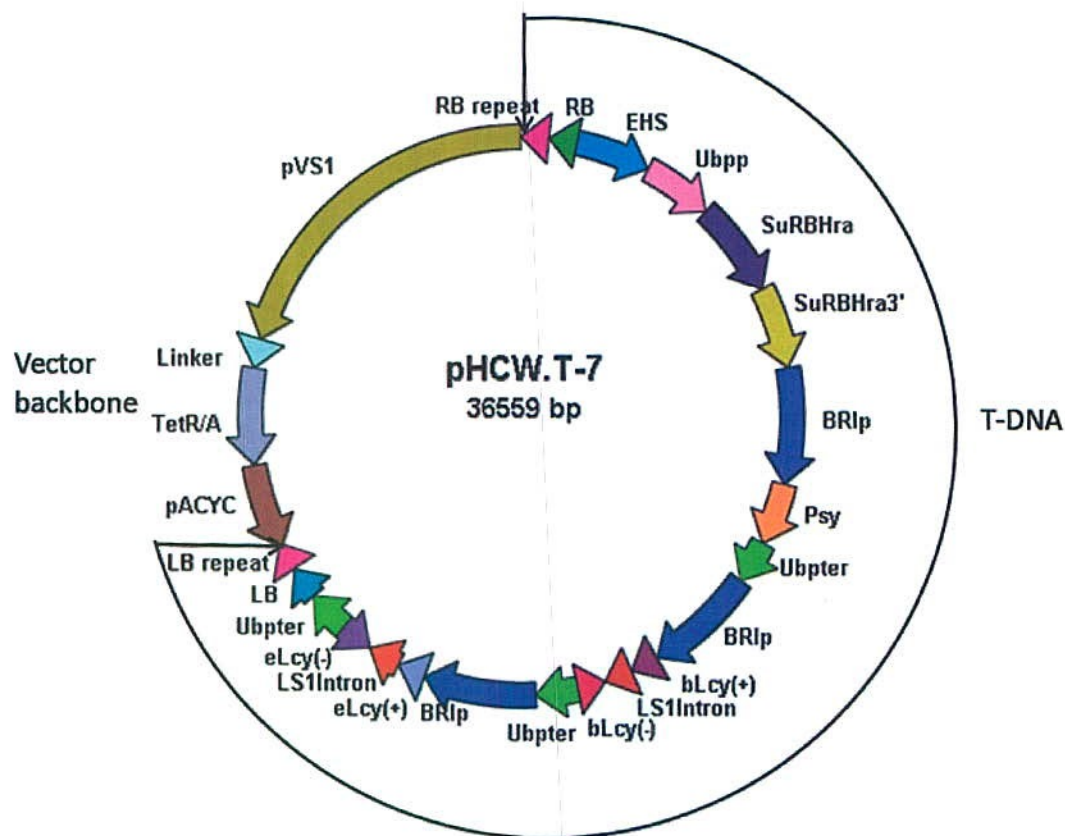
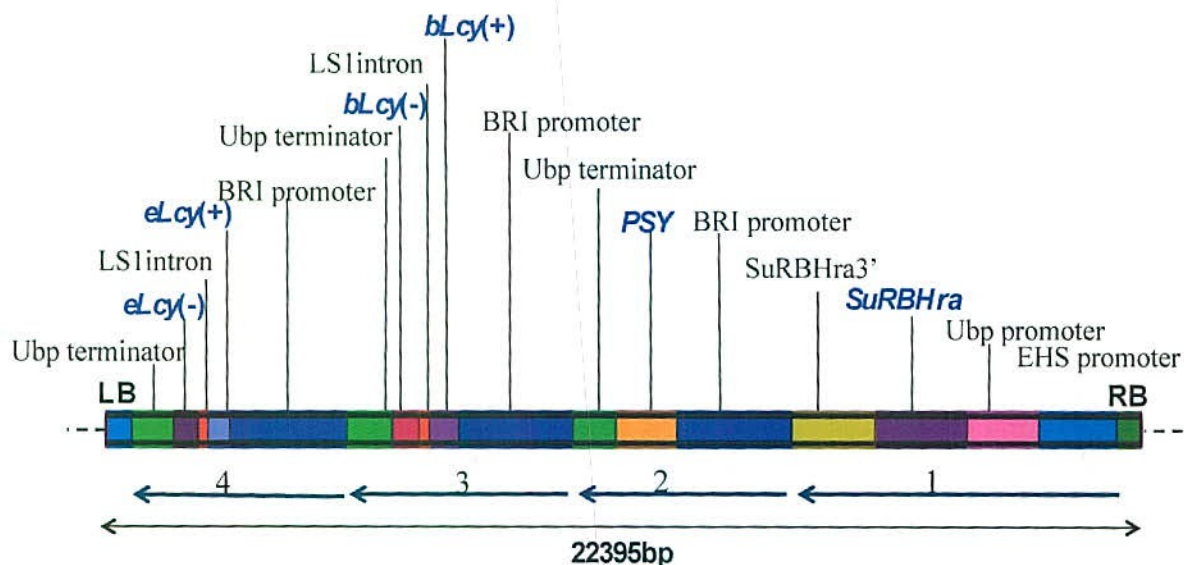




Figure II-5. Schematic diagram of the T-DNA of pHCW.T-7



Numbered arrows (1-4) indicate the four expression cassettes and respective regulatory elements in pHCW.T-7.

#### II.B.1. GENETIC ELEMENTS OF pHCW.T-7 T-DNA

The 22.4 kb T-DNA of plasmid pHCW.T-7 has four expression cassettes. The organization and orientation of the four expression cassettes are depicted in Figure IV-5 and are described below. The gene of interest in individual expression cassettes is labeled in blue text. The location of each of the genetic element on the T-DNA (base pair position) and its size are presented in Table IV-1.

- EHS-Ubpp-*SuRBHra*-*SuRBHra3'* expression cassette (plant selectable marker) is composed of a constitutive fusion promoter EHS-Ubpp. EHS-Ubpp is a fusion of the promoter/5'UTR sequence of the epoxide hydrolase (*EHS*) gene and the promoter/5'UTR of the tetrameric ubiquitin (*Ubi*) gene. Both promoters were isolated from pineapple (*Ananas comosus*). *SuRBHra* is a mutant acetolactate synthase (*ALS*) gene from tobacco (*Nicotiana tabacum*) (Lee et al., 1988). *SuRBHra* is highly resistant to the herbicide, chlorsulfuron, the basis for our selection of transformed pineapple plants. The *SuRBHra3'* corresponds to the 3'untranslated region of the *ALS* gene from tobacco which serves as a transcription terminator. The fused promoters were shown to be strongly expressed in pineapple tissues (Wintz and Firoozabady, 2011), hence were used to express the gene for selection with chlorsulfuron.
- BRIp-PSY-Ubpt* expression cassette is composed of a fruit-enhanced promoter sequence of the *BRI* gene of pineapple (*A. comosus*) (Moyle et al., 2005), the full-

length coding sequence of the phytoene synthase (*PSY*) gene from tangerine (*Citrus unshiu*) (Ikoma et al., 2001) and the 3' UTR/flanking region of the tetrameric *Ubi* gene from pineapple (*A. comosus*) which serves as a transcription terminator (Wintz and Firoozabady, 2011).

- c) BRIp-*bLcy* RNAi-Ubpter expression cassette is composed of the fruit-enhanced promoter sequence of *BRI* gene of pineapple (*A. comosus*) (Wintz and Firoozabady, 2011), a partial coding sequence of lycopene  $\beta$ -cyclase (*bLcy*) gene from pineapple (*A. comosus*) (Singh et al, 2003; Kim et al, unpublished; Pecker et al, 1996; Hugueney et al. 1995) in the sense orientation, the intron 2 sequence of *ST-LSI* gene from potato (*Solanum tuberosum*) (Eckes et al., 1986), a partial coding sequence of lycopene  $\beta$ -cyclase (*bLcy*) gene from pineapple (*A. comosus*) in the anti-sense orientation, and a terminator sequence corresponding to the 3' UTR/flanking region of the tetrameric *Ubi* gene from pineapple (*A. comosus*) (Wintz and Firoozabady, 2011).
- d) BRIp-*eLcy* RNAi-Ubpter expression cassette is identical to the BRIp-*bLcy* RNAi-Ubpter except that the *bLcy* RNAi portion is replaced with the partial coding sequence of the lycopene  $\epsilon$ -cyclase (*eLcy*) gene from pineapple (*A. comosus*) in the sense and anti-sense orientation.

BRIp drives expression of the *PSY*, the *bLcy* RNAi cassette, and the *eLcy* RNAi cassette in pineapple fruits. The rationale behind choosing the BRIp was based on published data by Moyle et al., 2005, in which the authors showed fruit-enhanced expression of *BRI* during pineapple fruit development and  $\beta$ -glucuronidase (*GUS*) gene expression in pineapple fruits (Firoozabady et al., unpublished results) .



Table II-1. Description of genetic elements in pHCW.T-7

| Genetic Element                   | Size (bp) | Position      | Description                                                                                                                                                                                                                                                                                                                                                                                         |
|-----------------------------------|-----------|---------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| RB                                | 547       | 22395 - 21849 | Right border region of T-DNA from Ti plasmid of <i>Agrobacterium tumefaciens</i> . Short direct repeat that flanks the T-DNA and is required for transfer of the T-DNA into plant cells (Depicker et al., 1982).                                                                                                                                                                                    |
| <b>Selectable marker cassette</b> |           |               |                                                                                                                                                                                                                                                                                                                                                                                                     |
| EHS                               | 1625      | 21848 - 20224 | Promoter region of epoxide hydrolase gene from pineapple (Wintz and Firoozabady, 2011). When fused with Ubpp, provides constitutive expression in pineapple (Wintz and Firoozabady, 2011).                                                                                                                                                                                                          |
| Ubpp                              | 1536      | 20223 - 18688 | Promoter region of the tetrameric ubiquitin gene from pineapple (Wintz and Firoozabady, 2011) Provides constitutive expression in pineapple (Wintz and Firoozabady, 2011).                                                                                                                                                                                                                          |
| <i>SuRBHra</i>                    | 1995      | 18687 - 16693 | <i>ALS</i> gene from the mutant tobacco line Hra encodes the enzyme acetolactate synthase (Lee et al., 1988, GenBank ID: X07645.1). Catalyzes the conversion of two pyruvate molecules to acetolactate and carbon dioxide, the first committed step in biosynthesis of essential branched chain amino acids. This enzyme is insensitive to sulfonylurea and functions as a plant selectable marker. |
| <i>SuRBHra3'</i>                  | 1763      | 16692 - 14930 | Terminator sequence of native acetolactate synthase gene from tobacco (Townsend et al., 2009, GenBank ID: FJ649655). Provides 3' untranslated region (3'UTR) and polyadenylation site for termination of transcription.                                                                                                                                                                             |
| Intervening sequence              | 49        | 14929 - 14881 | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                       |
| <b>Functional gene cassettes</b>  |           |               |                                                                                                                                                                                                                                                                                                                                                                                                     |
| BRIp                              | 2452      | 14880 - 12429 | Promoter region of bromelain inhibitor gene from pineapple (Wintz and Firoozabady, 2011). Provides fruit-enhanced expression of the target gene.                                                                                                                                                                                                                                                    |
| Intervening sequence              | 8         | 12428 - 12421 | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                       |
| <i>PSY</i>                        | 1311      | 12420 - 11110 | <i>PSY</i> gene from tangerine encodes the enzyme phytoene synthase (Ikoma et al., 2001, GenBank ID: AB037975). Catalyzes conversion of geranylgeranyl pyrophosphate to phytoene (Romer et al., 2002).                                                                                                                                                                                              |
| Intervening sequence              | 17        | 11109 - 11093 | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                       |
| Ubpter                            | 940       | 11092 - 10153 | Terminator sequence of the tetrameric ubiquitin gene from pineapple (Wintz and Firoozabady, 2011). Provides 3' untranslated region and polyadenylation site for termination of transcription.                                                                                                                                                                                                       |
| Intervening sequence              | 23        | 10152 - 10130 | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                       |
| BRIp                              | 2452      | 10129 - 7678  | Promoter region of bromelain inhibitor gene from pineapple (Wintz and Firoozabady, 2011). Provides fruit-enhanced expression of the target gene.                                                                                                                                                                                                                                                    |

|                      |      |             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
|----------------------|------|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Intervening sequence | 12   | 7677 - 7666 | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| <i>bLcy(+)</i>       | 619  | 7665 - 7047 | A partial coding sequence of lycopene $\beta$ -cyclase gene ( <i>bLcy</i> ) from pineapple (Wintz and Firoozabady, 2011) in the sense orientation. This region was isolated from pineapple based on nucleotide sequence homology to lycopene $\beta$ -cyclase genes from the plant sources: <i>Zea mays</i> PS1 gene (GenBank ID: AY206862, Singh et al., 2003), <i>Citrus unshiu</i> (GenBank ID: AY166796, Kim et al., unpublished), <i>Solanum lycopersicum</i> (GenBank ID: X86452, Pecker et al., 1996) and <i>Capsicum annuum</i> (GenBank ID: X86221, Huguency et al., 1995). Provides RNAi suppression of endogenous lycopene $\beta$ -cyclase.      |
| Intervening sequence | 10   | 7046 - 7037 | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| LSI Intron           | 190  | 7036 - 6847 | Intron 2 sequence of <i>ST-LSI</i> gene from potato (Eckes et al., 1986, GenBank ID: X04753.1). Provides stability to the inverted repeat structure in <i>Escherichia coli</i> (Hirai and Kodama, 2008) and facilitates hairpin RNA duplex formation in plants (Wesley et al., 2002).                                                                                                                                                                                                                                                                                                                                                                        |
| Intervening sequence | 10   | 6846 - 6837 | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| <i>bLcy(-)</i>       | 619  | 6836 - 6218 | A partial coding sequence of lycopene $\beta$ -cyclase gene ( <i>bLcy</i> ) from pineapple (Wintz and Firoozabady, 2011) in the anti-sense orientation. This region was isolated from pineapple based on nucleotide sequence homology to lycopene $\beta$ -cyclase genes from the plant sources: <i>Zea mays</i> PS1 gene (GenBank ID: AY206862, Singh et al., 2003), <i>Citrus unshiu</i> (GenBank ID: AY166796, Kim et al., unpublished), <i>Solanum lycopersicum</i> (GenBank ID: X86452, Pecker et al., 1996) and <i>Capsicum annuum</i> (GenBank ID: X86221, Huguency et al., 1995). Provides RNAi suppression of endogenous lycopene $\beta$ -cyclase. |
| Intervening sequence | 13   | 6217 - 6205 | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| Ubptter              | 940  | 6204 - 5265 | Terminator sequence of the tetrameric ubiquitin gene from pineapple (Wintz and Firoozabady, 2011). Provides 3'untranslated region and polyadenylation site for termination of transcription.                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| Intervening sequence | 41   | 5264 - 5224 | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| BRip                 | 2452 | 5223 - 2772 | Promoter region of bromelain inhibitor gene from pineapple (Wintz and Firoozabady, 2011). Provides fruit-enhanced expression of the target gene (Wintz and Firoozabady, unpublished).                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| <i>eLcy(+)</i>       | 504  | 2771 - 2268 | A partial coding sequence of lycopene $\epsilon$ -cyclase gene ( <i>eLcy</i> ) from pineapple in the sense orientation (Wintz and Firoozabady, unpublished). This region was isolated from pineapple based on nucleotide sequence homology to lycopene $\epsilon$ -cyclase genes from the plant sources: <i>Oryza sativa</i> (japonica cultivar-group) (GenBank ID: AK072015, Kikuchi et al., 2003) and <i>Adonis palaestina</i> (GenBank ID: AF321536, Cunningham and Gantt, 2001). For RNAi suppression of endogenous lycopene $\epsilon$ -cyclase.                                                                                                        |



|                         |       |               |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
|-------------------------|-------|---------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Intervening sequence    | 30    | 2267 – 2238   | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| LS1 Intron              | 190   | 2237 – 2048   | Intron 2 sequence of ST-LS1 gene from potato (Eckes et al., 1986, GenBank ID: X04753.1). Provides stability to the inverted repeat structure in <i>Escherichia coli</i> (Hirai and Kodama, 2008) and facilitates hairpin RNA duplex formation in plants (Wesley et al., 2002).                                                                                                                                                                                                                                                                                 |
| Intervening sequence    | 10    | 2047 – 2038   | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| <i>eLcy(-)</i>          | 504   | 2037 – 1534   | A partial coding sequence of lycopene $\epsilon$ -cyclase gene ( <i>eLcy</i> ) from pineapple (Wintz and Firoozabady, unpublished) in the antisense orientation. This region was isolated from pineapple based on nucleotide sequence homology to lycopene $\epsilon$ -cyclase genes from the plant sources: <i>Oryza sativa</i> (japonica cultivar-group) (GenBank ID: AK072015, Kikuchi et al., 2003) and <i>Adonis palaestina</i> (GenBank ID: AF321536, Cunningham and Gantt, 2001). Provides RNAi suppression of endogenous lycopene $\epsilon$ -cyclase. |
| Intervening sequence    | 2     | 1533 – 1532   | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| Ubpter                  | 940   | 1531 – 592    | Terminator sequence of the tetrameric ubiquitin gene from pineapple (Wintz and Firoozabady, 2011). Provides 3'untranslated region and polyadenylation site for termination of transcription.                                                                                                                                                                                                                                                                                                                                                                   |
| Intervening sequence    | 9     | 591 – 583     | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| LB                      | 582   | 582 – 1       | Left border region of T-DNA from Ti plasmid of <i>Agrobacterium tumefaciens</i> . Short direct repeat sequence that flanks the T-DNA and is required for transfer of the T-DNA into plant cells (Barker et al., 1983).                                                                                                                                                                                                                                                                                                                                         |
| <b>Plasmid backbone</b> |       |               |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| p15A                    | 1851  | 36559 - 34709 | The p15A origin of replication (Chang and Cohen, 1978) for maintenance of the plasmids in <i>Escherichia coli</i> .                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| TetR/A                  | 1956  | 34708 - 32753 | The tetracycline gene (TetR/A) from plasmid RP1 (Waters et al., 1983) as an <i>Escherichia coli</i> selectable marker.                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| Intervening Sequence    | 65    | 32752 - 32688 | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| pVS1                    | 10292 | 32687 - 22396 | pVS1 origin of replication (Itoh et al., 1984) for maintenance of the plasmids in <i>Agrobacterium tumefaciens</i> (Itoh et al., 1984).                                                                                                                                                                                                                                                                                                                                                                                                                        |

## II.C. PLASMID VECTOR pHCWflACC3'-2

Event EF2-114 was produced by *Agrobacterium tumefaciens*-mediated transformation using two plasmids pHCW.T-7 (Figure IV-4) and pHCWflACC3'-2 (Figure IV-6). The size of the pHCWflACC3'-2 expression plasmid is approximately 25.8 kb.

Figure II-6. Circular map of pHCWflACC3'-2

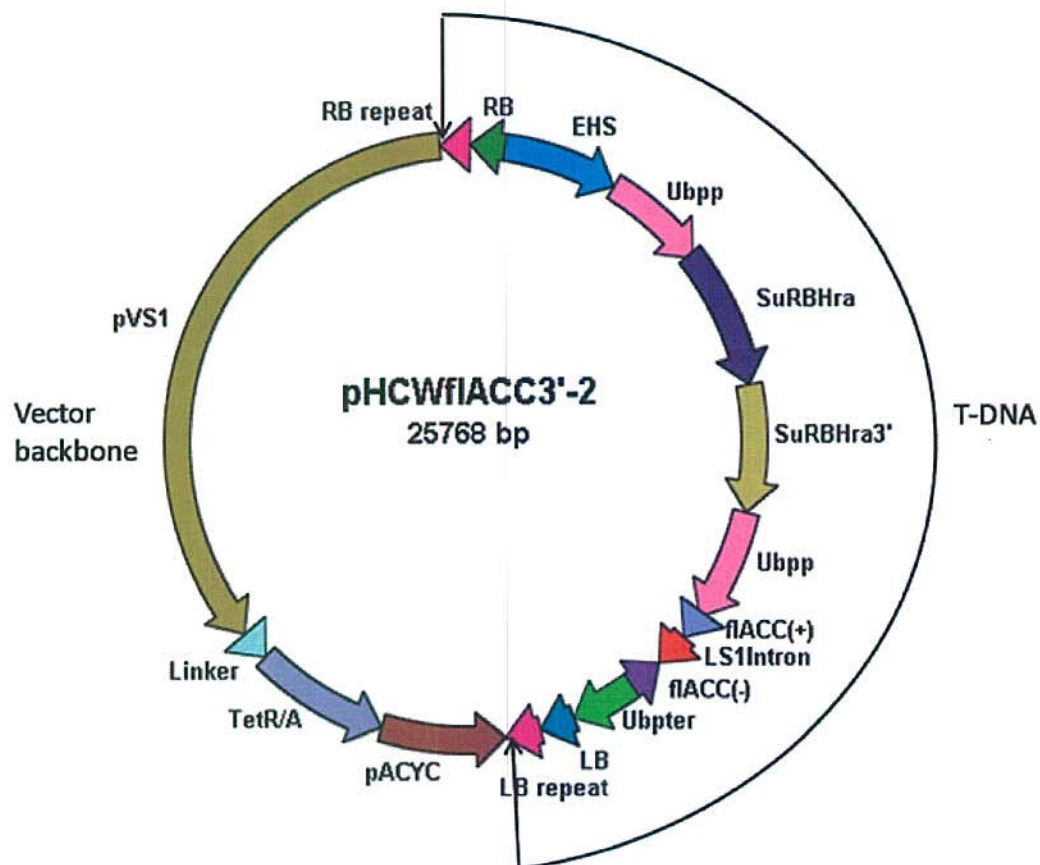
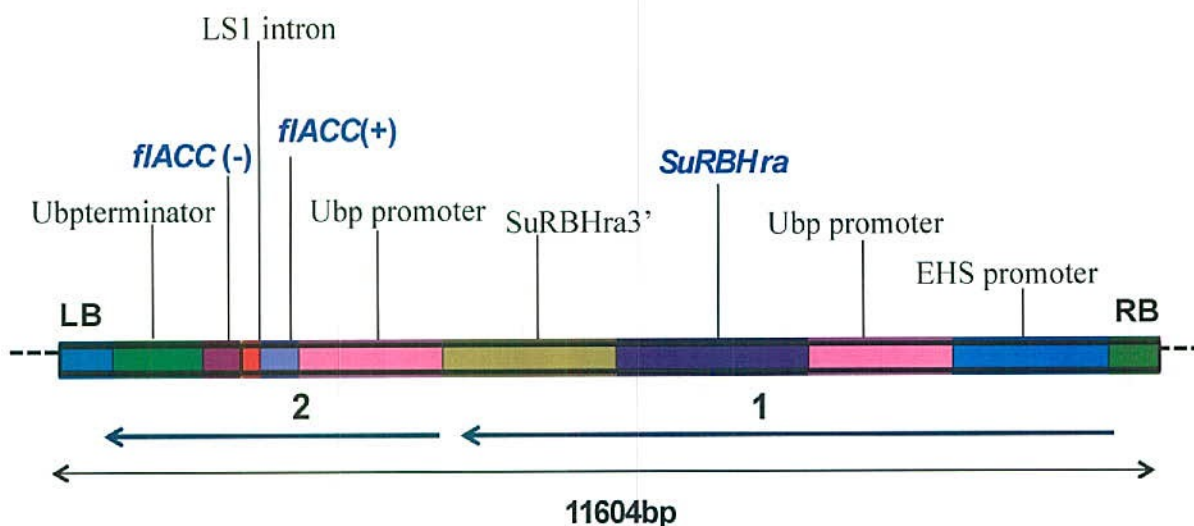




Figure II-7. Schematic diagram of the T-DNA of pHCWflACC3'-2



Numbered arrows (1-2) indicate the two expression cassettes and respective regulatory elements in pHCWflACC3'-2

#### II.C.1. GENETIC ELEMENTS OF pHCWflACC3'-2 T-DNA

The 11.6 kb T-DNA of the pHCWflACC3'-2 plasmid has two expression cassettes. The organization and orientation of the two expression cassettes are depicted in Figure IV-7 and are described below. The gene of interest in individual expression cassettes is labeled in blue text. The location of each of the genetic element on the T-DNA (base pair position) and its size are presented in Table IV-2.

- a) EHS-Ubpp-*SuRBHra*-*SuRBHra*3' expression cassette (plant selectable marker) is same as previously described for pHCW.T-7 (Section IV.B.1).
- b) Ubpp-*flACC*3' RNAi-Ubpter expression cassette is composed of a promoter/5'UTR from the ubiquitin (*Ubi*) gene of pineapple (*A. comosus*), a portion of the *flACC*3' gene from pineapple (*A. comosus*) in the sense orientation, the intron 2 sequence of *ST-LSI* gene from potato (*S. tuberosum*) (same as in pHCW.T-7), a portion of the *flACC*3' gene from pineapple (*A. comosus*) in the anti-sense orientation, and a terminator sequence corresponding to the 3' UTR/flanking region of pineapple *Ubi* gene.

Table II-2. Description of genetic elements in pHCW $\Delta$ ACC3'-2

| Genetic Element                   | Size (bp) | Position      | Description                                                                                                                                                                                                                                                                                                                                                                                              |
|-----------------------------------|-----------|---------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| RB                                | 547       | 11604 - 11058 | Right border region of T-DNA from Ti plasmid of <i>Agrobacterium tumefaciens</i> . Short direct repeat that flanks the T-DNA and is required for transfer of the T-DNA into plant cells (Depicker et al., 1982).                                                                                                                                                                                         |
| <b>Selectable marker cassette</b> |           |               |                                                                                                                                                                                                                                                                                                                                                                                                          |
| EHS                               | 1625      | 11057 - 9433  | Promoter region of epoxide hydrolase gene from pineapple (Wintz and Firoozabady, 2011). When fused with Ubpp, provides constitutive expression in pineapple (Wintz and Firoozabady, 2011).                                                                                                                                                                                                               |
| Ubpp                              | 1536      | 9432 - 7897   | Promoter region of the tetrameric ubiquitin gene from pineapple (Wintz and Firoozabady, 2011). Provides constitutive expression in pineapple (Wintz and Firoozabady, 2011).                                                                                                                                                                                                                              |
| SuRBHra                           | 1995      | 7896 - 5902   | An <i>ALS</i> gene from the mutant tobacco line Hra, encodes the enzyme acetolactate synthase (Lee et al., 1988, GenBank ID: X07645.1). Catalyzes the conversion of two pyruvate molecules to acetolactate and carbon dioxide, the first committed step in biosynthesis of essential branched chain amino acids. This enzyme is insensitive to sulfonyleurea and functions as a plant selectable marker. |
| SuRBHra3'                         | 1763      | 5901 - 4139   | Terminator sequence of native acetolactate synthase gene from tobacco (Townsend et al., 2009; GenBank ID: FJ649655). Provides 3'untranslated region and polyadenylation site for termination of transcription.                                                                                                                                                                                           |
| Intervening sequence              | 42        | 4138 - 4097   | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                            |
| <b>Functional gene cassette</b>   |           |               |                                                                                                                                                                                                                                                                                                                                                                                                          |
| Ubpp                              | 1536      | 4096 - 2561   | Promoter region of the tetrameric ubiquitin gene from pineapple (Wintz and Firoozabady, 2011). Provides constitutive expression in pineapple (Wintz and Firoozabady, 2011).                                                                                                                                                                                                                              |
| Intervening sequence              | 11        | 2560 - 2550   | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                            |
| <i>ΔACC(+)</i>                    | 400       | 2549 - 2150   | A portion of the meristem ACS gene ( <i>ΔACC3'</i> ) from pineapple (Botella et al., 2000, GenBank ID: DT335956; Wintz and Firoozabady, 2011) in the sense orientation. Provides RNAi suppression of endogenous gene.                                                                                                                                                                                    |
| Intervening sequence              | 14        | 2149 - 2136   | Sequence used in DNA cloning.                                                                                                                                                                                                                                                                                                                                                                            |
| LS1 Intron                        | 190       | 2135 - 1946   | Intron 2 sequence of <i>ST-LS1</i> gene from potato (Eckes et al., 1986, GenBank ID: X04753.1). Provides stability to the inverted repeat structure in <i>Escherichia coli</i> (Hirai and Kodama, 2008) and facilitates hairpin RNA duplex formation in plants (Wesley et al., 2002).                                                                                                                    |



| Genetic Element         | Size (bp) | Position      | Description                                                                                                                                                                                                                 |
|-------------------------|-----------|---------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Intervening sequence    | 6         | 1945 - 1940   | Sequence used in DNA cloning.                                                                                                                                                                                               |
| <i>flACC(-)</i>         | 400       | 1939 - 1540   | A portion of the meristem ACS gene ( <i>flACC3'</i> ) from pineapple (Botella et al., 2000, GenBank ID: DT335956; Wintz and Firoozabady, 2011) in the anti-sense orientation. Provides RNAi suppression of endogenous gene. |
| Intervening sequence    | 8         | 1539 - 1532   | Sequence used in DNA cloning.                                                                                                                                                                                               |
| Ubpter                  | 940       | 1531 - 592    | Terminator sequence of the tetrameric ubiquitin gene from pineapple (Wintz and Firoozabady, 2011). Provides 3'untranslated region and polyadenylation site for termination of transcription.                                |
| Intervening sequence    | 9         | 591 - 583     | Sequence used in DNA cloning.                                                                                                                                                                                               |
| LB                      | 582       | 582 - 1       | Left border region of T-DNA from Ti plasmid of <i>Agrobacterium tumefaciens</i> . Short direct repeat sequence that flanks the T-DNA and is required for transfer of the T-DNA into plant cells (Barker et al., 1983).      |
| <b>Plasmid backbone</b> |           |               |                                                                                                                                                                                                                             |
| p15A                    | 1851      | 25768 - 23918 | The p15A origin of replication (Chang and Cohen, 1978) for maintenance of the plasmids in <i>Escherichia coli</i> .                                                                                                         |
| TetR/A                  | 1956      | 23917 - 21962 | The tetracycline gene (TetR/A) from plasmid RP1 (Waters et al., 1983) as an <i>Escherichia coli</i> selectable marker.                                                                                                      |
| Intervening Sequence    | 65        | 21961 - 21897 | Sequence used in DNA cloning.                                                                                                                                                                                               |
| pVS1                    | 10292     | 21896 - 11605 | pVS1 origin of replication (Itoh et al., 1984) for maintenance of the plasmids in <i>Agrobacterium tumefaciens</i> (Itoh et al., 1984).                                                                                     |

## II.D. REGULATORY SEQUENCES

The expression cassettes in pHCW.T-7 and pHCWflACC3'-2 contain promoters and transcription termination sequences derived from pineapple (*Ananas comosus*).

EHS-Ubpp is a fusion promoter consisting of the promoter/5'UTR sequence of the epoxide hydrolase (*EHS*) gene and the promoter/5'UTR of the tetrameric ubiquitin (*Ubi*) gene. Epoxide hydrolases are a class of enzymes that catalyze the hydration of chemically reactive epoxides to their corresponding dihydrodiol products (Fretland and Omiecinski, 2000). In plants, epoxide hydrolases play an important role in oleic acid metabolism (Blée, 1998). Oleic acids are major constituents of cutins, which comprise the cuticles that protect plants against dehydration. In addition, they also serve as signal molecules upon pathogen attack (Schweizer et al., 1996). In pineapple fruits and aerial parts, enhanced expression of an epoxide hydrolase gene (GenBank ID: AY098527) has been reported (Neuteboom et al., 2002).

Ubiquitin is a protein found in all eukaryotic cells and is implicated in processes such as protein turnover, chromatin structure, cell cycle control, DNA repair, and response to pathogen attack and other stresses (Vierstra, 1996). Ubiquitin promoter-based vectors have been used successfully in transforming a number of different monocot species (Wilmink et al., 1995), including several cereals (McElroy and Brettell, 1994). In pineapple, a tetrameric ubiquitin gene (GenBank ID: AY098526) has been shown to be constitutively expressed (Neuteboom et al., 2002).

Both promoters were isolated by Wintz and Firoozabady (2011) from pineapple. Overall, the activity of the EHS-Ubpp fusion promoter in pineapple tissues has been shown to be higher than or equal to the single Ubpp promoter and much higher than single EHS promoter by a transient gene expression assay system using the  $\beta$ -glucuronidase (*GUS*) gene (Wintz and Firoozabady, 2011 and Firoozabady et al., unpublished results). In Event EF2-114, EHS-Ubpp drives expression of the selectable marker gene.

BRlp is a fruit-enhanced promoter sequence of the *BRI* gene of pineapple (Moyle et al., 2005). The physiological function of *BRI* is thought to regulate the coexistent proteinase activity in the cell, although the detailed function is not clear. In pineapple, under the acidic condition of vacuoles, *BRI* blocks the activity of bromelain and/or other proteinases and protects other proteins from proteolysis (Sawano et al., 2002). In Event EF2-114, BRlp drives specific expression of *PSY*, *bLcy* RNAi, and *eLcy* RNAi cassettes in pineapple fruits. The rationale behind choosing the BRlp was based on published data by Moyle et al., 2005, in which the authors showed fruit-enhanced expression of *BRI* during pineapple fruit development.

Ubpp is the promoter/5' UTR of the tetrameric ubiquitin (*Ubi*) gene isolated from pineapple (Wintz and Firoozabady, 2011). In Event EF2-114, Ubpp drives constitutive expression of the *flACC3'* RNAi cassette in pineapple (Wintz and Firoozabady, 2011 and Firoozabady et al., unpublished results).



Ubpter is the 3' UTR/flanking region of the tetrameric *Ubi* gene from pineapple (*A. comosus*) that serves as a transcription terminator (Wintz and Firoozabady, 2011).

The ST-LS1 intron is the Intron 2 sequence of the *ST-LS1* gene from potato (*Solanum tuberosum*) (Eckes et al., 1986, GenBank ID: X04753.1), which functions to stabilize the inverted repeat sequences in *Escherichia coli* and facilitates hairpin-RNA duplex formation in plants (Hirai and Kodama, 2008; Wesley et al, 2001).

## II.E. T-DNA BORDERS

The right border sequence in plasmids pHCW.T-7 and pHCWfIACC3'-2 includes a 25 bp repeat region originally isolated from *Agrobacterium tumefaciens* pTiT37 (Depicker et al., 1982), which acts as an initiation point of T-DNA transfer into plant cells. Similarly, the left border sequence includes a 25 bp repeat region originally isolated from *Agrobacterium tumefaciens* pTiA6 (Barker et al., 1983) that delimits and acts as an end point of T-DNA transfer into plant cells.

## II.F. GENETIC ELEMENTS OUTSIDE THE T-DNA BORDERS

The transformation vectors are derived from a binary plasmid, pALS1301 (Firoozabady et al., 2006). The genetic elements outside of the T-DNA in plasmids pHCW.T-7 and pHCWfIACC3'-2 are identical. The vector backbone consists of three key elements: the pVS1 origin of replication (Itoh et al., 1984) for maintenance of the plasmids in *Agrobacterium*, the tetracycline gene (TetR/A) from plasmid RP1 (Waters et al., 1983) as an *Escherichia coli* selectable marker, and the p15A origin of replication (Chang and Cohen, 1978) for maintenance of the plasmids in *Escherichia coli*. Cloning of the individual genetic elements and assembling of the transformation vectors were performed using standard recombinant molecular biology techniques (Maniatis et al., 1982; Sambrook and Russell, 2001).

## II.G. DESCRIPTION OF THE TRANSFORMATION SYSTEM

Event EF2-114 was developed through *Agrobacterium*-mediated transformation of pineapple (*Ananas comosus*) variety MD2 based on the method described by Firoozabady (2011). Briefly, longitudinal sections of *in vitro* shoots (Firoozabady and Gutterson, 2003) were prepared and cultured. Leaf base sections were prepared from the longitudinal sections and co-transformed with two *Agrobacterium tumefaciens* GV3101(pMP90) strains (Koncz and Schell, 1986) carrying the binary plasmids pHCW.T-7 (Figure IV-4) and pHCWfIACC3'-2 (Figure IV-6), respectively.

Following co-cultivation, the leaf base sections were transferred to recovery media containing carbenicillin to eliminate *Agrobacterium* and later were transferred to selection media containing

the antibiotics vancomycin and cefotaxim to eliminate residual *Agrobacterium*, and chlorsulfuron (CS), an herbicide, to select for transformed plant cells and to produce transformed organogenic shoot meristems. A total of eight transfers were done with increasing selection pressure, from sub-lethal to lethal levels of CS to produce pure transgenic tiny buds. The step up selection pressure was essential as the weak constitutive promoter did not support, immediately after the recovery period, survival of transformed cells in the presence of lethal concentrations of CS, which further developed into transformed shoot buds. Tiny bud clusters were transferred twice to media containing CS and cefotaxim to further grow and multiply transformed shoots and shoot buds. Shoots and shoot buds were transferred to agar-solidified media to normalize transformed shoots and shoot buds, and then transferred to liquid media with CS every 4-6 weeks to multiply and elongate the transformed shoots. PCR analyses confirmed the presence of the introduced genes in tissue culture-derived shoots.

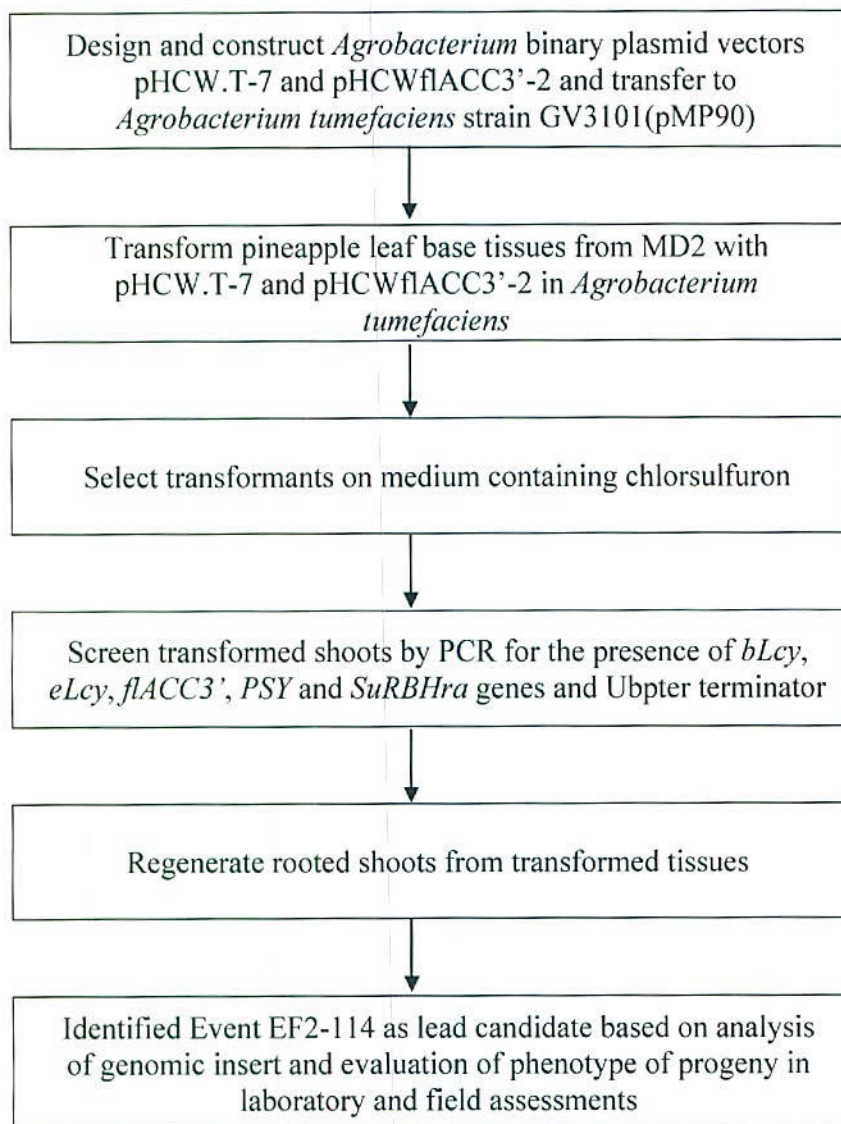
Transgenic T<sub>0</sub> plants were shipped to Corporación de Desarrollo Agrícola Del Monte (CDADM), Buenos Aires, Costa Rica for evaluation. At CDADM, plants were grown in a greenhouse for 15-20 weeks before transfer to soil for confined field trials (Costa Rica Ministry of Agriculture Permit #GB-122-05). Plants were grown according to standard agronomic production practices until they produced fruits. Fruits were cut to assess internal flesh color and quality characteristics (°Brix, citric acid, ascorbic acid and pH). Only plants that had fruits with internal pink/red color were advanced for further propagation in the field or in tissue culture. Meristem cultures were produced primarily from the crown (and occasionally from the slip or the ratoon) of T<sub>0</sub> plants. The T<sub>1</sub> propagules were grown for further evaluation and were subsequently used for molecular and phenotypic assessments. Molecular analyses (PCR and Southern) confirmed the presence of the introduced genes in both tissue culture-derived as well as field-propagated plants. The major steps in development of Event EF2-114 are depicted in Figure IV-8.

## II.H. DEVELOPMENT OF TEST AND CONTROL MATERIALS

Figure IV-9 lists the genotypes and descriptions of Event EF2-114 and non-transgenic MD2 control plant materials used in the studies described in this submission. The methods and conditions of cultivation of test and control materials used in these studies are described in Appendix A.



Figure II-8. Process of development of Event EF2-114



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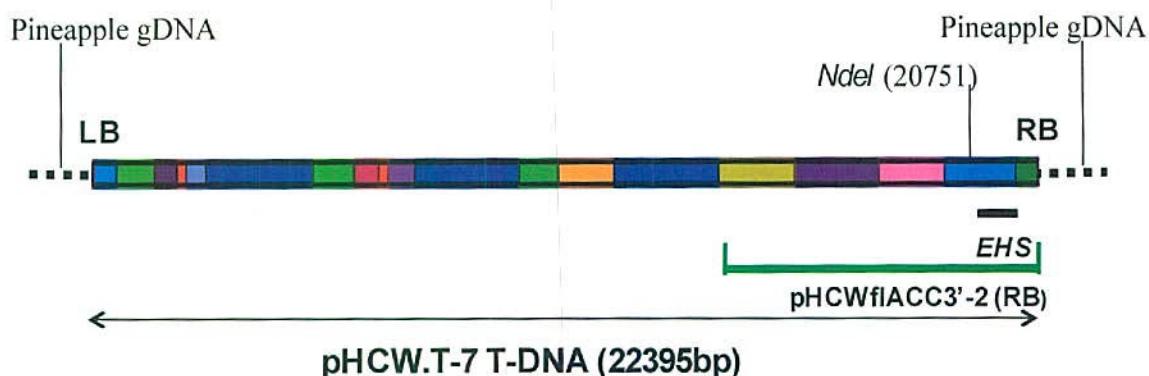
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### Generational Stability

In order to assess the stability of the inserted DNA in Event EF2-114 across generations, Southern blot analysis was performed using DNA obtained from four generations of Event EF2-114 plants. For reference, the propagation diagram of Event EF2-114 is presented in Figure IV-9. The specific generations tested are also indicated in Figure IV-9.

The Southern blot analysis included genomic DNA from the leaf tissues of pineapple plants from MD2 and from four generations (Figure IV-9: T<sub>1</sub>-TC, T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub>) of Event EF2-114. A Southern blot with genomic DNA digested with *Nde*I and probed with the *EHS* probe (Figure V-20, Table V-10) was used for this analysis. It should be noted that this portion of the two T-DNAs is identical in the two plasmids and as mentioned previously, the *EHS* probe is expected to hybridize to both.

**Figure II-9. Generational stability – Schematic map of pHCW.T-7/pHCWflACC3'/NdeI**



**Table II-3. Generational stability - pHCW.T-7/pHCWflACC3'-2 T-DNA - Size(s) of expected and observed hybridization bands**

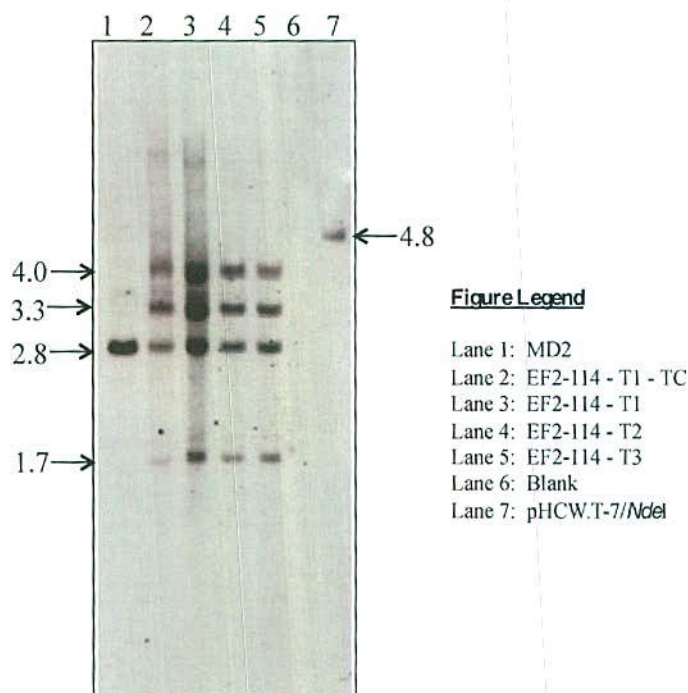
| Restriction enzyme on T-DNA/gDNA   | Probe      | Probe position and Size (bp) | Expected bands in EF2-114 (kb) | Observed bands in EF2-114 (kb)   | Expected band in control plasmid (kb) |
|------------------------------------|------------|------------------------------|--------------------------------|----------------------------------|---------------------------------------|
| <i>Nde</i> I (20751)/ <i>Nde</i> I | <i>EHS</i> | 20779 - 21772 (993)          | Endogenous & >1.6              | 1.7, 2.8 (Endogenous), 3.3 & 4.0 | 4.8                                   |

The result of the Southern analysis is shown in Figure V-21. The 2.8 kb band present in MD2 in Lane 1 in Figure V-21 is the result of the probe hybridizing to the endogenous *EHS*. There are three additional bands hybridizing at 1.7, 3.3 and 4.0 kb. All the three bands are >1.6 kb as



expected. On one hand, from this Southern using a single restriction digest, it could be interpreted that there are three integrations from the right border, cumulative of both the plasmids. On the other hand, the Southern with two restriction enzymes (Figure V-6, *NcoI/BamHI*) confirmed the presence of a total of eight integrations from the two plasmids. This shows that in the single digest, some of the hybridizing bands may be migrating together due to rearrangements with tandem or inverted repeats T-DNA structures. However, for generational stability analysis, maintenance of the same hybridization pattern in various generations is more important than the co-migration of the bands. The hybridization pattern seen in Figure V-21 Lane 2, 3, 4, and 5 corresponding to T<sub>1</sub>-TC, T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub> generations respectively is identical confirming the stability of the introduced T-DNA(s). Thus, this analysis confirmed the stability of the T-DNA insertions without any modification across the four generations tested.

**Figure II-10. Southern blot – Generational stability**



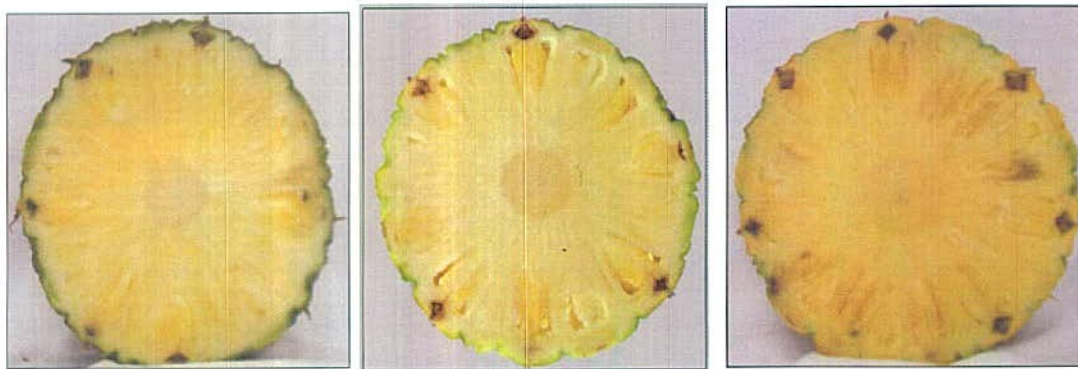
#### **Generational stability for fruit internal color:**

We also examined the internal color of the fruits over six generations of propagation (T<sub>0</sub>, T<sub>1</sub>-TC, T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub>, T<sub>4</sub> and T<sub>5</sub>). While the parental variety MD2 has a golden flesh color, Event EF2-114 fruit exhibits pink/red color in the flesh due to accumulation of lycopene. In all generations, the pink color and uniformity was similar to the initial plants of Event EF2-114 (T<sub>0</sub>). Pictures of samples from three representative fruits for each generation are

presented in Figure V-22. For statistical analyses of internal color, see Appendix H, Table H-2.

**Figure II-11. Generational stability for fruit internal color**

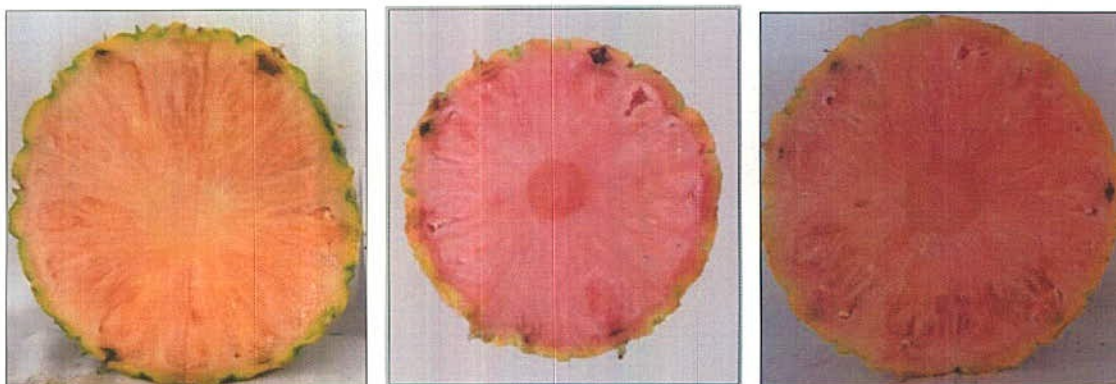
**MD2**



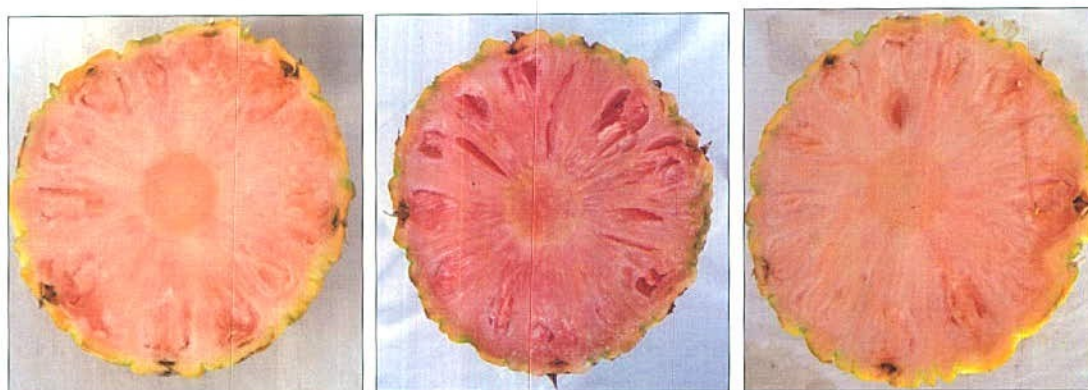
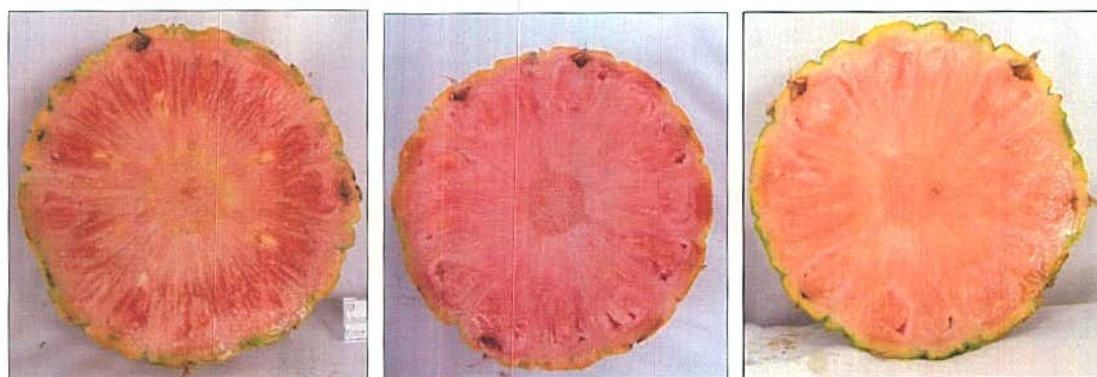
**T<sub>0</sub> Generation**



**T<sub>1</sub>-TC Generation**



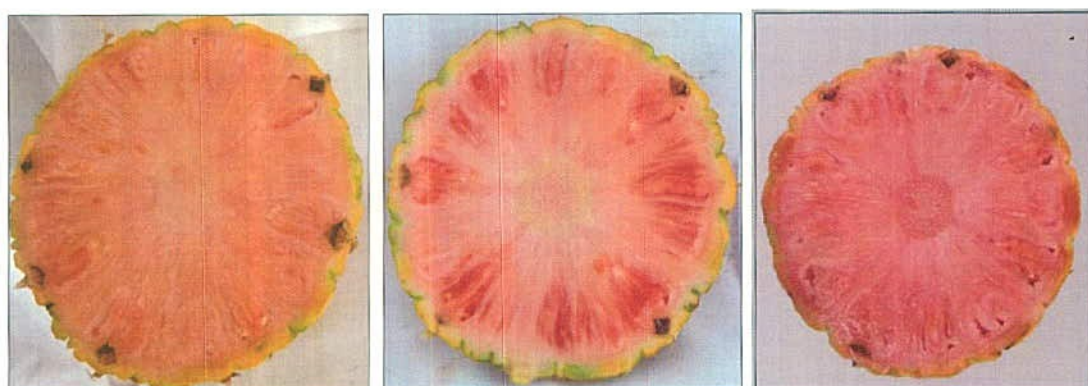


**T<sub>1</sub> Generation****T<sub>2</sub> Generation****T<sub>3</sub> Generation**

### T<sub>4</sub> Generation



### T<sub>5</sub> Generation



#### II.I.1. SUMMARY OF GENERATIONAL STABILITY ANALYSIS

Southern hybridization data of genomic DNA of pineapple plants from four generations with *EHS* probe established that the T-DNAs from both the transformation plasmids remained stable across four generations of Event EF2-114 plants. Also, the internal color of the fruits remained stable over six generations of propagation tested.

#### II.J. CHARACTERIZATION OF EVENT EF2-114 EXPRESSION PRODUCTS

Pineapple tissues are recalcitrant to extraction of proteins and nucleic acids. The difficulty in obtaining high quality proteins and nucleic acids from pineapple fruit tissues, in particular, is mainly due to their low concentrations over fresh weight, to the high activity of proteases and nucleases, and to high levels of interfering compounds such as polyphenols and polysaccharides (Song et al., 2006). In order to produce sufficient quantities of PSY and SuRBHra proteins to perform safety studies recommended by Codex guidance, attempts were made to overexpress the



*PSY* and *SuRBHra* genes in *Escherichia coli*. The genes were cloned into pQE-60 (Qiagen, Valencia, CA) expression vectors. The pQE-60 vector carries a Tn-5 promoter/lac operator system, inducible by IPTG (isopropyl thiogalactopyranoside) and a C-terminus histidine tag for nickel affinity purification. The *PSY* protein failed to express in this system. The *SuRBHra* protein was localized exclusively in the inclusion bodies in *Escherichia coli* and purification required harsh denaturing conditions, such as the use of 8M urea. Proteins purified under such conditions are not suitable for use in *in vitro* digestibility, heat stability, or acute toxicity assays.

Multiple attempts were made to assess the expression levels of the full-length introduced proteins, *SuRBHra* and *PSY*, in Event EF2-114 using Western blots (data not shown). For *PSY* expression by Western, pineapple fruit samples were sent to Dr. Ralf Welsch (University of Freiburg, Freiburg, Germany), whose laboratory has expertise working with enzymes of the carotenoid biosynthetic pathway. The Welsch lab had previously raised anti-*PSY* antibodies against the *Arabidopsis* *PSY* (GenBank ID: NM\_121729), which were shown to cross-react with the *Arabidopsis* *PSY* antibodies (Maass et al., 2009). *Arabidopsis* *PSY* has 80% similarity with the tangerine *PSY* at the amino acid level. Since our attempt to express *PSY* protein in *Escherichia coli* failed, pineapple fruit samples were sent to the Welsch laboratory and Western blots were performed using the anti-*PSY* antibodies from *Arabidopsis*. The antibodies cross-reacted poorly to the target protein in the blots, resulting in very low signal-to-noise ratio.

For Western blot analysis of *SuRBHra* in Event EF2-114, antibodies raised against the protein purified under denaturing conditions from *E. coli* inclusion bodies were used. The antibodies cross-reacted poorly to the target protein in the blots resulting in very low signal-to-noise ratio. Accordingly, alternative methods were necessary for assessing *SuRBHra* and *PSY* expression in Event EF2-114.

Similarly, multiple attempts were made to assess expression of *SuRBHra* and *PSY*, and suppression of *bLcy* and *eLcy* in Event EF2-114 using Northern blot analysis. Northern blots generally require 5-10 µg of total RNA for each lane of the blots, and isolation of this quantity from pineapple tissues, particularly from fruits proved difficult. Therefore, Real-Time quantitative Reverse Transcription PCR (qRT-PCR) was chosen as a method of choice to evaluate expression of the introduced genes in Event EF2-114 due to the technical difficulties in extracting sufficient, high quality proteins and RNA from pineapple tissues, and the lack of antibodies needed for analyses.

qRT-PCR has become one of the most widely used methods for detection and/or comparison of gene expression levels. qRT-PCR is attractive because it requires minute quantities (ng) of template RNA and uses fluorescent reporter molecules to monitor the amplification products during each cycle of the PCR reaction. qRT-PCR combines nucleic acid amplification and detection steps into one homogeneous assay with a large dynamic range of detection and tremendous sensitivity. Unlike Northern hybridizations, the need for post-handling of RNA is eliminated in qRT-PCR, thus making it a more accurate method of choice for comparing gene expression levels. qRT-PCR differs from classical PCR by the measurement of the amplified PCR



product at each cycle throughout the PCR reaction. In practice, a video camera records in real-time the light emitted by the fluorochrome incorporated into the newly synthesized PCR product.

## II.K. EXPRESSION OF EVENT EF2-114 PRODUCTS

As previously described in Section IV, Event EF2-114 was developed by *Agrobacterium tumefaciens*-mediated transformation of two plasmids, pHCW.T-7 and pHCWfIACC3'-2. Plasmid pHCW.T-7 carries three genes, *PSY*, *bLcy* and *eLcy*, for the manipulation of carotenoids. Expression of all the three genes is under the control of the fruit-enhanced BRIp promoter. Plasmid pHCWfIACC3'-2 carries a portion of the flowering control gene, *fIACC3'*, as an RNAi construct, the expression of which is under the control of the constitutive Ubp promoter. Both expression plasmids also carry the *SuRBHra* gene as a plant selectable marker, the expression of which is under the control of a constitutive EHS-Ubpp fusion promoter. Various analyses were carried out in order to assess the expression level of the introduced genes in Event EF2-114.

For the present study, RNA samples were prepared in the laboratory of Del Monte Fresh Produce (Richmond, CA) and qRT-PCR was performed by Syd Labs, Inc., (Malden, MA). In Event EF2-114, qRT-PCR was performed for the following genes relative to native pineapple  $\beta$ -actin or bromelain inhibitor (*BRI*) gene expression:

1. *PSY* overexpression
2. *SuRBHra* constitutive expression
3. *bLcy* suppression
4. *eLcy* suppression
5. *fIACC3'* suppression and
6. *BRI* native expression.

Gene expression in the non-transgenic, parental MD2 was used as a comparator. The pineapple  $\beta$ -actin gene was used as a normalization control in all qRT-PCR analyses. Details of the sample collection and protocol for isolation of total RNA from pineapple leaves and fruits are described in Appendix B. The qRT-PCR protocol used by Syd Labs is described in Appendix B.

In the present analysis, transcript levels in Event EF2-114 were normalized to pineapple  $\beta$ -actin transcript levels and expressed relative to the content in MD2. Data represent the mean of three technical replicates from one biological replicate or nine technical replicates from three biological replicates.

### II.K.1. *PSY* EXPRESSION

Several attempts were made to detect expression of the introduced *PSY* gene in Event EF2-114 by qRT-PCR, taking into consideration both the spatial and temporal nature of the *BRI* promoter. All attempts resulted in Ct values >35 and, thus, the *PSY* transcript levels were below the threshold level of detection.



The lack of detectable *PSY* transcripts in Event EF2-114 led to the assumption that the activity of the endogenous pineapple *PSY* gene(s) might be contributing to the formation of sufficient phytoene in the carotenoid biosynthetic pathway in the fruits leading to accumulation of lycopene (pink/red color) in the fruits. To evaluate this assumption, efforts were made to detect the endogenous *PSY* transcript levels in both Event EF2-114 and MD2 fruits. Recently, two fruit-specific, partial *PSY* genes (*UT1* with 342 bp of coding region and *UT2* with 214 bp of coding region) have been identified through massively parallel sequencing of the pineapple fruit transcriptome (Ong, et al., 2012; V. S. Kumar, personal communication). Using the sequences of *UT1* and *UT2* genes, specific primers were designed and qRT-PCR analysis was performed with RNA isolated from the fruits at 106, 131 and 154 days after forcing (DAF). The results are summarized in Table VI-5 and Figure VI-3.

As seen in Table VI-5 and Figure VI-4, the endogenous *UT1* and *UT2* transcript levels were very similar in Event EF2-114 relative to MD2, supporting the assumption that the flux of phytoene in the carotenoid biosynthetic pathway is maintained through the expression of the native *PSY* genes in the fruit, thereby decreasing or eliminating the need to overexpress exogenous *PSY* to accumulate lycopene.

Lack of expression of the introduced tangerine *PSY* gene under control of the strong fruit-enhanced BRI promoter has raised various possibilities, including transcriptional gene silencing (TGS) and/or posttranscriptional gene silencing (PTGS) in Event EF2-114 fruits. The outcome of TGS is inhibition of transcription of the transgene and the outcome of PTGS is degradation of the transgene RNA (Paszowski and Whitham, 2001). Since qRT-PCR measures steady-state level of transcripts, it is not clear at this juncture if the transcription of the heterologous *PSY* was not initiated or was inhibited in the nucleus, or the transcribed RNA was degraded before it reached the cytoplasm.

There are several studies associating methylation of promoter sequences leading to TGS and methylation of the transcribed region leading to PTGS in transgenic plants (Vaucheret et al., 1998). In the transformation plasmid, pHCW.T-7, the BRI promoter is repeated three times. If promoter methylation of BRI promoter had played a role in silencing of the *PSY*, it would be safe to assume that it would have affected all three instances. However, this does not appear to be the case since RNAi-mediated suppression of *bLcy* and *eLcy* driven by the identical promoter sequence in the same construct has worked as expected (Table VI-5, Figure VI-3).

Small interfering RNAs (siRNA) in the sense and antisense orientations specific for the transcribed transgene sequences have also been detected and shown to play an important role in PTGS (Hamilton and Baulcombe, 1999) in transgenic plants. Similarly, a correlation between siRNAs specific for the promoter region and TGS has been described (Mette et al., 2000; Sijen et al., 2001).

Several studies lent support to the hypothesis that silencing is triggered by threshold concentrations of transgene transcript or another product of transgene expression (Lindbo et al., 1993). For



example, the frequency of silencing was positively correlated with the promoter strengths of introduced genes (Que et al., 1997) and silencing was more pronounced in homozygous than in corresponding hemizygous plants (summarized in Vaucheret et al., 1998). This might be true with the silencing of the tangerine *PSY* transgene in Event EF2-114. One could assume that transcription from the two native *PSY* genes in pineapple fruits (*UT1* and *UT2*) might have contributed to the accumulation of *PSY* transcripts to threshold levels in the fruits, triggering the silencing of transcription of the transgene. The RNA threshold sensing mechanism may play an important role as a genome surveillance system capable of defending a genome against molecular parasites, such as transposons and viruses (Schubert et al., 2004).

Other possibilities for lack of detectable expression of the tangerine *PSY* in Event EF2-114 include the number and arrangement of the transgene copies and the influence of genomic regions in the vicinity of the T-DNA insertion sites (Butaye et al., 2005). Based on the Southern analysis, there are four copies of the *PSY* gene from two integration sites of the pHCW.T-7 plasmid. Detailed characterization of 132 independent transgenic *Arabidopsis* lines carrying various reporter genes revealed no case of silencing as a result of site of T-DNA integration (Schubert et al., 2004). However, in the same analysis, gene silencing was observed when more than a certain number of identical transgenes (5-12 copies) were present in the genome.

#### II.K.2. *SuRBHra* EXPRESSION

*SuRBHra* expression was examined both in the leaves and fruits of Event EF2-114 and non-transgenic MD2. Samples for analysis included RNA from one biological replicate of leaves and RNA from three biological replicates of fruits harvested at 154 DAF. qRT-PCR assays were run on three technical replicates for each biological replicate.

The primers/probe set designed for this analysis was based on the sequence of the introduced *SuRBHra* gene. The primers/probe could not detect any corresponding endogenous *ALS* transcript in MD2 control, unless there is an identical *SuRBHra* gene. For this reason, a fold change in *SuRBHra* expression relative to MD2 control could not be calculated. Instead, the Ct values as such were used to express the data. The Ct values of  $\beta$ -actin were used as a positive control to validate the assay.

The qRT-PCR data for leaves and fruits are shown Tables VI-1 and VI-2, respectively. As seen from Table VI-1 and VI-2, while the *SuRBHra* transcripts were not detectable in MD2 leaves or fruits,  $\beta$ -actin expression was detectable. The close average Ct value for *SuRBHra* in Event EF2-114 leaves (Table VI-1, 23.93) versus fruits (Table VI-2, 20.18) shows the constitutive nature of *SuRBHra* expression.

*SuRBHra* expression level in fruits was also compared to the expression level of another highly expressed gene in pineapple fruits, the bromelain inhibitor (*BRI*), for the purpose of relative



quantification. *SuRBHra* expression levels were found to be 25 fold lower in fruits relative to *BRI* expression in the same sample (Table VI-3 and VI-4). Low-level *SuRBHra* expression may, therefore, be just adequate to serve as a selectable marker without accumulating to significant concentration in fruit.

**Table II-4. *SuRBHra* expression in Event EF2-114 leaves**

| Gene                            | Replicates | MD2          | EF2-114      |
|---------------------------------|------------|--------------|--------------|
| <i><math>\beta</math>-Actin</i> | Rep1       | 22.69        | 22.41        |
|                                 | Rep2       | 22.34        | 22.34        |
|                                 | Rep3       | 22.44        | 22.59        |
|                                 | <b>Ave</b> | <b>22.49</b> | <b>22.45</b> |
|                                 | Stdv       | 0.18         | 0.13         |
| <i>SuRBHra</i>                  | Rep1       | ND           | 24.03        |
|                                 | Rep2       | ND           | 23.91        |
|                                 | Rep3       | ND           | 23.85        |
|                                 | <b>Ave</b> | ND           | <b>23.93</b> |
|                                 | Stdv       | ND           | 0.09         |

Table II-5. *SuRBHra* expression in Event EF2-114 fruits

| Gene                            | Replicates | MD2   | EF2-114 |
|---------------------------------|------------|-------|---------|
| <i><math>\beta</math>-Actin</i> | Rep1       | 20.43 | 20.49   |
|                                 | Rep2       | 20.32 | 20.37   |
|                                 | Rep3       | 20.32 | 20.50   |
|                                 | Rep4       | 20.69 | 20.68   |
|                                 | Rep5       | 20.53 | 20.75   |
|                                 | Rep6       | 20.63 | 20.69   |
|                                 | Rep7       | 20.84 | 21.00   |
|                                 | Rep8       | 20.83 | 20.80   |
|                                 | Rep9       | 20.75 | 20.87   |
|                                 | Ave        | 20.59 | 20.68   |
|                                 | Stdv       | 0.20  | 0.20    |
| <i>SuRBHra</i>                  | Rep1       | ND    | 19.81   |
|                                 | Rep2       | ND    | 19.79   |
|                                 | Rep3       | ND    | 20.10   |
|                                 | Rep4       | ND    | 20.10   |
|                                 | Rep5       | ND    | 20.12   |
|                                 | Rep6       | ND    | 20.22   |
|                                 | Rep7       | ND    | 20.47   |
|                                 | Rep8       | ND    | 20.37   |
|                                 | Rep9       | ND    | 20.64   |
|                                 | Ave        | ND    | 20.18   |
|                                 | Stdv       | ND    | 0.28    |

Table II-6. *BRI* and *SuRBHra* expression in Event EF2-114 fruits

| Sample  | Replicates | <i><math>\beta</math>-Actin</i> | <i>BRI</i> | <i>SuRBHra</i> |
|---------|------------|---------------------------------|------------|----------------|
| EF2-114 | Rep1       | 20.49                           | 15.24      | 19.81          |
|         | Rep2       | 20.37                           | 14.76      | 19.79          |
|         | Rep3       | 20.50                           | 15.26      | 20.10          |
|         | Rep4       | 20.68                           | 15.73      | 20.10          |
|         | Rep5       | 20.75                           | 15.68      | 20.12          |
|         | Rep6       | 20.69                           | 15.33      | 20.22          |
|         | Rep7       | 21.00                           | 15.49      | 20.47          |
|         | Rep8       | 20.8                            | 15.55      | 20.37          |
|         | Rep9       | 20.87                           | 15.71      | 20.64          |
|         | Ave        | 20.68                           | 15.42      | 20.18          |
|         | Stdv       | 0.20                            | 0.29       | 0.28           |



Table II-7. Comparison of BRI and SuRBHra expression in Event EF2-114 fruits

| Gene           | Sample  | GOI, Ave Ct | Actin, Ave Ct | $\Delta$ ctGOI- $\beta$ -Actin | $\Delta\Delta$ Ct | Fold change |
|----------------|---------|-------------|---------------|--------------------------------|-------------------|-------------|
| <i>BRI</i>     | EF2-114 | 15.42       | 20.68         | -5.26                          | 0.00              | 1.00        |
| <i>SuRBHra</i> |         | 20.18       | 20.68         | -0.50                          | 4.76              | 0.04        |

### II.K.3. SUPPRESSION OF *bLcy* AND *eLcy*

Lycopene is converted by lycopene  $\beta$ -cyclase (*bLcy*) and lycopene  $\epsilon$ -cyclase (*eLcy*) to produce  $\beta$ -carotene and  $\alpha$ -carotene, which are precursors of abscisic acid and lutein, respectively. To accumulate lycopene in pineapple fruits, RNAi constructs containing both *bLcy* and *eLcy* genes under the control of fruit-enhanced BRIp promoters were introduced in Event EF2-114. qRT-PCR analysis of Event EF2-114 and MD2 fruits harvested at 106, 131 and 154 DAF were performed to examine suppression of *bLcy* and *eLcy*. Fruits were analyzed at three different developmental stages to capture the range of expression and, thus, to detect suppression of both *bLcy* and *eLcy*. The results of analyses are presented in Table VI-5 and Figure VI-3.

The data shows that expression levels of *bLcy* were reduced by 86%, 83% and 93% at 106, 131 and 154 DAF respectively, relative to MD2.

Based on our Southern analysis, it was known that *bLcy* is encoded by a single gene in the MD2 (Figure V-12, Lane 1) genome. It is evident from the strong down-regulation of *bLcy* in all three developmental stages of Event EF2-114 fruits that the *bLcy* RNAi construct efficiently suppressed the endogenous gene in a fruit-enhanced manner.

RNA expression level of *eLcy* was reduced by 88% at 106 DAF and subsequently reduced by 68% and 66% at 131 and 154 DAF, respectively. Based on our Southern analysis, *eLcy* appears to be coded by two genes in MD2 (Figure V-6, Lane 1). At present, the sequence of only one of the three genes is known, which was used in the construction of the *eLcy* RNAi construct for the present study. The homology between the sequence of the *eLcy* gene in our RNAi construct and the other gene is not known and how each of these two genes is regulated at the temporal and spatial levels is also not known. The 68% and 66% reduction at 131 and 154 DAF might be due to the differences in the nucleotide sequences between the two genes affecting the efficacy of suppression.

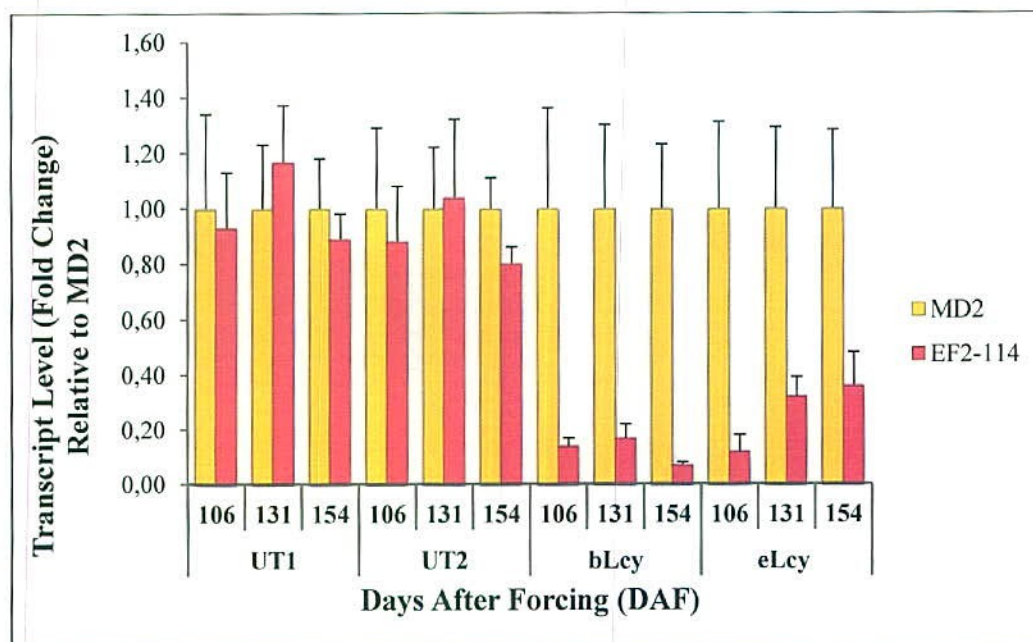
Expression of the endogenous pineapple *UT1* and *UT2* genes and efficient suppression of *bLcy* and *eLcy* gene expression in Event EF2-114 resulted in the accumulation of lycopene (mean 21 ppm) and a three-fold reduction of  $\beta$ -carotene (from 28.1 IU/100 g in MD2 to 8.9 IU/100 g in Event EF2-114) in the fruits. Efficient suppression of *bLcy* and *eLcy* by RNAi technology, leading

to higher lycopene content has been reported in transgenic tomato line (Ma et al., 2011). In this case, the tomato *bLcy* and *eLcy* genes were on two separate transformation plasmids. Ronen et al. (2000) have shown an increase in  $\beta$ -carotene content and a decrease in lycopene content in transgenic tomato lines by overexpression of the tomato *bLcy* gene. Our qRT-PCR results from Event EF2-114 fruits are in agreement with these reports.

**Table II-8. Relative expression/suppression of *UT1*, *UT2*, *bLcy* and *eLcy***

| Gene        | DAF | MD2  | STDV-MD2 | EF2-114 | STDV-114 |
|-------------|-----|------|----------|---------|----------|
| <i>UT1</i>  | 106 | 1.00 | 0.34     | 0.93    | 0.20     |
|             | 131 | 1.00 | 0.23     | 1.17    | 0.20     |
|             | 154 | 1.00 | 0.18     | 0.89    | 0.09     |
| <i>UT2</i>  | 106 | 1.00 | 0.29     | 0.88    | 0.20     |
|             | 131 | 1.00 | 0.22     | 1.04    | 0.28     |
|             | 154 | 1.00 | 0.11     | 0.80    | 0.06     |
| <i>bLcy</i> | 106 | 1.00 | 0.36     | 0.14    | 0.03     |
|             | 131 | 1.00 | 0.30     | 0.17    | 0.05     |
|             | 154 | 1.00 | 0.23     | 0.07    | 0.01     |
| <i>eLcy</i> | 106 | 1.00 | 0.31     | 0.12    | 0.06     |
|             | 131 | 1.00 | 0.29     | 0.32    | 0.07     |
|             | 154 | 1.00 | 0.28     | 0.36    | 0.12     |

**Figure II-12. Relative expression/suppression of *UT1*, *UT2*, *bLcy* and *eLcy***





#### II.K.4. SUPPRESSION OF *flACC3'*

In order to reduce natural, precocious flowering in pineapple, the RNAi construct containing the meristem-specific sequence of *flACC3'* was introduced into MD2. In plasmid pHCWflACC3'-2, expression of the *flACC3'* hairpin structure is under transcriptional control of the constitutive Ubpp promoter. Although a constitutive promoter drives expression of the RNAi construct, the *flACC* sequence is specific to meristem ACS (*flACC3'*) and, thus, its expression is expected to down regulate expression of the cognate endogenous ACS gene.

From the Southern analyses, the introduced *flACC* gene in Event EF2-114 genome (Figure V-8, Lane 2), we have not observed the flowering control phenotype in field evaluations of Event EF2-114. Several attempts were also made to evaluate the expression of *flACC3'* both in MD2 and Event EF2-114 meristematic tissues by qRT-PCR (Data not shown). The Ct values obtained in these analyses were >35 and, thus, expression levels were below the threshold level of detection. Without the detectable expression of *flACC3'* in MD2, it was not possible to assess the effect of RNAi on flowering control in Event EF2-114.

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### III. COMPOSITIONAL ASSESSMENT OF EVENT EF2-114

Safety assessments of biotech crops typically include comparisons of the composition of edible tissues of the GM crop to that of conventionally bred counterparts (Codex Alimentarius, 2003). When available, compositional assessments are performed using the principles and analytes outlined in the OECD consensus documents for the subject crop. However, unlike major commodities, neither OECD, nor ILSI has published a consensus document or database on pineapple composition. Therefore, for purposes of this assessment, the published food science literature, FDA and Health Canada were consulted to develop a set of compositional parameters for comparative analysis of Event EF2-114 and its conventional counterpart, MD2.

The information provided in this section also addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 44, 45, 46, and 47 for assessment of composition, metabolites, and effects of food processing.

#### III.A. NUTRIENTS OF PINEAPPLE

The pineapple fruit is delicious, healthy and nutritious; it has vitamins, minerals, fiber and enzymes that are good for the digestive system and helps in maintaining ideal weight and balanced nutrition. Pineapple has minimal fat (saturated fat) and sodium and is a good source of vitamin C, potassium and dietary fiber (Purseglove, 1972; Morton, 1987; Mateljan, 2007). Pineapple can be eaten raw or used in cooking. The edible part of the pineapple fruit (60% of the fresh fruit) is made up of 85% water, 0.4% protein, 14% sugar, 0.1% fat and 0.5% fiber (Purseglove, 1972). The sugar content varies considerably during the ripening process and is cultivar dependent. Pineapple fruit contains antioxidants, namely vitamins A and C, which are thought to reduce the oxidative damage caused by free radicals and chelating metals. It also contains the protease enzyme complex, bromelain, which is thought to aid digestion (Tochi et al., 2008).

Table VII-1 illustrates the nutrient content of pineapple varieties Smooth Cayenne and MD2, compared to apple, orange and banana (USDA ARS, 2009; Health Canada, 2008).



Table III-1. Nutrient composition of fresh pineapple

| Nutrient (per 100 g edible tissue) | Unit | Pineapple: Smooth Cayenne, USDA <sup>1</sup> | Pineapple: MD2             |                 | Apple                  |                 | Orange            |                 | Banana            |                 |
|------------------------------------|------|----------------------------------------------|----------------------------|-----------------|------------------------|-----------------|-------------------|-----------------|-------------------|-----------------|
|                                    |      |                                              | USDA <sup>1</sup>          | HC <sup>1</sup> | USDA <sup>1</sup>      | HC <sup>1</sup> | USDA <sup>1</sup> | HC <sup>1</sup> | USDA <sup>1</sup> | HC <sup>1</sup> |
| Non-edible proportion              |      | 42% (core, crown, parings)                   | 49% (core, crown, parings) | NR <sup>2</sup> | 23% (core, stem, skin) | NR <sup>2</sup> | 27% (peel, seeds) | NR <sup>2</sup> | 36% (skin)        | NR <sup>2</sup> |
| Water                              | g    | 87                                           | 86                         | NR <sup>2</sup> | 87                     | NR <sup>2</sup> | 87                | NR <sup>2</sup> | 75                | NR <sup>2</sup> |
| Energy                             | kJ   | 190                                          | 215                        | 212             | 200                    | 217             | 197               | 197             | 371               | 372             |
| Protein                            | g    | 0.55                                         | 0.53                       | Tc <sup>1</sup> | 0.27                   | Tc <sup>1</sup> | 0.94              | 0.76            | 1.09              | 0.85            |
| Total lipid (fat)                  | g    | 0.13                                         | 0.11                       | Tc <sup>1</sup> | 0.13                   | Tc <sup>1</sup> | 0.12              | Tc <sup>1</sup> | 0.33              | Tc <sup>3</sup> |
| Carbohydrate                       | g    | 11.82                                        | 13.5                       | 13.41           | 12.76                  | 13.77           | 11.75             | 11.45           | 22.84             | 22.88           |
| Fiber (total dietary)              | g    | NR <sup>2</sup>                              | 1.4                        | 1.3             | 1.3                    | 1.9             | 2.6               | 1.8             | 2.4               | 1.8             |
| Total sugars                       | g    | 8.29                                         | 10.32                      | NR <sup>2</sup> | 10.1                   | NR <sup>2</sup> | 9.35              | NR <sup>2</sup> | 12.23             | NR <sup>2</sup> |
| Sucrose                            | g    | 4.59                                         | 6.47                       | NR <sup>2</sup> | 0.82                   | NR <sup>2</sup> | NR <sup>2</sup>   | NR <sup>2</sup> | 2.39              | NR <sup>2</sup> |
| Glucose (dextrose)                 | g    | 1.76                                         | 1.7                        | NR <sup>2</sup> | 3.25                   | NR <sup>2</sup> | NR <sup>2</sup>   | NR <sup>2</sup> | 4.98              | NR <sup>2</sup> |
| Fructose                           | g    | 1.94                                         | 2.15                       | NR <sup>2</sup> | 6.03                   | NR <sup>2</sup> | NR <sup>2</sup>   | NR <sup>2</sup> | 4.85              | NR <sup>2</sup> |
| Starch                             | g    | NR <sup>2</sup>                              | NR <sup>2</sup>            | NR <sup>2</sup> | NR <sup>2</sup>        | NR <sup>2</sup> | NR <sup>2</sup>   | NR <sup>2</sup> | 5.38              | NR <sup>2</sup> |
| Calcium                            | mg   | 13                                           | 13                         | 13              | 5                      | 6               | 40                | 40              | 5                 | 5               |
| Iron                               | mg   | 0.25                                         | 0.28                       | 0.24            | 0.07                   | 0.14            | 0.1               | 0.08            | 0.26              | 0.25            |
| Magnesium                          | mg   | 12                                           | 12                         | 12              | 4                      | 5               | 10                | 10              | 27                | 27              |
| Phosphorus                         | mg   | 9                                            | 8                          | 9               | 11                     | 11              | 14                | 14              | 22                | 22              |
| Potassium                          | mg   | 125                                          | 108                        | 107             | 90                     | 107             | 181               | 181             | 358               | 358             |
| Sodium                             | mg   | 1                                            | 1                          | 1               | 0                      | 1               | 0                 | 0               | 1                 | 1               |
| Zinc                               | mg   | 0.08                                         | 0.12                       | NR <sup>2</sup> | 0.05                   | NR <sup>2</sup> | 0.07              | NR <sup>2</sup> | 0.15              | NR <sup>2</sup> |
| Copper                             | mg   | 0.081                                        | 0.113                      | NR <sup>2</sup> | 0.031                  | NR <sup>2</sup> | 0.045             | NR <sup>2</sup> | 0.078             | NR <sup>2</sup> |
| Manganese                          | mg   | 1.593                                        | 0.818                      | NR <sup>2</sup> | 0.038                  | NR <sup>2</sup> | 0.025             | NR <sup>2</sup> | 0.27              | NR <sup>2</sup> |
| Vitamin C (total ascorbic acid)    | mg   | 16.9                                         | 56.4                       | 56.1            | 4                      | 4.3             | 53.2              | 53.4            | 8.7               | 8.5             |
| Vitamin B1 Thiamin                 | mg   | 0.078                                        | 0.08                       | NR <sup>2</sup> | 0.019                  | NR <sup>2</sup> | 0.087             | NR <sup>2</sup> | 0.031             | NR <sup>2</sup> |
| Vitamin B2 Riboflavin              | mg   | 0.029                                        | 0.033                      | NR <sup>2</sup> | 0.028                  | NR <sup>2</sup> | 0.04              | NR <sup>2</sup> | 0.073             | NR <sup>2</sup> |
| Vitamin B3 Niacin                  | mg   | 0.47                                         | 0.507                      | NR <sup>2</sup> | 0.091                  | NR <sup>2</sup> | 0.282             | NR <sup>2</sup> | 0.665             | NR <sup>2</sup> |
| Pantothenic acid                   | mg   | 0.193                                        | 0.217                      | NR <sup>2</sup> | 0.071                  | NR <sup>2</sup> | 0.25              | NR <sup>2</sup> | 0.334             | NR <sup>2</sup> |
| Vitamin B6                         | mg   | 0.106                                        | 0.114                      | NR <sup>2</sup> | 0.037                  | NR <sup>2</sup> | 0.06              | NR <sup>2</sup> | 0.367             | NR <sup>2</sup> |
| Folate                             | mcg  | 11                                           | 19                         | 20              | 0                      | 0               | 30                | 30              | 20                | 20              |
| Choline                            | mg   | 5.6                                          | 5.4                        | NR <sup>2</sup> | 3.4                    | NR <sup>2</sup> | 8.4               | NR <sup>2</sup> | 9.8               | NR <sup>2</sup> |



| Nutrient (per 100 g edible tissue) | Unit | Pineapple: Smooth Cayenne, USDA <sup>1</sup> | Pineapple: MD2    |                 | Apple             |                 | Orange            |                 | Banana            |                 |
|------------------------------------|------|----------------------------------------------|-------------------|-----------------|-------------------|-----------------|-------------------|-----------------|-------------------|-----------------|
|                                    |      |                                              | USDA <sup>1</sup> | HC <sup>1</sup> | USDA <sup>1</sup> | HC <sup>1</sup> | USDA <sup>1</sup> | HC <sup>1</sup> | USDA <sup>1</sup> | HC <sup>1</sup> |
| Vitamin A                          | IU   | 52                                           | 57                | NR <sup>2</sup> | 38                | NR <sup>2</sup> | 225               | NR <sup>2</sup> | 64                | NR <sup>2</sup> |
| $\beta$ -Carotene                  | mcg  | 31                                           | 34                | 34              | 17                | 27              | 71                | 71              | 26                | 26              |
| Vitamin E ( $\alpha$ -tocopherol)  | mg   | NR <sup>2</sup>                              | 0.02              | NR <sup>2</sup> | 0.05              | NR <sup>2</sup> | 0.18              | NR <sup>2</sup> | 0.1               | NR <sup>2</sup> |
| Vitamin K (phylloquinone)          | mcg  | 0.7                                          | 0.7               | NR <sup>2</sup> | 0.6               | NR <sup>2</sup> | 0                 | NR <sup>2</sup> | 0.5               | NR <sup>2</sup> |

1 USDA = USDA ARS, 2009; HC = Health Canada, 2008

2 Tc = Trace

3 NR = Not reported

### III.B. NUTRIENT COMPOSITION OF EVENT EF2-114

Event EF2-114 is genetically engineered to accumulate lycopene in the fruits by overexpression of a phytoene synthase gene and suppression of lycopene  $\beta$ -cyclase and lycopene  $\epsilon$ -cyclase genes. Genetic manipulation of carotenoid biosynthesis pathway has been reported in diverse food and non-food plants including rice, potato, tomato, carrot, canola, *Lotus japonicus* flowers, tobacco, and *Arabidopsis* (Giuliano et al., 2008). Ma et al. (2011) reported that suppression of lycopene  $\beta$ -cyclase and lycopene  $\epsilon$ -cyclase genes in tomato resulted in several fold increase in lycopene accumulation and significant reduction in  $\beta$ -carotene and lutein.

The intended and unintended effects of the introduced genes were evaluated by comparing the nutrient composition of Event EF2-114 and MD2 fruits. For this, plants were grown under identical climatic conditions (Appendix A section A.2) and cultural practices in a completely randomized block design with three replicates with unequal numbers, consisting of a total of 2,541 Event EF2-114 plants (606+1,477+458) and 145 MD2 plants (25+61+59). Fruits in the same ripening stage (shell color) and size were harvested on October 22, 2013 as different ripening stages affect some of the nutritional composition of the fruits (Ramsaroop and Saulo, 2007; Shamsudin et al. 2007). A total of 45 Event EF2-114 shippable fruits (i.e., without damages) and 33 MD2 shippable fruits were harvested at random from all replicates and shipped by express courier to the US for analyses (ABC Research Laboratories, Gainesville, FL). At ABC Research Laboratories, 18 fruits each of Event EF2-114 and MD2 were randomly selected for compositional analyses (see also Appendix A section A.1 and Appendix E for details).

A total of 19 analytes were evaluated in Event EF2-114 and MD2 fruits, including lycopene,  $\beta$ -carotene,  $\alpha$ -carotene, lutein, proximates, sugars, various amino acids, potassium and vitamin C. These components were chosen for analysis because 1) proximates are considered important measures of fruit quality and nutrition, 2) fruit sugars are important factors for flavor, nutrition and



sweetness, 3) certain amino acids involved in biosynthesis of the essential, branched-chain amino acids (methionine, leucine, isoleucine, and valine) can potentially be affected by the *SuRBHra* gene product (Van Dyk et al., 1998), and 4) pineapple is an excellent source of vitamin C and a very good source of potassium. Additionally, the intended effect of the genetic modification is the accumulation of lycopene in fruit tissues. Accordingly, components of carotenoid biosynthesis were examined to determine the effects of the introduced genes on the relative proportion of lycopene,  $\beta$ -carotene,  $\alpha$ -carotene and lutein.

The nutrient composition of Event EF2-114 was evaluated by analyzing fruits produced under identical condition as MD2. MD2, the parental variety of Event EF2-114, has a genetic background identical to Event EF2-114 except for the introduced genes and is, therefore, a suitable comparator variety for nutrient composition. Since MD2 is the predominant fresh market pineapple variety with about 98% market share in North America, no other fresh market pineapple varieties were included in this comparative study.

Analyses of key nutrients demonstrate that Event EF2-114 is substantially equivalent to MD2 with the following exceptions: Event EF2-114 fruits accumulate lycopene and contain lower  $\beta$ -carotene as a result of the genetic modification; and contain higher potassium levels compared to MD2 fruit ( $P < 0.001$ ). Minor differences between Event EF2-114 and MD2 fruits were noted in certain other nutrient components (ash, carbohydrates, moisture, sucrose, vitamin C, leucine) ( $P < 0.01$ ), however, these differences are within the reported range of nutrients in pineapple varieties and are considered to be biologically insignificant.

The results of nutrient composition analyses for Event EF2-114 and MD2 control fruits are summarized in Tables VII-2 and VII-3 and Figure VII-1. The analyses demonstrate that there are no significant differences in nutrients selected for comparison in Event EF2-114 and MD2, except for lycopene,  $\beta$ -carotene and potassium ( $P < 0.001$ ).

**Carotenoids (lycopene,  $\beta$ -carotene,  $\alpha$ -carotene, lutein):** Lycopene concentration in Event EF2-114 fruit varied between 14.3 and 32.9 ppm (avg. 21.3 ppm). The accumulation of lycopene resulted in production of pink/red-colored flesh in the fruits of Event EF2-114 (Figure VII-2). To our knowledge, there has been no report of the presence of lycopene in any type of pineapple fruits. Traditional varieties, Champaka (CH) and Smooth Cayenne (SC), are similar to MD2 for nutrient composition with the exception of having lower concentration of vitamin C. The USDA (2013a and 2013b) reported the amount of lycopene in MD2, CH, and SC as 0 ppm and Health Canada (2008) also reported the amount of lycopene in pineapple cubes as 0 ppm (Table VII-4). Charoensiri et al. (2009) reported the amount of lycopene in Sriracha pineapple, a Southeast Asian variety, to be 0 ppm. Lycopene concentrations in Event EF2-114 are within the range of lycopene produced in other foods with a history of safe use. For example, lycopene concentrations are 26 ppm for tomato (calculated as  $4631 \mu\text{g per } 180 \text{ g} = 26 \text{ ppm}$ ), 45 ppm for watermelon, and 14 ppm for grapefruit (USDA, 2013c, Release 25, NDB no. 11529, 9326, 9112, respectively). Health Canada (2008) also reported the same lycopene concentrations for these three products.



The mean value for  $\beta$ -carotene concentration in Event EF2-114 was 8.9 IU/100 g (or 5.34  $\mu$ g/100 g), which is significantly lower than the mean value seen in MD2 (28.1 IU/100 g = 16.86  $\mu$ g/100 g). (Note that 1 IU of  $\beta$ -carotene = 0.6  $\mu$ g of  $\beta$ -carotene, for comparison to other crops mentioned below). This effect is predictable since the conversion of lycopene to  $\beta$ -carotene is decreased by suppressing lycopene  $\beta$ -cyclase (see Section IV.A.1 for the strategy used in manipulation of the carotenoid biosynthesis pathway). For comparison, the USDA database reports the  $\beta$ -carotene concentration in MD2 and CH/SC as 34 IU/100 g and 31 IU/100 g, respectively (USDA, 2013a; USDA, 2013b; Table VII-4) and Health Canada reports the  $\beta$ -carotene concentration in pineapple cubes as 34 IU/100 g (Health Canada, 2008). Charoensiri et al. (2009) reported the amount of  $\beta$ -carotene to be below detection level for Sriracha pineapple.

Overall, pineapple is not a major source of  $\beta$ -carotene (pro-vitamin A). Sweet potato, carrot, cantaloupe and apricot produce 9444, 8285, 2020 and 1094  $\mu$ g/100 g of  $\beta$ -carotene, respectively, according to USDA (2013d) report, and 9382, 8225, 2009 and 1094  $\mu$ g/100 g of  $\beta$ -carotene, respectively, according to Health Canada (2008) report. Consequently, reduction of  $\beta$ -carotene content in Event EF2-114 is not expected to have a significant impact on dietary  $\beta$ -carotene (pro-vitamin A) uptake.

The  $\alpha$ -carotene content was also measured in the fruits of MD2 and Event EF2-114, however the concentration was below the detection level (<10 IU/100 g) (Table VII-2, footnote, data not shown). The USDA database (USDA, 2013a; USDA, 2013b; USDA, 2013f) reports the concentration of  $\alpha$ -carotene in MD2, CH, and SC to be 0 IU/100 g.

Lutein concentration in general is extremely low in pineapple. The USDA database reports that lutein concentration was zero for all varieties including MD2, CH and SC (USDA, 2013a; USDA, 2013b; USDA, 2013e; Table VII-4). For comparison, foods high in lutein/zeaxanthin are kale (19,700  $\mu$ g/100 g), spinach (15,690  $\mu$ g/100 g), summer squash (2,248  $\mu$ g/100 g), sweet corn (949  $\mu$ g/100 g), pepper (431  $\mu$ g/100 g), tangerine (163  $\mu$ g/100 g) and orange (129  $\mu$ g/100 g) (USDA, 2013e).

Lutein is produced by conversion of  $\alpha$ -carotene (Hirschberg, 2001). In Event EF2-114, as the conversion of lycopene to  $\alpha$ -carotene is decreased by suppressing lycopene  $\epsilon$ -cyclase (see Section IV.A.1), we were curious if lutein concentration is actually reduced in Event EF2-114 compared to MD2 (see also above for the rationale). With the analyses used, we detected lutein at very low concentrations (two fruits of Event EF2-114 had 1.5 and 1.8 and two of MD2 had 2.2 and 2.5  $\mu$ g lutein per 100 g fruit). We had previously looked at lutein content in a few samples of other events and obtained similar results. This lower lutein concentration in Event EF2-114 was predictable.

With respect to the changes seen in carotenoids accumulation in our studies, similar results were reported by Ma et al. (2011), in which the suppression of lycopene  $\beta$ -cyclase and lycopene  $\epsilon$ -cyclase genes in tomato increased lycopene and reduced  $\beta$ -carotene and lutein accumulation.



**Potassium:** Potassium concentration was significantly higher in Event EF2-114 (156 mg/100 g) than in MD2 (129 mg/100 g); the mean value for Event EF2-114 was out of the range of values measured for MD2. A higher potassium value in Event EF2-114 could be considered a desirable trait.

Potassium concentration is variable among varieties of pineapple. For example, the USDA reported (USDA, 2013a, USDA, 2013b) that MD2 produces 108 mg/100 g and CH/SC 125 mg/100 g of potassium (Table VII-4) and, in another report, all varieties produce 109 mg/100 g of potassium (USDA, 2013g). Health Canada (2008) reported that pineapple cubes produce 107 mg/100 g of potassium (Table VII-4). FAO (2012) reported production of 205 mg/100 g in West African pineapple fruits. Othman (2011) reported that pineapple fruit variety Mbezi (grown in Tanzania) produces 411 mg/100 g-fw-pineapple. Of the four macro elements (calcium, sodium, magnesium, potassium) and metals (chromium, manganese, zinc, iron, copper, aluminum, cadmium, lead) measured, potassium was the predominant minerals element in the pineapple fruits. The potassium content of Mbezi pineapple fruits measured was higher than the potassium contents for East African pineapple fruits reported by West, et al. (1988) i.e. 150 – 280 mg/100 g-fw.

The difference for the amount of potassium seen between Event EF2-114 and MD2 can be contributed to environmental factors and perhaps potassium uptake from soil. In tomato, there has been correlation between lycopene content and potassium content in the fruit. Also, the lycopene content increased linearly with increasing potassium level in the plant nutrient solution (Serio et al. 2007). Taber et al. (2008) found that concentration of potassium in fruits of high lycopene tomato 'Fla. 8153' was significantly ( $P < 0.01$ ) correlated to the fruit carotenoids, indicating a possible role for potassium nutrition in increasing lycopene and reducing  $\beta$ -carotene in the fruits. Therefore, it is possible that accumulation of lycopene in Event EF2-114 increased (is correlated with higher) level of potassium in the fruit.

Types and sources of potassium fertilizers affect quality, nutrients and post-harvest attributes of pineapple and other fruits (Quaggio et al. 2009; Ganeshamurthy et al. 2011). Total soluble solids and ascorbic acid content of the 'Smooth Cayenne' pineapple fruit pulp varied significantly as a function of potassium rates and sources. Total titratable acidity increased in response to potassium application. The use of potassium sulfate resulted in a better fruit sugar/acid ratio, especially at higher potassium rates. Fruit firmness declined with increased storage period, and this effect was greater with no potassium application. Post-harvest characteristics of pineapple fruits were more affected by potassium rates than by potassium sources (Quaggio et al. 2009).

High rate of potassium fertilizer is needed to achieve not only highest total fruit production but also the greatest percentage of fruits suitable for marketing and maintaining good quality with high mineral content. Applied potassium influences fruit size, appearance, color and consumer acceptance such as fruit aroma and taste. Lack or deficiency of potassium produces plants highly susceptible to diseases and pests and other environmental stresses. Other beneficial effects of



potassium include high juice content, high vitamin C content, uniformity and acceleration of ripening of fruits and resistance to bruising or physical breakdown during shipping and storage (Ganeshamurthy et al. 2011).

Overall, pineapples consumed in the US are not major sources of potassium; consumption of pineapple annually in the US is limited to ~6 lb (~3 lbs flesh) per capita (Figure II-4). Agri-Food Trade Service (2012) reported that consumption of pineapple in the US was on the average 2.2 kg per capita during 2006-2011 (Table VII-5). Then we calculated as how much pineapple and other fruits would contribute to daily intake of potassium for adults (based on daily consumption values and the potassium content of each fruit). Therefore, there is a limmeted role of pineapple as potassium source in the US with an average of 0.1% of the daily adequate intake for an adult (Table VII-6). Potassium concentrations in banana and white beans, the highest reported for a food crop, are 358 mg/100 g and 561 mg/100 g, respectively (USDA, 2013g).

**Vitamin C:** Vitamin C is a major nutrient component of pineapple. In this analysis, the mean value for vitamin C concentration was lower in Event EF2-114 compared to that of MD2 (40.3 mg/100 g vs. 46.8 mg/100 g). Levels of vitamin C for Event EF2-114 and MD2 were within the ranges normally seen in commercially produced MD2. For example, although statistically significant differences ( $P < 0.01$ ) were observed for vitamin C (Table VII-2), the differences were small and the mean values were within the ranges of values reported for MD2.

MD2 and Event EF2-114 exhibit similar ascorbic acid content when measurements were performed with much larger sample sizes and over multiple years (see Appendix F, Internal Quality of Event EF2-114). In another experiment conducted between July 2011 and July 2013, there was no statistically significant difference in ascorbic acid levels between Event EF2-114 and MD2 (see Appendix G, Plant and Fruit Characteristics of Event EF2-114).

Vitamin C concentration in pineapple fluctuates dramatically. Quaggio et al. (2009) showed that potassium is the foremost nutrient required for pineapple crop and potassium fertilizer rates and sources would affect ascorbic acid concentrations in SC fruits. Padio Seda et al. (1994) showed significant loss in vitamin C in pineapple at high temperatures of 30-40 °C. Cock and Leal (2012) showed that vitamin C concentration of 'Perola' (P) increases in relation to age of the plant at the time of flower induction (number of months after planting), while the vitamin C content in SC remained constant after different flower induction times. This was consistent with the results obtained by Cock et al. (2010) for P, SC and Favo De Mel, while for Jupí cultivar they found a positive relationship between age of the plant at the time of flower induction and vitamin C concentration. In our analyses, the fruits tested by ABCR were induced for flowering at 13 months after planting, while plants in experiment of July 19 planting (Appendix G) were induced for flowering at 18-18.5 months after planting. This age differences and harvesting time difference



(June/July vs. October) could account for differences in vitamin C concentrations within and between MD2 and Event EF2-114.

Lee and Kader (2000) reported that content of vitamin C in fruits and vegetables can be influenced by various factors such as:

1. Genotypic differences.
2. Pre-harvest climatic conditions (i.e., temperature and light intensity during growing season, the higher the intensity of light during the growing season, the greater is vitamin C content in plant tissue).
3. Cultural practices (i.e., application of fertilizers and agrochemicals and irrigation. Nitrogen fertilizers at high rate tend to decrease the vitamin C content in many fruits and vegetables; vitamin C content of many crops can be increased with less frequent irrigation).
4. Maturity and harvesting methods (mechanical injuries such as bruising, surface abrasions, and cuts can result in accelerated loss of vitamin C).
5. Postharvest handling procedures: Temperature management after harvest is the most important factor to maintain vitamin C of fruits and vegetables; losses are accelerated at higher temperatures and with longer storage durations. However, some chilling sensitive crops such as pineapple show more losses in vitamin C at lower temperatures. Conditions favorable to water loss after harvest result in a rapid loss of vitamin C. Destruction of ascorbic acid can occur before any visible symptoms of chilling. The retention of vitamin C is lowered by bruising, and other mechanical injuries, and by excessive trimming. The loss of vitamin C after harvest can be reduced by storing fruits and vegetables in reduced O<sub>2</sub> and/or up to 10% CO<sub>2</sub> atmospheres; higher CO<sub>2</sub> levels can accelerate vitamin C loss.

Taking into consideration the results obtained by Pardio Sedas et al. (1994) and Lee and Kader (2000), it is possible that the Event EF2-114 fruits were subjected to higher or lower temperature during shipment to ABCR laboratory and this might account for differences in vitamin C concentrations between MD2 and Event EF2-114.

**Other nutrients:** Levels for all other nutrients analyzed from Event EF2-114 and MD2 were within the ranges normally seen in commercially produced MD2. For example, although statistically significant differences ( $P < 0.01$ ) were observed for ash, carbohydrates, moisture, sucrose and leucine (Table VII-3), the differences were small and the mean values were within the ranges of values reported for MD2. Also, large variations have been reported in pineapple fruit variety Mbezi for ash, moisture content, titratable acidity (TA), fat, fiber, sugars and total soluble solids (TSS) (Othman, 2011). Joomwong and Sornsrivichai (2005) reported large variations during the year for most of the traits measured including TA, TSS, TSS/TA ratio, texture and pH for the fruit of SC pineapple. Wardy et al. (2009) reported large variations for vitamin C and sugars and

less variation for acidity, TSS and sensory indexes for MD2 (different stages post-harvest), SC and Sugarloaf pineapples. Othman et al. (2006) found large variations for TSS, TA, fructose, glucose, sucrose, total sugars, etc. for pineapple variety Gandul fruits with or without treatment with Fruitone. Cock and Leal (2012) and Cock et al., (2010) found the age at the time of forcing resulted in large variations for vitamin C, pH, Brix/Acid ratio within different cultivars with the exception of SC for vitamin C.

Table VII-4 shows a summary of our results compared to the data published by USDA (2013a and 2013b), FAO (2012) and Health Canada (2008) for pineapple nutrient compositions. Also, see Appendix E – Materials and Methods for Compositional Analysis of Event EF2-114, Appendix F – Internal Quality of Event EF2-114, Appendix G – Plant and Fruit Characteristics of Event EF2-114 and Appendix H – Sensory Analysis of Event EF2-114 for comparison of different characteristics of Event EF2-114 and MD2.

### III.C. COMPOSITION ASSESSMENT CONCLUSIONS

Taking all the analyses into consideration, we conclude that Event EF2-114 is similar to its parental variety, MD2 with respect to nutrient composition, with the exception of lycopene,  $\beta$ -carotene and potassium. Other differences in nutrient composition between the two varieties are within the range of natural variations of MD2 and insignificant within the context of the American diet.



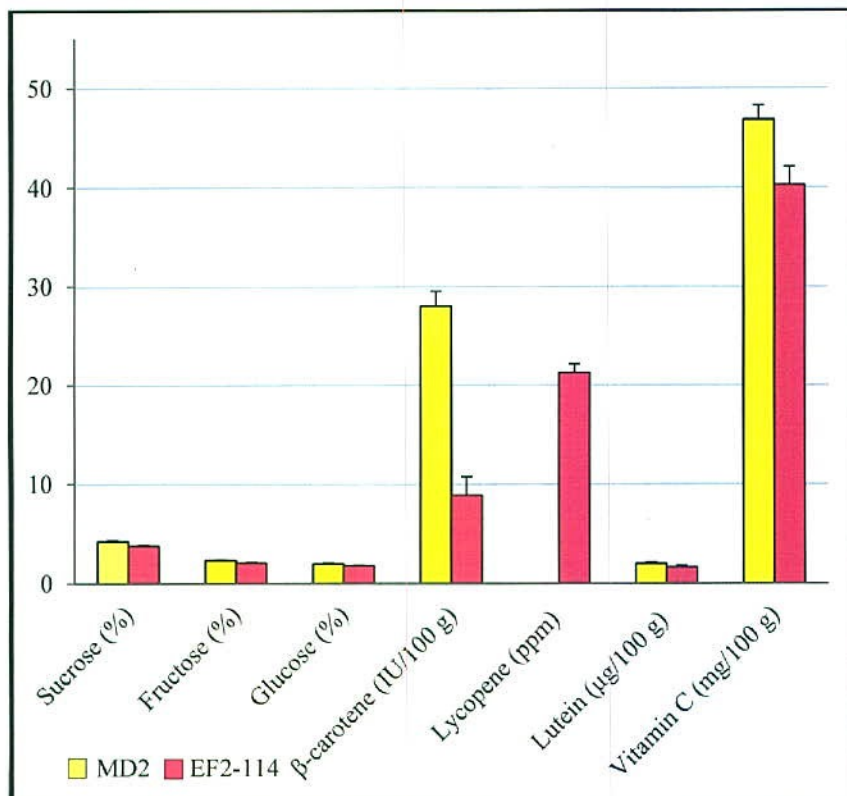
Table III-2. Carotenoids, vitamin C, and potassium levels in MD2 and Event EF2-114

| Nutrient composition         | MD2 |      |             |     | Event EF2-114 |      |             |     | P-value |
|------------------------------|-----|------|-------------|-----|---------------|------|-------------|-----|---------|
|                              | N   | Mean | Range       | SE  | N             | Mean | Range       | SE  |         |
| Lycopene (ppm)               | 18  | 0.0  | 0.0 – 0.0   | 0.0 | 18            | 21.3 | 14.3 – 32.9 | 0.9 | <0.000  |
| $\beta$ -carotene (IU/100 g) | 18  | 28.1 | 20.8 – 39.5 | 1.5 | 18            | 8.9  | 5.0* - 32.8 | 1.9 | <0.000  |
| Vitamin C (mg/100 g)         | 18  | 46.8 | 36.0 - 55.8 | 1.4 | 18            | 40.3 | 28.3 - 56.2 | 1.8 | 0.008   |
| Potassium (mg/100 g)         | 18  | 129  | 117 - 142   | 1.7 | 18            | 156  | 101 - 204   | 6.6 | 0.0003  |

\* Limits of detection (LOD) for  $\beta$ - and  $\alpha$ -carotene were 10 IU/100 g, hence the  $\beta$ -carotene values for the samples below LOD were considered to be 5 IU/100 g (mean of 0 and 10).  $\alpha$ -carotene was also measured in the fruits and the values were below LOD for all samples tested.

N = Number of fruits analyzed. SE = Standard error. Student's *t* test was performed to compare the analytes. Results significantly different at  $p < 0.001$  are shown in bold.

Figure III-1. Carotenoids, vitamin C and sugars levels in MD2 and Event EF2-114



Standard errors are represented by the bars.

Table III-3. Proximates, sugars, and amino acid levels in MD2 and Event EF2-114

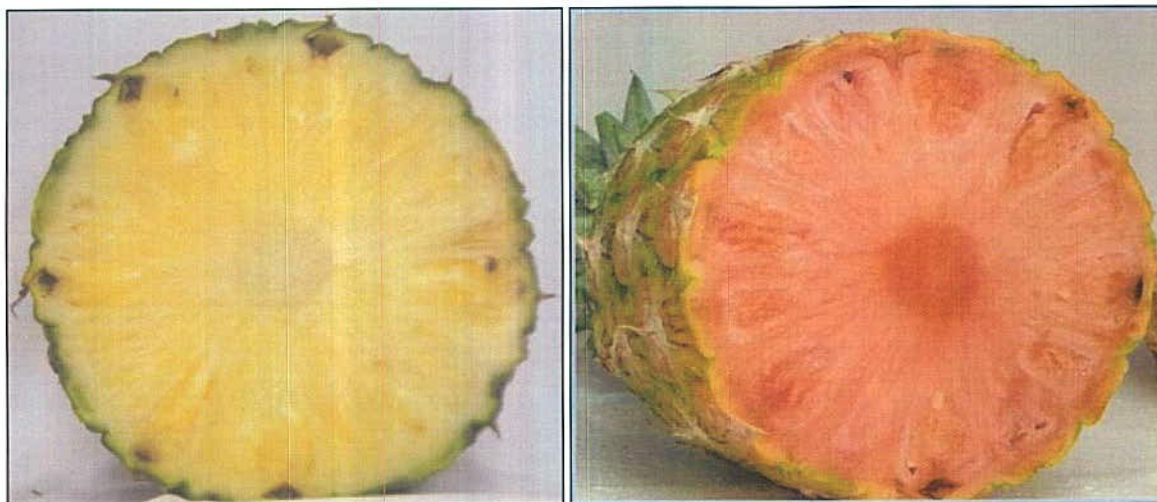
| Analyte                 | MD2 |       |               |       | Event EF2-114 |       |               |       | P-value |
|-------------------------|-----|-------|---------------|-------|---------------|-------|---------------|-------|---------|
|                         | N   | Mean  | Range         | SE    | N             | Mean  | Range         | SE    |         |
|                         |     |       |               |       |               |       |               |       |         |
| Proximates              |     |       |               |       |               |       |               |       |         |
| Total dietary fiber (%) | 18  | 2.8   | 1.9 - 3.3     | 0.11  | 18            | 2.9   | 1.7 - 3.8     | 0.15  | 0.509   |
| Ash (%)                 | 18  | 0.27  | 0.23 - 0.32   | 0.01  | 18            | 0.31  | 0.20 - 0.42   | 0.01  | 0.001   |
| Carbohydrates (%)       | 18  | 12.0  | 10.1 - 13.4   | 0.18  | 18            | 11.2  | 10.0 - 12.7   | 0.18  | 0.002   |
| Fat (%)                 | 18  | 0.22  | 0.14 - 0.31   | 0.01  | 18            | 0.17  | 0.10 - 0.25   | 0.01  | 0.016   |
| Moisture (%)            | 18  | 86.9  | 85.5 – 88.8   | 0.18  | 18            | 87.8  | 86.5 – 89.0   | 0.18  | 0.002   |
| Protein (%)             | 18  | 0.58  | 0.37 - 0.73   | 0.02  | 18            | 0.54  | 0.35 - 0.78   | 0.03  | 0.370   |
| Sugars                  |     |       |               |       |               |       |               |       |         |
| Sucrose (%)             | 18  | 4.26  | 3.54 - 5.08   | 0.11  | 18            | 3.81  | 3.09 - 4.50   | 0.09  | 0.004   |
| Fructose (%)            | 18  | 2.34  | 1.59 - 3.12   | 0.09  | 18            | 2.07  | 1.51 - 2.68   | 0.07  | 0.026   |
| Glucose (%)             | 18  | 2.00  | 1.33 - 2.74   | 0.08  | 18            | 1.80  | 1.26 - 2.47   | 0.06  | 0.063   |
| Amino acids             |     |       |               |       |               |       |               |       |         |
| Leucine (%)             | 18  | 0.034 | 0.020 - 0.051 | 0.002 | 18            | 0.026 | 0.017 - 0.040 | 0.002 | 0.004   |
| Isoleucine (%)          | 18  | 0.025 | 0.010 - 0.040 | 0.002 | 18            | 0.029 | 0.012 - 0.041 | 0.002 | 0.240   |
| Methionine (%)          | 18  | 0.016 | 0.004 - 0.044 | 0.003 | 18            | 0.008 | 0.004 - 0.012 | 0.001 | 0.011   |
| Valine (%)              | 18  | 0.031 | 0.023 - 0.049 | 0.002 | 18            | 0.033 | 0.018 - 0.049 | 0.002 | 0.399   |

N = Number of fruits analyzed. SE = Standard error. Nutrient levels are shown in % FW. Student's *t* test was performed to compare the analytes. Results significantly different at  $P < 0.01$  are shown in bold.



**Figure III-2. Flesh color in MD2 and Event EF2-114 fruits**

Event EF2-114 fruit (right) exhibits pink/red color in the flesh due to accumulation of lycopene while parental variety MD2 (left) has a golden flesh color.



**Table III-4. Nutrient composition of Event EF2-114 and MD2 compared to published data**

| Nutrient composition         | Experimental Data <sup>1</sup> |               | Published Literature Data |                  |                                                                        |                             |
|------------------------------|--------------------------------|---------------|---------------------------|------------------|------------------------------------------------------------------------|-----------------------------|
|                              | Event EF2-114                  | MD2 (Control) | MD2 (USDA, 2013a)         | SC (USDA, 2013b) | SC (FAO, 2012)                                                         | Pineapple, cubes (HC, 2008) |
| Total dietary fiber (%)      | 2.9                            | 2.8           | 1.4                       | NR <sup>2</sup>  | 1.3                                                                    | 1.3                         |
| Ash (%)                      | 0.31                           | 0.27          | 0.2                       | 0.27             | 0.3                                                                    | NR <sup>2</sup>             |
| Carbohydrates (%)            | 11.2                           | 12            | 13.5                      | 11.8             | 12                                                                     | NR <sup>2</sup>             |
| Fat (%)                      | 0.17                           | 0.22          | 0.11                      | 0.13             | 0.2                                                                    | Tc <sup>4</sup>             |
| Moisture (%)                 | 88                             | 87            | 86                        | 87               | 86                                                                     | NR <sup>2</sup>             |
| Protein (%)                  | 0.54                           | 0.58          | 0.53                      | 0.55             | 0.4                                                                    | Tc <sup>4</sup>             |
| Sucrose (%)                  | 3.81                           | 4.26          | 6.47                      | 4.59             | NR <sup>2</sup>                                                        | NR <sup>2</sup>             |
| Fructose (%)                 | 2.07                           | 2.34          | 2.15                      | 1.94             | NR <sup>2</sup>                                                        | NR <sup>2</sup>             |
| Glucose (%)                  | 1.8                            | 2             | 1.7                       | 1.76             | NR <sup>2</sup>                                                        | NR <sup>2</sup>             |
| $\beta$ -carotene (IU/100 g) | 9                              | 28            | 34                        | 31               | 61 $\beta$ -carotene equivalents <sup>3</sup> (30.5 $\beta$ -carotene) | 34                          |
| Lycopene (ppm)               | 21                             | 0             | 0                         | 0                | NR <sup>2</sup>                                                        | 0                           |
| Lutein (mcg/100 g)           | 1.7                            | 2.3           | 0                         | 0                | NR <sup>2</sup>                                                        | NR <sup>2</sup>             |
| Vitamin C (mg/100 g)         | 40.3                           | 46.8          | 56.4                      | 16.9             | 30                                                                     | 56                          |
| Leucine (%)                  | 0.026                          | 0.034         | NR <sup>2</sup>           | NR <sup>2</sup>  | NR <sup>2</sup>                                                        | NR <sup>2</sup>             |
| Isoleucine (%)               | 0.029                          | 0.025         | NR <sup>2</sup>           | NR <sup>2</sup>  | NR <sup>2</sup>                                                        | NR <sup>2</sup>             |
| Methionine (%)               | 0.008                          | 0.016         | NR <sup>2</sup>           | NR <sup>2</sup>  | NR <sup>2</sup>                                                        | NR <sup>2</sup>             |
| Valine (%)                   | 0.033                          | 0.031         | NR <sup>2</sup>           | NR <sup>2</sup>  | NR <sup>2</sup>                                                        | NR <sup>2</sup>             |
| Potassium (mg/100 g)         | 156                            | 129           | 108                       | 125              | 205                                                                    | 107                         |

<sup>1</sup> Mean values

<sup>2</sup> NR = Not reported

<sup>3</sup> Estimation: 1 mg  $\beta$ -carotene equivalents = 1 part  $\beta$ -carotene + 0.5 part  $\alpha$ -carotene + 0.5 part  $\beta$ -cryptoxanthin

<sup>4</sup> Tc = Trace

HC, 2008 = Health Canada, 2008



Table III-5 Fresh fruit consumption in the United States (kg per capita, 2006-2011)

| Fruits                            | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | Mean |
|-----------------------------------|------|------|------|------|------|------|------|
| Banana                            | 11.4 | 11.8 | 11.4 | 11.6 | 11.7 | 11.6 | 11.6 |
| Apples                            | 7.8  | 7.2  | 7.1  | 6.9  | 7.0  | 7.0  | 7.2  |
| Oranges, tangerines and mandarins | 5.7  | 4.4  | 5.7  | 5.6  | 5.7  | 5.6  | 5.5  |
| Grapes                            | 3.8  | 4.0  | 4.1  | 4.2  | 4.1  | 4.0  | 4.0  |
| Strawberries                      | 2.6  | 2.7  | 2.7  | 2.8  | 2.8  | 2.7  | 2.7  |
| Pineapple                         | 2.2  | 2.2  | 2.2  | 2.2  | 2.2  | 2.2  | 2.2  |
| Cherries                          | 0.5  | 0.5  | 0.4  | 0.4  | 0.4  | 0.4  | 0.4  |

Source: Agri-Food Trade Service, 2012

Table III-6. Potassium (K) supply from fruit consumption sources in the US

| Fruits                            | Daily consumption* (g) | K content <sup>Δ</sup> (mg/100g) | Average daily intake per capita (mg) | K supply according to daily value <sup>■</sup> |
|-----------------------------------|------------------------|----------------------------------|--------------------------------------|------------------------------------------------|
| Banana                            | 31.7                   | 358                              | 113.6                                | 2.4%                                           |
| Apples                            | 19.6                   | 107                              | 21.0                                 | 0.4%                                           |
| Oranges, tangerines and mandarins | 14.9                   | 173                              | 25.8                                 | 0.5%                                           |
| Grapes                            | 11.1                   | 203                              | 22.4                                 | 0.5%                                           |
| Strawberries                      | 7.4                    | 153                              | 11.4                                 | 0.2%                                           |
| Pineapple                         | 6.0                    | 109                              | 6.6                                  | 0.1%                                           |
| Cherries                          | 1.2                    | 222                              | 2.6                                  | 0.1%                                           |

Sources: (\*) Estimated from data presented in Table VII-5

(Δ) USDA (2013g)

(■) Calculated using 4,700 mg per day as the adequate intake of potassium for adults (USDA and U.S. Department of Health and Human Services, 2010).

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